Stress and body condition predict haemosporidian parasitaemia in birds from Cerrado, southeastern Brazil

Regiane da Silva Rodrigues[1,*], Victor Aguiar de Souza Penha[2], Rafael Yukio Miwa[3], Joaquim Olinto Branco[1] & Oswaldo Marçal Junior[3]

Stress may be defined as a change in the homeostasis of an individual. However, if long-lasting, stress may produce significant detrimental effects to individuals, such as increasing the susceptibility to haemosporidian parasites. Therefore, we aimed to study whether and how stress and body condition predict the probability of hosts having haemosporidian parasites and increased parasite load. We captured birds in an area of Cerrado *stricto sensu* in southeastern Brazil and used microscopy techniques to assess haematological parameters, as well as infections by protozoans, and confirmed all infections using nested PCR. We used the heterophil/lymphocyte ratio and global leucocytes as a proxy of individual stress and the scaled mass index as a body condition metric. We captured 68 individuals from five bird species: Ruddy Grounddove *Columbina talpacoti*, Great Kiskadee *Pitangus sulphuratus*, Lesser Elaenia *Elaenia chiriquensis*, Flavescent Warbler *Myiothlypis flaveola* and Browncrested Flycatcher *Myiarchus tyrannulus*. We did not find support for parasite prevalence being predicted by body condition or haematological variables. However, we found that birds with a higher heterophil/lymphocyte ratio, lower body condition and lower global leukocyte count, were more likely to have a higher parasite load (haemosporidian parasitaemia). Our results suggest that birds with chronic stress and poor health may be more susceptible to greater infection intensity and/or recrudescence episodes by malarial parasites due to previous immunosuppression.

Key words: *Plasmodium*, *Haemoproteus*, malarial parasites, scaled mass index, global leukocytes, heterophil, lymphocytes

[1]Graduate Program in Ecology and Natural Resources, Federal University of São Carlos, São Carlos, São Paulo, Brazil;
[2]Graduate Program in Ecology and Conservation, Federal University of Paraná, Curitiba, Paraná, Brazil;
[3]Graduate Program in Ecology and Conservation of Natural Resources, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil;
*corresponding author (regianedsr@gmail.com)

Free-living birds may face several stressors, such as food deprivation and adverse climate conditions (Vleck *et al.* 2000). During stressful situations, there is an adrenocortical response, increasing glucocorticoids in the bloodstream, such as corticosterone (Mougeot *et al.* 2010). Physiological changes caused by increased corticosterone levels may increase the cardiovascular tone and regulate the immune system (Sapolsky *et al.* 2000). However, once corticosterone persists in the body, caused by prolonged exposure to the stressor,

there may be negative consequences to the individual, such as decreased reproductive success and increased susceptibility to predation (Butler *et al.* 2010, Gormally *et al.* 2019). Hence, stress may be defined as a change to homeostasis (Romero 2012). Several metrics may be used to quantify stress in birds, such as the level of circulating corticosterone (Lin *et al.* 2004), corticosterone deposited in feathers (Bortolotti *et al.* 2008) and stress haematological indices. The heterophil/lymphocyte (H/L) ratio has been used as a robust and a long-

lasting index of stress effects in birds (Gross & Siegel 1983), such as parasite prevalence. However, the evidence for such an effect is mixed, with some studies showing no association between malarial parasites and H/L index (Granthon *et al.* 2017), whereas others studies found a positive relationship, with elevated H/L ratio in infected birds (Wojczulanis-Jakubas *et al.* 2012).

Avian malaria parasites are vector-borne protists (Haemosporida, genera *Plasmodium* and *Haemoproteus*) and they may be transmitted to vertebrates via mosquito vectors (Valkiūnas 2005). They are considered an ideal model for host-parasite studies, for example because they are nearly globally distributed (Ricklefs *et al.* 2016). Ganser *et al.* (2020), studied the relationship between haemosporidian parasites and life-history traits in bird species across savannas in Africa, and they found that bird species that invest more in parental care, which can be considered as a stress source to birds (DuRant *et al.* 2013), were more susceptible to haemosporidian parasite infection, when compared to avian species lacking parental care. Indeed, during the breeding season, birds have been shown to be more susceptible to acquiring infection by haemosporidian parasites (Rodrigues *et al.* 2020), indicating that birds dealing with a stressful environment are more likely to acquire haemosporidian infections. However, most studies showed an indirect relationship between stressful sources and probability of being infected by haemosporidian parasites and did not consider more direct measurements of stress, such as the H/L ratio. Here, we analysed the relationship between H/L ratio and the probability of haemosporidian infection and infection intensity in five bird species.

Haemosporidian parasites have also been linked to decreased plumage coloration in passerine birds (Penha *et al.* 2020) and decreased body condition (Marzal *et al.* 2013), which is associated with the health status of individuals (Peig & Green 2009). Therefore, less conspicuous coloration may indicate an individual's decreased ability to acquire and compete for resources and, thus, their greater susceptibility to haemosporidian parasite infections (Penha *et al.* 2020). Likewise, body condition, which is a measure of health status, indicates current condition and may be used to assess the fitness of individuals (del Cerro *et al.* 2010). Since individuals in poor condition may be less active and, thus, less likely to compete for and acquire resources, they may be more susceptible to infection by parasites. Ribeiro *et al.* (2020) studied the relationship between haemosporidian parasite infection probability and body condition, and found that individuals with lower body condition were indeed more susceptible to acquiring haemosporidian parasites.

In this study, we investigate whether individuals that suffer from stress and have a poorer body condition are more likely to have a greater prevalence and intensity of haemosporidian parasites. To this end, we measured haemosporidian infection prevalence and parasitaemia and related these to H/L ratio, global leukocytes and body condition. We assumed that individuals that suffer from stress would have a higher H/L index, increased global leukocytes and lower body condition, and that these measures would be associated with high haemosporidian infection prevalence and parasitaemia.

METHODS

Study area
We conducted the study at Clube Caça e Pesca Itororó Reserve (18°59'S, 48°18'W), an area of 640 ha located 8 km from the urban centre of Uberlândia, Minas Gerais, Brazil. The vegetation comprises of several phytophysiognomies, however, there is a predominance of Cerrado *sensu strictu*, comprising of dense shrub vegetation with trees from 2 to 8 m high and understory dominated by grasses and perennial herbs (Del-Claro & Marquis 2015). It is characterized by a seasonal tropical climate, with average annual rainfall and temperature of 22°C and 1550 mm precipitation (Bächtold *et al.* 2012).

Capture and collecting samples
Individuals of different species were captured with mist nets (10 × 2.5 m, 38 mm mesh) at different points in the reserve from April to October 2016, in the middle of the dry season. This period corresponds to the months in which most bird species are not breeding, in order to exclude the stress caused by reproductive effort. The most abundant bird species in the study area and of which at least five individuals were captured, were used here in this study. Every individual was banded following the protocol described by the National Center for Research and Conservation of Wild Birds (CEMAVE, Brazil) and biological samples were collected with permission from the Ministry of Environment of Brazil (CEMAVE/IBAMA: 4064-1, SISBIO: 51261-1, 50372-2). The bird's body masses were obtained using a dynamometer scale (Pesola) and morphometric measurements of the right tarsus with an MTX digital calliper. A blood sample was collected

through branchial venipuncture in the left wing, using a disposable sterile insulin needle (30 × 3 mm), and a small amount of blood was collected using a heparinized capillary tube (Campbell 2015). We used a few drops of the blood to make two or three smears, dried in the field at room temperature, fixed in absolute methanol for one minute and stained with Giemsa dye for one hour (Valkiūnas 2005). The remaining blood was placed in microtubes containing 90% alcohol for subsequent DNA extraction and haemosporidian parasite investigation by nested PCR (see below). The tubes and slides were identified with the ring number and date of collection.

Detection of haemosporidian parasites and haematological indices

The stained slides were examined under an optical microscope at 100× magnification in immersion oil, analysing 100 fields per slide (area of approximately 13.2 µm), in which the global leukocyte (GL from hereon) count was performed and the types of white blood cells were determined, i.e. lymphocytes, heterophile, eosinophils, basophils and monocytes. The GL was the overall quantity of white blood cells. To obtain the heterophile/lymphocyte ratio (H/L from hereon), our proxy for stress, we counted the total number of leukocytes in 50 random slide fields, and the H/L ratio was determined by dividing the number of heterophils by the number of lymphocytes (Gross & Siegel 1983). Furthermore, the number of immature red cells and red cells infected by haemosporidian parasites were quantified and classified. For this, we checked c. 4000 erythrocytes per individual. Subsequently, parasitaemia was calculated by estimating the proportion of infected red blood cells, shown as number per 10,000 cells (Valkiūnas 2005).

For blood stored in microtubes, we used molecular detection of the haemosporidian parasites causing avian malaria. We first extracted the DNA using a standard phenol-chloroform protocol followed by isopropanol precipitation. The extracted DNA was examined for the presence of *Plasmodium* and *Haemoproteus* infections by nested PCR, using 343F (59GCTCACGCAT-CGCTTCT39) and 496R (59GACCGGTCATTTTCTTTG-39) primers (Fallon *et al.* 2003). We considered an individual to be infected if it was positive for both PCR and microscopy. All results obtained from the PCR analysis were confirmed by the microscopy.

Scaled mass index

To assess body condition, we used the scaled mass index (SMI from hereon) as proposed by Peig & Green

(2009). The SMI was calculated using the following equation:

$$\hat{M} = M_i \left[\frac{L_0}{L_i} \right] b_{SMA} \, ,$$

where M_i is the body mass and L_i is the linear body measurement of individual i, L_0 is an arbitrary value of the L (in this case, we used the arithmetic mean of the right tarsus from the captured birds species), and finally, b_{SMA} is the scaling exponent estimated by the standardized major axis (SMA) regression of body mass (M) on the linear measurement (L). The b_{SMA} is calculated indirectly by dividing the slope from an OLS regression by the Pearson's correlation coefficient (Labarbera 1989). The SMI was determined using R software (R Core Team 2016).

Statistical analysis

We assessed all variables for normality assumptions by visually inspecting the distribution in a histogram. We also calculated the Spearman correlation coefficient between all the explanatory variables, namely SMI, H/L and GL. We used a threshold of 0.8 of r_s to exclude variables with a high correlation. We found no strong correlations among our variables, so we included all of them in the statistical analysis. We corrected all the explanatory variables on a logarithmic scale because they had a log-normal distribution. Two separate models were developed, each with a different response variable: (1) prevalence of haemosporidian infections (present or absent) and (2) parasitaemia (proportion of infected cells). The explanatory variables were the same in both models, namely SMI, H/L and GL. We first ran a generalized linear mixed model (GLMM) with the 'glmer' function from the 'lme4' package (Bates *et al.* 2015) with a binomial distribution. With this model we intended to test whether the probability of individuals being infected would be predicted by stress level as well as body condition. In the second model, we use the 'glmmTMB' function (Brooks *et al.* 2017) with zero-inflated and beta distribution, to control for the high number of uninfected individuals. With this model, we wanted to test whether parasitaemia is predicted by stress and body condition. Species identity was included as a random effect in both models to account for differences between species. We sequentially removed non-significant variables from the model using the 'drop1' function of the 'lme4' package (Bates *et al.* 2015), until a model was reached that contained only significant variables, based on their *P*-value (< 0.05). We used the 'effect' function from the 'effects' package (Fox & Hong 2009) with the best statistical

model to generate the predicted values for the figures. All statistical analyses were performed using R software (R Core Team 2016).

RESULTS

We captured 68 individuals belonging to five different bird species and three different families, namely Columbidae, Parulidae and Tyrannidae. We found that 38.2% of individuals were infected by haemosporidian parasites from either *Plasmodium* or *Haemoproteus* genera (Table 1).

Ruddy Ground-doves *Columbina talpacoti* had the highest SMI average (40.6 ± 6.7) and Great Kiskadees *Pitangus sulphuratus* the lowest (5.62 ± 1.9). The species with the highest haemosporidian parasite prevalence were Ruddy Ground-dove (55%) and Lesser

Table 1. Species name, family, parasitaemia (mean number of infected red-blood cells for 10,000 erythrocytes ± SD), H/L (mean ± SD), SMI (mean ± SD), GL (mean ± SD) and prevalence.

Family/Species	Parasitaemia	H/L	SMI	GL	Prevalence
Columbidae					
Ruddy Ground-dove	433 ± 570	0.68 ± 0.31	40.66 ± 6.70	23.63 ± 6.49	54.54%
Parulidae					
Flavescent Warbler	31 ± 88	0.24 ± 0.19	6.24 ± 2.27	25.87 ± 20.95	12.50%
Tyrannidae					
Lesser Elaenia	200 ± 325	0.57 ± 0.91	8.21 ± 2.03	16.14 ± 8.76	35.71%
Great Kiskadee	50 ± 122	0.80 ± 0.54	5.62 ± 1.90	32.83 ± 16.98	16.60%
Brown-crested Flycatcher	42 ± 113	0.05 ± 0.03	9.06 ± 5.24	47.28 ± 38.40	14.28%

Table 2. Parameter estimates, standard errors and *P*-values for the generalized linear mixed models, in which, in the first, the prevalence of parasites was used as the response variable and in the second, the parasitaemia. The second model was corrected for zero-inflation, showing the variation and the *P*-value for this parameter. In all models, the following explanatory variables were used: GL, H/L and SMI. The species identity was included as a random effect, showing the variation and standard deviation as well. All variables with statistical significance are marked with an asterisk.

Dependent variable: Prevalence of haemosporidian parasites (binomial family)

Parameter	Estimate	SE	*P*
Intercept	–3.07	1.97	0.11
SMI	0.49	0.31	0.11
H/L	0.33	0.26	0.21
GL	0.48	0.54	0.37

Random variable:	**Variance**		
Species	0.0004		

Dependent variable: Parasitaemia (beta family)

Parameter	Estimate	SE	*P*
Intercept	7.81	0.43	<0.01[*]
SMI	–0.68	0.03	<0.01[*]
GL	–0.05	0.02	0.04[*]
H/L	0.23	0.01	<0.01[*]

Random variable:	**Variance**		
Species	0.86		

Zero-inflated	**Estimate ± SE**	*P*	
Intercept	0.47 ± 0.24	0.05	
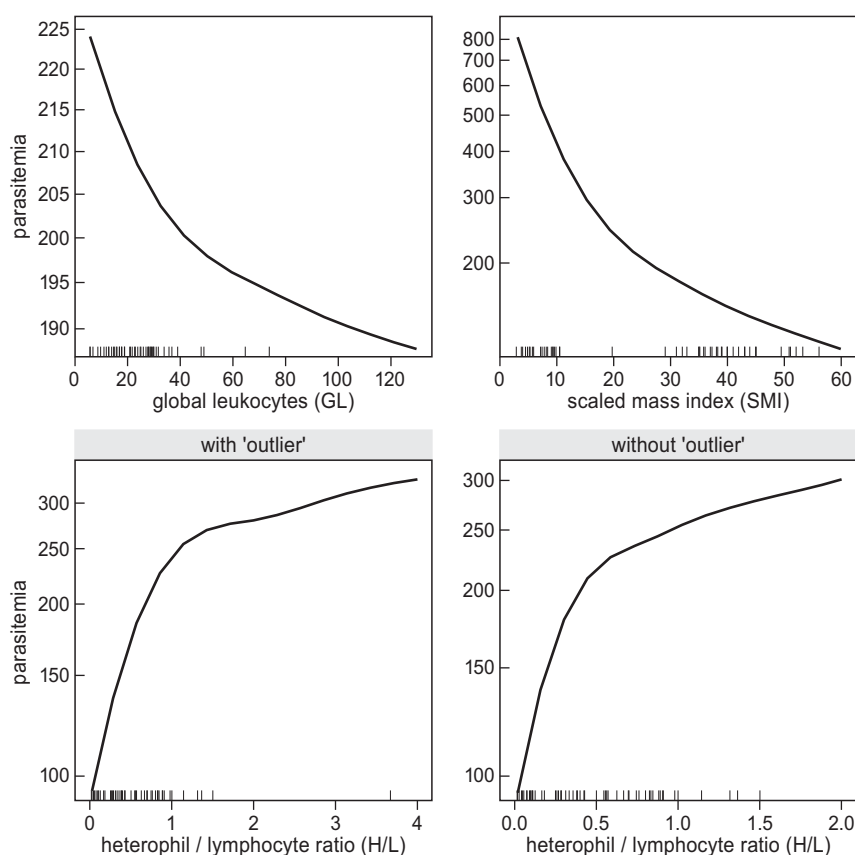
Figure 1. Generalized linear mixed models results between parasitaemia (response variable), and GL (upper left), and SMI (upper right) and H/L with (bottom left) and without possible 'outlier' (bottom right). Solid lines represent the predicted values for the best statistical model and small vertical lines at the bottom of each graph represent the distribution of each explanatory variable.

Elaenia *Elaenia chiriquensis* (35%), whereas the Flavescent Warbler *Myiothlypis flaveola* (12%) had the lowest prevalence, which also reflected the same pattern for parasitaemia (Table 1). Ruddy Ground-doves also had the highest H/L (0.68 ± 0.31). In contrast, Brown-crested Flycatchers *Myiarchus tyrannulus* had the lowest H/L (0.05 ± 0.03) and the highest GL (47.28 ± 38.40).

We found no significant association between haemosporidian parasite prevalence and our explanatory variables (Table 2). However, SMI ($P < 0.01$; Table 2), H/L ($P = 0.04$; Table 2) and GL ($P < 0.01$; Table 2) predicted parasitaemia. We found a negative relationship between parasitaemia and SMI and GL, indicating that the lower the body condition and immune response as measured by the GL, the greater the parasitaemia (Figure 1). On the other hand, we found a positive relationship between parasitaemia and H/L, indicating that the greater the stress, the greater the parasitaemia (Figure 1).

We evaluated the possibility that an individual of the Lesser Elaenia could potentially be an outlier for the H/L (value = 3.6), with a value that was much higher than the mean, which was 0.55 ± 0.53. Therefore, we reran the model without that value, and found similar results. H/L was still significant after removing the possible outlier (Drop1 results: AIC = 7548.2, $P < 0.001$; Estimate = 0.25 ± 0.01 (±SD), $P < 0.001$; Figure 1).

DISCUSSION

We found no evidence that physiological variation analysed in the present study (H/L ratio, SMI and GL) in avian hosts explains haemosporidian parasite prevalence. However, we found evidence that birds with higher levels of stress and lower body condition (assessed by the H/L, GL and SMI) were more likely to have higher parasitaemia.

We found that as GL decreased, the intensity of infections increased. Changes in the number of leukocytes may reflect diseases that are already in progress in the bird host. Also leukocytes levels in the bloodstream may suggest systemic stress caused by malnutrition, excessive exercise and/or significant weight loss (Owen & Moore 2006, Campbell 2015). Immunity can also be compromised during periods of high stress for birds and, for that reason, individuals may become more susceptible to pathogens or parasitic infections (Moore & Gotelli 1996). Therefore, our results suggest that lower levels of white blood cells, associated with increased stress, may be an indicative that individuals will be more susceptible to haemosporidian parasite infections, as shown by higher parasitaemia in our study.

It is well known that avian malaria infection has two phases: the acute and the chronic phase of infection. The acute phase shows a steep increase of the parasite parasitaemia until it reaches a peak (also known as crisis), in which birds may show clinical symptoms (Lapointe et al. 2012). In the chronic phase, the parasitaemia gradually decreases, reaching chronically low levels of infection. Lachish et al. (2011) have demonstrated that the costs of an acute infection by haemosporidian parasites may be highly detrimental and long-lasting, in which avian individuals may become anaemic, lethargic and even non-active. Therefore, the majority of captured avian individuals, if infected, may be in a chronic phase of infection, since acute-phase individuals may show little activity (Dimitrov et al. 2015) and, therefore, are less likely to be captured in mist nets. Consequently, we believe that the analysed hosts in the present study are likely to be in a chronic phase of infection. Also, even in Ruddy Ground-doves, the captured species that had the highest mean values of infected red-blood cells, there was very low infection intensity (mean 4% of parasitaemia). As stated by Valkiūnas (2005), this value is below the limit level of parasitaemia capable of weakening the birds to the point of becoming inactive. Hence, chronic phase levels of parasitaemia are generally low and sometimes undetectable, with sporadic episodes of recrudescence (Cubas et al. 2014), which is the steep increase of parasites in the peripheral blood during chronic infections (Valkiunas 2005). Increased stress in birds leads to higher levels of corticosterone that suppress their immune system (Lapointe et al. 2012). In turn, once exposed to sources of stress that suppress the immune system, we hypothesize that individuals may suffer from recrudescence episodes, which may even occur in animals that have been clinically cured and free of parasites for long periods (Cubas et al. 2014). Therefore, individuals with long-lasting stress, measured by an increase in H/L may indeed more susceptible to higher parasitaemia.

Here we used the H/L ratio, which has been shown to be a reliable index of chronic stress assessment for birds, whether in captivity or natural environments, and it is associated with injuries, reproductive cycle and seasonal changes (Gross & Siegel 1983, Campbell 2015). Heterophils are highly phagocytic granulocytes and capable of a broad spectrum of antimicrobial activity, due to their cytoplasmic granules containing lysosomes and non-lysosomal enzymes used for bactericidal activity (Harmon 1998, Campbell 2015), and when they enter tissues during episodes of inflammation, they produce reactive oxidants that can be harmful to the host (Becker et al. 2004, Krams et al. 2013). The reduction in lymphocytes is mainly associated with excess corticosteroids and increased stress, as well as immunosuppression processes (Ots et al. 1998, Clark et al. 2009). We found a positive association between H/L and parasitaemia and hypothesize that birds with ongoing bacterial infections (characterized by the largest number of heterophiles), and/or immunosuppression conditions, may become less resistant and more susceptible to new infections or episodes of recrudescence by haemosporidian parasites. Wojczulanis-Jakubas et al. (2012) evaluated the leukocyte profile of parasitized and non-parasitized individuals by haemosporidian protists and found high levels of heterophiles and a higher H/L ratio for infected individuals. While they attributed this increase in H/L ratio to the presence of the parasites, such an increase in parasite prevalence could also be caused by higher H/L ratio, as we hypothesize in this study.

Many studies have correlated changes in the body condition of birds with factors such as climatic seasonality, variation in precipitation, stage of the reproductive cycle (Møller et al. 2003, Norte et al. 2009, Sánchez-Guzmán et al. 2018), parasite infections (Figuerola et al. 1999), territorial defence (Whiteman & Parker 2004) and immune response (Merino et al. 2000). Generally, such studies show that body condition tends to respond negatively to stressors. Since we evaluated individuals from peri-urban populations, one may expect that food deprivation, increased risk of predation and other unfavourable conditions may increase stress, and, for that reason, have a greater impact on body condition, where individuals with a lower body condition index may suffer higher levels of parasitaemia. Atkinson et al. (2000), showed that heavier birds tend to have higher survival than lighter

ones when suffering from infections with haemosporidian parasites. This suggests that the general body condition may predict the survival of hosts to acute infections. Therefore, a decrease in body condition can also be a determining factor for surviving the acute phase infection, or during lower peaks of parasitaemia in the chronic stage of infection. Furthermore, Ilgunas *et al.* (2019) demonstrated that malarial infections may influence the probability of hosts having access to energy-rich resources throughout the infection period. Our result also corroborates the findings of Dutra *et al.* (2017), who observed a decrease in the body condition index in individuals infected with *Leucocytozoon* and *Plasmodium*, with a marked decrease in total plasma proteins, which can be used as a biochemical measure of the individual's nutritional reserves.

In conclusion, we found that the general condition of birds can influence the count of hemoparasites in peripheral blood, where those suffer from stress and with a poorer body condition tend to be more susceptible to higher intensities of parasitaemia. We showed that the lower the GL and the SMI, and the higher the H/L, the greater the intensity of the parasite count in the peripheral blood. This might indicate that chronic stress directly affects an individual's immune response and makes individuals more likely to have a recrudescence episode with an increased haemosporidian parasite load in peripheric blood, or to have increased difficulty fighting off haemosporidian parasite infections during the acute phase. We would like to emphasize the need for further studies, investigating the relationship between decreased health and susceptibility to parasites in experimental studies, mainly investigating the corticosterone levels and the response to oxidative stress throughout periods of stress. We also highlight the fact that our results should be analysed with care, since the haemosporidian parasites may also affect stress and body condition in avian hosts. Therefore, we suggest that future studies specifically take a closer look at the directionality of these effects, to further analyse whether the increased parasite load may be a cause or effect of a decreased body condition, or increased GL and H/L.

ACKNOWLEDGEMENTS

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SAMENVATTING

Wanneer vogels langdurig stress ondervinden, kan dat invloed hebben op hun gevoeligheid voor bepaalde bloedparasieten, zoals malaria. Wij hebben in de boom- en struiksavanne (cerrado) van Zuid-Brazilië bij 68 individuen van vijf vogelsoorten (Steenduif *Columbina talpacoti*, Grote Kiskadie *Pitangus* sulphuratus, Kleine Elenia *Elaenia chiriquensis*, Amazonezanger *Myiothlypis flaveola* en Cayennetiran *Myiarchus tyrannulus*) onderzocht hoe stress en lichaamsconditie van invloed zijn op de gevoeligheid voor een besmetting met deze parasieten. Wij vonden geen verband tussen lichaamsconditie en bloedvariabelen (maat voor stress) enerzijds en de mate van voorkomen van parasieten anderzijds. Wel vonden wij dat individuen met een hoge verhouding tussen bepaalde bloedcellen (heterofiele granulocyten/witte bloedlichaampjes ratio), een lagere lichaamsconditie en een kleiner aantal witte bloedlichaampjes een hogere parasietenbelasting hadden. Deze resultaten wijzen erop dat vogels met een slechte gezondheid en chronische stress vatbaar zijn voor een hevigere besmetting en/of verergering van een eerdere besmetting met bloedparasieten als gevolg van een eerder ontstane verslechtering van het immuunsysteem.