Research Paper

Does human disturbance affect physiological traits of Two-banded Plovers nesting on an urban beach?

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ABSTRACT. In coastal Patagonia, Argentina, shorebird breeding habitats overlap with high levels of human recreational activities, such as dog-walking and driving motorized vehicles. We assessed physiological traits related to body condition, nutritional metabolites, cellular immunity, and stress responses of adult Two-banded Plovers (*Charadrius falklandicus*) in northern Chubut, Patagonia, Argentina. We examined blood samples from 46 individuals captured at an urban ("disturbed" treatment, n = 28) and a rural beach ("control" treatment, n = 18) between 2016 and 2017. We studied (1) body condition by size-correcting body mass to wing-chord lengths, (2) nutritional metabolites, such as the haematocrit, glucose, and concentrations of triglycerides, cholesterol, and total proteins, (3) cellular immunity by counting total leukocytes, and the percentages of heterophils, lymphocytes, eosinophils, and monocytes, and (4) stress responses by obtaining the heterophil/lymphocyte ratio and quantifications of plasma baseline corticosterone. We determined the overall support of predictor variables: treatment, day of the year (DOY), interaction DOY × treatment, year, and sex on a multivariate assessment using MANOVA. Body condition and triglycerides decreased, and monocytes increased, during the breeding season at the control site (DOY × treatment). Body condition significantly differed by sex, with males exhibiting lower values than females. Triglycerides, cholesterol, and total leukocyte count significantly differed between years, showing higher values in 2017. The multivariate model showed the greatest variation only explanatory in DOY (n = 0.67, P < 0.001) and year (n^2 = 0.62, P = 0.001). Overall, birds exhibited depleted body condition and triglycerides throughout the season at the control site: a pattern likely biased by the capture date. Contrary to the disturbed site, most samples at the control site were collected from mid-to-late breeding season each year, and most birds were likely renesting. Sex differences observed for body condition suggest greater energy-costly reproduction activities for males, including territory defense and courtship behaviors. No significant differences were found in stress responses, and based on observations in the field, birds may tolerate human disturbance. Habituation was a previously suggested coping mechanism for reducing the costs of repeated exposure to non-lethal stimuli, such as human activity. Given that no treatment effects on physiological traits were observed, beyond triglycerides, our results may still help to improve the decisions of wildlife managers toward protecting beach-nesting areas by preventing impacts to nests, chicks, and pairs during their breeding season.

Les perturbations provoquées par les humains affectent-elles les caractéristiques physiologiques des pluviers des Falkland nichant sur une plage urbaine?

RÉSUMÉ. Sur les côtes de la Patagonie, en Argentine, les habitats de reproduction des oiseaux se superposent à de nombreuses activités de loisirs humaines, comme les promenades de chiens et la conduite de véhicules à moteur. Nous avons évalué les caractéristiques physiologiques liées à l'état du corps, aux métabolites de la nourriture, à l'immunité cellulaire et aux réponses au stress chez les pluviers des Falkland adultes (*Charadrius falklandicus*) au nord du Chubut, dans la région de Patagonie, en Argentine. Nous avons examiné des échantillons sanguins prélevés sur 46 individus capturés sur une plage urbaine (traitement « perturbé », n = 28) et sur une plage rurale (traitement « témoin », n = 18) de 2016 à 2017. Nous avons étudié (1) l'état de l'organisme en corrigeant la masse corporelle en fonction de la taille selon la longueur de la corde des ailes, (2) les métabolites de la nourriture, comme l'hématocrite, la glycémie et les concentrations de triglycérides, le cholestérol et les protéines totales, (3) l'immunité cellulaire en comptant le nombre total de leucocytes et les pourcentages d'hétérophiles, de lymphocytes, d'éosinophiles et de monocytes et (4) les réponses au stress en obtenant le ratio hétérophiles/lymphocytes et les quantifications initiales de corticostérone dans le plasma. Nous avons déterminé le support global des variables prédictives : traitement, jour de l'année (JDA), interaction JDA x traitement, année et sexe sur une analyse multidimensionnelle en utilisant la procédure MANOVA. L'état du corps et les triglycérides diminuaient tandis que les monocytes augmentaient au cours de la saison de reproduction sur le site témoin (JDA x traitement). L'état du corps variait considérablement selon le sexe, les mâles présentant une valeur moindre que les femelles. Les triglycérides, le cholestérol et le nombre total de leucocytes variaient énormément selon les années, affichant des valeurs plus élevées en 2017. Le modèle multidimensionnel présentait la plus importante variation uniquement explicative en termes de JDA (n = 0,67 P < 0,001) et d'année (n^2 = 0,62, P = 0,001). Globalement, l'état du corps et les triglycérides des oiseaux étaient amoindris tout au long de la saison sur le site témoin : un modèle probablement biaisé par la date de capture. Contrairement au site perturbé, la plupart des échantillons du site témoin avaient en effet été prélevés du milieu à la fin de la saison de reproduction chaque année et la plupart des oiseaux faisaient probablement un deuxième nid. Les différences observées en termes d'état

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du corps entre les deux sexes suggèrent des activités de reproduction plus énergivores chez les mâles, notamment des comportements de défense du territoire et de parade nuptiale. Aucune différence majeure n'a été constatée en termes de réponse au stress et selon les observations effectuées sur le terrain, les oiseaux pourraient tolérer les perturbations humaines. L'accoutumance est un mécanisme de défense qui a déjà été suggéré pour réduire l'énergie à dépenser en cas d'exposition répétée à des stimuli non mortels tels que l'activité humaine. Dans la mesure où on n'a observé aucun effet lié au traitement sur les caractéristiques physiologiques en dehors des triglycérides, nos résultats pourraient malgré tout améliorer les décisions des gestionnaires de la faune pour protéger les zones de nidification sur les plages en évitant les conséquences sur les nids, les oisillons et les couples pendant la période de reproduction.

INTRODUCTION

The frequency and intensity of human-wildlife interactions have increased over the last century and are more prevalent in areas associated with nature-based tourism and recreation (Madden 2004). Because human-wildlife interactions have been associated with negative consequences for wild animal populations, it is critical to identify species' vulnerability to anthropogenic stressors and associated environmental changes (Tablado et al. 2021). A changing environment could disrupt the ability of organisms to cope with novel stressful situations, including disturbance, extreme climatic events, new diseases, predators, and other "labile perturbation factors" (LPF, sensu Wingfield 2003). Physiological mechanisms, such as the stress responses mediated by glucocorticoids can regulate the ability of animals to cope with new stimuli that ultimately can optimize the fitness of individuals in challenging environments (Angelier and Wingfield 2013). In our current context of global change, therefore, the assessments of physiological responses of wild populations to anthropogenic changes are relevant to investigate how organisms respond to different stressors and, ultimately, being able to provide early alerts of their health condition (Palacios et al. 2018, Sueiro et al. 2020).

Shorebird populations inhabiting coastal areas are highly susceptible to human activities on both their breeding (Borneman et al. 2016, Kwon et al. 2021) and nonbreeding grounds (Schlacher et al. 2013, Gibson et al. 2018). Increasing human recreational activities including dog-walking and driving motorized vehicles during spring and summer usually overlap with the shorebird breeding season, affecting both their occurrence and relative abundance (Lafferty et al. 2013). Likewise, the human disturbance has been associated with myriad consequences to shorebirds, including reduced reproductive performance, effect on body condition, true survival, and site fidelity (Lord et al. 2001, Gibson et al. 2018). In the long term, the consequences of human disturbance are driving changes in demography and population trends (Faulhaber et al. 2016). Previous studies have demonstrated that human disturbance is associated with shifts in reproductive behavior (Sabine et al. 2008, Borneman et al. 2016), but behavior is also intertwined with physiological processes (Weston 2019). In brief, environmental variation inherently modulates both behavior and physiology, but the degree to which behavior reactions to human disturbance are linked to physiological responses, and vice versa, is largely unknown for most shorebird species (but see Felton et al. 2018, Charuvi et al. 2020). The health status of birds can be assessed through certain blood and biochemistry parameters, such as hematocrit, blood glucose, and plasma concentrations of triglycerides, cholesterol, and total proteins (Brown 1996). Avian hematocrit level or the relative proportion of red blood cells compared with total blood volume,

constitutes a tool to diagnose anemia indicated by a reduction in the oxygen-carrying capacity (Dawson and Bortolotti 1997). Glucose is used by birds to produce energy mainly through cellular oxidation (Klasing 1998), which is regulated by pancreatic hormones and reflects the level of carbohydrates ingested in response to an individual energy need (Whittow 2000). Synthesis of fatty acids takes place mainly in the liver, and the concentration of avian plasma lipids, such as triglycerides and cholesterol circulating in the blood is influenced by factors, such as age, sex, reproductive stage, health status, nutrition quality, and energy demands (Griminger 1986). Total proteins, composed mainly of albumins, are associated with food intake and energy reserves, and globulins are related to immunity (Brown 1996).

In addition, counts of leukocytes (white blood cells) from blood smears provide a reliable tool to assess several functions and processes of the innate immune system (Davis et al. 2008). Heterophils and lymphocytes constitute the majority (~80%) of leukocytes (Rupley 1997). Heterophils are the primary phagocytic leukocyte and proliferate in response to infections, inflammation, and stress (Maxwell and Robertson 1998), whereas lymphocytes are involved in immunological activity modulating immunoglobulin production (Campbell 1995). The remaining ~20% of the leukocytes are a combination of eosinophils (related to inflammation process and parasites defense; Maxwell 1987), basophils (related to early inflammation and immediate hypersensitivity reactions; Maxwell and Robertson 1995), and monocytes (phagocytic activity associated with infections; Davis et al. 2004). The relative proportion of heterophils to lymphocytes (hereafter, H/L-ratio) is a well-known measure indicative of stress (Davis et al. 2008). The H/L ratio is positively related to the magnitude of the stressor and circulating glucocorticoids, which can also be influenced by infections (Davis et al. 2008). Glucocorticoids, such as corticosterone in birds, are crucial to mobilize energy through various metabolic pathways, leading to behavioral and physiological reactions (Sapolsky et al. 2000). Corticosterone, a hormone indicative of acute stress (Wingfield et al. 1998), interacts to regulate glucose and triglyceride levels (Remage-Healey and Romero 2001), and modulate the abundance of peripheral leukocytes (Müller et al. 2011).

In this study, we assessed parameters related to body condition, nutritional status, cellular immunity, and stress responses of nesting adult Two-banded Plovers (*Charadrius falklandicus*, hereafter, TBPLs) in northern Patagonia, Argentina. Using samples from individuals captured during incubation at an urban (disturbed) and a rural (control) beach, we examined (1) body condition as a measure of an individual structural size by regressing body mass against the wing chord length; (2) nutritional status by measuring the hematocrit, blood glucose, and plasma concentrations of triglycerides, cholesterol, and total

Fig. 1. Map of the study area in northern Chubut province, Patagonia, Argentina. The box indicates the location of Puerto Madryn city, and the grey dots are both study areas. Playa Parana, the disturbed site, is located near Puerto Madryn city, and Playa Blanca, the control site, is located within Peninsula Valdés Natural Protected Area.
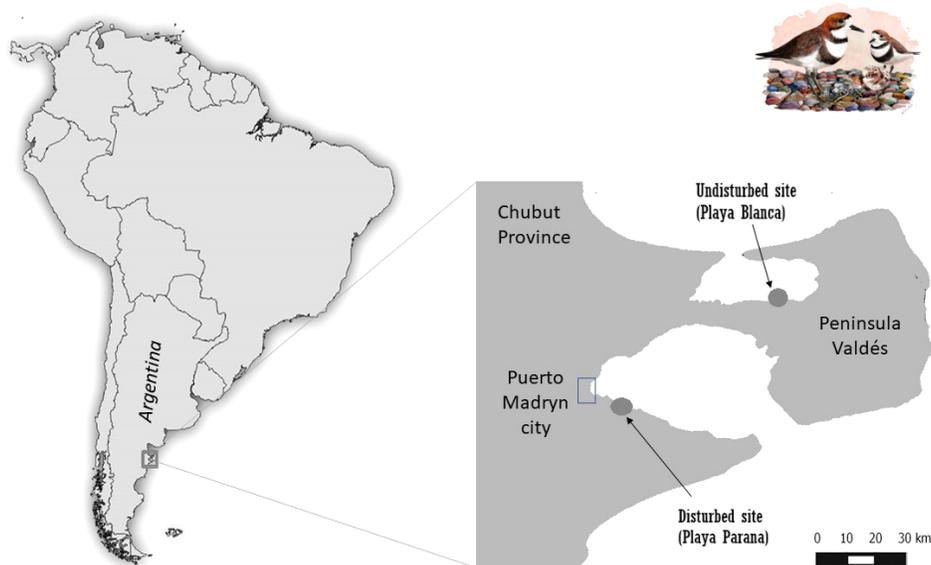
proteins; (3) cellular immunity by counting total leukocytes, and the percentages of heterophils, lymphocytes, eosinophils, basophils, and monocytes; and (4) stress responses by calculating the H/L-ratio and obtaining plasma levels of baseline corticosterone. We hypothesized that human disturbance would have negative effects on physiological performance, and predicted that individuals nesting on an urban beach would have lower physical condition and exhibit lower values of glucose, triglycerides, cholesterol, and total proteins, and elevated counts of total leukocytes, H/L-ratios, and corticosterone concentrations than birds nesting on a rural beach.

METHODS

Study area
We conducted the study on beaches of the northeast coast of Chubut, Patagonia, in southern Argentina (Fig. 1). The study area supports many waterbird species, such as gulls, oystercatchers, and plovers, which commonly breed during the austral spring and summer (September–January). Many reproductive areas for waterbirds are also frequently used for tourism and other types of human recreation (Yorio 1999). During the last decade, the nearby city of Puerto Madryn (42.7666°S, 65.0333°W) annually received an average of 230,000 tourists. In 2016 and 2017, there were about 202,000 and 185,000 visitors, respectively, to the city; where ~33% and 40% of these totals, respectively, concentrated during spring and summer (Secretaria de Turismo 2016, 2017).

We performed fieldwork during the breeding seasons of 2016 and 2017 in two beach areas where TBPLs regularly nested and reared chicks (Hevia 2020). One of the study sites, Playa Parana, is a small-gravel beach located 6 km southeast of Puerto Madryn with a high rate of human activity. Visitors (locals and tourists) include pedestrians with unleashed dogs, fishermen, runners, bikers, and divers (Hevia 2020). Driving motorized vehicles is one of the most common human activities observed across the whole beach (~2 km). The site can be reached by visitors from Puerto Madryn in 10 minutes by car, resulting in significant human disturbance. For example, the beach can hold as many as 5000 people, 2000 vehicles, and 300 unleashed dogs during peak use (GDH, *personal observation*). Therefore, plover nests located at Playa Parana (hereafter, disturbed site) were subject to "high" levels of visitor pressure. Our second study site, Playa Blanca, is a sandy-vegetated and isolated beach located about 100 km northeast of Puerto Madryn. The site is about 3 km long, and access to the site requires local landowner permission and therefore does not receive visitors. Plover nests at Playa Blanca (hereafter, control site) were subject to "low" levels of visitor pressure.

Study species and data collection
The Two-banded Plover is a Neotropical shorebird species, endemic to southern South America, and widely distributed across Argentina, Chile, Uruguay, and Brazil (Wiersma et al. 2020). The species has a biparental care strategy during incubation (St Clair et al. 2010a). Females typically lay three eggs in a small-rudimentary scrape supplied with tiny pieces of dry leaves, algae, or shells taken from the surrounding environment (Hevia 2013, 2020). The egg-laying stage takes about 5 days, and the incubation period is approximately 27 days (Hevia 2013).

At the study sites, we conducted nest surveys and searched for adults vocalizing and displaying distraction behaviors, such as broken wings and rodent runs. We captured adults using a wire mesh "fall-door" trap (0.2 m x 0.4 m) placed on nests with

Table 1. Values of the mean (± SE) and median (min-max) for hematological, nutritional, cellular immunity, and stress parameters of adult Two-banded Plovers (*Charadrius falklandicus*) at both study areas (disturbed and control site), during both breeding seasons (2016 and 2017) in Chubut, Patagonia, Argentina. Note p-values > 0.05 for all parameters, except for Triglycerides (P = 0.001).

Parameter	Site	n	Mean±SE	Median (range)	Site	n	Mean±SE	Median (range)
Haematocrit (%)	Disturbed	28	49.4±0.5	50.0 (42–55)	Control	18	48.1±0.6	48.5 (41–52)
Glucose (mg/dl)	Disturbed	28	259.4±4.5	260.0 (218–310)	Control	18	242.3±8.5	243.5 (189–343)
Triglycerides (mg/dl) †	Disturbed	27	89.1±6.8	72.0 (68–221)	Control	14	109.3±22.2	71.5 (68–350)
Cholesterol (mg/dl) †	Disturbed	28	204.8±11.7	177.5 (68–303)	Control	16	201.6±14.6	186.0 (69–300)
Total proteins (g/dl)	Disturbed	28	6.3±0.1	6.3 (4.5–8.5)	Control	18	5.9±0.1	6.0 (5–7.5)
Total leukocytes (n)	Disturbed	28	50.9±7.6	40.5 (13–167)	Control	18	45.0±5.6	37.0 (11–116)
Eosinophils (%)	Disturbed	28	35.4±3.2	33.6 (10.5–67.9)	Control	18	31.2±2.9	27.2 (15.7–56.5)
Monocytes (%)	Disturbed	28	4.3±0.6	3.6 (0–13.3)	Control	18	7.1±1.8	3.9 (0–27)
Heterophils (%)	Disturbed	28	15.5±1.8	13.9 (1.9–36)	Control	18	16.1±2.3	15.8 (1.9–42)
Lymphocytes (%)	Disturbed	28	44.7±3.2	42.7 (13.8–71)	Control	18	45.5±3.2	46.2 (20.9–75.5)
H/L-ratio	Disturbed	28	0.44±0.1	0.27 (0.04–1.8)	Control	18	0.4±0.1	0.31 (0.04–1.7)
Cort (ng/ml) ‡	Disturbed	18	7.3±0.9	6.4 (1.5–13.1)	Control	9	5.7±0.7	5.0 (2.5–9.2)

† Total sample sizes of triglycerides and cholesterol differ between sites because of the limited detectable read range reported by the device (Roche Accutrend® Plus).

‡ Radioimmunoassay (RIA) for plasma baseline corticosterone was feasible for 27 samples.

complete clutches (3 eggs). We operated the trap between sunrise and noon during low winds and no rainfall. Once birds were captured, we controlled handling time to avoid trapping effects on stress responses, particularly relevant for measures related to acute stress, such as corticosterone levels (Romero and Romero 2002). Therefore, we recorded the handling time as the period elapsed between the capture of the bird (trap fell) and obtaining a blood sample (Davis 2005, Romero and Reed 2005). For all samples (n = 46), this time was 1–5 minutes (mean = 2.4 ± 0.1), which allowed reliable measures of stress responses (H/L-ratio and plasma levels of baseline corticosterone).

In each breeding season, we took a blood sample once from each bird. In two years (2016–2017), we collected a total of 18 samples at the control site and 28 at the disturbed site. After capture, we collected blood immediately into heparinized micro-capillary tubes (0.075 ml total volume per tube). We made punctures for blood extraction from the brachial vein of the wing using a 27G disposable needle (D'Amico et al. 2017). To collect the amount of blood required for measuring all physiological parameters, each blood sample was 2–4 micro-capillary tubes (0.15–0.30 ml). The samples represented 2.5–5% of the average total blood volume (2.48 ml) to average body mass (64 g). We employed a conservative and safe method by calculating an average blood volume related to species-specific body mass and limiting the sample volume to less than 10% of total blood volume (Voss et al. 2010). We stored micro-capillary tubes on ice (4 °C) for about 3–4 hours until centrifugation in the lab, and prepared blood smears with a drop of fresh blood, air-dried, and fixed with absolute ethanol for 3 minutes (D'Amico et al. 2017).

TBPL body sizes are on average 18 cm (Wiersma et al. 2020). We weighed each bird immediately after blood extraction using a 100 g Pesola scale (± 1 g). Body mass ranged from 58 to 70 g (mean = 64 g ± 0.3; n = 46). We used a stop-end ruler (± 1 mm) to measure the wing length (flattened and straightened) from the wrist to the tip of the longest primary (Gratto-Trevor 2018). Wing lengths varied from 116 to 133 mm (mean = 127.3 mm ± 0.5; n = 46). Last, we marked individually each bird with a field-readable alphanumeric orange flag on the upper leg. The color scheme

identification followed the Pan American Shorebird Protocol (PASP) for banding shorebirds across the Western Hemisphere (Howes et al. 2016). After banding, we released birds near the capture site.

Laboratory analysis

We centrifuged capillary tubes containing bird blood samples for 12 minutes at 12,000 rpm (Cavour VT 1224), and we measured the hematocrit with a ruler (JP Selecta, Spain). We used an Accutrend Plus point-of-care device for the quantitative determination of glucose, triglycerides, and cholesterol concentrations (Roche Diagnostic, Germany). The reflectance photometric measurement was performed using meter-specific test strips for each blood parameter (Reportable ranges: Glucose, 20–600 mg/dL; Triglycerides, 70–600 mg/dL; and Cholesterol, 150–300 mg/dL). Specifically, the method for chemical detection of triglycerides used by the Accutrend Plus® meter and other commercially available instruments is identical (Irvine et al. 2017, Araújo et al. 2022). In the first place, erythrocytes are separated from the plasma before measuring triglycerides within the test strip. Then, triglycerides are cleaved by an esterase into glycerol and free fatty acids. Two additional enzymatic steps convert glycerol into hydroacetone phosphate and hydrogen peroxide, which in the presence of peroxidase, the hydrogen peroxide oxidizes an indicator to a dye. Thus, Accutrend point-of-care devices do not differentiate between free glycerol and triglycerides (Araújo et al. 2022). Triglyceride concentration values are measured by providing means of reflectance photometry. Furthermore, triglyceride samples that fell below the detectable range and were reported by the meter as "Low," were excluded from the analysis (n = 5, see Table 1). We measured concentrations of total proteins with a veterinary digital refractometer (Misco). We stained blood smears with Tinción 15 (Biopur SRL) and examined them in a Zeiss light microscope (D'Amico et al. 2017). We obtained total leukocyte counts by scanning monolayer fields with similar erythrocyte densities for all samples (Campbell 1995) and we estimated by counting the number in a monolayer field and multiplying by the number of fields that were scanned until reaching 100 leukocytes (Lobato et al. 2005). We obtained the

percentage of each leukocyte type from a sample of 100 leukocytes under 1000x magnification (oil immersion), and we classified cells as basophils, heterophils, eosinophils, lymphocytes, or monocytes (Campbell 1995). We calculated the H/L-ratio as an index from heterophil and lymphocyte counts (Davis et al. 2008). We measured corticosterone plasma levels by radioimmunoassay (RIA) following procedures from Wingfield et al. (1992). We followed a protocol for DNA sex determination described by van der Velde et al. (2017).

Statistical analysis

We performed data analysis and modeling using program R v.4.1.2 (R Core Team 2021). For each physiological trait, we tested for homoscedasticity and normality of residuals using Levene's test and the Shapiro-Wilk test, respectively. We transformed five variables given they did not meet normality. Total leukocyte counts, H/L-ratio, and corticosterone were log-transformed, whereas heterophil and monocyte proportions were arcsine-square-root-transformed. Triglycerides and cholesterol failed to pass normality even after log transformation, but we included them in the model because running an identical one without them did not meaningfully influence a multivariate analysis. As we observed low levels of basophils in the samples (on average, less than 0.1% of total leukocyte counts), we excluded them. We calculated body condition as size-corrected body mass for all individuals as the residual mass on wing-chord lengths (Owen and Moore 2008).

We calculated Pearson correlations for all pairwise combinations of physiological traits (Appendix 2). For example, body condition was correlated positively with plasma total proteins (r = 0.33, P = 0.02) and negatively with monocytes (r = -0.34, P = 0.02), while the H/L-ratio showed a high correlation with heterophils (r = 0.90, P < 0.0001) and lymphocytes (r = -0.58, P < 0.0001) as expected. Because heterophils was the only trait with the highest correlation among all pairwise combinations, we decided to exclude them from our model. We used multivariate multiple regression to simultaneously model the univariate associations among a suite of predictor variables and a set of physiological traits while considering the potential covariances among physiological variables. For predictor variables, we included bivariate covariates for (1) treatment (disturbed/control), (2) year (2016/2017), and (3) sex (male/female), and continuous covariates for (4) handling time (minutes), (5) day of the year (DOY), and (6) an interaction between DOY and treatment. We defined treatment given the level of human disturbance pressure on incubating TBPLs, whether their nests were located on an urban (disturbed) or rural (control) beach. In our model, we included year, sex, and handling time as nuisance variables in an attempt to account for or "control" for variation in physiological traits separate from the impacts of human disturbance. We included DOY to account for seasonal variation in physiological traits during the breeding season. At the disturbed site, we conducted bird captures between 14 September and 25 November in 2016 (n = 16), and between 9 October and 29 December in 2017 (n = 12); whereas at the control site, captures were made between 12 October and 23 November in 2016 (n = 11), and between 11 October and 29 December in 2017 (n = 7). Last, we included the interaction between DOY and treatment to explore patterns of physiological traits from individuals at each of the sites during the breeding seasons. All continuous variables were z-standardized.

Inspections of univariate associations (ANOVA) between each predictor variable and each physiological trait were explanatory if the associated 95% confidence intervals did not overlap zero. Last, we determined the overall support for each predictor variable on the global physiological set with multivariate analysis of variance (MANOVA) using Pillai's trace multivariate test (Pillai 1955) and report the partial eta test statistic (n^2) and its associated p-value as evidence for a predictor variable multivariate explanatory power. Here, n^2 represents the proportion of the variance that was explained by a specific predictor variable ($SS_{predictor} / [SS_{predictor} + SS_{error}]$).

RESULTS

During incubation, we captured, weighed, bled, and banded 46 adult TBPLs in the breeding seasons of 2016 and 2017. We obtained blood samples from 28 individuals at the disturbed site (18 females and 10 males) and 18 individuals at the control site (12 females and 6 males). Each physiological trait measured from samples collected at each study site is listed in Table 1, and values of the mean (± SE), median, and ranges are included from both years.

Effects of predictor variables on body condition, nutritional parameters, and immunity

Except for handling time, all predictor variables were significative for one or more physiological traits given the standardized mean values and 95% confidence intervals did not overlap zero (P < 0.05; Fig. 2). We observed significant effects of DOY for body condition (β = -1.88, 95% CI: -3.18 – -0.59, P = 0.005), and the interaction DOY × treatment (β = 2.07, 95% CI: 0.70 – 3.50, P = 0.004). Triglyceride concentrations differed with DOY (β = -1.05, 95% CI: -1.66 – -0.57, P < 0.001), treatment (β = -0.77, 95% CI: -1.31 – -0.19, P = 0.01), and the interaction DOY × treatment (β = 0.80, 95% CI: 0.25 – 1.43, P = 0.01). Both traits, triglycerides, but particularly body condition, showed a significantly sharp decrease at the control site during the breeding season (Fig. 3). Body condition also significantly differed between the sexes (β = -1.29, 95% CI: -2.52 – -0.06, P = 0.04), with males exhibiting lower values than females. Monocytes were the only cellular immunity parameter that significantly differed as an effect of DOY (β = 1.22, 95% CI: 0.47 – 1.64, P < 0.001), and DOY × treatment (β = -0.96, 95% CI: -1.45 – -0.20, P = 0.003), increasing throughout the nesting season at both beaches; however, we noted a sharp rise at the control site (Fig. 4). Several physiological parameters differed between the years of capture. Triglycerides (β = 0.86, 95% CI: 0.21 – 1.44, P = 0.009), cholesterol (β = 0.75, 95% CI: 0.03 – 1.51, P = 0.04), and total leukocyte counts (β = 1.18, 95% CI: 0.56 – 1.85, P < 0.001) were all significantly higher in 2017 than in 2016.

Multivariate assessment

Overall support for each predictor variable on the global physiological set with multivariate analysis of variance was only explanatory for DOY (n^2 = 0.67, P < 0.001), and year (n^2 = 0.62, P = 0.001), and both explained the greatest amount of variation in multivariate space, whereas DOY × treatment were marginally explanatory (n^2 = 0.45, P = 0.06).

Fig. 2. Univariate associations of each predictor variable (Treatment, DOY, DOY × Treatment, Year, and Sex) with standardized mean values of physiological traits measured from Two-banded Plovers (*Charadrius falklandicus*) at both disturbed and control sites in Chubut province, Patagonia, Argentina. Note that standardized mean values are shown only for significant regressions of body condition, triglycerides, cholesterol, total leukocyte counts, and monocytes. Blue error bars are 95% confidence intervals that did not overlap zero (P < 0.05).
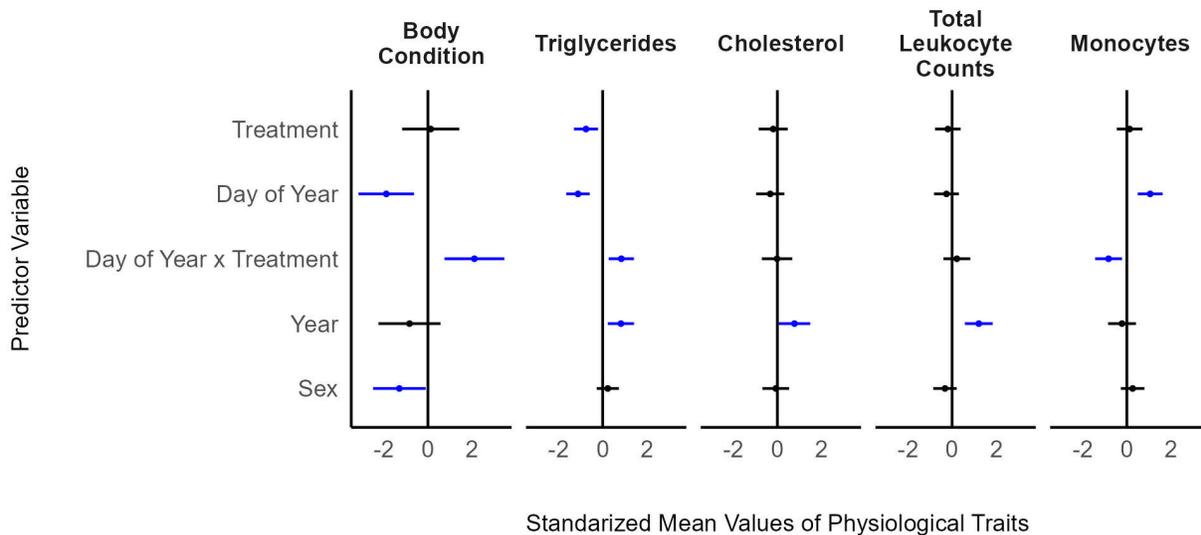

DISCUSSION

Adult TBPL captured at the disturbed site did not significantly exhibit lower physical condition or elevated stress responses during the breeding season. Instead, significant effects of the interaction DOY × treatment were observed for body condition and triglycerides among adults at the control site, with substantial declines during the breeding season. The breeding season period of TBPLs was nearly four months, and searching for nests and capturing birds were not always logistically feasible for the entire season. A potential explanation for the observed patterns of body condition and triglycerides between sites may have been differences in capture dates. Birds at the disturbed site were captured earlier in the breeding season from mid-September to mid-November. In contrast, a majority of sampled females (n = 12) and males (n = 6) at the control site were captured from early November to late December (mid-to-late breeding seasons of 2016 and 2017), which was likely after their first nesting attempt. If most birds were attending their second or third nesting attempt when captured at the control site, then the observed patterns of body condition and significant declines in triglyceride concentrations might have been due to birds attending late breeding attempts. In general, the probability of producing multiple nesting attempts or replacement clutches can be affected by many factors, including the body condition of laying females, nest success, and the timing of the breeding season (Amat et al. 1999). The laying of new clutches is important in accounting for individual lifetime productivity (Fraga and Amat 1996) and might be also particularly relevant in TBPLs, where the pressure from human disturbance and nest predation levels constrain the Patagonian population (Hevia 2013, 2020).

Body condition also significantly differed between the sexes, as TBPL males exhibited lower body condition than females.

Energy-costly reproduction activities could impact, for instance, daily energy expenditures (DDE; Cresswell et al. 2004) and basal metabolic rates (BMR; Gutiérrez et al. 2011), therefore, body mass loss can be a strategy to reduce maintenance costs. Based on observations in the field, TBPL males dedicate more time establishing, defending, and scraping within their territory boundaries, in addition to performing various courtship behaviors. The activities exhibited by males could imply costs associated with reducing time, for example, to feed efficiently. A daily trade-off of food intake/energy expenditure has been documented in other shorebirds, and frequently mirrored in lower body condition (Jenni-Eiermann and Jenni 1994). Additionally, during the incubation period, foraging efficiency is also constrained by the need for synchronized incubation duties, an aspect of relevance given TBPLs are biparental. As a result, parental investment (in nest attendance) can also have an impact on the body condition of biparentally incubating shorebirds (Bulla et al. 2014).

Triglycerides, cholesterol, and total leukocyte counts also differed significantly by year, and values were higher for TBPLs sampled in 2017. In general, shorebirds must cope with year-to-year variation in seasonal changes during each breeding season, while accessing sufficient food, and managing predator and human disturbance pressure, in addition to facing local inclement weather conditions (Weiser et al. 2018). Plasma lipids, such as triglycerides and cholesterol may reflect the current metabolic state of a reproductive bird because of daily food intake, but other seasonal processes, such as the molting of feathers can also affect lipid metabolism (Jenni and Jenni-Eiermann 1996). Variations related to age-nesting experience (Reid 1988), and reproductive effort (Hirshfield and Tinkle 1975), are also expected to contribute to seasonal changes in bird nutritional states and immunity. In this sense, the age-specific nesting experience of young versus old

Fig. 3. Estimated regression lines of the interaction DOY × Treatment for standardized measurements of body condition, haematocrit, glucose, triglycerides, cholesterol, and total proteins obtained from adult Two-banded Plovers (*Charadrius falklandicus*) blood samples. Regression lines are shown for control (blue lines) and disturbed (red lines) sites. Significant parameters for the interaction DOY × treatment are shown with an asterisk (P < 0.05).
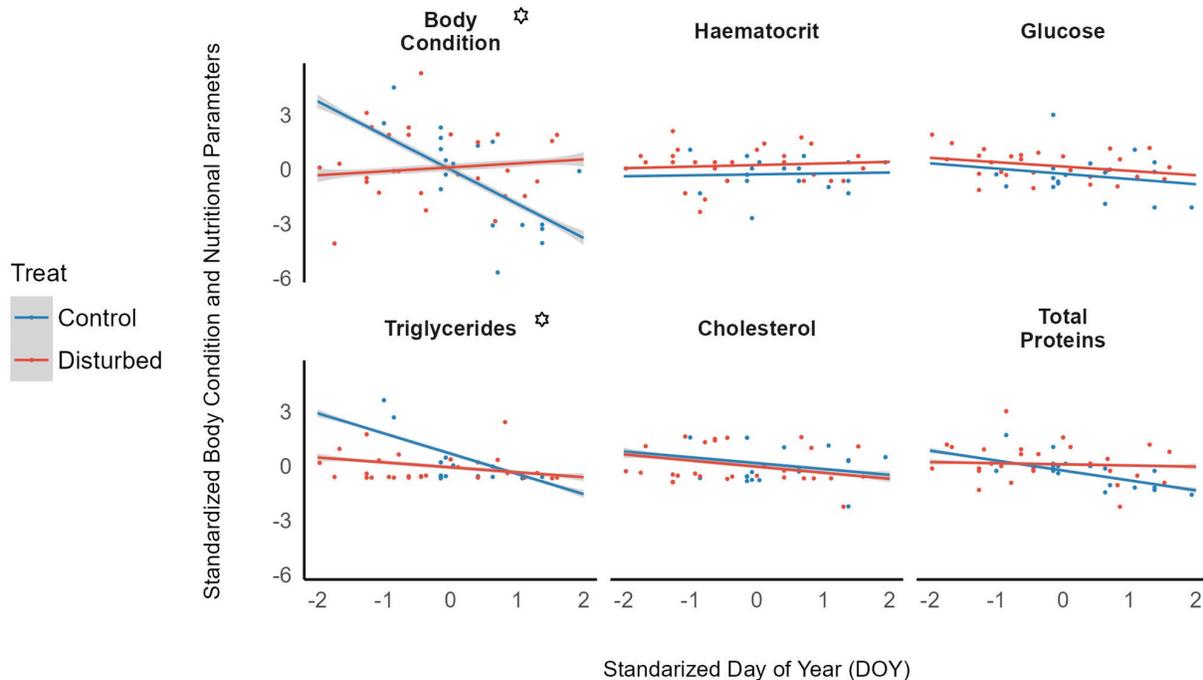

adult TBPLs, the ability of females to replace clutches or produce multiple nesting attempts during the season, in addition to access to a greater quantity or better quality of prey items, constitute aspects that could explain a better nutritional state of TBPLs sampled in 2017. Certainly, future physiological assessments concerning foraging ecology should be considered to increase understanding of seasonal changes across breeding seasons.

No significant differences in stress responses, either in the H/L-ratios or plasma corticosterone baseline levels, were observed in TBPLs. However, chronic stress has been observed, for example, in birds inhabiting urban, rural, and mixed habitats (Chávez-Zichinelli et al. 2013, Foltz et al. 2015). Specifically, we did not find a site effect on TBPL corticosterone concentrations, consistent with previous findings in marine iguanas (*Amblyrhynchus cristatus*) not being chronically stressed in a high touristic site in Galapagos. Instead, iguanas had elevated corticosterone levels when exposed to El Niño or oil spill events (Romero and Wikelski 2002). Based on observations at our study sites, TBPLs generally flushed from nests in the disturbed site at lower distances than in the control site, suggesting that some individuals may have gained the ability to tolerate human disturbance as a coping mechanism in a coastal recreational beach. The Patagonian population of TBPLs may also behave similarly to their congeners at Malvinas (Falkland) Islands, and habituation was suggested as a mechanism for reducing the costs of non-lethal stimuli, such as human activity (St Clair et al. 2010b). Furthermore, repeated exposure to non-lethal stimuli can allow individuals to distinguish risk from genuine predators more

effectively, consistent with observations of lower flushing distances at sites with high levels of human activity (St Clair et al. 2010b). In birds, recent studies have highlighted that a flexible and rapid emergence of behavioral tolerance may force them to adjust their behavior in small-scale and recently urbanized areas (Tryjanowski et al. 2020). Tolerance should allow birds to "adjust" their behavior by performing flexible responses while facing the same stressors for long periods. In agreement with our observations of TBPLs nesting at the disturbed site, in other shorebird species, such as American Oystercatchers (*Haematopus palliatus*), birds that also breed during spring and summertime on recreational beaches are forced to spend a larger proportion of their daily time off-nest while displaying alert behaviors (Felton et al. 2018). The ability to adjust behaviors to cope with non-lethal stimuli remains unclear for Patagonian TBPLs, but some experimental studies using technological devices have explored physiological and behavioral responses in incubating shorebirds (Bulla et al. 2014, Felton et al. 2018, Charuvi et al. 2020). Nevertheless, physiological mechanisms mediated by glucocorticoids can regulate the ability of animals to cope with novel stressful situations, including human disturbance, extreme climatic events, and new diseases (Wingfield 2003), and optimize the fitness of individuals in challenging environments (Angelier and Wingfield 2013). Ultimately, the ability of birds to modulate their tolerance to human disturbance pressure may play a key role in the facilitation of wildlife-human coexistence (Tryjanowski et al. 2020).

Previous comparative studies among biparental shorebird species have shown that parental care may take a diversity of forms and

Fig. 4. Estimated regression lines of the interaction DOY × Treatment for standardized measurements of total leukocyte counts, eosinophils, monocytes, lymphocytes, H/L ratio, and corticosterone obtained from adult Two-banded Plovers (*Charadrius falklandicus*) blood samples. Regression lines are shown for control (blue lines) and disturbed (red lines) sites. Significant parameters for the interaction DOY × treatment are shown with an asterisk (P < 0.05).
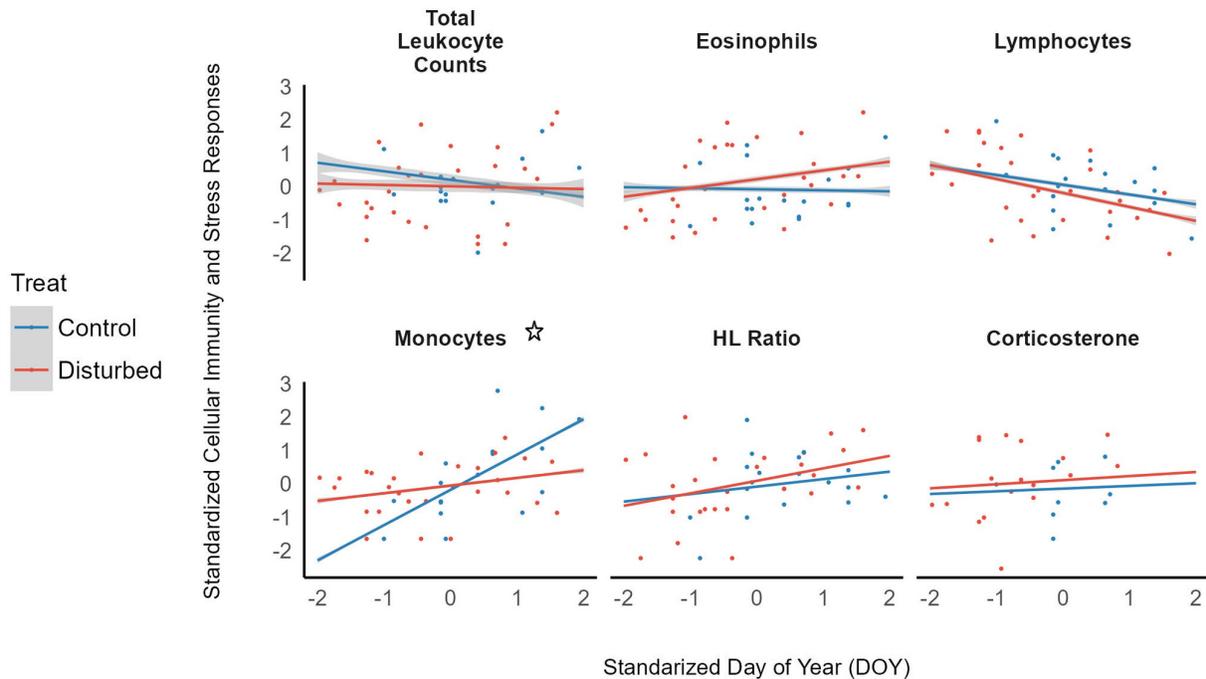
is also flexible during incubation (Bulla et al. 2017). TBPLs have a biparental care strategy, with a strong diel sex role and a female-biased incubation pattern in the resident population of Malvinas (Falkland) Islands (St Clair et al. 2010a). During their study, St Clair et al. (2010a) observed an unequal contribution by females and males during nest attendance consistent with unequal daytime and night-time periods, with females incubating during daylight and males at night (St Clair et al. 2010a). Likewise, Patagonian TBPL females also incubate mostly during daylight and males during dark hours, based on preliminary data from temperature loggers deployed in nests (Hevia, Bulla, Sandercock, D'Amico, *unpublished data*). St Clair et al. (2010a) observed that incubation shifts were triggered by changes in light levels at sunset and sunrise, therefore, a latitudinal breeding range should constrain parental investment, such as the constancy of incubation by females and males (St Clair et al. 2010a). In this sense, data collection using loggers in Patagonian TBPL nests will determine incubation patterns to help answer questions related to nest attendance. For example, is there a more equal parental investment between the sexes at our study sites because daylight-night lengths are more equal than in Malvinas (Falkland) Islands? Could site differences be related to sex-biased foraging opportunities and consequently impact body condition and nutritional parameters? It has also been noted that parental care can be flexible (Bulla et al. 2017). Single parents of biparental shorebirds can partially or fully compensate for incubation investment while the partner is absent (Bulla et al. 2019). Can TBPLs potentially incubate uniparentally as the partner who deserts may have extra mating opportunities? Questions about

flexibility in incubation strategies have no answers yet, but are guiding the next steps for further research on Patagonian TBPLs. Although a lot of the breeding biology work has been done on Nearctic shorebird species, there is still much to be learned about endemic species of Neotropical shorebirds. Long-term research efforts would help to enhance our knowledge of the population ecology of Two-banded Plovers and other poorly known Neotropical plovers.

CONCLUSION

Regardless of the lack of treatment effects on physiological traits and a low sample size, our study results could still benefit management decisions by encouraging land managers to protect TBPL breeding habitats that are subject to human disturbance. Simple management actions toward the protection of beach-nesting areas could have a direct conservation impact on breeding shorebirds. Symbolic fencing, for example, can restrict access to pedestrians and vehicles in specific beach areas, as a previous study case showed for TBPLs during the breeding season (Hevia and Bala 2018). From a conservation perspective, physiological assessments are a useful tool that benefits the decision making of wildlife managers (Coristine et al. 2014, Madliger et al. 2016). Conservation physiology is an integrative discipline that constantly incorporates feedback from physiologists and decision makers (Wikelski and Cooke 2006), and as such, many conservation efforts rely on physiological approaches to assessing the general-health state of wild birds.

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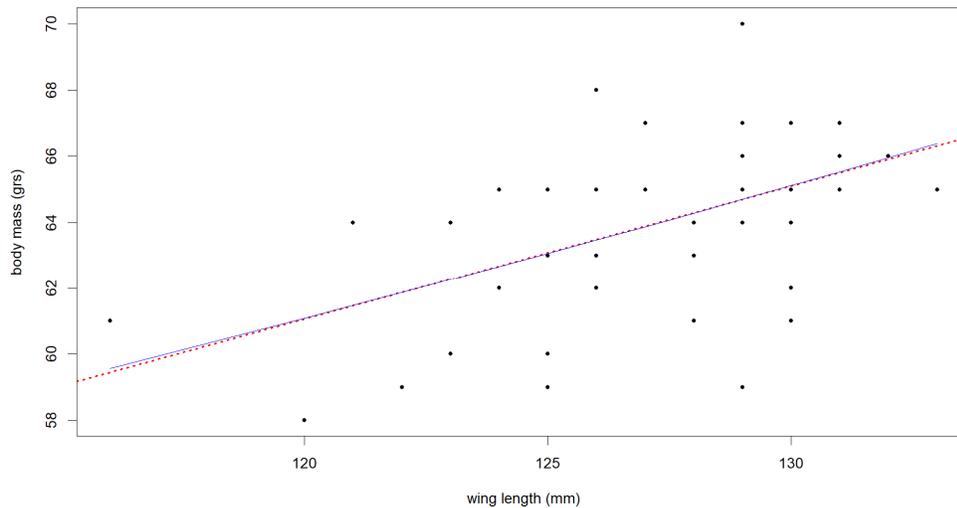
Appendix 1.

Besides the linear model, a higher-order term for wing length of adult TBPLs was evaluated. Both linear and quadratic estimation model, with their respective estimates, the R2 adjusted, and p-values are shown below. Overall, both models are significant (linear, P < 0.001; quadratic, P = 0.001) but the quadratic term for the wing length does not improve the relationship. In fact, the slope coefficient for the quadratic term is not significantly different from zero and the adjusted R-square for the quadratic model is lower than the linear model. The relationships are shown in the figure below (red dashed line = linear, blue solid line = quadratic). Given a quadratic term for the wing length does not improve the relationship with body mass, we have opted to retain a linear approach as the better model. Then, the trait body condition was calculated as size-corrected body mass for all individuals as the residual mass on linear wing-chord lengths.

Model	Estimate ± Std. Error	t-value	Pr(>|t|)
Intercept	37.19 ± 331.90	0.112	0.911
phys$Wing	0.01097 ± 5.29	0.002	0.998
I(phys$Wing^2)	0.001568 ± 0.02	0.074	0.941

Linear Adjusted R-square: 0.2473
Quadratic Adjusted R-squared: 0.2299

Appendix 2.

Pearson correlations for all pairwise combinations of physiological traits from TBPLs blood samples. Note, for example, body condition was correlated positively with plasma total proteins (r = 0.33, P = 0.02) and negatively with monocytes (r = -0.34, P = 0.02), while the H/L-ratio showed a high correlation with heterophils (r = 0.90, P < 0.0001) and lymphocytes (r = -0.58, P < 0.0001) as expected.

This page is a scatterplot matrix (pairs plot) showing pairwise correlations among the variables body_cond, HTO, GLU, TGL, CHOL, TP, WBC, EOSI, HETE, LINF, MONO, HL_RATIO, and CORT. The upper triangle displays correlation coefficients, the diagonal shows density distributions, and the lower triangle shows scatterplots.

Row \ Col	body_cond	HTO	GLU	TGL	CHOL	TP	WBC	EOSI	HETE	LINF	MONO	HL_RATIO
CORT	-0.110	0.124	-0.072	0.010	0.179	0.166	-0.096	0.123	-0.010	-0.085	-0.077	0.070
HL_RATIO	-0.116	-0.091	0.201	-0.175	-0.017	-0.164	0.348*	-0.010	0.896***	-0.583***	0.150	
MONO	-0.339*	0.170	-0.189	-0.083	-0.119	-0.321*	-0.071	-0.081	0.054	-0.301*		
LINF	0.087	0.202	-0.022	0.163	-0.014	-0.008	-0.514***	-0.731***	-0.243			
HETE	-0.128	-0.084	0.310*	-0.133	-0.090	-0.136	0.126	-0.387**				
EOSI	0.143	-0.193	-0.118	-0.081	0.129	0.200	0.452**					
WBC	0.099	-0.187	-0.026	0.013	0.165	-0.012						
TP	0.335*	-0.067	0.243	0.025	-0.045							
CHOL	0.015	0.074	-0.090	0.219								
TGL	0.248.	0.074	-0.200									
GLU	-0.010	0.030										
HTO	-0.004											