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Are levels of digestive enzyme activity related to the natural diet in passerine birds?

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ABSTRACT

Digestive capabilities, such as the rates nutrient hydrolysis and absorption, may affect energy intake and ultimately feeding behavior. In birds, a high diversity in gut biochemical capabilities seems to support the existence of a correlation between the morphology and physiology of the intestinal tract and chemical features of the natural diet. However, studies correlating the activity of digestive enzymes and the feeding habits at an evolutionary scale are scarce. We investigated the effect of dietary habits on the digestive physiological characteristics of eight species of passerine birds from Central Chile. The Order Passeriformes is a speciose group with a broad dietary spectrum that includes omnivorous, granivorous and insectivorous species. We measured the activity of three enzymes: maltase, sucrase and aminopeptidase-N. Using an autocorrelation analysis to remove the phylogenetic effect, we found that dietary habits had no effect on enzymatic activity. However, we found that granivorous and omnivorous species had higher levels of disaccharidase activities and insectivores had the lowest. The major difference in enzymatic activity found at the inter-specific level, compared to the reported lower magnitude of enzyme modulation owing to dietary acclimation, suggests that these differences to some extent have a genetic basis. However, the lack of a clear association between diet categories and gut physiology suggested us that dietary categorizations do not always reflect the chemical composition of the ingested food.

Key terms: birds, diet, intestinal enzymes, Passeriformes, food habits, adaptation.

INTRODUCTION

Different foods can require different digestive processing strategies. This simple fact has allowed the evolution of a great diversity in vertebrate digestive systems (Stark, 2005). Digestive capabilities, such as nutrient hydrolysis and absorption rates, affect rates of energy intake and ultimately feeding behavior and energy allocation (Karasov, 1990; Martínez del Rio, 1990; Gatica et al., 2006; Karasov and Martinez del Río, 2007). Furthermore, the study of digestive processes constitutes a mechanistic bridge between the physiological processes that occur in the digestive tract and feeding and nutritional ecology (Diamond, 1991; Karasov, 1996). From this perspective, several studies have been conducted to determine the interaction between the characteristics of diet and physiology at different levels of organization and ontogenetic stages (Penry and Jumars, 1986; Martinez del Rio and Karasov, 1990; Martinez del Rio et al., 1992; Hume, 1998; Lopez-Calleja et al., 1997; Caviedes-Vidal and Karasov, 2001; Sabat and Veloso, 2003; Sassi et al., 2007; Naya et al., 2008). In turn, it is well known that diet has the potential to shape several aspects of the animal phenotype on a short-term scale, from the molecular and biochemical, to the systemic, organismal and behavioral levels (Sibly and Calow, 1986; Martínez del Rio and Stevens, 1989; Weiner, 1992; Bozinovic and Muñoz-Pedreros, 2005; Silva et al., 2005; Naya et al., 2009).

In birds, the digestive enzymes located in the brush border membrane of enterocytes play a significant role in the ability to efficiently digest dietary nutrients. Because complex carbohydrates and proteins must be degraded into their constituent monomers before they can be absorbed (Eckert, 2001; Chang and Karasov, 2004), the levels of those membrane-bound enzymes have the potential to be good predictors of the different digestive strategies of bird species. Among these enzymes, disaccharidases sucrase-isomaltase, maltase-glucoamilase and trehalase, are responsible for catalyzing the final step of carbohydrate digestion; and exopeptidase aminopeptidase-N (APN) degrades oligopeptides into amino acids (Martínez del Rio, 1990; Martínez del Rio et al., 1995).

Several studies have shown the existence of variability in the activity of digestive enzymes at both the inter-and intra-specific levels (Martinez del Río and Stevens, 1989; Afik, 1995, Sabat, 2000; Meynard et al., 1999; Sabat and González, 2003; Schondube and Martinez del Rio, 2004). For example, in songbirds from the genus *Cinclodes*, the lack of sucrase activity seems to be related to the highly specialized carnivorous diet (Sabat, 2000; Sabat and Gonzalez, 2003). Schondube and Martínez del Río (2004) found that the activity of APN in the passerine *Diglosa baritula* is significantly less than expected for body size, which could be explained by the low intake of proteins in this nectarivore passerine. Furthermore, Meynard et al. (1999), found a higher activity of maltase in the hervibore passerine *Phytotoma rara*, an expected result given the high carbohydrate diet of this animal. On the other hand, it has been documented that the activity of brush border enzymes can be strongly affected by the levels of specific substrates in both the field and the laboratory (Martinez del Río et al., 1995; Sabat et al., 1999; Caviedes-Vidal et al., 1994; Karasov et al., 1997; Sabat et al., 1998; Caviedes-Vidal et al., 2000; Brzek et al., 2009).

Thus, although that expression of some digestive enzymes in birds seems to be the result of dietary transitions and

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should reflect the regular presence and concentration of nutrients in the species natural diet, this hypothesis has not been tested directly (eg, Meynard et al., 1999; Martinez del Rio and Stevens, 1989; Martínez del Río, 1990; Sabat, 2000; Sabat & González, 2003). Moreover, the presence of a particular enzyme (e.g. sucrase), the response of those enzymes to dietary acclimation, and the levels of enzymatic activity seem to depend on the phylogenetic origin of the species (Karasov and Levey 1990; Caviedes-Vidal et al., 1994; Martinez del Río, 1990; Levey et al., 1999; Schondube and Martinez del Río, 2004). Unfortunately, few studies have documented the association between enzymatic activity and dietary habits at an evolutionary scale in an appropriate phylogenetic context. In this vein, studies that have evaluated or incorporated the effect of phylogeny on intestinal enzyme activity in vertebrates are very scarce (see Shondube et al., 2001 for an example in mammals), and to the best of our knowledge no studies has been conducted to date on birds (see Karasov and Martinez del Rio, 2007).

The main objective of this study was to explore the effect of dietary habits on hydrolytic activity of the digestive enzymes sucrase, maltase and APN in eight species of songbirds (Order Passeriformes) of central Chile. We used songbirds because this speciose group includes species with widely contrasting dietary habits, including species that feed only on insects, seeds, or mixtures (Dykstra and Karasov, 1992; Lopez-Calleja, 1995; Sabat et al., 1998; Jaksic, 2001). This variation in food habits provides a unique opportunity to study the effect of dietary shifts on the activity of intestinal enzymes.

Many comparative studies rely on the analysis of changes occurring in a dependent variable (i.e. physiology) with respect to an independent variable (i.e. diet; Felsenstein, 1985; Garland et al., 1992; Garland and Adolph, 1994). However, these interspecific comparisons always contain a phylogenetic effect that could affect the analysis. In our work, we adopted a statistical approach to study physiological adaptation, which considers the evolutionary relationships among species. Based on the phylogeny constructed by Sibly and Ahlquist (1990) we evaluated the effect of taxonomic affiliation on the activity of intestinal enzymes. Thus, we predicted that the levels of enzyme activity should correlate with dietary habits and not with taxonomic affiliation. In other words, we predicted that APN levels should be lower in granivorous birds and disaccharidases maltase and sucrase levels lower in insectivorous birds. We predicted intermediate levels of both enzymes in omnivorous birds.

METHODS

Collection of individuals

Birds were captured at two localities in central Chile: Quebrada de la Plata (33º30'S, 70º54'W) and San Carlos de Apoquindo (33°23'S, 70°30'W). Both sites have a Mediterranean climate with cool wet winters and relatively hot dry summers. Eight species of songbirds were captured with mist nets from November 2008 to April 2009. We captured birds only in spring and summer to avoid the putative seasonal variation in physiological parameters. We ascribed each bird species according to dietary habits (Table I) obtained from literature (Dykstra and Karasov, 1991; Lopez-Calleja, 1995; Sabat et al., 1998; Jaksic, 2001).

Enzyme Activity Measurement

Immediately after capture, birds were killed by thoracic compression (American Ornithologists' Union, 1988). The small intestine was immediately excised, flushed with ice-cold saline (0.9%), measured (0.1 cm) and weighed (0.001g) before storage at -50°C, for subsequent enzyme determinations. In the laboratory, tissues were thawed and homogenized for 30 s at maximum setting in a Ultra Turrax T25 homogenizer (Janke & Kunkel, Breisgau, Germany) in 20 volumes of 0.9 % NaCl solution. Disaccharidase activity was determined according

TABLE I

Body mass, small intestine mass (Im), small intestine length (Il), total hydrolytic activities and food habits for eight passerine species from central Chile. Values of total enzyme activities and small intestine length were corrected by body mass elevated to the allometric exponent from the equations: APN = $0.08mb^{1.17}$; Maltase = $0.65mb^{1.32}$; Sucrase = $0.04mb^{0.96}$; Il = $4.16mb^{0.39}$, resulting from the regression for all individuals. S = seeds, O = omnivore, F = fruit, I = insects. EMB = Emberizidae, ICT= Icteridae, TYR = Tyrannidae, FUR= Furnariidae. Different letters denotes significant differences among treatments after ANCOVA, at $P < 0.05$. Values are mean ± SE.

Species	N	Diet	Family	Body mass (g)	Im (g)	Il (cm $g^{-0.39}$)	Maltase (UI $g^{-1.32}$)	Sucrase (UI $g^{-0.96}$)	APN (UI $g^{-1.17}$)
Phrygilus fruticeti	7	S	EMB	36.62 ± 1.25	0.98 ± 0.14	4.32 ± 0.39^a	0.74 ± 0.23^a	0.06 ± 0.02^a	0.09 ± 0.02^a
Diuca diuca	23	S	EMB	32.71 ± 0.48	0.76 ± 0.05	4.26 ± 0.09^a	0.74 ± 0.12^a	0.07 ± 0.01^a	0.07± 0.01^a
Zonotrichia capensis	10	O (S/I)	EMB	20.57 ± 0.55	0.48 ± 0.04	4.19 ± 0.12^a	0.95 ± 0.29^a	0.06 ± 0.01^a	0.06 ± 0.01^a
Curaeus curaeus	10	O (S/I)	ICT	89.65 ± 3.25	2.21 ± 0.13	3.55 ± 0.16^b	0.48 ± 0.07^a	0.06 ± 0.12^a	0.05 ± 0.003^a
Elaenia albiceps	12	O (I/F)	TYR	13.56 ± 0.48	0.25 ± 0.03	3.12 ± 0.12^c	1.37 ± 0.26^b	0.21 ± 0.05^b	0.06 ± 0.01^a
Asthenes humicola	9	I	FUR	21.31 ± 0.31	0.43 ± 0.04	3.82 ± 0.11^a	0.39 ± 0.06^a	0.02 ± 0.003^a	0.07 ± 0.01^a
Anairetes palurus	11	I	TYR	5.51 ± 0.17	0.10 ± 0.01	4.12 ± 0.21^a	0.39 ± 0.06^c	0.03± 0.01^a	0.06 ± 0.01^a
Leptasthenura aegithaloides	5	I	FUR	7.8 ± 0.32	0.16 ± 0.02	4.41 ± 0.23^a	0.86 ± 0.15^a	0.03 ± 0.01^a	0.12 ± 0.01^b

to the method of Dahlqvist (1964) as modified by Martínez del Río (1990). Briefly, tissue homogenates (100 mL) were incubated at 40°C with 100 mL of 56 mmol L^{-1} sugar solutions in 0.1 M Maleate/NaOH buffer, pH 6.5. After 10 min, reactions were stopped by adding 3 mL of a stop solution (a bottle of Glucose LS (Valtek) in 250 mL 0.1 mol L^{-1} TRIS/HCl, pH 7). Absorbance was measured at 505 nm with a Shimadzu UV-1700 spectrophotometer after 18 min at 20°C. APN assays were done using L-alanine-p-nitroanilide as a substrate. Briefly, 100 mL of homogenate diluted with 0.9% NaCl solution was mixed with 1 mL of assay mix (2.04 mmol L^{-1} L-alanine-p-nitroanilide in 0.2 mol L^{-1} NaH2PO4/Na2HPO4, pH 7). The reaction was incubated at 40°C and arrested after 10 min with 3 mL of ice-cold acetic acid 2N, and absorbance was measured at 384 nm. On the basis of absorbance constructed for glucose and p-nitroanilide, standardized intestinal enzymatic activities were calculated. We estimated the concentration of protein in the homogenate using the commercial Coomassie Plus Protein Assay Reagent (Pierce). Absorbances were read at 595 nm and crystalline bovine serum albumin was used as standard. However, because total protein was highly positively correlated with the mass of tissue, and also in order to avoid an underestimation of enzyme activities, data are presented as total hydrolytic activity (μmol min^{-1}) and activity per unit of intestinal wet mass (μmol min^{-1} g^{-1}wet tissue). Martinez del Rio et al. (1995) provide justification for our choice of standardization.

Statistical analysis

Differences between species in enzyme activities and small intestine morphology were evaluated by analysis of covariance (ANCOVA), using body mass as a covariate. To test for specific differences we used a *post hoc* Fisher test. We performed a phylogenetic autocorrelation analysis (PA; Cheverud and Dow, 1985) to remove the effects of phylogenetic inertia, using a phylogenetic tree based on Sibley and Alquist's (1990; Figure 1) hypothesis. It has been reported that this method is consistent with other phylogenetic correction approaches, such as, independent contrasts and phylogenetic generalized least squares regressions (Whiters et al 2006). With the phylogenetic residuals obtained from PA analysis, we performed a Kruskal-Wallis ANOVA to test if dietary category affects the mass-independent activity of intestinal enzymes and the morphology of the small intestine. We also performed a series of regression analyses between physiological and morphological variables against body mass, using all individuals. In addition, in order to determine the possible relationship between enzymatic activities, a Spearman rank correlation was performed between sucrase and maltase activity and between sucrase and aminopeptidase-N activity, using the mean value of each species. Results are given as mean ± S.E. Statistical analyses were performed using Statistica 7® (1997) for Windows.

RESULTS

The protein content of the homogenates did not differ significantly among species ($F_{9,60}$ = 1.85, p = 0.08, average 12.9 ± 0.7 mg protein/g wet tissue). Hence, any changes observed in enzyme specific activities (i.e., UI or UI/mg wet tissue) indicate changes in enzyme activity rather than in tissue water and/or protein content. In addition, although we present enzyme activity as summed (total) activity and also standardized by intestinal wet mass, our data can be compared to those of other studies that normalized activity to protein content, using the equation derived from the regression between the protein content of the small intestine and the wet mass of these tissues (Figure 2).

All enzymatic activities were significantly and positively correlated with body mass (Figure 3). The allometric relationship relating total APN, sucrase, and maltase activities

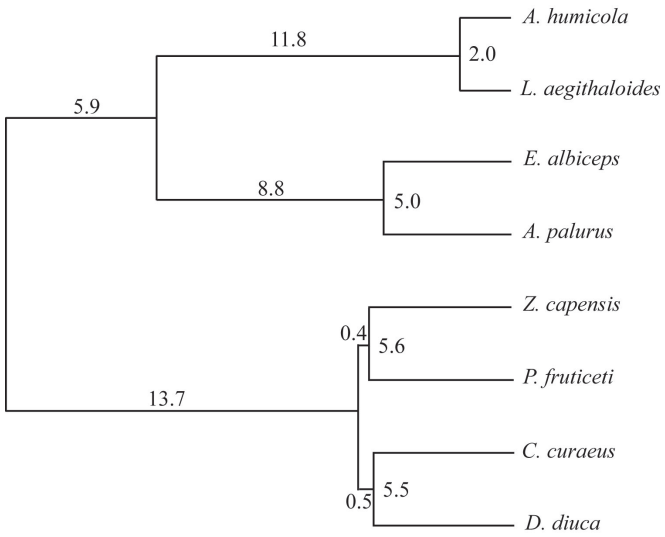

Figure 1. Phylogenetic tree of passerines birds based on Sibley & Ahlquist (1990), used to obtain the phylogenetic residual by phylogenetic autocorrelation analysis for the physiological and morphological variables.

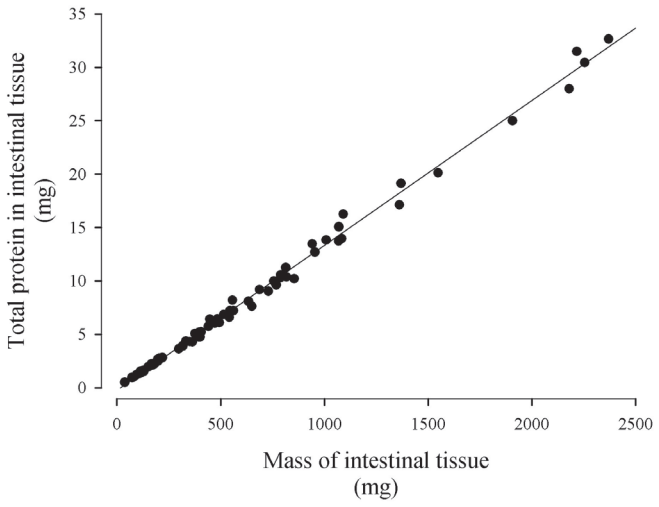

Figure 2. Relationship between mass of small intestine (in mg wet tissue) and total protein measured (in mg protein): Line represents linear regression. Estimated relationship: mg protein in tissue = 0.0136 ± 0.0001 * tissue mass - 0.23 ± 0.09; r2 = 0.99, p < 0.05.

(μmol min^{-1}) with body mass were: APN = $0.06mb^{1.17 \pm 0.1}$ ($r^2 = 0.62$, $p < 0.05$); Sucrase = $0.04mb^{0.96 \pm 0.15}$ ($r^2 = 0.33$, $p < 0.05$); maltase = $0.54mb^{1.32 \pm 0.12}$ ($r^2 = 0.58$, $p < 0.05$). When we explored the association among the three enzymes, we found that sucrase and maltase (UI/g wet tissue) were positively and significantly correlated among species ($r_s = 0.81$, $p = 0.01$; Figure 4). In contrast, sucrase and APN activities (UI/g wet tissue) were not significantly correlated ($r_s = 0.43$, $p = 0.29$; Figure 4).

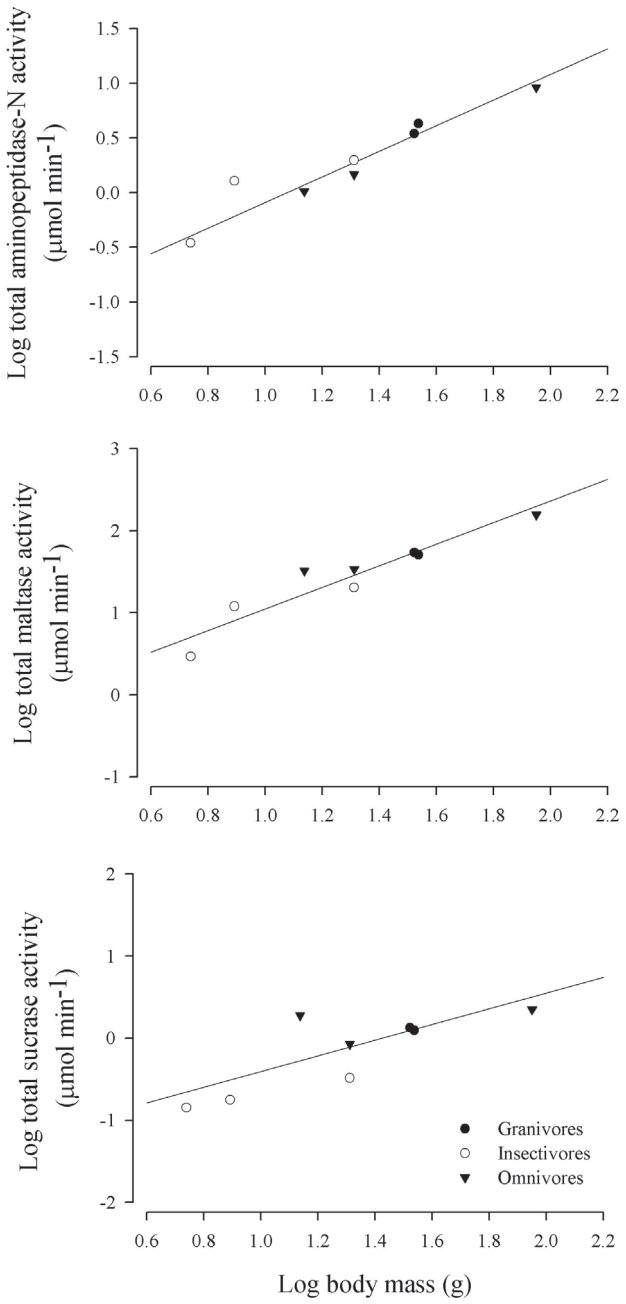

Figure 3. Enzyme activity as a function of body mass: Panels show the allometric relationships for aminopeptidase-N, maltase and sucrase total activities. Each point represents the mean value of a species; however the line represents linear regression obtained with all individuals.

Dietary category had no significant effect on APN activity ($H_{2,8} = 0.22$, $p = 0.89$), maltase activity ($H_{2,8} = 1.81$, $p = 0.41$) or sucrase activity ($H_{2,8} = 2.00$, $p = 0.37$). Additionally, the small intestine mass and length were not affected by dietary category ($H_{2,8} = 1.11$, $p = 0.57$ and $H_{2,8} = 3.13$, $p = 0.21$, respectively). Table I shows details on the total enzymatic activity and small intestine morphology of the studied species. The analysis of covariance revealed an interspecific variation in the activity of total APN ($F_{7,77} = 3.51$, $p = 0.003$), maltase ($F_{7,77} = 4.40$, $p < 0.001$), sucrase ($F_{7,77} = 8.60$, $p < 0.001$) and small intestine length ($F_{7,77} = 7.82$, $p < 0.001$). The mass of the small intestine did not show interspecific variation ($F_{7,77} = 0.89$, $p = 0.52$). The *post hoc* Fisher test revealed that the species *Elaenia albiceps* had the highest sucrase activity ($p < 0.05$, Table I). This species also exhibited the shortest small intestine ($p < 0.05$, Table I). In addition, *Curaeus curaeus* and *Elaenia albiceps* showed the highest maltase activity ($p < 0.05$, Table I). Finally, the highest level of APN was found in *Leptasthenura aegithaloides* ($p < 0.05$, Table I).

DISCUSSION

Interspecific comparisons are commonly used to identify physiological or biomechanical modifications that have accompanied the evolutionary process, and with some frequency are used to infer genetic changes in response to natural selection (Harvey and Page1, 1991; Garland and Carter, 1994). However, because species usually are non-independent statistically, traditional statistical methods, like ANOVA or ANCOVA, have been criticized (Felsenstein, 1985; Garland et al., 1993; Garland, 1994). However, McNab (2009) discussed two major problems with phylogenetic analysis. The first relates to the concept of independent character. McNab (2009) pointed out that if we only considered the evolution of a trait, the species are non-independent, but if physiological rates are considered, the important issue is that the physiological trait must match environmental conditions to allow survival, irrespective if they were attained by ancestry or convergence. The second problem pointed out by McNab (2009) was related to the lack of complete and consensus cladograms. This is a major problem because phylogenetic analysis assumes a well-known phylogeny (e.g. topology and branch lengths are known), which in most cases are unknown or are subject to controversy. To control for putative phylogenetic effects, in this study we evaluated the rates of enzymatic activities and the morphology of the gut using phylogenetic autocorrelation to remove the effects of phylogenetic proximity. Our main result revealed that both morphological and biochemical features of the small intestine differed among the studied species, but neither gut morphology nor the activities of digestive enzymes were affected by food habits. However, these results should be taken with caution because the analysis we used could be biased by the low statistical power associated with the few data obtained by this procedure. As well, the morphology of the small intestine was also affected by species, being shorter in the omnivorous species *Elaenia albiceps*.

Our results revealed that the allometric exponent of the relationship between the hydrolytic activity of sucrase and body mass was 0.96. The scaling exponent of maltase and APN were 1.32 and 1.17 respectively. These exponents are slightly higher than the values reported for others passerines (Shondube and Martinez del Rio, 2004). Probably our small

sample size and the high representation of small birds in our sample led to an overestimation of the allometric exponent. Nevertheless, the allometric exponent of the small intestine length (0.39) was very similar to that found previously (0.34) for passerines (Ricklefs, 1996). We also found a positive and significant correlation between mass-specific sucrase and maltase, which has been reported previously for other bird species (e.g., Martinez del Rio, 1990; Biviano et al., 1993; Sabat et al., 1998; Meynard et al., 1999; Schondube and Martinez del Río, 2004). In many bird species, maltase activity is the result of the action of sucrase-isomaltase and maltase-glucoamilase complex. Thus, an increase in sucrase-isomaltase expression is translated into increased maltase activity (Afik et al., 1995). In addition, we found no correlation among disaccharidase and APN activities among bird species. As pointed out by Diamond (1991), maintaining molecular machinery to assimilate nutrients may be costly, and the unused intestinal membrane bound proteins may take up space and biosynthetic energy. Thus, as we already predicted (Sabat et al., 1998), a trade-off in the ability to efficiently utilize either protein-rich insect diets or carbohydrate-rich seed diets would be expected. In this vein, the absence of a positive correlation among disaccharidases and aminopeptidases could be viewed as a support of the existence of such trade-offs. However, our results contrast with those found by Sabat et al. (1998), who reported a positive and significant correlation among the three enzymes in *Z. Capensis* and *Diuca diuca* (see also Naya et al., 2005 for a study of an ectotherm vertebrate). Furthermore, the above mentioned studies were conducted at an intraspecific level, whereas our results come from inter-species comparisons. This discrepancy may reveal a difference between interspecific and intraspecific approaches. Unfortunately, no additional studies on the correlation between disaccharidases and aminopeptidases have been reported for bird species at the inter-specific level, which prevents making a more general conclusion about the existence of such a trade-off.

Even though hydrolytic activities were not significantly affected by the dietary category, our results suggest that the activity of the enzymes could be affected by the probable concentrations of the specific substrates. In this vein, the levels of enzymatic activity observed in some species seems to support the hypothesis of the existence of a functional match between the rates of enzymatic activity and the dietary substrates present in natural diets. For example, we found that species that are described as omnivores, such *Elaenia albiceps,* showed the highest activities for sucrase compared to the others species. As well, it has been reported that *E. albiceps* often visits flowers of the Proteaceus tree *Embothrium ccoccineum* to consume nectar. These flowers have sucrose concentrations up to 40 % of the total sugars in nectar, representing a significant amount of energy intake for birds (Smith-Ramirez and Armesto, 1998). In addition, *E. albiceps* has been reported as occasional consumers of fruits (Rozzi et al., 1996). Accordingly, the sucrase activity of *E. albiceps* was 11.3 times higher than the activity of *Asthenes humicola*, species described as insectivore. In addition, *E.albiceps* exhibited the highest maltase activity. The activities of these enzymes were 4.2 times higher than the activities of the insectivore *Anairetes palurus*. Finally, *Leptathenura aegithaloides* an insectivore species, showed the highest APN activity, being 1.5 times higher than the activity of the granivore *Diuca diuca*. Thus, some of our results suggest that transitions in diet could have been accompanied by physiological changes, but the correspondence between diet and enzyme activity levels remains ambiguous. In this vein, enzymatic activity is not the only variable that influences the efficiency with which animals hydrolyze and assimilate nutrients. Hence, comparison of only the activities of different enzymes in the same individuals or populations may not be appropriate to predict foraging preferences and digestive abilities. As pointed out by Karasov (1996), nutrient utilization is determined by morphological and physiological traits, such as the levels of enzyme activity and carriers and the retention time of food. Thus, it is possible that even when enzymatic activities for a particular substrate are low, animals may still efficiently break down substrates if the retention time is sufficiently low (Afik et al., 1995). Thus, physiology and diet

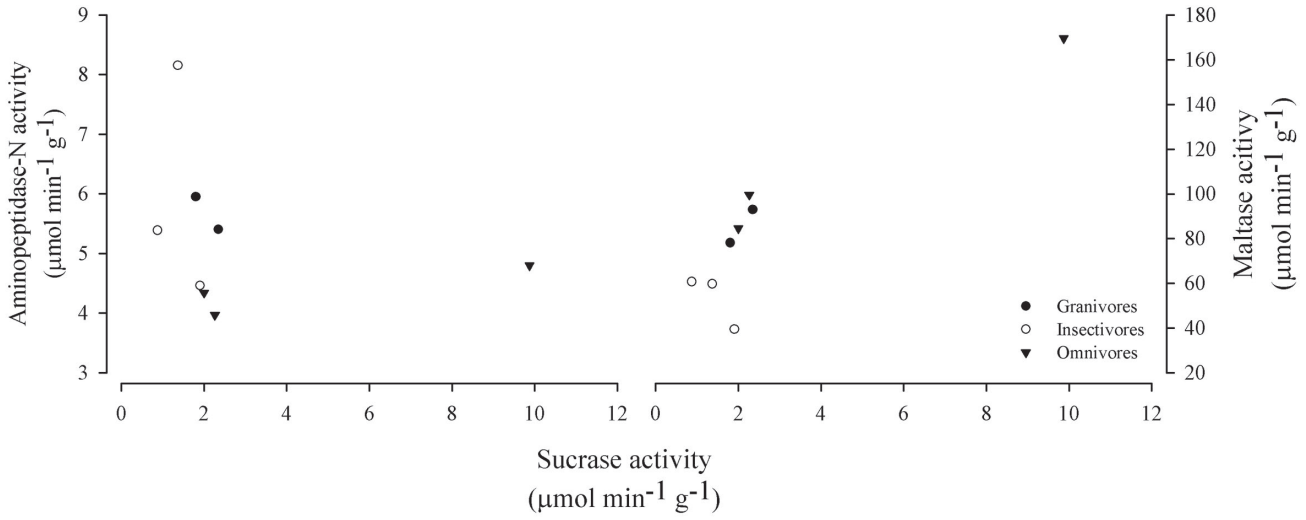

Figure 4. Relationship among intestinal enzymes: Sucrase and maltase activities were positively correlated whereas the activities of aminopeptidase-N and sucrose were not correlated. Each point represents the mean values of a species.

show many complex interactions with each other (Karasov and Diamond, 1988). For example, Karasov and Levey (1990) suggested that frugivorous birds tend to have higher rates of intestinal uptake of sugars and amino-acids in order to compensate for shorter digestion time. Perhaps the activity of intestinal enzymes follows a similar pattern. Although we have no data on residence time of food for our bird species, we proposed a negative association between the digestive residence time of our species and specific activity of enzymes (UI/ g wet tissue).

What could explain the lack of association between digestive enzyme levels and dietary habits in passerine birds? As pointed out by several authors (McNab, 1992; Cruz-Neto et al., 2001; Cruz-Neto and Bozinovic, 2004) the use of dietary categorization on the outcome of comparative studies is problematic because in some cases there is a misallocation of the species, given the dietary category. We suggest that probably our analyses could be biased by the erroneous assignment of dietary categories for some of our model species. Indeed, in some cases the feeding habits for the species could be poorly known or biased by the underestimation of seasonal and/or geographical differences in dietary habits (Klasing, 1998; Karasov and Martinez del Río, 2007; Sabat et al., 2009). On the other hand, dietary categories do not necessarily reflect the dietary chemical composition. For example, a species defined as a seed-eating specialist (granivores), could nevertheless experience a wide range of variability in protein content and sugar types among different seeds. Consequently, different granivorous species might also exhibit different levels and types of specific substrates. In this vein, a study that incorporates more species and an analysis of the composition of bird diets would be more appropriate to elucidate if specific activities of brush border enzymes are adjusted to the chemical composition of natural diets. For example, the incorporation of dietary information based on a continuous scale, such as the percentage of proteins or the ratio between nitrogen and carbon in the gut content, might be used to get a more precise characterization of bird diets (see Muñoz-Garcia and Williams, 2005). Besides, the isotopic composition of tissues has been used as a quantitative variable in comparative studies differentiating among species with distinct dietary habits (see Shondube et al., 2001). As pointed out by Sabat et al. (2009), this procedure will resolve a common methodological problem when dietary categories are used instead of some continuous variable. For example, this could avoid the common failure of traditional dietary analysis, such as the loss of information about dietary variability in the field (see Klaasing, 1998), which may mask any significant association between physiological traits and food habits.

Even though we did not find an apparent association between diet and biochemistry of the intestinal tract, we observed an obvious inter-specific difference in gut physiology and morphology. Studies on birds have documented that inter and intra-specific variations in physiological traits are adaptative and reflect adjustments generated for natural selection, phenotypic plasticity or to a combination of these two sources of phenotypic variation. The hydrolytic activity of intestinal enzymes can often be modulated by the presence of their specific dietary substrates (Martinez del Rio and Stevens, 1989; Martinez del Rio et al., 1992; Sabat et al., 1995). However, the magnitude of enzymatic activity changes in passerine birds as a product of acclimation (in the laboratory) or acclimatization (in the field) seems to be much lower than differences we found among species. For instance, the magnitude of modulation of maltase between diets with the highest and lowest levels of activities is 2.6 in adult Pine warblers (*Dendroica pinus*; Levey et al., 1999), 1.5 in adult *Diuca diuca* and *Zonotrichia capensis* (Sabat et al., 1998) and 1.4 in the starling (*Sturnus vulgaris*), whereas the variation in APN among diets ranges from 1.2 (Pine warblers; Levey et al., 1999) to 1, 7 in *Z. capensis*, *Diuca diuca* and Yellow-rumped warblers and starlings (Afik et al., 1995; Martinez del Río et al., 1995; Sabat et al., 1998). The magnitude of variation between the highest and lower level of activity of enzymes in our sample was ca. 11 times for maltase and 2 for APN. Thus, the highest magnitude of difference of disaccharidase and protease activities among passerine species probably indicates that the observed activities could be the result of evolutionary change (Afik et al., 1995; Shondube et al., 2001). Nevertheless, recently, Brzek (2009) documented that specific maltase activity can be modulated during development in nestlings of house sparrows (*Passer domesticus*), but not in adult birds (Cavides-Vidal et al., 2000), suggesting that the magnitude of modulation in enzyme activities can be largely underestimated when using adult animals as experimental subjects. Unfortunately, few studies on the physiological diversity of birds have partitioned phenotypic variation (Biviano et al., 1993; Brzek et al., 2009), and hence it remains unknown how much of intra-specific variability in enzyme activities has a genetic basis or is attributable to phenotypic plasticity.

In summary, our results indicate that dietary categories based on traditional analysis do not allow inferring a functional relationship between the diet of birds and digestive function. Thus, ongoing studies should use explicit phylogenetical methods coupled with dietary information based on a continuous scale variable (e.g., dietary nitrogen) to test the hypothesis of a correlated evolution between physiological features and diet in birds.

On the other hand, the observed interspecific differences in digestive biochemical capabilities suggest they could be the result of an evolutionary change. However, we cannot put aside the putative role that developmental plasticity could play in generating the observed pattern. Thus, the effect of developmental plasticity on digestive enzyme activities in species with different dietary habits should be conducted to test this hypothesis and to test the proposed existence of a correspondence between dietary variability and phenotypic flexibility. In this vein, there is remarkably paucity of research on the developmental plasticity of important physiological traits, and further studies on this topic should be conducted to obtain a better understanding of physiological modifications that have accompanied the evolution of dietary habits in birds.

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