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Seasonal changes in digestive enzymes in five bird species

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Running title: Seasonal changes in enzymatic activity

Seasonal changes in digestive enzymes in five bird species

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Abstract

Most animals must cope with seasonal fluctuations in environmental conditions, including variations in food availability and composition. Accordingly, it is expected that most species should exhibit reversible seasonal phenotypic adjustments in their physiology. Here we assessed seasonal variation in the activity of three digestive enzymes (sucrase, maltase and aminopeptidase-N) in one omniviorous (*Zonotrichia capensis* (Muller, 1776)), three granivorous (*Carduelis barbata* (Molina, 1782), *Diuca diuca* (Molina, 1782) and *Phrygilus fruticeti (*von Kittlitz, 1833)), and one insectivorous (*Leptasthenura aegithaloides* (von Kittlitz, 1830)) bird species. Based on the adaptive modulation hypothesis, we predicted that the omnivorous species should exhibit the largest seasonal variation in the activity of their digestive enzymes, in relation to granivorous and insectivorous species. We found that *Z. capensis* adjusts total activities of disaccharidases, total sucrase activity varied between seasons in *C. barbata*, and total activity of aminopeptidase-N only changed seasonally in *L. aegithaloides*. Moreover, this last species modified the tissue-specific activity of both disaccharidases as well as the wet mass of their intestine. Taken together, our results suggest that seasonal dietary changes occur in most of the species, regardless the trophic categories at which they belong. Consequently, a better knowledge of the diet, and it seasonal variation, is necessary to better account for the results recorded in this study.

Keywords: passerine, diet, digestive enzymes, birds, seasonal changes, *Zonotrichia capensis*, *Carduelis barbata, Diuca diuca*, *Phrygilus fruticeti*, *Leptasthenura aegithaloides,*

Black-chinned Siskin, Plain-mantled Tit-Spinetail, Common Diuca-Finch, Mourning Sierra-Finch, Rufous-collared Sparrow

Introduction

Most animals must face seasonal fluctuations in environmental conditions, including variations in food availability and composition. In this sense, it is expected that animals that do not migrate should exhibit reversible (seasonal) phenotypic adjustments in their physiology in order to face environmental variations. This type of response, which has been termed phenotypic flexibility (Piersma and Drent 2003), is an important determinant of fitness allowing organisms to respond to environmental variation at shorter than evolutionary time scales (de Jong 1995; Bicudo et al. 2010; Piersma and van Gils 2011). The response of both morphology and physiology of the digestive tract is a good example of how phenotypic flexibility allows organisms to cope with dietary switching (Starck 1999; Naya et al. 2007; Karasov et al. 2011). The flexibility of the digestive system includes changes in gut size, enterocytes features (e.g., size and microvilli length), nutrient uptakes rate, and enzymatic activity (Zaldúa and Naya 2014). For the particular of digestive enzymes, modulation has been well documented in the laboratory, revealing that enzymatic activity respond to changes in internal demands as well as to a variety of nutritional factors (Karasov 1996; Piersma and Lindström 1997; Starck 1999; McWilliams and Karasov 2001; Naya et al. 2007).

Birds exhibit a great variation in digestive morphological and biochemical traits (Stark 2005), including intra and inter-specific variation in several membrane-bound intestinal enzymes, such as sucrase, maltase and aminopeptidase-N (Martínez del Rio and Stevens 1989; Afik et al. 1995; Sabat 2000; Meynard et al. 1999; Sabat and Gonzalez 2003; Shondube and Martínez del Rio 2004). In a recent study, Ramirez-Otarola et al. (2011) reported that percentage of nitrogen in the diet was negatively correlated with maltase

activity for 16 bird species, concluding that interspecific differences in biochemical capacity are associated with nutrient contents of each species' natural diet. Furthermore, previous studies about dietary modulation of sucrase, maltase and aminopeptidase-N, suggest that exposure to contrasting diets affects the extraction efficiency, adjusting the rates of enzymatic hydrolysis, and uptake to the contents in diet (reviewed by Karasov 1996). Also, these studies conclude that generalist birds usually have greater levels of modulation of digestive enzymes than specialist species (e.g., Martínez del Rio 1990; Hernandez and Martínez del Rio 1992; Afik and Karasov 1995). However, to date most studies conducted in birds did not accounted for the putative effect that seasonal changes in natural diet could exert on the hydrolysis rate of tissue-specific digestive enzymes. In this sense, some studies conducted in hibernating ectotherms indicate that the tissue-specific and total activities of sucrase, maltase, and aminopeptidase-N are up-regulated during summer, in parallel to the annual feeding cycle of these species (Naya et al. 2009*a*, *b*). In addition, Liu et al. (2013) showed that in Mongolian gerbils, the tissue-specific and total activities of sucrase, maltase, and aminopeptidase-N were up-regulated during winter, demonstrating seasonal adjustments in the activity of these enzymes also occur in mammals.

In this study, we examine seasonal changes in the activity of sucrase, maltase and aminopeptidase-N in five species of passerine birds, with fairly contrasting dietary habits: the rufous collared sparrow (*Zonotrichia capensis*) is an omnivorous species that mainly predate on seeds and insects; the black-chinned siskin (*Carduelis barbata*), the diuca finch (*Diuca diuca*), and the mourning sierra-finch (*Phrygilus fruticeti*), are three granivorous species; and, finally, the plain-mantled tit-spinetail (*Leptasthenura aegithaloides*) is an

insectivorous species (Lopez-Calleja 1995; Jaksic 2001). Based on the adaptive modulation hypothesis, we expected that the omnivorous species should exhibits the largest seasonal variation in the activity of their digestive enzymes, in relation to species belonging to the other two trophic categories.

Material and Methods

Study area and collection of individuals

Individuals of *Z. capensis* (n = 6), *C. barbata* (n = 7), *D. diuca* (n = 13), *P. fruticeti* (n = 6) and *L. aegithaloides* (n = 3) were captured at Quebrada de la Plata (33º 31' S, 70º 50' W) during the austral winters of 2012 and 2013. For seasonal comparisons, we used data published by Ramirez-Otarola et al. (2011) and Sabat et al. (2013) obtained from individuals of the same species collected in the same site, but during summer months (*Z. capensis*: n = 14, *C. barbata*: n = 9, *D. diuca*: n = 11, *P. fruticeti*: n = 5, *L. aegithaloides*: n = 5). These samples sizes for summer months, were increased by the inclusion of two new specimens of *P. fruticeti* and five of *Z. capensis* that were captured in the austral summer of 2013. The study site has a Mediterranean climate with cool and wet winters and relatively dry and hot summers (di Castri and Hajek 1976). The highest temperatures are found from December to March (austral summer) with a mean temperature of 18.9 ºC and maximum daily temperature ranging from 26.9ºC to 29.7ºC. The lowest temperatures are found form June to August (austral winter) with a mean temperature of 8.3ºC and maximum daily temperature ranging from 14.9ºC to 16.7ºC. Annual mean precipitation is 367 mm, concentrated in June-August (208 mm), and during summer it is minimal representing only

3% of the annual total (12 mm) (http://www.worlclimate.com; http://worldweather.wmo.int).

All the individuals were captured with mist nets, and immediately after capture birds were killed by thoracic compression (American Ornithologist Union, 1988). Then, the small intestine was rapidly excised, flushed with ice-cold saline (0.9%), measured (0.1 cm) and weighed (0.001 g), before storage at -50 °C for enzyme activity determinations. All tissues were maintained frozen for no more than three weeks until the enzyme determinations were performed. The carcasses of the animals were immediately stored in sealed plastic bags and frozen at -20ºC for further incineration by the Hygiene and Safety Committee of the Faculty of Sciences of the University of Chile.

Enzyme activity measurements

We measured the activity of three intestinal enzymes that act on carbohydrates and proteins: sucrase-isomaltase (EC 3.2.1.48), maltase-glucoamilase (EC 3.2.1.20) and aminopeptidase-N (EC 3.4.11.2). Sucrase-isomaltaseis responsible for the hydrolysis of sucrose, while maltase-glucoamilase catalyzes the hydrolysis of maltose. We used both enzymes as indicators of the ability of digest and assimilate carbohydrates (Martínez del Rio 1990; Martínez del Rio et al. 1995). The exopeptidase aminopeptidase-N participates in the final digestion of the small peptides generated from the hydrolysis of proteins by gastric and pancreatic proteases (Martínez del Rio 1990; Martínez del Rio et al. 1995). We used this enzyme as an indicator of the birds' capacity to digest proteins.

For biochemical assays, tissues were thawed and the whole small intestine was homogenized (30 s at maximum setting in a ULTRA TURRAX T25 homogenizer Janke

and Kunkel, Breisgau, Germany) in 20 volumes of 0.9% NaCl solution. Disaccharidases activity was determined according to the method of Dahlqvist (1964) as modified by Martínez del Rio (1990). Briefly, tissue homogenates (35 µL) were incubated at 40°C with 35 µLof 56 mmol L^{-1} sugar solutions in 0.1 M Maleate/NaOH buffer, pH 6.5. After 10 min, reactions were stopped by adding 1 mL of a stop developing solution (one bottle of Glucose LS (VALTEK) in 250 mL 0.1 mol L^{-1} TRIS/HCl, pH 7). Absorbance was measured at 505 nm in a Multiskan Go Thermo Scientific multiplate reader after 18 min at 20°C. Aminopeptidase-N assays were done using L-alanine-p-nitroanilide as a substrate. Briefly, 25µL of homogenate diluted with 0.9% NaCl solution were mixed with 0.5 mL of assay mix (2.04 mmol L^{-1} L-alanine-pnitroanilide in 0.2 mol L^{-1} NaH2PO4/Na2HPO4, pH 7). The reaction was incubated at 40°C and arrested after 10 min with 1.5 mL of ice-cold acetic acid 2N, and absorbance was measured at 384 nm in a Multiskan Go Thermo Scientific multiplate reader. On the basis of absorbance constructed for glucose and p-nitroanilide, total intestinal enzymatic activities were calculated. We measured the concentration of protein in the homogenate using the commercial Coomassie Plus Protein Assay Reagent (Pierce). Because total protein was highly positively correlated with the mass of tissue (r^2= 0.90 $P < 0.001$), data are presented as total (µ mol min^{-1}) and tissue-specific (µ mol min^{-1} g^{-1}) hydrolytic activities.

Data analysis

Differences between seasons in body mass and tissue-specific enzymatic activities were evaluated separately for each species using a one-way analysis of variance (ANOVA). Seasonal differences in small intestine size and total enzymatic activity were evaluated separately for each species using a one-way analysis of co-variance (ANCOVA), with body

mass as covariate. Prior to all statistical analyses, data were examined for assumptions of normality and homogeneity of variance using Kolmogorov-Smirnov and Levene tests, respectively. In some cases, data were log-transformed to meet the assumptions of the analyses (e.g., total maltase activity in *Z. capensis*). Interactions between the covariate (body mass) and the factor (season) were tested using a parallelism test, and a separate slope ANCOVA model was used when necessary (e.g., intestine wet mass in *L. aegithaloides*). Results are given as absolute mean ± 1 S.E. (body size, tissue-specific enzymatic activities) or least square adjusted mean ± 1 S.E. (intestine size, total enzymatic activities). Statistical significance was established at the 0.05 level, and all the analyses were performed using Statistica 7® (1997) for Windows.

Results

Body mass and wet mass of the intestine only changed between seasons for the insectivorous species, *L. aegithaloides* (Table 1). For this species, body mass was greater during winter than during summer ($F_{1,6}$ = 7.4, P = 0.03), while the opposite pattern of variation was observed for small intestine wet mass ($F_{1,4}$ = 16.8, P = 0.01). Small intestine length did not change between seasons for any of the five species analyzed (Table 1). As for enzymatic determinations, tissue-specific sucrase activity in *L. aegithaloides* ($F_{1,6}$ = 15.9, P < 0.01), and total sucrase activity in the omnivorous species (*Z. capensis*, $F_{1,17}$ = 6.8, P = 0.02), was higher during winter than during summer (Fig. 1). By contrast, tissue-specific and total activity of sucrase was higher during summer in one of the three granivorous species (*C. barbata*, $F_{1,13}$ = 8.1, P = 0.01 and $F_{1,12}$ = 5.2, P = 0.04,

respectively) (Fig. 1). Similarly, tissue-specific and total activity of maltase in *Z. capensis* ($F_{1,18}$ = 4.49, P = 0.05 and $F_{1,18}$ = 8.7, P = 0.01, respectively), and tissue-specific maltase activity in *L. aegithaloides* ($F_{1,6}$ = 80.1, P < 0.01), was higher during winter than during summer (Fig. 1). Neither tissue-specific nor total activity of this enzyme changed between seasons for any of the three granivorous species (Fig. 1). Finally, tissue-specific and total activity of N-aminopeptidase only changed between seasons in *L. aegithaloides* ($F_{1,6}$ = 23.8, P < 0.01 and $F_{1,5}$ = 8.1, P = 0.04, respectively), being higher during summer than during winter (Fig. 1).

Discussion

It has been proposed that animals that face fluctuations in their diets should modulate, rather than maintain high levels of specific enzymes, because the metabolic costs of synthesizing and maintaining large amounts of digestive enzymes (Karasov and Hume 1997). According to this hypothesis (i.e., the adaptive modulation hypothesis), the transport of monosaccharides or amino acids should be positively related to the dietary level of these substrates, and the modulation of the digestive traits should be present in omnivorous species or in species with important variation in their diets (Karasov and Diamond 1983; Ferraris and Diamond 1989; Buddington et al. 1991; Karasov 1992). Hence, for the particular set of species here analyzed, we expected to find markedly seasonal variations in enzymatic activity in the omnivorous species (*Z. capensis*), which switch their diet between summer and winter, but not in insectivorous and granivorous species with little dietary variation among seasons. However, we only found partial support for this idea. That is,

while we found that *Z. capensis* effectively adjusts total activities of sucrase and maltase, we also found that total sucrase activities varied between seasons for one granivorous species (*C. barbata*) and that total activity of aminopeptidase-N only changed in the insectivorous one (*L. aegithaloides*). Moreover, this last species was the only one that modified the tissue-specific activity of both disaccharidases as well as the wet mass of their intestine. Thus, it could be say that the insectivorous species was the one that showed the largest amount of seasonal flexibility for the digestive traits here assessed.

As for the direction of the changes, we find that total activities of sucrase and maltase were higher during winter than during summer in *Z. capensis*. This pattern is not surprising since this species is a generalist that consumes seed and insects. Given that the consumption of insects by *Z. capensis* is higher during summer when the abundance of insects is the highest (Lopez-Calleja 1995; Sabat et al. 1998), the increase in the activity of disaccharidases in winter would be related to a seed-based diet during this season. In addition, it is known that in the laboratory the rufous sparrow modulates the activities of their digestive enzymes when fed on synthetic diets (Sabat et al. 1998). For *C. barbata* we found that total sucrase activity was higher during winter than during summer. This species is characterized by a high consumption of seeds during summer (Ramírez-Otárola et al. 2011), and is supposed to show little dietary variation through the year. However, the diet of this species had not been extensively studied during winter, and the enzymatic adjustments recorded here suggest that some minor seasonal changes, probably related with the incorporation of different types of seed, may occur. For *L. aegithaloides* we found that both tissue-specific and total aminopeptidase-N activity was up-regulated during summer months, when the size of the intestine was larger. This produced an amplification of the

overall change in the activity of this enzyme, reaching an increase of 670% over winter values. This adjustment was by far the largest adjustment recorded for all the five species analyzed. In addition, at winter, when the size of the intestine was smaller, tissue-specific activity of both disaccharidases were up-regulated, causing that overall activity of these enzymes did not change seasonally. Finally, neither tissue-specific nor total enzymatic activity was affected by season in two of the three granivorous species (*D.diuca* and *P. fruticeti*). These results agree with dietary data. In fact, the diuca finch consumes 97% of seed during summer and 100% in winter, with no significance differences between seasons (Lopez-Calleja 1995; Sabat et al. 1998). Although dietary information for *P. fruticeti* is scarcer, data obtained for a few individuals collected during summer indicate that it consumes 99% seeds (Ramirez-Otarola et al. 2011) whereas in winter seed consumption is about 98% (Lopez-Calleja 1995).

Unfortunately, our study does not include information regarding the current diet that individuals consumed at the time of capture and we were only able to make inferences about the effect of the diet based on information obtained from the literature. This is important to consider since some of the analyzed species may present a variation in the diet in shorter periods (weeks or months, eg, *Z. capensis*, see Sabat et al. 1998) or even present an interannual variation, which limits the conclusions that can be derived from a comparison between the biochemical characteristics with the information of the diet obtained in different individuals and time.

The adaptive modulation hypothesis is based on that enzyme activities are modulated by specific dietary substrates, but also depends on the phylogenetic origin. For instance, non-passerine birds appears to have the ability to modulate the activity of their

disacharidasses, but usually do not modulate the activity of their proteases (see the review of McWhorter et al. 2009). The case of passerines is more complex because some species appears to be able to up-regulate amino peptidases, but not sucrase and maltase (see Sabat et al. 1998; Afik et al. 1995; Caviedes-Vidal et al. 2000), whereas others appears to exhibit significant up-regulation of maltase activity by dietary starch (Levey et al. 1999). In this vein, Brzec et al. (2013) experimentally demonstrated that these differences between different passerines species may be explained by the fact that disaccharidase activities (but not aminopeptidases) may be both up-regulated by dietary carbohydrates and inhibited by the lipid content of the diet. Thus, in our case species that seems to be strictly granivorous (e.g., *C. barbata*), but indeed exhibited changes in disacharidase activities along the year may include seeds that vary in its lipid content. The same rationale may be applied to the insectivore *L. aegithaloides*, that is, this species may include some preys that are rich in lipids during summer (such as insect larvae) and hence the activity of dicsaccharidases might decreases at this time. However, the opposite pattern observed for aminopeptidase-N is puzzling, and suggest that other characteristics of the dietary substrates, such as protein quality, may be playing a role in enzyme regulation. For instance, Rios et al. (2012) reported both seasonal and dietary adjustments in intestinal esterase activities in *Z. capensis* and *D. diuca*, which was related to the diversity of unsaturated/saturate fatty acids present in the diet.

To sum up, seasonal fluctuations may impose challenges to species since they usually have cope with fluctuations in both food availability and quality. To solve this problem animals could migrate or could adjust their phenotypic traits. The five species analyzed in the present study do not migrate so they have to cope with seasonal variation in

diet with some others strategies, like de modulation of membrane bound intestinal enzymes. In line with this, we found that three of the five species analyzed showed seasonal modulation of intestinal enzymes, and that these species belong to different trophic categories. By contrast, the remaining two species (*D. diuca* and *P. fruticeti*) did not change the activities of these enzymes between seasons, which agree with the constancy in the composition of their diet along the year. Alternatively, the variation found in digestive traits may be related to the effect of climatic variation *per se* (e.g., environment temperature) or to the effect of the interaction between climatic variables and diet. In fact, due to the climatic seasonality, bird species that do not migrate often have to face changes in the environmental temperature, which means that in the cold seasons the animals increase the thermoregulation energy requirements, and therefore food consumption (eg, Mc Williams et al. 1999). In this sense, it has been described that many endotherms, including birds, respond with increases in tissue-specific enzymatic activities and total summed activities (Mc Williams et al. 1992) as a result of seasonality in the field (Liu et al. 2013), under cold conditions in the laboratory (del valle et al. 2004), or to face changes in the availability of food (Lee et al. 2002, Killpack and Karasov 2012). Thus, we cannot discard that the complex pattern of seasonal variation reported here –whereas some species increase mass-specific enzymatic activities during winter, others during summer, and others did not change at all– may be the result of the combined effect of climatic variables, such as temperature, and changes in food supply and consumption due to seasonality (Stein et al. 2004). In any case, it is clear that a better knowledge of the diet and its specific substrates as well as their seasonal variation, for practically all the species analyzed is necessary to better account for the results obtained in the present study.

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Tables

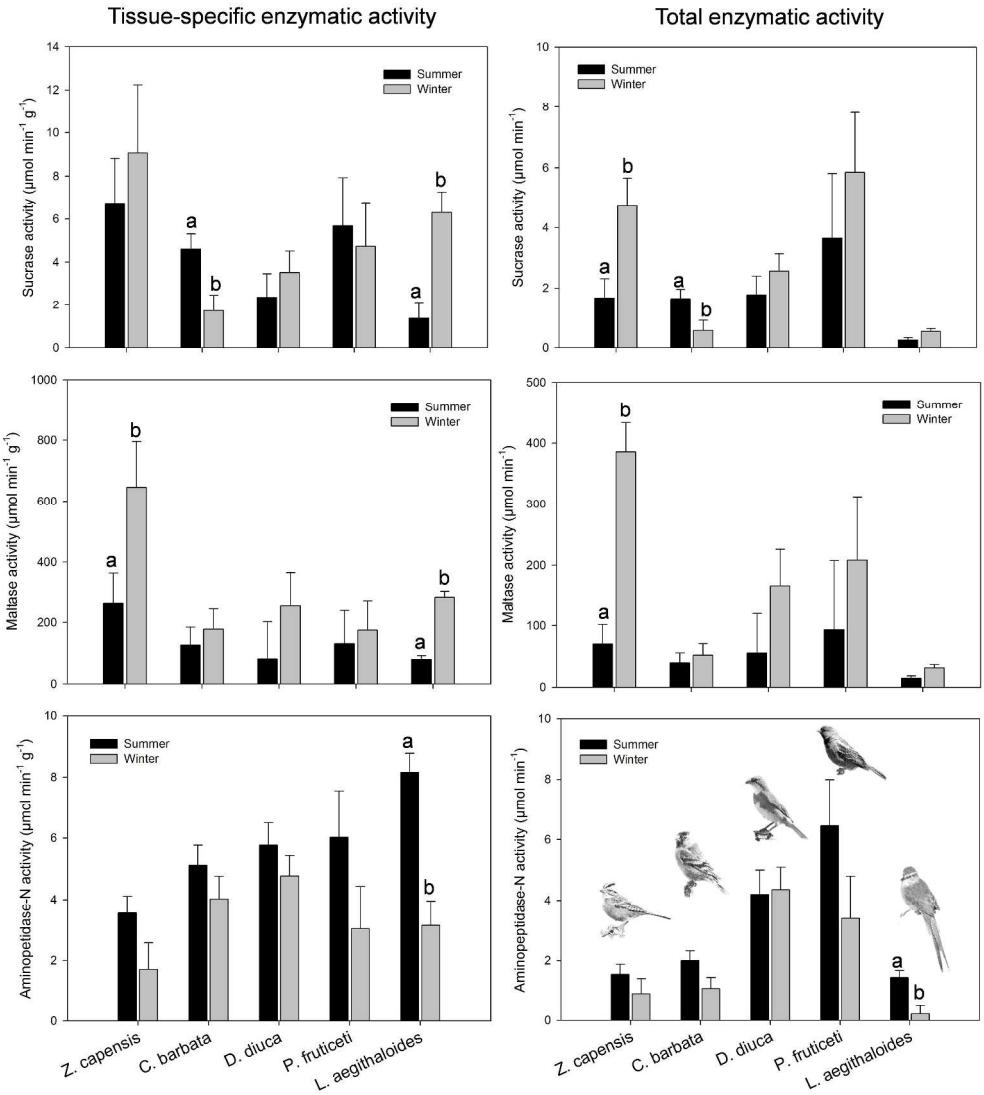
Table 1. Body mass (X ± 1SE), small intestine length (X ± 1SE) and small intestine wet mass (X ± 1SE), for each species in each season*.

Species	Body mass (g)		Small intestine length (cm)		Small Intestine wet mass (g)	
	Summer	Winter	Summer	Winter	Summer	Winter
Z. capensis	20.6 (0.5)	22.6 (0.8)	13.7 (0.3)	14.4 (0.5)	0.41 (0.04)	0.50 (0.06)
C. barbata	14.3 (0.7)	13.0 (0.8)	24.1 (1.1)	23.1 (1.2)	0.35 (0.04)	0.28 (0.04)
D. diuca	33.74 (1.1)	31.3 (1.0)	17.9 (0.5)	16.5 (0.5)	0.73 (0.06)	0.77 (0.06)
P. fruticeti	35.4 (3.4)	33.5 (3.1)	16.8 (1.3)	18.9 (1.2)	0.88 (0.08)	0.99 (0.07)
L. aegithaloides	7.8 (0.4)[a]	9.4 (0.5)[b]	9.94 (1.8)	9.0 (2.1)	0.16 (0.01)[a]	0.13 (0.01)[b]

Different letters denote a significant difference between winter and summer ($P < 0.05$). *Summer results mainly correspond to a previous data published by Ramirez-Otarola et al. (2011) and Sabat et al. (2013). We completed the sample size by the inclusion of two new specimens of *P. fruticeti* and five of *Z. capensis* that were captured in the austral summer of 2013."

Figure Legends

Figure 1. Specific and total activities of sucrase, maltase and aminopeptidase-N in five passerine birds collected during summer and winter. Summer enzymatic activities correspond to data reported by Ramirez-Otarola et al. (2011) plus specimens captured in the austral summer of 2013. See text for details.



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