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ORIGINAL RESEARCH

Seasonal changes in diet, immune function, and oxidative stress in three passerines inhabiting a Mediterranean climate

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Abstract

Oxidative status and immune function are energy-demanding traits closely linked to diet composition, particularly resource availability and nutritional value. In seasonal environments, nutrient availability and diet quality fluctuate, potentially influencing these traits. However, limited evidence exists regarding these dietary effects on immune function in seasonal environments. In this study, we employed stable isotope analysis to assess the impact of seasonal changes in niche width and trophic level (*i.e.*, $\delta^{15}N$) on two immune variables (hemolysis and hemagglutination scores) and two oxidative status parameters (lipid peroxidation and total antioxidant capacity) in three passerine species: *Zonotrichia capensis* (omnivorous), *Troglodytes aedon* (insectivorous), and *Spinus barbatus* (granivorous). We found that hemolysis scores varied seasonally in *Z. capensis*, with higher values in winter compared to summer. Total antioxidant capacity (TAC) also increased during the winter in *Z. capensis* and *S. barbatus*. The isotopic niche width for *Z. capensis* and *S. barbatus* was smaller in winter than in summer, with the omnivorous species exhibiting a decrease in $\delta^{15}N$. Despite the seasonal shifts in ecological and physiological traits in *Z. capensis*, we identified no correlation between immune response and TAC with trophic level. In contrast, in the granivorous *S. barbatus*, the lower trophic level resulted in an increase in TAC without affecting immunity. Our findings revealed that dietary shifts do not uniformly impact oxidative status and immune function across bird species, highlighting species-specific responses to seasonal changes. This underscores the importance of integrating ecological and evolutionary perspectives when examining how diet shapes avian immunity and oxidative balance.

Keywords Diet · Seasonality · Immunity · Oxidative stress · Antioxidant · Birds

Introduction

An organism's ability to maintain homeostasis and survive depends largely on its immune system, which provides defense against parasites and pathogens a (Janeway et al. 2005; Abbas et al. 2012). Maintaining a well-functioning immune system comes at an energetic cost (King and Swanson 2013). As such, an investment in immune defense may reduce resources needed for other biological functions like growth and reproduction (Bonneaud et al. 2003; Brommer 2004; Brzęk and Konarzewski 2007; King and Swanson 2013). Moreover, in addition to the energetic costs, immune function can result in self-damage or immunopathology, including the generation of reactive oxygen species (Lambeth 2007; Stahlschmidt et al. 2015). Thus, the optimal functioning of the immune system would primarily depend on environmental and ecological factors that influence nutritional balance (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Norris and Evans 2000). Accordingly,

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both field and experimental studies suggest that the capacity of organisms to mount an immune response is affected by environmental conditions where food availability and diet quality are key factors (Buehler et al. 2008, 2009; Hawley and Altizer 2011; Martin et al. 2011; Schultz et al. 2020).

In seasonal environments, resource quantity (availability) and quality (nutritional value) vary temporally in a somewhat predictable fashion and organisms must be able to adjust their physiology to handle such environmental variation (Klaassen 1995; Nelson et al. 2002; Foster and Kreitzman 2010; Versteegh et al. 2012a). Indeed, Gasparini et al. (2006), indicates that variation in immune function appears when shifts in food resources occur seasonally. For example, resource scarcity is an accurate predictor of seasonal declines in innate immunity (e.g., complement activity), particularly in birds (Gasparini et al. 2006; Jackson et al. 2020; Schultz et al. 2020). A second physiological attribute that may be influenced by seasonal variation in resource availability (van de Crommenacker et al. 2011) and quality (Carter et al. 2021; Cohen et al. 2009) is oxidative status, defined as the balance between pro-oxidants and antioxidants (Costantini 2014; Halliwell and Gutteridge 2015). Oxidative status is of special interest to ecologists and eco-physiologists because of its strong relationship with animal health (Barja and Herro 2000; Halliwell and Gutteridge 2015; Smith et al. 2016; Costantini 2019; Vágási et al. 2019) and by extension with fitness (Cohen et al. 2008; Costantini 2014; Speakman et al. 2015; Vágási et al. 2019). For example, the potent antioxidant vitamin E is often provided as a dietary supplement to maintain redox balance in poultry (Gallus gallus domesticus; Surai et al. 2019). While an increase in foraging activity during periods of low resource availability resulted in an increase in the production of pro-oxidants in the Seychelles warbler (Acrocephalus sechellensis; van de Crommenacker et al. 2011).

While experimental studies (Jimenez et al. 2020; Hawley and Altizer 2011; Martin et al. 2011; Schultz et al. 2020) have examined the link between diet and changes in immune function and/or shifts in oxidative status, it is unknown if seasonal adjustments in those physiological conditions are related to more complex dietary variables such as the diversity of consumed resources or trophic level. Animals living in seasonal environments likely experience an expansion of their dietary niche width when preferred resources are scarce, which may also result in a concomitant shift in trophic position (Jaeger et al. 2010; Maldonado et al. 2017; Sanchez-Hernandez et al. 2020). Characterization of seasonal niche variation is important to assess if these more complex dietary features are linked with immune and oxidative status.

Stable isotope analysis (SIA) is a widely used tool in studies of foraging ecology and is especially useful to quantify dietary variation and temporal shifts in diet composition at both the population and individual level (Hobson and Clark 1992; Kelly 2000; Peterson and Fry 1987; Newsome et al. 2015). Here, we use SIA to evaluate the relationship between immune function and oxidative state with dietary variation and trophic level in three passerines belonging to different foraging guilds: the granivorous Black-chinned siskin (*Spinus barbatus*), the omnivorous rufous-collared sparrow (*Zonotrichia capensis),* and the insectivorous house wren (*Troglodytes aedon*). Previous studies show that these species at our study site in central Chile shift their diets seasonally, which in some cases are associated with morphological and physiological adjustments (Novoa 1993, Swansson 2010; Ramirez-Otarola et al. 2018). We hypothesized that the constitutive immune variables and antioxidant capacity are enhanced in summer relative to winter due to increased food (insect and seed) availability during the warmer months. We decided to assess the hemolysis and hemagglutination scores, two components of constitutive innate immunity that represent the first line of defense against infection (Ochsenbein and Zinkernagel 2000, Panda and Ding 2015, Reyneveld et al. 2020). We used these indices because they reflect an individual's ability to eliminate foreign cells and, therefore, fight infection (Matson et al. 2005). Additionally, these two immune variables are not only related with survival, but also to other fitness components like fecundity and mate selection (Møller and Haussy 2007; Parejo and Silva 2009; Roast et al. 2020). Studying how immune function and oxidative status varies according to environmental variables that influence resource availability and quality can help us to understand the potential effects of environmental change on the adaptive strategies of birds and their population dynamics.

Materials and methods

Sample collection. We captured adult birds belonging to three passerine species with different dietary habits in austral summer (January) and winter (August) of 2021, including 68 (summer: $n = 41$; winter: $n = 27$) *Zonotrichia capensis* (omnivore), 51 (summer: $n = 29$; winter: $n = 22$) *Troglodytes aed*on (insectivore), and 34 (summer: $n = 14$; winter: $n = 20$) *Spinus barbatus* (granivore). Birds were captured using mist nets, between 8:00 and 11:00 am, at Quebrada de la Plata (33°30′S, 70°54′O, 800 m.a.s.l.) in central Chile, a Mediterranean locality characterized by hot and dry summers and cold winters (Di Castri and Hajek 1976). Sites were shortly monitored to choose sampling sites and mist nets were installed in shaded areas, left undisturbed, and checked every 15 min. After capture, bird body mass was measured to the nearest 0.01 g using a precision scale (Bel Italia ES 1001), then ca. 50–70 µl of blood was collected from the brachial vein with heparinized capillaries and stored on ice

for transport to the laboratory. We decided to measured body mass and not body condition because mass is a valuable metric for comparing individuals and populations that is not influenced by factors such as feather density or molt stage, which can influence body condition scores (Kalnins et al. 2022). Moreover, body mass is correlated with various fitness traits, including reproductive success and survival in numerous bird species (Downs et al. 2019, Kalnins et al. 2022; Milenkaya et al. 2015; Ruhs et al. 2020, Scanes 2016). After blood collection, the birds were released at the site where they were captured. The entire duration from when the animal was first restrained until blood collection was finished did not surpass 2 min. At the laboratory, samples were centrifuged for 15 min at 18,000*g* to separate plasma from red blood cells (M240R, BOECO Germany). The time that elapsed between taking the blood sample and its centrifugation in the laboratory did not exceed 4 h. Plasma samples were stored at − 80 °C for subsequent analysis.

Immune variables. To quantify the constitutive immune function of birds, we measured two parameters in plasma samples using the hemolysis–hemagglutination assays (Matson et al. 2005): (1) natural antibody-mediated hemagglutination and (2) complement-mediated hemolysis titers. Given our limited sample size, we adjusted the original protocol by reducing the plasma volume from 25 µL to 10 µL. As a result, we proportionally adjusted the quantity of all reagents to ensure appropriate scaling (Brust et al. 2022; Names et al. 2021; Pigeon et al. 2013). We used a 96-round bottom well plate for the hemolysis–hemagglutination assay. We added 10 µl of phosphate-buffered saline (PBS) from row 2 to row 12, and then added 10 µl of plasma samples into the first and second row, followed by serial dilutions from row 2 to row 12. We then added 10 µl of 1% rabbit red blood cells (Rockland Immunochemicals Inc.) to each well and mixed the plate gently. We covered the plate with parafilm and incubated it for 90 min at 37 °C. After incubation, plates were tilted at an angle of 45° at room temperature for 20 min before we measured the hemagglutination titer. Plates were then maintained at room temperature for 70 additional minutes before measuring hemolysis score. We expressed the hemagglutination and the hemolysis titer as the negative log2 of the last dilution in which each function was still observable. Samples were randomized with respect to each ID and the same person evaluated all scans.

Oxidative status. We assessed oxidative status by measuring two biomarkers in blood plasma samples: (1) total antioxidant capacity (TAC) that measures the presence of molecular antioxidants, and (2) lipid peroxidation as a measurement of oxidative damage. We estimated TAC concentrations using a commercial assay kit (Oxiselect STA-360, CellBioslabs, Inc). Due to the limited samples sizes of plasma, we performed TBARS and TAC assays using diluted samples at a 1:10 ratio. For dilutions, we use phosphate-buffered saline (PBS) as dilution solution according to the protocol provided by the manufacturer (CellBioslabs, Inc). We transferred 200 µl of each diluted sample to a 96-well microplate and then added 180 µl of reaction buffer to each well and mixed thoroughly. The initial absorbance of each sample was read at 490 nm. Next, 50 µl of copper ion reagent was added to initiate the reaction, and then the reactions were incubated for 5 min in an orbital shaker. After the incubation period, 50 µl of a stop solution (Part 236004, Cell BiosLAb, Inc) was added to each well to cease the reaction. Final absorbance was read at 490 nm. The final concentration was calculated according to the manufacturer's suggestion using the equation obtained from the regression between absorbance and concentration from the standard curve, and then multiplied by the dilution factor. The standard curve was elaborated using a uric acid standard.

Plasma lipid peroxidation was measured by spectrophotometric determination of Thiobarbituric Acid Reactive Substances (TBARS) with a commercial assay kit (OxiSelect STA- 330, Cell BiosLAb, Inc.) TBARS allows one to quantify malondialdehyde (MDA) concentration in blood plasma. The thiobarbituric acid reacts with MDA forming a fluorescent compound that can be detected in a spectrophotometer (Dasgupta and Klein 2016). We transferred 100 µl of diluted each blood plasma sample to separate microcentrifuge tubes, added 100 µl of SDS Lysis Solution, and mixed thoroughly. The tubes were incubated for 5 min at room temperature. We then added 250 µl of TBA reagent to each tube and mixed thoroughly, which was incubated for 45 min at 95 °C. After the incubation period, each tube was allowed to cool down to room temperature. Finally, we transferred 200 µl of each microcentrifuge tube to a 96-well microplate and read the absorbance at 532 nm. The standard curve contained MDA concentrations ranging from 0 to 125 µM. The final concentration was calculated according to the manufacturer's suggestion using the equation obtained from the regression between absorbance and concentration from the standard curve, and then multiplied by the dilution factor. For the elaboration of the standard curve we used an MDA standard.

Stable isotope analysis

Upon capture, we obtained a whole blood sample from the brachial vein, which was transferred to a clean microscope slide and allowed to dry in the field then transported to the laboratory. In the laboratory, the samples were dried at 60 °C in an oven for an additional week, after which the dried whole blood sample were scraped off the slide using a razor blade and weighed (0.5–0.6 mg) into tin capsules. Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) values of whole blood reflect recent dietary inputs ~ 30–60 days prior to sample collection (Bauchinger and MacWilliams 2009; Newsome et al. 2015), so the isotopic composition of this tissue is a robust

proxy for seasonal diet (Hobson and Clark 1992; Martınez del Rio et al. 2009; Vander Zanden et al. 2015; Maldonado et al. 2017). Stable isotope values are expressed using standard delta (δ) notation as deviations in parts per thousand (‰) according to the equation: $\delta X = (R_{sample}/R_{Standard} - 1) \times 1000$, where X corresponds to ^{15}N or ^{13}C and R_{sample} and $R_{standard}$ correspond to the molar ratios of $^{15}N/^{14}N$ or $^{13}C/^{12}C$ of the sample and reference material, respectively. Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values were measured with a Costech 4010 elemental analyzer (Valencia, CA) coupled to a Thermo Scientific Delta V Plus isotope ratio mass spectrometer (Bremen, Germany) at the University of New Mexico Center for Stable Isotopes (Albuquerque, NM, USA) Stable isotope values are reported in standard delta notation (δ) as deviations in parts per thousand (‰) according to the following equation: $\delta X = (Rsample/Rstandard - 1) \times 1000$, where X represents ^{15}N or ^{13}C and Rsample and Rstandard correspond to the $^{15}N/^{14}N$ and $^{13}C/^{12}C$ molar ratios of the sample and reference material, respectively. Analyses were normalized to three in-house laboratory reference materials that were calibrated against IAEA N1, IAEA N2, and USGS 43 for $\delta^{15}N$ and NBS 21, NBS 22, and USGS 24 for $\delta^{13}C$. Analytical precision was calculated from the analysis of reference materials was ± 0.1‰ for both $\delta^{13}C$ and $\delta^{15}N$. To determine the relative trophic level (TL) of individuals in each species, we followed the method described by Post (2002) using the formula: $TL = (1 + [\delta^{15}Nanimal - \delta^{15}Nproducers]/\Delta^{15}N)$, where $\delta^{15}Nanimal$ represents the nitrogen isotope values of tissue samples collected from the consumer, $\delta^{15}Nproducers$ is the isotopic signature of primary producers at the base of the food web, and $\Delta^{15}N$ is the trophic discrimination factor. We used a mean $\Delta^{15}N$ value of 3.4‰, which may slightly underestimate the relative trophic position of birds at higher trophic levels as trophic discrimination increases with increasing dietary protein content. (Martinez del Rio et al. 2009). Stable isotopes analysis was carried out in the Laboratory of Biogeochemistry and Applied Stable Isotopes (LABASI), Pontificia Universidad Católica de Chile.

Statistical analysis

Statistical analyses were performed in R through the RStudio interface (RStudio version 2022.07.2 + 576) (Posit 2023). All data were examined for assumptions of normality and homogeneity of variance using Kolmogorov–Smirnov and Levene tests prior to statistical analysis. When the variables were not normally distributed, we logarithmically transformed data for statistical analysis.

We performed an analysis of variance (ANOVA) separately for each species to evaluate differences between seasons in body mass, immune parameters, trophic level, and oxidative variables. In some cases, we used a non-parametric equivalent test to evaluate significant differences between seasons because the data were not normally distributed. To evaluate seasonal changes in the $\delta^{13}C$ and $\delta^{15}N$ values of whole blood obtained in winter and summer, we performed a multivariate analysis of variance (MANOVA), with isotope values as the dependent variables and season as the independent factor. $\delta^{13}C$ and $\delta^{15}N$ values of plants collected at our field site did not significantly differ between winter and summer (supplementary material), so we did not correct for (baseline) seasonal variation in the isotopic composition of the base food of the food web. To evaluate the relationship between isotopic signature and immunological and oxidative variables, we performed analysis of spearman correlations, because the data were not normally distributed.

We estimated the isotopic niche width of each species using standard ellipse areas corrected for small sample size (SEA_C; Jackson et al. 2011). To compare isotopic niche widths among seasons, we constructed Bayesian standard ellipse area (SEA_B) with Markov chain Monte Carlo simulations (10,000 iterations) using Stable Isotope Bayesian Ellipses in R (SIBER; Jackson et al. 2011), which allowed us to estimate the probability that isotopic niches were larger or smaller than one another.

Results

We found a significant increase in body mass in winter compared with summer in both *T. aedon* ($F_{1,28} = 9.52$, $p = 0.01$) and *S. barbatus* ($F_{1,5} = 15.5$, $p = 0.01$; Table 1); no differences in body mass were observed between seasons in *Z. capensis* ($F_{1,38} = 2.89$, $p = 0.09$; Table 1). Regression analysis showed that immunological variables and oxidative parameters were unaffected by body mass for any species (see Table S1).

Hemolysis scores in *Z. capensis* were significantly higher in winter than summer ($F_{1,48} = 6.01$, $p = 0.02$; Fig. 1a), while no seasonal variation was observed in *T. aedon* ($F_{1,26} = 0.05$, $p = 0.82$) and *S. barbatus* ($F_{1,5} = 0.43$, $p = 0.54$). Hemagglutination scores did not differ between seasons for any species (Fig. 1b); *Z capensis*: $F_{1,48} = 0.12$, $p = 0.74$; *S. barbatus*: $F_{1,5} = 1.57$, $p = 0.27$; *T. aedon*: $F_{1,26} = 0.18$, $p = 0.68$. Blood

Table 1 Mean (± SE) body mass of *Z. capensis*, *T. aedon,* and *S. barbatus* captured in summer and winter

Species	Body mass (g)	
	Summer	Winter
Zonotrichia capensis	20.6 ± 0.5[a]	21.7 ± 0.2[a]
Troglodytes aedon	9.7 ± 0.2[a]	10.5 ± 0.2[b]
Spinus barbatus	14.3 ± 0.7[a]	15.2 ± 0.4[b]

Different letters denote significant differences

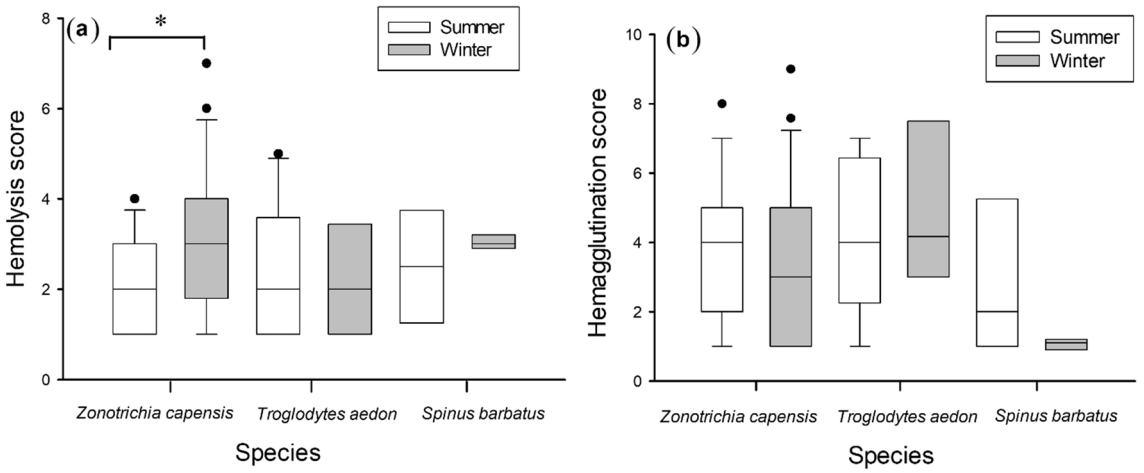

Fig. 1 Hemolysis and hemagglutination scores of *Z. capensis, T. aedon* and *S. barbatus* captured in summer (open boxes) and winter (closed boxes). In panel (**a**), the hemolysis score was higher in winter than in summer for *Z. capensis*; whereas for the other species, the hemolysis score did not show significant differences between the two seasons. In panel (**b**), the hemagglutination score did not exhibit significant differences between summer and winter for any of the species. Error bars represents the 95% confidence interval, the bottom and top of the box corresponds to the 25th and 75th percentiles, the line inside the box represent the 50th percentile (median), and outliers are shown as closed circles

plasma TAC were higher in winter than summer (Fig. 2a) for *Z. capensis* ($F_{1,7} = 5.31$, $p = 0.02$) and *S. barbatus* ($F_{1,5} = 11.41$, $p = 0.02$, while no seasonal differences were observed in *T. aedon* ($F_{1,8} = 0.02$, $p = 0.89$). Lipid peroxidation did not differ between seasons for any species (Fig. 2b); *Z. capensis*: $F_{1,38} = 1{,}23$, $p = 0.27$; *S. barbatus*: $F_{1,5} = 0.53$, $p = 0.58$; *T. aedon*: $F_{1,8} = 0.09$, $p = 0.78$.

We found significant differences between seasons in the isotopic niche of all three species (MANOVA; Z. capensis: $F_{1,30} = p = 0.002$; *T.aedon*: $F_{1,32} = 9.2$, $p < 0.001$; S. barbatus: $F_{1,29} = 7.57$, $p = 0.002$). Analysis showed that *Z. capensis* had higher $\delta^{15}N$ values in summer (ANOVA; $F_{1,59} = 8.46$, $p = 0.005$) (Fig. 3). Instead, $\delta^{15}N$ values in *T. aedon* (ANOVA; $F_{1,32} = 5.33$, $p = 0.16$) and *S. barbatus* ($F_{1,30} = 1.42$, $p = 0.24$) did not differ between seasons. The $\delta^{13}C$ values were higher during summer than in winter in *S. barbatus* (ANOVA; $F_{1,30} = 15.05$, $p = 0.0005$) and *T. aedon* (ANOVA; $F_{1,33} = 22.02$, $p < 0.001$). The correlation

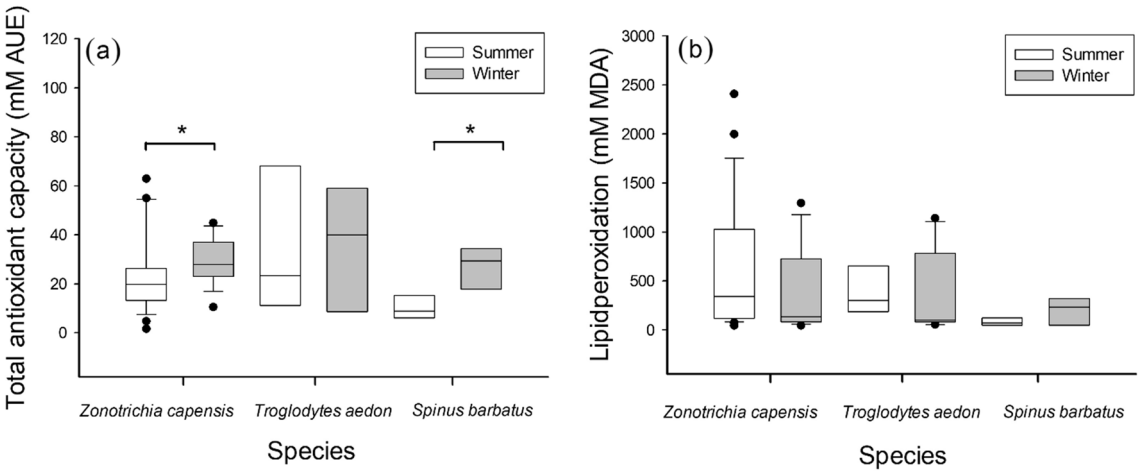

Fig. 2 "Hemolysis and hemagglutination scores of Z. capensis, T. aedon, and S. barbatus captured in summer (open boxes) and winter (closed boxes). In panel (**a**), the hemolysis score was higher in winter than in summer for *Z. capensis*; whereas for the other species, the hemolysis score did not show significant differences between the two seasons. In panel (**b**), the hemagglutination score did not exhibit significant differences between summer and winter for any of the species. Error bars represents the 95% confidence interval, the bottom and top of the box corresponds to the 25th and 75th percentiles, the line inside the box represent the 50th percentile (median), and outliers are shown as closed circles."

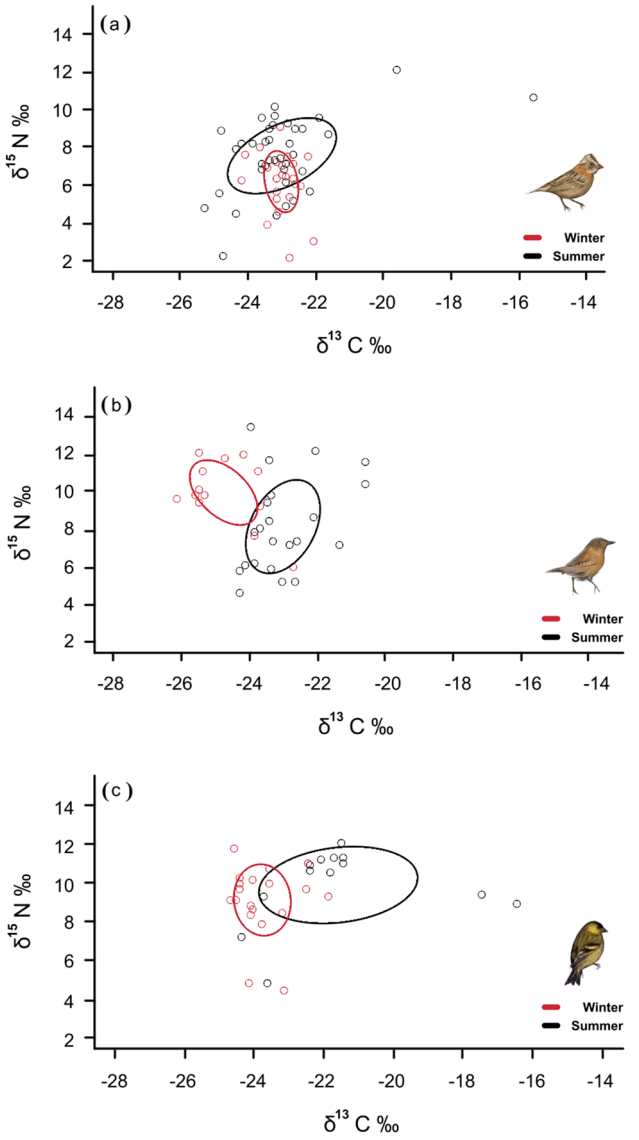

Fig. 3 Standard ellipses (SEA_C) estimated from carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values of blood collected in summer (black) and winter (red) for **a** *Zonotrichia capensis*, **b** *Troglodytes aedon*, and **c** *Spinus barbatus* captured in central Chile. Each point represents an individual and ellipses represent the 95% ellipse area. Numbers next to each ellipse correspond to the size ($‰^2$) of each ellipse. The isotopic niche width of *Z. capensis* and *S. barbatus* is smaller during winter, while there are no significant differences in the isotopic niche width between seasons for *T. aedon*, suggesting a relatively consistent trophic niche throughout the year

analysis revealed that neither the hemolysis nor the hemagglutination score showed a significant correlation with tissue $\delta^{15}N$ values of any species (Table 2). As for the oxidative status variables, we observed a significant and negative correlation between the levels of TAC and the mean $\delta^{15}N$ value of S. barbatus during winter; however, we did not find significant correlations for the other species and seasons (Table 2).

Table 2 Correlation analysis between the innate immunological and oxidative variables and isotopic signature $\delta^{15}N$ in summer and winter for *Z. capensis*, *T. aedon,* and S. barbatus

Species	Season	Variable	$\delta^{15}N$	
Zonotrichia capensis	Summer	Hemolysis	$r=0.11$	$p=0.51$
		Hemagglutination	$r=-0.08$	$p=0.65$
		Lipid peroxidation	$r=-0.03$	$p=0.91$
		TAC	$r=-0.26$	$p=0.22$
	Winter	Hemolysis	$r=-0.17$	$p=0.44$
		Hemagglutination	$r=-0.08$	$p=0.72$
		Lipid peroxidation	$r=0.19$	$p=0.91$
		TAC	$r=-0.09$	$p=0.74$
Troglodytes aedon	Summer	Hemolysis	$r=0.37$	$p=0.24$
		Hemagglutination	$r=0.09$	$p=0.79$
		Lipid peroxidation	$r=0.41$	$p=0.49$
		TAC	$r=-0.21$	$p=0.75$
	Winter	Hemolysis	$r=0.55$	$p=0.1$
		Hemagglutination	$r=0.03$	$p=0.93$
		Lipid peroxidation	$r=-0.09$	$p=0.85$
		TAC	$r=-0.59$	$p=0.16$
Spinus barbata	Summer	Hemolysis	$r=0.04$	$p=0.6$
		Hemagglutination	$r=-0.17$	$p=0.26$
		Lipid peroxidation	$r=-0.03$	$p=0.6$
		TAC	$r=0.02$	$p=0.6$
	Winter	Hemolysis	$r=-0.21$	$p=0.74$
		Hemagglutination	$r=-0.2$	$p=0.8$
		Lipid peroxidation	$r=-0.3$	$p=0.62$
		TAC	$\mathbf{r=-0.85}$	$\mathbf{p=0.001}$

Significant correlations are presented in bold

Isotopic niches of *Z. capensis* and *S. barbatus* were smaller in winter than summer ($p<0.05$, Table 3), while the isotopic niche of *T. aedon* was similar in size between seasons (Table 3, Fig. 3). The degree of overlap in isotopic niche between seasons was 20% for *Z. capensis*, 17% for *T. aedon,* and 24% for *S. barbatus* (Fig. 3). Finally, *Z. capensis* occupy a higher trophic level in summer than in winter ($p<0.05$, Table 3), while the trophic level of *T. aedon* and *S. barbatus* remains constant between seasons (Table 3).

Table 3 Mean (±SD) $\delta^{13}C$ and $\delta^{15}N$ values, trophic level and associated estimates of standard ellipse areas of blood collected from *Z. capensis*, *T. aedon,* and *S. babartus* in summer and winter

Species	$\delta^{13}C$ (‰)	$\delta^{15}N$(‰)	SEA_B (‰^{2})		*p*	Trophic level	*F*, df, *p*
			Mean	95% CI			
Zonotrichia capensis							
Summer (*n* = 41)	− 23.0 ± 1.6	7.6 ± 1.9	9.1	6.3–12.1	0.009	2.3 ± 0.09[a]	$F_{1,63} = 6.02$, $p = 0.02$
Winter (*n* = 27)	− 23.1 ± 0.5	6.1 ± 1.6	2.6	1.6–3.7		1.7 ± 0.47[b]	
Troglodytes aedon							
Summer (*n* = 29)	− 23.0 ± 1.1	8.2 ± 2.5	8.3	5.0–12.0	0.11	2.2 ± 0.11[a]	$F_{1,41} = 2.61$, $p = 0.11$
Winter (*n* = 22)	− 24.5 ± 1.1	10 ± 1.7	5.3	2.6–8.5		2.7 ± 0.16[a]	
Spinus barbatus							
Summer (*n* = 14)	− 21.6 ± 2.3	9.9 ± 1.9	14.9	6.7–23.2	0.002	2.5 ± 0.3[a]	$F_{1,31} = 0.014$, $p = 0.91$
Winter (*n* = 20)	− 23.7 ± 0.8	9.1 ± 1.8	4.9	2.8–7.1		2.6 ± 0.27[a]	

Samples sizes are noted in parentheses next to season of collection. Different letter denotes significant differences between seasons

Discussion

Some studies have proposed that variation in bird immunity among seasons is primarily driven by a trade-off between reproduction and molt (Sheldon and Verhust 1996; Martin 2005; Moreno-Rueda 2010; Hegemann et al. 2012), or possibly due to higher parasite loads in the wet versus dry season in tropical environments (Horrocks et al. 2015). Other studies have proposed that seasonal shifts in immune function are the result of changes in resource availability and/or quality (Gasparini et al. 2006; Jackson et al. 2020). We tested the hypothesis that immune function and oxidative status shift seasonally and are correlated with dietary breadth and trophic level and found partial support for our predictions.

Contrary to *S. barbatus* and *T. aedon*, which showed seasonal shifts in only one dietary niche variable, the omnivorous species *Z. capensis* exhibited significant changes in both niche breadth and trophic position (Table 3). Specifically, *Z. capensis* exhibited a decrease in dietary diversity and relative trophic level during the winter. Our findings are consistent with those reported by Lopez-Calleja (1995), who observed that *Z. capensis* reduces its consumption of insects during winter, leading to a narrower trophic niche breadth. This decrease in insect consumption is likely a result of the reduced insect abundance during the winter season (Lopez-Calleja 1995). In contrast, *S. barbatus* exhibited a reduction in its isotopic niche during winter, but this change was not accompanied by a shift in $\delta^{15}N$ or relative trophic level (Table 3), suggesting that this species maintains a consistent trophic position throughout the year (Ramirez-Otarola et al. 2011; Fuentes-Castillo et al. 2016). In contrast, insectivorous *T. aedon* exhibited no seasonal change in $\delta^{15}N$ values, isotopic niche width, or trophic level (Table 3). The observed results could be the result of this species' diet being dominated by insects in both summer and winter (Ramirez-Otarola et al. 2011).

The analysis of immune variables revealed that only *Z. capensis* exhibited seasonal differences in immune capacity, as indicated by hemolysis scores. Considering this finding and the observed seasonal changes in the trophic niche and trophic level of *Z. capensis*, higher seed intake during winter may be linked to increased consumption of specific nutrients, such as vitamins, carbohydrates, and fatty acids (Klasing 1998; Twining et al. 2016; Al-Khalaifah and Al-Nasser 2021). These nutrients have been shown to enhance T-cell development (Humprey and Rudrappa 2008) and modulate humoral and cellular immunity in poultry (Bhanja et al. 2015). However, the correlation analyses between $\delta^{15}N$ and hemolysis and hemagglutination scores did not reveal any significant associations. Therefore, the observed seasonal change in hemolysis scores may not be directly related to shifts in resource use between seasons and may be more related to life history and environmental conditions, which have been shown to influence immune function (Bowden et al. 2007; Hasselquist et al. 2007; Moore and Siopes 2002; Nelson and Demas 1996; Nelson et al. 2002; Pap et al. 2015; Schultz et al. 2020; Weil et al. 2015). For example, previous studies on both captive and wild birds reported that immune function is enhanced in winter due to abiotic conditions such as photoperiod (Nelson and Demas 1996; Nelson et al. 2002; Moore and Siopes 2002; Weil et al. 2015), parasitism (Hasselquist et al. 2007; Pap et al. 2015) or decreases in ambient temperature (Bowden et al. 2007; Schultz et al. 2020). Our Mediterranean study site is characterized by marked seasonality with hot/dry summers and cold/wet winters, environmental cues that are known to trigger metabolic responses in these passerine species (Maldonado et al. 2009) and, therefore, could also account for the observed seasonal variation in immune response. Furthermore, the absence of seasonal changes in the immune variables in *T. aedon* and *S. barbatus* may be attributed to the same factors mentioned previously. These could include lower abundance of parasites (Pap et al.

2010a) or hormone levels (e.g., melatonin) (Martin et al. 2008; Pap et al. 2010b).

Based on previous studies, omnivorous birds are more susceptible to a broader range of pathogens due to their diverse food sources in comparison to strictly granivorous or insectivorous species (Bandelj 2015; Dolnik et al. 2010). Conversely, it has been observed that the presence of pathogens varies between seasons in both tropical (Ferreira Junior et al. 2017) and temperate habitats (Tinsley et al. 2011). Therefore, to what extent the omnivorous *Z. capensis* is susceptible to pathogens, which would necessitate a more efficient and adaptable immune system to counter the pathogens encountered in each season, is a topic that needs further attention.

The TAC levels in *Z. capensis* and *S. barbatus* were highest in winter, and lipid peroxidation remained constant between seasons in all species (Fig. 2). Additionally, correlation analysis revealed that $\delta^{15}N$ values were not correlated with either TAC or lipid peroxidation levels in any season in *Z. capensis*. In contrast, we observed a significant negative correlation between TAC and $\delta^{15}N$ values in *S. baratus* during the winter season. These findings suggest that higher consumption of plant-derived resources in *S. barbatus* may be associated with elevated TAC levels, but this association appears to occur only in the winter months. We hypothesize that *S. barbatus* may focus on just a few preferred high-quality plant resources in winter, resulting in narrow isotopic niches relative to summer (MacArthur and Pianka 1966), which is consistent with the observation of higher body mass in birds during this season (Table 1). This reduction in resource diversity may yield an increase in the consumption of specific nutrients and result in an increase in the expression of exogenous antioxidants that would enhance TAC. For example, birds cannot synthesize the essential fatty acid linoleic acid (Griminger 1986) and must acquire this compound from their diet (Lands 2016). Most seeds are rich in this nutrient (Bewly and Black 1982) and studies show that diets rich in linoleic acid enhance the enzymatic-origin antioxidant capacity in rats (Kim et al. 2005), pigeons (Xu et al. 2020), and chickens (Zhang et al. 2008).

On the other hand, we acknowledge that observed seasonal changes in TAC in *Z. capensis* and *S. barbatus* may be the result of other factors independent of diet because concentrations of antioxidants in birds are affected by multiple variables including hormone levels, life history traits, and ambient temperature (Lin et al. 2008; Cohen et al. 2008; Cohen et al. 2009a; Norte et al. 2009). For example, Xia and Moller (2018) demonstrated that long-lived bird species have higher TAC than short-lived species. In contrast to the other two species, we did not observe any seasonal changes in lipid peroxidation or antioxidant capacity in *T. aedon* (Fig. 2a). Finally, changes in TAC concentration were not related to shifts in hemolysis scores in summer or winter for *Z. capensis* and *S. barbatus* (Supplementary Material), suggesting these variables may represent different aspects of health in these species.

In conclusion, our study findings indicate that seasonal variation in the diet of two of the three study species were not correlated with changes in some components of the constitutive innate immunity. The seasonal change in hemolysis score observed in *Z. capensis* is an interesting result because this immune variable is related to survival, fecundity, and mate selection (Møller and Haussy 2007; Parejo and Silva 2009; Roast et al. 2020), Thus, the modifications of hemolysis score between seasons in *Z. capensis* may be a benefit in terms of increased fitness. On the other hand, in the case of *S. barbatus,* the antioxidant capacity was found to be related to diet characteristics in winter. We hypothesize that this relationship is driven by seasonal variation in the consumption of specific nutrients that enhance the antioxidant capacity but not the immune function. However, our results should be interpreted with caution because we did not measure the nutritional content of diets available in summer and winter. Further evaluation of the relationship of resource nutritional quality, especially in terms of specific (essential) nutrients, and immune function and oxidative state is required to better understand of the effect of seasonal dietary variation on the performance and fitness of wild birds. Furthermore, it is important to acknowledge that the analysis of only two constitutive immune and oxidative variables may pose a limitation in interpreting our results comprehensively. We cannot rule out the possibility that other types of immune responses, such as cellular-mediated immune responses, might be affected by seasonal changes in diet. For instance, Gasparini et al. (2006) reported a seasonal decline in IgY levels in *Rissa tridactyla* chicks, highlighting the importance of analyzing other variables to better understand the impact of seasonal changes in diet quality and availability on immune function and oxidative status. Finally, we are fully aware that physiological attributes can be influenced not only by ecological factors (e.g., diet composition) but also by phylogeny (Bloomerg and Garland 2002). Due to limitations in the number of available species for this study, which do not allow for phylogenetic informed analyses, we have chosen to focus solely on the dietary aspect. Nevertheless, caution must be exercised when interpreting the results obtained, as there may be underlying effects of phylogenetic history on immune function. Therefore, further investigations that consider the potential effects of phylogenetic relationships between species are necessary to gain a more comprehensive understanding of the seasonal changes in natural diet, immune function, and oxidative status.

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Availability of data and materials The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest Authors declare that there are no conflicts of interest.

Ethical approval Animals were captured with permits from SAG, Chile (No. 6406/2021). All protocols were approved by the Institutional Bioethics Committee of the Universidad Mayor.

Consent to participate Not applicable.

Consent for publication Not applicable.

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