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Title: Aminopeptidase activity is related to the amino acids composition of the food in passerine birds

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ABSTRACT

Background. Passerine birds exploit different kinds of feeding habits and they have to face seasonal changes in food availability. Therefore, the composition of the principal nutrient in their food differs from the usual. In consequence the digestive function – enzyme hydrolysis and absorption – have to adapt to these nutrients. These changes in digestive physiology could respond to the adaptive modulation hypothesis which postulated that the activities of digestive enzymes should match the levels of their substrates in their diet so energy is not wasted on enzymes that are no need. Thus, we decide to measure intestinal enzymes activities of two species of passerine birds that differ in natural diet. Overall we hypothesized that species with different feeding habits present enzyme activity according to the mainly component of the diet (e.g., carbohydrates, proteins). Our prediction is that the individuals will present enzyme activity proportionally to the primary components of the diets.

Methods. We select for study: red ovenbirds (*Furnarius rufus*), which are strict insectivores and zebra finches (*Taeniopygia guttata*), which are specialist granivores. We complete the analysis with publish data for house sparrows (*Passser domesticus*) feed on high starch from the literature. To examine intestinal enzyme activities, we measured the activity of two disaccharidases (sucrase-isomaltase and maltase-glucoamilase) and one dipeptidase (aminopeptidase-N).

Results. The average intestinal activity of sucrase shows that the omnivorous *P. domesticus* presents almost 4 times more activity than the granivorous *T. guttata* and more than 11 times than the insectivorous *F. rufus*. This difference is also reflected in the total sucrase hydrolytic capacity where *P. domesticus* has roughly 10 times more than the other two birds. Surprisingly in *F. rufus* we found maltase and aminopeptidase activity while sucrase activity was close to zero. In the case of the average activity of maltase for the omnivorous *P. domesticus* is approximately 40 % more than the granivorous *T. guttata* and more than 5 times than the insectivorous *F. rufus*. Although the total maltase hydrolytic capacity of *P. domesticus* is 5 times more than *T. guttata* and *F. rufus*. The average of aminopeptidase-N activity for *F. rufus* and *T. guttata* almost doubled the *P. domesticus* ones. Also *F. rufus* roughly doubles the other two birds in total aminopeptidase hydrolytic capacity.

Discussion. This study has shown that exist a relationship between the levels of amino acids in the diet and the total aminopeptidase capacity, but in the case of carbohydrates this relationship is not evident.

INTRODUCTION

Birds possess the capacity to exploit a broad diversity of resources and they have to face seasonal changes in food availability. The switch to available food modifies the predominant nutrients in their food intake. Consequently the consumption of food with

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different nutrients requires that the optimization of the digestive function – enzyme hydrolysis and absorption – is adapted to these nutrients (Karasov & Martínez del Río 2007). In several vertebrates was reported a modulation of disaccharidases activity correlated with substrates in their diets (Biviano et al. 1993; Harpaz & Uni 1999; Hernández & Martínez del Río 1992; Sabat et al. 1995). These changes in digestive physiology could respond to the adaptive modulation hypothesis which postulated that activities of digestive enzymes should match the levels of their substrates in their diet so energy is not wasted on enzymes that are no need (Karasov 1992; Karasov & Diamond 1988). In birds, at the interspecific level it has been observed that the hydrolytic capacity of the individuals is related with the level of the substrate in the feeding habits (Kohl et al. 2011; Ramírez-Otarola & Sabat 2011). In consequence, we decide to measure intestinal enzymes activities of two species of passerine birds that differ in natural diet.

To examine intestinal enzyme activities, we measured the activity of two disaccharidases and one dipeptidase. Digestion of carbohydrates implicates glucosidase enzymes located on the brush border of the small intestine; between them we can find sucrase-isomaltase (EC 3.2.1.10) and maltase-glucoamilase (EC 3.2.1.3) (Hunziker et al. 1986; Palmer 1971). Maltase-glucoamilase hydrolyzes maltose in two molecules of glucose, while sucrase-isomaltase hydrolyzes sucrose in one molecule of fructose and another of glucose. In reference of protein digestion we have chosen to measure aminopeptidase-N (E.C. 3.4.11.2) that account for almost all peptidase activity in the brush border membrane (Maroux et al. 1973). This enzyme cleaves oligopeptides to produce dipeptides and amino acids (Sjostrom et al. 1978).

We select for study: red ovenbirds (REDO; *Furnarius rufus*), which are strict insectivores (Fraga 1980b) and zebra finches (ZEBF; *Taeniopygia guttata*), which are specialist granivores (Zann 1996a). We complete the analysis with publish data for the house

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sparrows (HOSP; *Passser domesticus*) feed on high starch from the literature (Caviedes-Vidal et al. 2000). We choose the high starch because represent most likely the natural diet (Anderson 2006).

Overall we hypothesized that species with different feeding habits present enzyme activity according to the mainly component of the diet (e.g., carbohydrates, proteins). Thus we predicted that the individuals will present enzyme activity proportionally to the primary components of the diets.

MATERIALS AND METHODS

Animals

Zebra finches (ZEBF) were purchased in San Luis, Argentina and house sparrows (HOSP) and red ovenbirds (REDO) were captured on the campus of Universidad Nacional de San Luis (UNSL), San Luis. HOSP and ZEBF were housed in cages indoors under constant environmental conditions (25 ± 1°C, relative humidity of 50 ± 10%) on a photoperiod of 14:10 (L:D) with water and food ad libitum (alpist, millet, vitamins and minerals). REDO were used on the same day of capture in order not to alter their eating habits. All animal procedures adhered to institutional animal use regulations and approved animal use protocols by the Animal Care and Use Committee of the UNSL, protocol number B212/15. Captured animals were approved by the Environmental Office of the state of San Luis, resolution number 75-PBD-2015.

Intestinal Enzyme Assays

Disaccharidases activities, sucrase-isomaltase and maltase-glucoamilase were assayed using the colorimetric method developed by Dahlqvist (Dahlqvist 1984) and modified by Martínez del Río (Martínez del Río 1990). Briefly, tissues were thawed at 4 °C and homogenized for 30 s using a manual homogenizer (Fisher ScientificTM Laboratory

Homogenizer, Model 125) in mannitol buffer (350 mM for birds) in 1 mM Hepes-KOH, pH 7.0. Aliquots of 40 L of diluted intestinal homogenates were incubated with 40 L of 56 mM sucrose or 56 mM maltose in 0.1 M maleate/NaOH buffer, pH 6.5, at 40 °C for 20 min. After 20 min of incubation the reaction was stopped by adding 1 mL of enzymatic glucose assay (Glucosa Liquid plus reagent-GT Laboratorios S.R.L.). Sample solutions were allowed to stand for 5 min at room temperature and the absorbance was measured at 505 nm and activity was determined using a glucose standard curve.

Aminopeptidase-N activity was assayed using L-alanine-p-nitroanilide as a substrate (Maroux et al. 1973). Aliquots of 10 µL of the intestinal homogenate were added to 1 mL assay solution (2.0 mM L-alanine-p-nitroanilide in 0.2 M phosphate buffer (NaH_2PO_4/Na_2HPO_4, pH 7). The reaction was incubated for 20 min at 40 °C and then stopped with 3 mL of chilled 2 M acetic acid. Absorbance was measured at 384 nm, and activity was determined using a p-nitroanilide standard curve.

On the basis of absorbance measurements and glucose and p-nitroanilide standard curve we calculated activities of each intestinal section normalized to the wet mass of the section. Activities of intestinal enzymes were expressed in micromoles per minute per gram of wet tissue.

We calculated the summed hydrolysis activity of the entire small intestine, an index of the total hydrolysis capacity, by multiplying activity per gram of wet tissue in each region by its respective mass, and summed over the three regions.

Statistics

Statistical analyses were conducted with SPSS and results are expressed as means ± 1 s.e.m. Total hydrolytic capacity were determined using ANOVA with Tukey's post hoc tests. As for enzymatic activity we use a repeated measures ANOVA with a between subject factor.

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142 The F-values of these and other analyses of variance are presented in the text with the relevant
143 degrees of freedom as subscripts. Significance was determined at $P < 0.05$.

144 **RESULTS**

145 The intestinal sucrase activity significantly varies among species and intestinal
146 segments ($F_{2,13} = 30.54$, $P < 0.001$, $F_{2,26} = 5.07$, $P = 0.014$, respectively; Fig.1 A). *P.*
147 *domesticus* presents significantly higher levels of enzyme activity all along the intestine
148 compare with the other two species ($P < 0.001$, for the three segments). Also only for this
149 specie the activity decays towards the distal segment ($P < 0.05$). In addition no differences
150 between *T. guttata* and *F. rufus* were detected ($P= 0.604$) despite the fact that *T. guttata* more
151 than doubles the activity for proximal and medial segments (See discussion).

152 The pattern of maltase activity also varies among species and intestinal segments ($F_{2,13}$
153 $= 8.51$, $P < 0.001$, $F_{2,26} = 13.54$, $P < 0.001$, respectively; Fig.1 B). *P. domesticus* and *T.*
154 *guttata* presents no differences between all segments ($P > 0.05$, for the three segments) and
155 also significantly higher levels of enzyme activity compare with the *F. rufus* ($P < 0.05$, for the
156 three segments for both species). Also only in *P. domesticus* the activity decays towards the
157 distal segment ($P < 0.05$).

158 The activity of aminopeptidase-N differs from the other two enzymes, the model
159 shows no differences between segments but significantly differences among species ($F_{2,26} =$
160 1.163, $P = 0.301$, $F_{2,13} = 4.897$, $P = 0.026$, respectively; Fig.1 C). The activity along the
161 intestine for *T. guttata* decays on the distal segment ($P < 0.05$), the opposite happens to *F.*
162 *rufus* were the activity rises on the distal segment ($P < 0.05$) and for *P. domesticus* the activity
163 stays without changes ($P > 0.05$). For the proximal and medial segment *T. guttata* shows
164 higher activity than the *P. domesticus* ($P < 0.01$ for both) but not different than *F. rufus*
165 (proximal $P = 0.108$, medial $P = 0.152$) and at the same time no differences were detected

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between *P. domesticus* and *F. rufus* (proximal $P = 0.319$, medial $P = 0.553$). For the distal segment *F. rufus* present higher activity than *P. domesticus* ($P > 0.05$) but no different from *T. guttata* ($P = 0.106$) and no differences was detected between *T. guttata* and *P. domesticus* ($P < 0.05$).

The total hydrolytic capacity for sucrase significantly varies among species ($P < 0.001$). *P. domesticus* presents more than 9 times higher capacity than *T. guttata* and *F. rufus* ($P < 0.001$ for both species) and no variation was detected between this last two species ($P = 0.994$; Fig. 2 A). For maltase the pattern is the same ($P < 0.001$). *P. domesticus* presents more than 4 times more capacity than *T. guttata* and *F. rufus* ($P < 0.001$ for both species) and no variation was detected between this last two species ($P = 0.981$; Fig 2 B). As for aminopeptidase-N capacity the pattern is different than the two others enzymes. *F. rufus* presents more than 2 times more capacity than *T. guttata* and *P. domesticus* ($P < 0.05$ for both species) and no variation was detected between this last two species ($P = 0.632$; Fig.2 C).

DISCUSSION

The average intestinal activity of sucrase shows that the omnivorous *P. domesticus* presents almost 4 times more activity than the granivorous *T. guttata* and more than 11 times than the insectivorous *F. rufus*. This difference is also reflected in the total sucrase hydrolytic capacity where *P. domesticus* has roughly 10 times more than the other two birds. Surprisingly in *F. rufus* we found maltase and aminopeptidase activity while sucrase activity was close to zero, so its biological significance is unclear. In our first enzyme assay we did not find activity of sucrase in the samples of *F.rufus*. In consequence we examine the sucrase activity from new homogenates with different dilutions and we did not obtain activity. In order to evaluate if the reagents were in good conditions we determine sucrase and maltase activity in samples of rats, house sparrows and zebra finchs. Also in this assay we added a

standard solution from glucose standard curve as a positive control. For these enzyme assays we found activity of both disaccharidases in the three samples. Finally, we decided to assay the three enzymes (sucrase, maltase, aminopeptidase) in samples of *F.rufus* and we obtained, again, values close to zero of sucrase activity in the three intestinal segments. These results are consistent with those of other studies which found little or no activity of sucrase in different species of passerine birds. Several studies focus on omnivorous and insectivorous passerines birds have described very low or total absence of activity of sucrase and/or maltase compared with other species of birds as shown in this study (Malcarney H.L. 1994; Martínez del Río 1990; Martínez del Río & Stevens 1989; Ramírez-Otarola et al. 2011; Sabat 2000; Sabat & Gonzalez 2003) (see Table 2). This phenomenon has been related to feeding preferences or to evolutionary process. In the case of feeding preferences, it refers that diet is determined by the digestive available machinery. In this context, our results may be explained that insectivorous diet of *F. rufus* is a consequence of the low activity of sucrase. However**,** Martínez del Río et al. (Martínez del Río & Stevens 1989) showed that when birds that lack of sucrase activity were fed with sucrose solutions developed signs of discomfort and osmotic diarrhea. Thus it can be suggested that the association between the absence of sucrase activity and diets is not merely a preference matter. It could be hypothesized that the illness observed in birds that lack of sucrase activity in consequence of sucrose feeding is an important factor to determinate their eating habits. Nonetheless more research on this topic needs to be undertaken. Another possible explanation for this might be that in birds take place evolutionary events that cause the lack of sucrase activity. For example, in hummingbirds occurred a transformation of a taste receptor function after their divergence from an insectivore ancestor. This evolutionary adaptation contributed to the acquisition of nectar-feeding behavior (Baldwin et al. 2014). In passerine birds, the lack of sucrase activity has been reported for species belonging to the Sturnidae-Muscicapidae lineage (Martínez del

Río 1990). Nevertheless, the data available add 8 families of *Passeriformes* birds that have zero or little activity of sucrase with different feeding habits (Table 2.). It can be therefore assumed that the lack of sucrase activity is not only observed in insectivorous passerine birds, but also affect species of birds that do not belong to the Sturnidae-Muscicapidae lineage. A further comparative work will reveal the relationship between feeding habits and evolutionary process that is governing the sucrase activity among birds.

The average activity of maltase for the omnivorous *P. domesticus* is approximately 40 % more than the granivorous *T. guttata* and more than 5 times than the insectivorous *F. rufus*. This shrink in the difference of the activity between *P. domesticus* and *T. guttata* (~400% to ~40%) could be associated to diet. Millet and alpist contain considerable amount of starch therefore *T. guttata* needs maltase to final breakdown of the starch. Although the total maltase hydrolytic capacity of *P. domesticus* is 5 times more than *T. guttata* and *F. rufus*. Also even though that *T. guttata* presents 3 times more activity of maltase than *F. rufus*, the total hydrolytic capacity is the same mainly due the differences in intestinal mass (see Table 1). The average of aminopeptidase-N activity for *F. rufus* and *T. guttata* almost doubled the *P. domesticus* ones. Also *F. rufus* roughly doubles the other two birds in total aminopeptidase hydrolytic capacity, this is mainly due to differences in activity and differences in intestinal mass (see Table 1).

As for the activity of three enzymes throughout the intestine we observed a tendency to diminish towards distal portion in birds that present disaccharidases activity (*P. domesticus, T. guttata*). While aminopeptidase activity does not present a uniform pattern among the species of birds studied. In the case of *F.rufus* aminopeptidase activity increases towards the distal portion and an opposite tendency is observed for the other two species. Aminopeptidase activity does not exhibit a predictable pattern throughout the intestine, as if it has been found for disaccharidases. These patterns may be related to feeding habits. Because, in insectivorous

240 birds or protein-rich diets they exhibit an increase aminopeptidase activity towards the distal
241 portion, whereas frugivorous, granivorous or omnivorous species show a pattern that
242 decreases towards the distal portion or remains constant throughout the entire intestine. The
243 present findings seem to be consistent with other research which found several pattern of
244 aminopeptidase activity among birds (Afik et al. 1995; Meynard et al. 1999; Witmer &
245 Martínez del Río 2001).

246 These findings suggest that exist a trend between L-proline and total hydrolytic
247 capacity. The reason for this is not clear but it may have something to do with the adaptive
248 modulation hypothesis which postulated that enzyme activity is related to the diet(Karasov
249 1992; Karasov & Diamond 1988). Moreover, there are several studies that support that the
250 intestinal enzyme activity responds to changes in the diet (Afik et al. 1995; Brzęk et al. 2010;
251 Caviedes-Vidal et al. 2000).

252 **CONCLUSIONS**

253 In conclusion, this study has shown that exist a relationship between the levels of
254 amino acids in the diet and the total aminopeptidase capacity, but in the case of carbohydrates
255 this relationship is not evident.

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360 **Tables and Figures**

361 **Table 1.** Animals attribute.

362 Letters P = proximal, M = medial, D = distal, intestinal segments.

Enzyme Assay Animals	***P. domesticus***	***T. guttata***	***F. rufus***
N (male/female)	4/2	4/1	2/3
Body mass (g)	26.0 ± 0.82	12.9 ± 0.40	46.7 ± 0.82
Small intestinal length (cm)	18.6 ± 0.9	9.7 ± 0.32	18.7 ± 0.55
Small intestinal width (cm)	None	P: 0.63 ± 0.02 M: 0.55 ± 0.03 D: 0.52 ± 0.04	P: 0.97 ± 0.02 M: 0.89 ± 0.004 D: 0.82 ± 0.02
Small intestinal mass (g)	1.4 ± 0.28	0.3 ± 0.02	1.2 ± 0.04
Diet	Omnivore (Anderson 2006)	Granivore (Zann 1996b)	Insectivore (Fraga 1980a)
References	(Caviedes-Vidal 2000)	This study	This study

363 Values are means ± 1 s.e.m.

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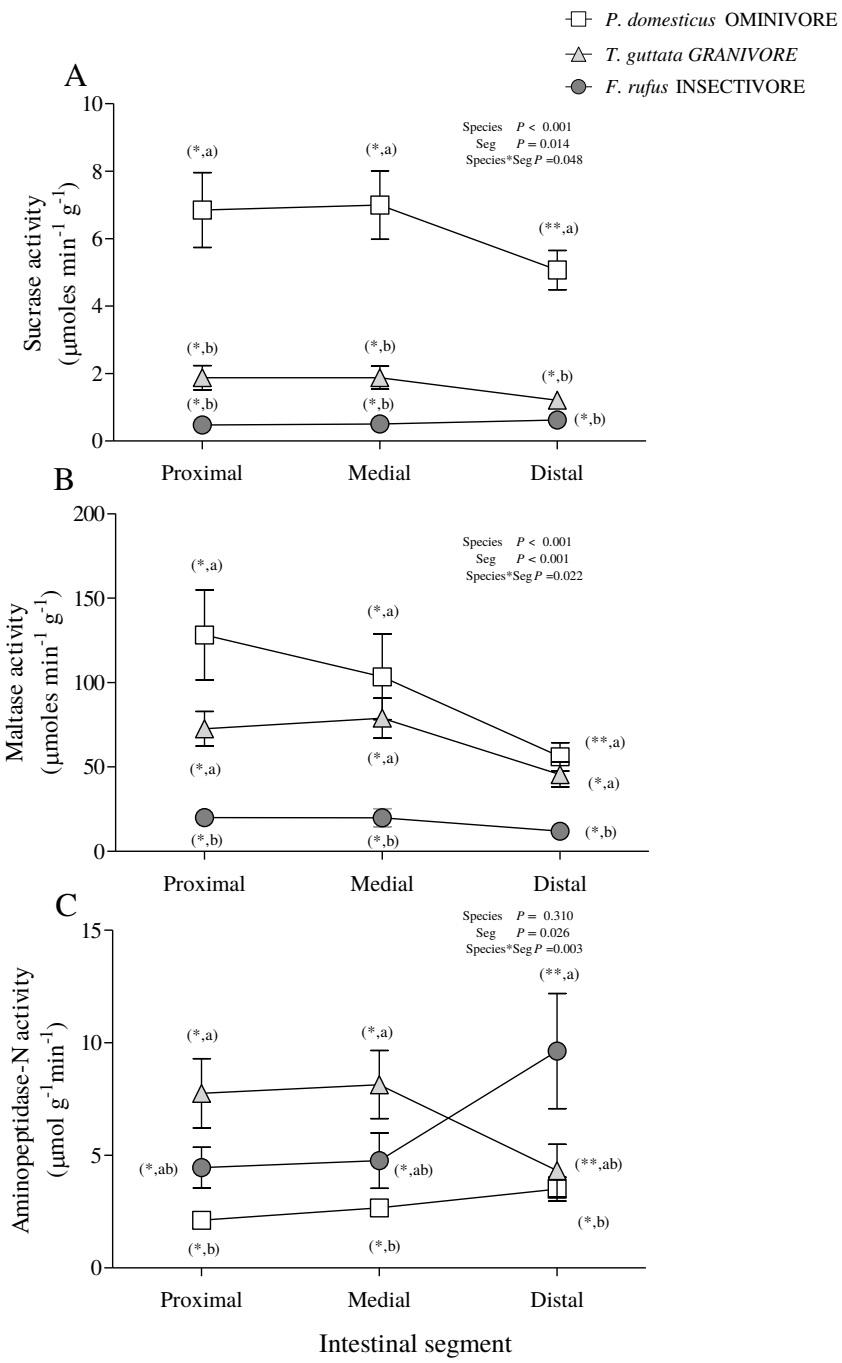

Figure 1. Intestinal enzymes activity of (A) sucrase, (B) maltase and (C) aminopeptidase-N for the three intestinal segments. Sample sizes were as follows: *P.domesticus n* =5, *T.guttata n* = 4, *F.rufus n*=5 (See table 1). *P*-values in the figures are for comparisons of species or intestinal regions. When species within an intestinal segment present the same letter they do not differ significantly. When segments within a species share an equal number of (*) they do not differ significantly. Data are means ± s.e.m.

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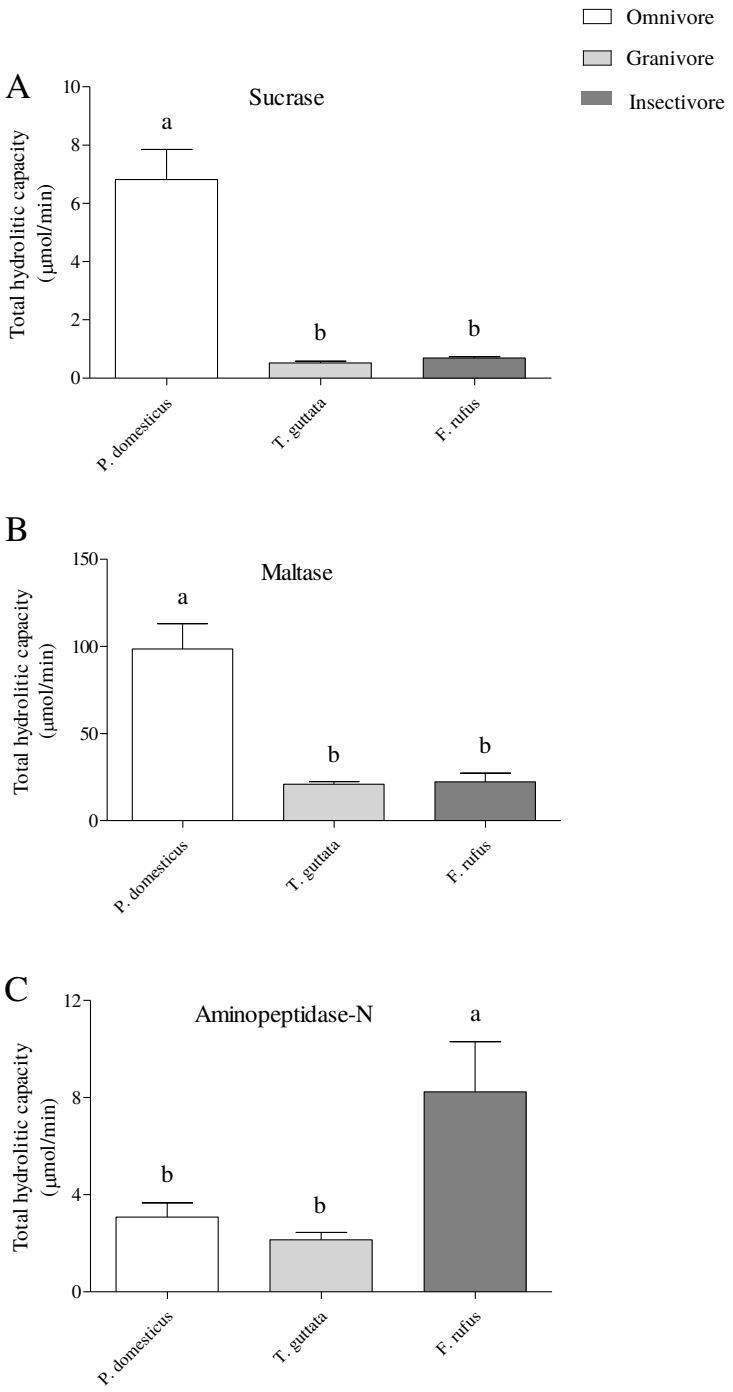

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372 **Figure 2.** Total hydrolytic capacity for (A) sucrase, (B) maltase and (C) aminopeptidase-N
373 for the three species *P. domesticus n* =5, *T. guttata n* = 4, *F. rufus n*=5 (See table 1). Data are
374 means ± s.e.m.; bars that share letters indicate no statistically significant difference ($P > 0.05$).

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375 **Table 2.** Summary of sucrase (< 1 mol min $^{-1}$) and maltase activity in passerine birds.

					Sucrase Activity		Maltase Activity		Ref.
Order	**Family**	**Genus**	**Scientific name**	**Diet**	Mmin $^{-1}$gprot^{-1}	Mmin $^{-1}$	Mmin $^{-1}$gprot^{-1}	Mmin $^{-1}$	
Passeriformes	Sturnidae	*Sturnus*	*S. vulgaris*	O	0		276±23	-	(Martínez del Río & Stevens 1989)
Passeriformes	Sturnidae	*Onychognathus*	*O. morio*	F	0		-	0.24±0.08	(Bizaaré et al. 2012)
Passeriformes	Tyrannidae	*Empidonax*	*E. difficilis*	I	-	0.33±0.12	-	7.15±2.51	(Martínez del Río 1990)
Passeriformes	Tyrannidae	*Anairetes*	*A.parulus*	I	-	0.16±0.04	-	3.56±0.73	(Ramírez-Otarola & Sabat 2011)
Passeriformes	Mimidae	*Dumetella*	*D. carolinensis*	O	0.50±0.43	0.04±0.05	102.03±31.03	28.96±14.12	(Malcarney et al. 1994)
Passeriformes	Mimidae	*Mimus*	*M. thenca*	O	0		-	116.55±26.21	(Gatica et al. 2006; Ramírez-Otarola & Sabat 2011)
Passeriformes	Emberizidae	*Zonotrichia*	*Z. capensis*	S/I	-	0.99±0.18	-	39.81±10.09	(Ramírez-Otarola & Sabat 2011)
Passeriformes	Troglodytidae	*Troglodytes*	*T. aedon*	I	-	0.10±0.05	-	8.06±2.31	(Ramírez-Otarola & Sabat 2011)

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Passeriformes	Estrildidae	*Taeniopygia*	*T. guttata*	S	-	0.52±0.06	-	20.90±1.59	This study
Passeriformes	Furnariidae	*Cinclodes*	*C. nigrofumosus*	C		0	4.78±0.60	-	(Sabat 2000)
Passeriformes	Furnariidae	*Cinclodes*	*C. patagonicus*	C/I		0	4.65±2.34	-	(Sabat 2000)
Passeriformes	Furnariidae	*Cinclodes*	*C. oustaleti*	C/I		0		-	(Sabat & Gonzalez 2003)
Passeriformes	Furnariidae	*Asthenes*	*A.humicola*	I	-	0.38±0.07	-	27.90±7.95	(Ramírez-Otarola et al. 2011)
Passeriformes	Furnariidae	*Leptasthenura*	*L. aegithaloides*	I	-	0.21±0.06	-	13.23±0.82	(Ramírez-Otarola et al. 2011)
Passeriformes	Furnariidae	*Furnarius*	*F. rufus*	I	-	0.69±0.04	-	22.29±4.92	This study
Passeriformes	Pycnonotidae	*Pycnonotus*	*P. tricolor*	F	-	0.26±0.03	-	0.13±0.16	(Bizaaré et al. 2012)
Passeriformes	Turdidae	*Catharus*	*C. aurantiirostris*	I/F	-	<0.05	-	5.47±1.12	(Martínez del Río 1990)
Passeriformes	Turdidae	*Catharus*	*C. minimus*	I/F	-	0.2	-	23.6	(Witmer & Martínez del Río 2001)
Passeriformes	Turdidae	*Catharus*	*C. guttatus*	I/F	-	0.2	-	26.9	(Witmer & Martínez del

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									Río 2001)
Passeriformes	Turdidae	*Catharus*	*C. ustulatus*	I/F	-	0.05	-	31.1	(Witmer & Martínez del Río 2001)
Passeriformes	Turdidae	*Hylocichla*	*H. mustelina*	I/F	-	0.3±0.04	-	29.9±4.6	(Witmer & Martínez del Río 2001)
Passeriformes	Turdidae	*Turdus*	*T. migratorius*	I/F	-	0.4±0.2	-	60.0±15.2	(Witmer & Martínez del Río 2001)
Passeriformes	Turdidae	*Turdus*	*T. rufopalliatus*	I	-	<0.05	-	9.45±2.15	(Martínez del Río 1990)
Passeriformes	Turdidae	*Turdus*	*T. falcklandii*	O		0	-	228.61±110.19	(Gatica et al. 2006; Ramírez-Otarola & Sabat 2011)
Passeriformes	Zosteropidae	*Zosterops*	*Z. virens*	F	-	0.34±0.15	-	0.17±0.04	(Bizaaré et al. 2012)

376 Values are means ± s.e.m

377 I= Insects, F= Fruits, S=Seeds, C= crustaceans and marine invertebrates, O= Omnivorous.