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Haemosporidian Infection Is Associated with the Oxidative Status in a Neotropical Bird

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Simple Summary: This study explored how blood parasites, known as haemosporidians, impact the oxidative status of Rufous-collared Sparrows, a common bird species in Chile. Oxidative status measures the balance between harmful molecules called reactive oxygen species (ROS) and the bird's ability to neutralize them with antioxidants. The study found that birds infected with haemosporidians had higher levels of ROS, indicating more oxidative stress than uninfected birds. While the overall antioxidant levels did not differ between infected and uninfected birds, the infected birds showed a greater imbalance in ROS/TAC, suggesting they suffered more oxidative damage. This was especially true for birds in their reproductive phase. These findings suggest that haemosporidian infection, combined with the demands of reproduction, may impose significant health costs on these birds. Understanding this could help us learn more about how infections affect wildlife, and the strategies parasites use to survive in their hosts.

Abstract: Haemosporidians are common blood parasites in wild bird populations, yet their effects on oxidative status remain understudied. Here, we measured the levels of thiobarbituric acid-reactive substances (TBARS) as an indicator of reactive oxygen species (ROS), total antioxidant capacity (TAC) as an indicator of non-enzymatic molecular antioxidants, and TBARS/TAC ratio as an indicator of oxidative status. We also used parasite genus-specific primers and PCR techniques to detect haemosporidians in 117 adults of Rufous-collared Sparrow (*Zonotrichia capensis*) from four locations in south–central Chile. Mixed-effect models were employed to compare oxidative indicators between infected and uninfected birds. Infected birds showed significantly higher TBARS levels, but no significant differences in TAC, leading to a higher TBARS/TAC ratio, especially in reproductive individuals. This suggests increased oxidative damage in infected birds, irrespective of sex or body condition. A positive relationship between TBARS and TAC was observed in both groups, but the antioxidant response was weaker in infected birds, indicating differential oxidative stress responses based on infection status. Body condition did not differ significantly between infected and uninfected individuals. These results demonstrate that haemosporidian infections impose oxidative costs on birds, potentially compounding the oxidative costs associated with reproduction.

Keywords: avian malaria; oxidative stress; passerine birds; *Zonotrichia capensis*

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1. Introduction

Parasites encompass various organisms, including helminths, arthropods, protozoa, bacteria, fungi, and viruses, that maintain a close relationship with their hosts [1]. Parasites can be found in almost every part of the world and thrive in various habitats [2]. This wide variety has been influenced by coevolutionary relationships with their hosts, leading to complex host physiological responses that may affect their oxidative status [3]. The oxidative status of a host reflects the balance between the production of reactive oxygen species (ROS) and antioxidant defenses [4–6]. ROS are natural byproducts of metabolic processes that, in excessive amounts, can harm cellular components such as proteins, lipids, and DNA, potentially leading to cellular dysfunction and a decline in overall organismal health [7–9]. Parasites can cause oxidative damage by triggering the overproduction of ROS as part of the host's immune response [3]. For example, neutrophils (heterophils in birds) phagocytize pathogens during the innate immune response, leading to an oxidative burst and increased ROS production due to the activation of the protein complex that generates superoxide anions [10]. These superoxide anions can be converted to other ROS species, such as the highly reactive hypochlorous acid (HOCl) catalyzed by myeloperoxidase (MPO), which is responsible for killing ingested pathogens [11,12]. The oxidative burst also increases the production of hydroxyl radicals (ROS), indirectly leading to the creation of thiobarbituric acid reactive substances (TBARS) through lipid peroxidation [13]. Thus, elevated levels of TBARS are commonly used as a biomarker for oxidative stress, including those induced by the immune response to infection [14].

In response to the harmful effects of increased ROS, hosts can acquire antioxidants such as carotenoids and vitamins from their diet and produce their antioxidant enzymes, including Superoxide Dismutase (SOD), Catalase, and Glutathione Peroxidase (GPx) [15]. On the other hand, parasites have developed various strategies to mitigate the harmful effects of increased ROS in their hosts. For instance, *Plasmodium falciparum* generates an antioxidant enzyme, glutathione reductase, to neutralize harmful ROS during the intraerythrocytic phase [16]. *Leishmania donovani* produces anti-inflammatory cytokines such as interleukin-10 (IL-10), which decreases the host's inflammatory response and reduces ROS levels [17,18]. Parasitic worms release molecules like thioredoxin peroxidase, which can scavenge ROS and safeguard the parasite from oxidative damage [19]. *Toxoplasma gondii* can manipulate host cell metabolism to avoid triggering excessive ROS production [20]. Furthermore, some parasites, such as *Brugia malayi*, can employ molecular mimicry to evade detection and destruction by the host's immune system, thereby avoiding the ROS produced during an immune response [21,22]. These strategies demonstrate the intricate interplay between host defense mechanisms and parasite survival tactics, enabling parasites to generate chronic infections and perpetuate their life cycles within their hosts.

The haemosporidians (*Plasmodium* spp., *Haemoproteus* spp., and *Leucocytozoon* spp.) are a group of blood parasites that are extremely common among birds and are primarily transmitted by blood-sucking arthropods [23]. Infection by haemosporidians may lead to weight loss, weakness, anemia, and occasionally mortality, particularly in populations of naïve hosts [24]. The impact of haemosporidians on the oxidative status of birds is still unclear. For example, some studies have found changes in reactive oxygen metabolites (ROM) levels [25,26] and enzymatic antioxidant activity [26,27], as well as of significantly higher TBARS levels and lower antioxidant activity (GPx, SOD, and GR) in infected individuals [28]. However, other studies did not find any effect of the infection by haemosporidians on ROM concentration [29] or enzymatic antioxidant activity [30], suggesting that the effect of haemosporidian infection on oxidative status may vary across populations and host species.

To increase our understanding of the relationship between haemosporidian infection and oxidative status in a neotropical bird, we characterized thiobarbituric acid-reactive substances (TBARS) as an indicator of ROS, total antioxidant capacity (TAC) as an indicator of non-enzymatic molecular antioxidants and TBARS/TAC ratio as an indicator of oxidative status in four populations of Rufous-collared Sparrows located in the center and south of

Chile (Figure 1; 33° S and 38° S). If the infection by haemosporidians is associated with oxidative status, we predict that infected individuals will exhibit higher TBARS levels and lower TAC levels, reflecting a greater TBARS/TAC unbalance, than uninfected individuals.

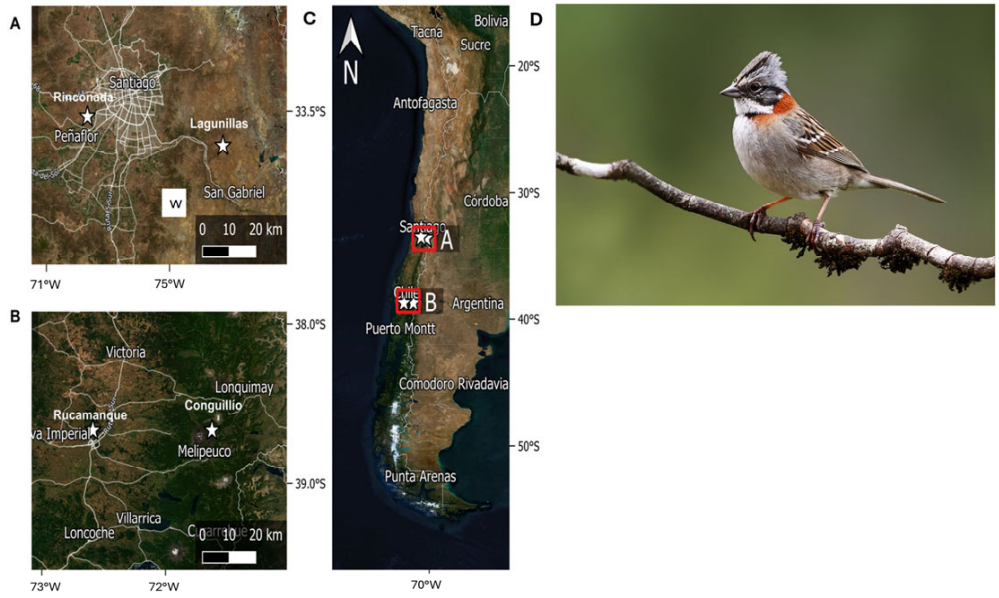

Figure 1. Figure showing the sites and species of study. The left map shows the four sampled sites: Rinconada (low) and Lagunillas (high) in the center (**A**) and Rucamanque (low) and Conguillío (high) in the south (**B**) of Chile ((**C**) white stars). The right map shows the extent of the Rufous-collared Sparrow's distribution in the country (geographical extent of the left panel). (**D**) Rufous-collared Sparrow. Photography by fotonaturaleza.

2. Materials and Methods

2.1. Study Species

The Rufous-collared Sparrow (Figure 1) is a small passerine (20 g) highly adaptable bird that thrives in diverse environments, ranging from sea level to altitudes exceeding 4000 m. It is a sedentary species; however, some individuals have been observed to perform short-altitudinal winter movements [31,32]. The species' ability to adjust to varied environmental conditions is attributed to intraspecific variations in behavior and physiology along both latitudinal [32–34] and altitudinal gradients [31,35,36]. Its habitat extends from the southernmost point of South America (55° S) to southern Mexico (10° N). These omnivorous birds consume mainly fruits, seeds, and insects [37]. They establish breeding territories during the Austral Spring. In the breeding season, bird sex is identified by observing incubation patches in females and male cloacal protuberance lengths [38]. Following the breeding season, from approximately January to March, they undergo molting and enter the non-breeding period from April to August, where sexual dimorphism cannot be determined [31,39]. Adult Rufous-collared Sparrows can be distinguished from young individuals by feather patterns and coloration differences. Adults typically exhibit a more defined and vibrant coloration, including a prominent rufous collar around the neck, a well-marked black facial mask, and a bright white or gray supercilium (eyebrow line). In contrast, the young have duller and less distinct feather patterns. Their plumage is often brownish, with streaked underparts and a more muted or absent rufous collar [39].

A previous study on the prevalence and diversity of haemosporidians in this species found a prevalence of 50.7% in central Chile [40]. Therefore, this species represents an excellent opportunity to investigate the impact of haemosporidians on the oxidative status of wild bird populations.

2.2. Bird Sampling

Adult birds were captured using mist nets between March and December of 2019 and 2020 at Lagunillas (33°21′ S, 70°17′ W, 2300–2700 m), Rinconada de Maipú (33°31′ S, 70°50′ W, ~450 m a.s.l.), Conguillío National Park (38°40′ S, 71°38′ W 1000–1800 m a.s.l.) and Rucamanque (38°40′ S, 72°36′ W ~400 m) for a total of 117 individuals. The dates for sampling were selected following the standardized observations suggested by Clark et al. (2019) to increase the likelihood of sampling both non-breeding and breeding individuals. A breeding individual was identified by the presence of incubation patches in females and cloacal protuberance lengths of over 5 mm in males [38]. We measured body mass with a 60 g balance scale (±0.1 g) and tarsus length with digital calipers to the nearest 0.1 cm for each bird captured. This allowed us to calculate a body condition index (BCI) based on the residuals of the regression between those measurements [41]. Before releasing, the birds were banded with individual metal bands (National Band and Tag Co., Newport, KY, USA and Split Metal Bird Rings, Porzana Ltd., Winchelsea, UK) and a small blood sample (c. 50–100 µL) was collected from the brachial vein using heparinized tubes. The samples were stored on ice (4 °C) for 5 h before reaching the laboratory. We also stored a blood sample (c. 20 µL) in FTA cards (Whatman, Buckinghamshire, UK) for the screening for haemosporidian parasites (see below) and molecular sexing by amplifying the CHD locus using the primers 2550F (5′-GTTACTGATTCGTCTACGAGA-3′) and 2718R (5′-ATTGAAATGATCCAGTGCTTG-3′; see Supplementary Materials for details) [42]. We performed molecular sexing on both breeding and non-breeding individuals.

2.3. Oxidative Status

The blood samples collected in heparinized tubes were centrifuged at 8000 rpm to separate the plasma, which was subsequently frozen at −80 °C until TBARS and TAC measurement [31,43,44]. TBARS was used as an indicator of oxidative damage and TAC as an indicator of non-enzymatic molecular antioxidants [31,43,44]. Both methods have been applied to diverse bird species, e.g., [45–47]. Plasma TAC levels were measured using the antioxidant capacity reduction method [48,49], which evaluates the reduction of the copper (II)-neocuproine complex to the copper (I)-neocuproine complex by antioxidants in the plasma, measured by colorimetry at 450 nm and compared with a Trolox standard curve [49]. The level of TBARS was determined by assessing the thiobarbituric acid concentration based on a reaction that evaluates the 1:2 adduct formed by malondialdehyde (MDA) and thiobarbituric acid (TBA), with the MDA-TBA adduct measured at 532 nm using colorimetry [50]. The TBARS/TAC ratio was used to estimate the oxidative status of individuals, with higher values indicating higher oxidative stress [46].

2.4. DNA Extraction and Screening for Haemosporidian Parasites

DNA was extracted from the collected blood stored on FTA cards using the Dried Blood Spot DNA Isolation Kit (Norgen Biotek Corp., Thorold, ON, Canada). A nested PCR protocol was performed to amplify a fragment of approximately 480 bp (excluding PCR primers) of parasites' mitochondrial DNA cytochrome b (cyt b) gene using genus-specific primers for *Leucocytozoon* spp., *Plasmodium* spp. and *Haemoproteus* spp., respectively [51]. Samples were screened at least twice to avoid false negatives. We included two positive controls for parasites and two negative controls (ddH_2O). Contamination was not detected.

2.5. Statistical Tests

Generalized mixed-effect models (GLMM) were used to compare the TBARS, TAC levels, and TBARS/TAC ratio between infected and non-infected birds. For each dependent variable, we formulated full models that included infection status ("infected" or "non-infected") as the explanatory variable. Reproductive status ("breeding" or "non-breeding") and the two-way interaction between infection and reproductive status were also included. BCI was included as a covariate, and sex ("female" or "male") as a cofactor. Locations and elevations of sampling sites were included as random effects. Akaike

Information Criterion was used to select the more suitable predictive model AIC [52]. GLMM was also used to compare and assess the relationship between TBARS and TAC between infected and non-infected individuals. The model includes TBARS as the dependent variable and TAC and the two-way interaction between TAC and infection status as explanatory variables. BCI and sex were included as covariable and cofactor, respectively. Locations and elevations of sampling sites were included as random effects. A linear mixed-effect model (LMM) was used to compare the BCI between infected and non-infected individuals. The model includes BCI as the response variable and infection status as the explanatory variables. Breeding status and sex were included as cofactors. Locations and elevations of sampling sites were included as random effects. We considered an effect to be statistically supported when $p < 0.05$. All analyses were performed in the R Studio statistical environment v.1.4.17.17 using the Base (version v. 2024.04.1) [53] and glmmTMB (version 1.1.-27.1) [54] packages.

3. Results

Of the 117 individuals, 71 (60.7%) were positive for haemosporidians. Within this group, 64 individuals presented infection by *Plasmodium* and *Haemoproteus* spp., and 46 by *Leucocytozoon* spp., with 39 individuals showing infection with the three haemosporidians. The more parsimonious model based on AIC for plasma TBARS level (with an AIC value of −1846.98) shows that TBARS levels were significantly higher in infected than non-infected birds regardless of sex, BCI, and breeding status (Table 1; Figure 2A). The best model based on the AIC for plasma TAC levels (with an AIC value of −927.16) indicates that TAC levels do not vary significantly between infected and non-infected individuals (Table 1; Figure 2B). Any significant variation in TAC levels was associated with reproductive status, sex, or BCI (refer to Table S1). Regarding the TBARS/TAC ratio, the best model based on AIC (with an AIC value of −768.24) indicates that this ratio is higher in infected birds than in non-infected birds (Table 1; Figure 2C). The interaction between infection status and reproductive status reveals that the TBARS/TAC ratio is significantly higher in infected breeding birds than in non-infected and infected non-breeding birds (Table 1; Figure 2D; please refer to Table S1 for all models). The relationship between TBARS and TAC and the interaction between TAC levels and infection status were significant (Table 2; Figure 3). Additionally, the body condition did not vary significantly between infected and non-infected individuals (Table 3).

Table 1. Generalized mixed-effect models (GMM) showing the differences in TBARS, TAC, and TBARS/TAC ratio of Rufous-collared Sparrows (n = 117) infected and uninfected with haemosporidian. Bold numbers indicate intervals that did not include zero.

Effect on TBARS Levels	**Estimate**	**SE**	**z Value**	***p*- Value**
Intercept	−8.51	0.14	−59.47	<0.00 **
Infection status [1]	0.25	0.11	2.33	0.02 *
Reproductive status [1]	−0.07	0.11	−0.65	0.52
Infection status [1]: Reproductive status [1]	−0.20	0.15	−1.35	0.18
Random effects				**σ**
Location: Elevation				0.05
Effect on TAC Levels	**Estimate**	**SE**	**z Value**	***p*- Value**
Intercept	−4.43	0.15	−29.59	<0.00 **
Infection status [1]	−0.04	0.10	−0.39	0.70
Reproductive status [1]	−0.09	0.11	−0.85	0.40
Infection status [1]: Reproductive status [1]	0.19	0.13	1.43	0.15
Random effects				**σ**
Location				0.05

Table 1. *Cont.*

Effect on TBARS/TAC Ratio	Estimate	SE	z Value	*p*- Value
Intercept	−4.06	0.10	−39.6	<0.00 ***
Infection status [1]	0.48	0.12	3.9	<0.00 ***
Reproductive status [1]	0.00	0.13	0.01	0.98
Infection status [1]: Reproductive status [1]	−0.53	0.17	−3.03	<0.00 ***
Random effects				**σ**
Location: Elevation				3.2×10^{-10}

[1] Parameter estimates and SE were estimated relative to the "infected" level in the variable infection status and the "non-breeding" level in the variable reproductive status. Significant codes: 0 ***; 0.001 **; 0.01 *.

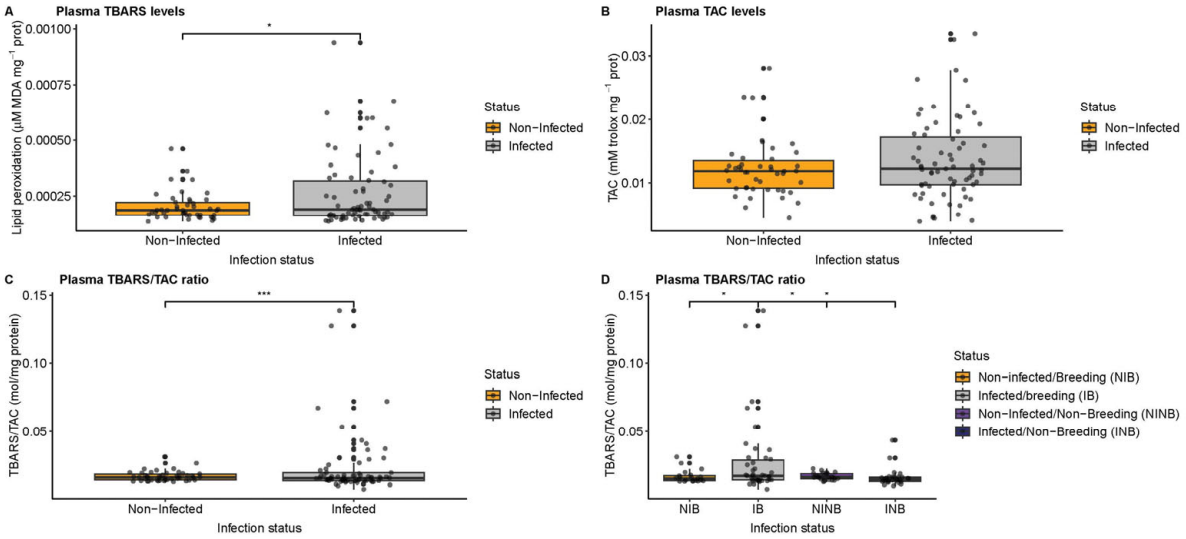

Figure 2. Differences in TBARS, TAC, and TBARS/TAC ratio between infected and non-infected individuals of Rufous-collared Sparrow (n = 117). The bold horizontal line inside the box represents the median score for TBARS (**A**), TAC (**B**), and TBARS/TAC ratio ((**C**) and (**D**), respectively). Vertical lines indicate standard error, and asterisks indicate significant differences among groups.

Table 2. Generalized mixed-effect model (GMM) showing the relationship between TBARS and TAC in Rufous-collared Sparrows (*n* = 117) infected and uninfected with haemosporidian. Bold numbers indicate intervals that did not include zero.

Effect on TBARS	Estimate	SE	z Value	*p*- Value
Intercept	−9.17	0.17	−53.18	<0.00 ***
TAC levels	47.21	12.54	3.76	<0.00 ***
Infection status [1]	0.53	0.18	2.95	<0.00 ***
Sex [1]	0.01	0.07	1.42	0.153
BCI	−0.01	0.01	−1.22	0.22
TAC levels: Infection status [1]	−25.99	12.98	−2.00	0.04 *
Random effects				**σ**
Location: Elevation				0.01

[1] Parameter estimates and SE were estimated relative to the "infected" level in the variable infection status and the "male" level in the variable sex. Significant codes: 0 ***; 0.01 *.

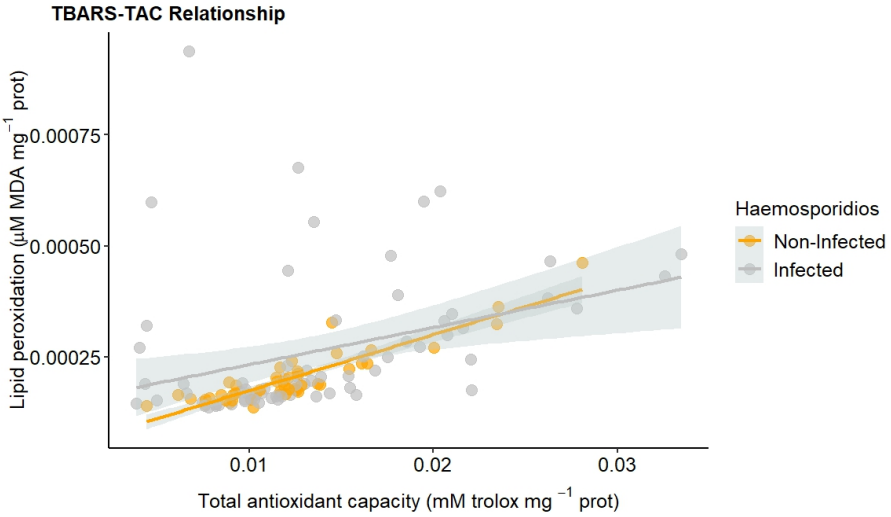

Figure 3. Relationship between TBAR and TAC in Rufous-collared Sparrow (n = 117) individuals infected and uninfected with Haemosporidian parasites. Model predictions are shown with 95% confidence intervals (lines and shaded area).

Table 3. Linear mixed-effect model (LMM) showing the relationship between body condition (BCI) and infection status for Haemosporidian parasites in Rufous-collared Sparrows (n = 117). Bold numbers indicate intervals that did not include zero.

Effect on Body Condition	Estimate	SE	z Value	*p*- Value
Intercept	−1.48	1.02	−1.44	0.18
Infection status [1]	1.07	0.81	1.33	0.19
Sex [1]	1.85	0.71	2.61	0.01 *
Breeding status [1]	0.07	0.74	0.10	0.92
Random effects				**σ**
Location: Elevation				1.7

[1] Parameter estimates and SE were estimated relative to the "infected" level in the variable infection status, the "male" in the variable sex, and the "non-breeding" level in the variable reproductive status, respectively. Significant codes: 0.01 *.

4. Discussion

The results show significantly higher TBARS levels in infected than non-infected birds, regardless of reproductive status, sex, and body condition. This suggests that the infection by haemosporidian parasites produces high ROS in the hosts, leading to enhanced lipid peroxidation and oxidative damage. The elevated TBARS levels in infected birds demonstrate the role of prooxidants in the host–parasite interaction. Parasitic infections often stimulate the host's immune system, producing ROS as a defense mechanism. While effective in pathogen destruction, these ROS can also cause collateral damage to host tissues, including the peroxidation of lipids in cell membranes [55]. The results are consistent with previous research, such as Jimenez-Peñuelas et al. (2023), who demonstrated increased TBARS levels in birds infected with haemosporidians [56]. This result also suggests that the haemosporidians would have a limited capacity to counteract the host's production of ROS.

The lack of variation in TBARS levels concerning reproductive status, sex, and body condition suggests that oxidative cost is a general physiological burden. Thus, it is plausible that the TBARS levels induced by parasitic infections could overshadow the effects of reproductive status, sex, and body condition. Our findings are consistent with studies in other avian species, such as those by van de Crommenacker et al. (2012), who reported increased TBARS levels in infected seabirds, suggesting a generalizable effect of parasitic infections on oxidative status across avian taxa [26].

The lack of significant differences in TAC levels between infected and non-infected birds suggests that infections by haemosporidian parasites may not deplete antioxidant reserves or overwhelm the antioxidant defenses of these birds. This observation aligns with studies by Schoenle et al. (2017) and Razavi et al. (2016), who found that parasitic infections did not necessarily lead to diminished antioxidant levels in bird species [29,57]. One possible explanation is that infected birds may upregulate their antioxidant defenses in response to increased ROS production, thereby maintaining their TAC levels [7]. Alternatively, it is possible that the severity or type of parasitic infection in our study was insufficient to impact the overall antioxidant capacity of the hosts markedly. Our findings are consistent with some studies, but differ from others. For instance, Jimenez-Gallardo et al. (2024) found that TAC levels could increase in response to parasitic infections, suggesting a compensatory upregulation of antioxidant defenses [58]. In contrast, other studies, such as that by Pap et al. (2015), report no changes in TAC levels between infected and non-infected individuals, suggesting that natural levels of chronic infection have a limited effect on oxidative stress physiology [59].

The significantly higher TBARS/TAC ratio in infected birds underscores the oxidative stress burden imposed by haemosporidian infection. The TBARS/TAC ratio serves as a useful index for evaluating the balance between pro-oxidant forces (indicated by TBARS) and antioxidant defenses (indicated by TAC) [7,31,43]. Our results suggest that the haemosporidian infection disrupts this balance, increasing oxidative damage. The pronounced TBARS/TAC imbalance in infected breeding birds highlights the interaction between reproductive effort and oxidative stress. Reproduction is an energetically costly process that can increase metabolic rates and ROS production, exacerbating oxidative stress [60]. The increased oxidative damage observed in infected breeding birds may be due to the combined oxidative costs of parasitic infection and reproductive activities. This finding aligns with studies by Alonso-Alvarez et al. (2004), who demonstrated that reproductive efforts could amplify oxidative stress [61]. Moreover, the positive and significant relationship between TBARS and TAC suggests a complex interaction where, as oxidative damage increases (TBARS), there is a compensatory upregulation of antioxidant defenses (TAC). The positive effect of TAC on TBARS was less pronounced in infected than non-infected individuals, suggesting that antioxidant capacity responds to oxidative stress differently depending on the status of haemosporidian infection. This could indicate a limit to how effectively infected individuals can bolster their antioxidant defenses in response to increasing oxidative damage, and might explain why non-infected individuals maintain lower TBARS levels despite facing oxidative challenges.

Some studies have found that birds in poor condition or with lower body fat reserves may be more susceptible to infections [62–64]. However, we did not find significant differences in body condition between infected and non-infected individuals. This result suggests that factors other than immediate body condition play a role in determining susceptibility to haemosporidian infection. For instance, genetic variability, particularly in immune-related genes such as the major histocompatibility complex (MHC), could influence the ability of certain individuals to resist or tolerate parasitic infections [65,66]. Furthermore, subtle differences in individual immune system efficiency and stress levels [67,68] may explain why infection status does not always align with observable body conditions. Therefore, while body condition is important, it alone may not fully reflect susceptibility to infection in wild bird populations.

While our results provide valuable insights into the oxidative costs of infection by haemosporidian, it is important to note some limitations that should be considered in future studies. First, using PCR to determine infection status provides information on the presence of haemosporidian DNA, but does not confirm whether the parasites were actively replicating or inducing a full-blown infection. As highlighted in other studies, a positive PCR result may reflect an abortive infection where the parasite is not necessarily active or causing harm to the host [69–71]. This limitation might explain why no significant difference was observed in body condition between infected and non-infected birds, as

birds with abortive infections could appear physically healthy despite harboring parasite DNA. Moreover, different haemosporidian lineages or haplotypes may exert varying degrees of oxidative damage on hosts [72,73]. Our study did not differentiate between these haplotypes, which may mask potential differences in the physiological impacts of infection. Finally, higher parasitemia levels usually cause more physiological stress, and variations in parasitic load might affect the level of oxidative damage [74].

In short, the results show the complex link between haemosporidian infection and oxidative damage in wild birds. Higher TBARS levels point to the oxidative costs of infection, especially in infected breeding birds, but stable TAC levels suggest an interesting strength in their antioxidant defenses. Future studies on how haemosporidian infection affects oxidative stress and antioxidant capacity, along with factors such as infection characteristics, host genetics, and overall health, could give more insight into the connection between the infection and oxidative status in wild birds.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/birds5030040/s1, DNA extraction and PCR methods; Table S1: all models for TAC, TBARS, and TBARS/TAC ratio with AIC value; Table S2: data set of this study.

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Data Availability Statement: The data supporting the results are included in the article as Supplementary Material (Table S2).

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