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Pancreatic and Intestinal Carbohydrases Are Matched to Dietary Starch Level in Wild Passerine Birds

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ABSTRACT

Evolutionary shifts in diet composition are presumably accompanied by simultaneous changes in digestive physiology. The adaptive modulation hypothesis predicts that activities of digestive enzymes should match the relative levels of their substrates in an animal's diet so that available membrane space and synthetic energy are not wasted on enzymes in excess of need. However, previous studies on captive passerine birds showed high intraspecific phenotypic flexibility only in proteases but not in carbohydrases in response to varying diet composition. In this study, we measured the activities of pancreatic, intestinal, and hepatic enzymes in six wild-caught passerine species. We predicted that if the adaptive modulation hypothesis holds during evolutionary shifts in diet composition in birds, then mass-specific activities of digestive enzymes should be correlated positively with the content of their relevant substrates in species' diets. Whereas mass-specific activities of proteases (aminopeptidase-N, trypsin, chymotrypsin, alanine aminotransferase) were not correlated with estimated dietary protein content, mass-specific activities of all studied carbohydrases (amylase, maltase, sucrase) were positively correlated with estimated dietary starch content. We conclude that activities of carbohydrases but not proteases are evolutionarily matched to diet composition in passerine birds. We hypothesize that the need for nitrogen and essential amino acids can prevent the evolution of a low activity of proteases, even in species feeding on a low-protein diet.

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Introduction

Passerine birds have diverse food habits, from species that are almost exclusively granivorous to species that eat primarily insects or even small vertebrates. With such variable diets, different species ingest different relative amounts of carbohydrate and protein. Large macromolecules of polysaccharides and proteins must be hydrolyzed before they can be absorbed through the intestine luminal wall into the blood system. This process usually includes two steps (Stevens and Hume 1995; Karasov and Hume 1997; Karasov and Martinez del Rio 2007): hydrolysis of nutrient macromolecules by enzymes released by the pancreas, products of which are subsequently hydrolyzed by intestinal brush border enzymes. In the case of proteins, relevant pancreatic enzymes are trypsin and chymotrypsin, and an important brush border enzyme is aminopeptidase-N. In the case of dietary starch, the relevant enzymes are pancreatic amylase and intestinal maltase and sucrase. Products of the latter step can be then absorbed across the intestinal epithelium and further processed by various postabsorptive processing enzymes in the liver (Karasov and Martinez del Rio 2007).

Diet composition is likely to act as a selective force on the digestive function of organisms (Karasov and Diamond 1988; Diamond 1993). Presumably, the activities of enzymes involved in digestion are adjusted to the amount of their substrates in an animal's typical diet (adaptive modulation hypothesis; Karasov and Diamond 1988; Diamond and Hammond 1992; Diamond 1993). If digestive enzyme levels are matched to relative levels of dietary substrate, then available energy/nutrients do not escape the digestive tract unabsorbed but, at the same time, available membrane space and synthetic energy are not wasted on enzymes in excess of need. Ideally, animals that switch among diet types should possess the ability to modulate enzyme levels to cope with the current content of nutrients in their diet. Indeed, short-term modulation of the activity of intestinal peptidase, aminopeptidase-N, is common in passerine birds experimentally fed diets with different protein content, for example, European starling (*Sturnus vulgaris*; Martinez del Rio et al. 1995), yellow-rumped warbler (*Dendroica coronata*; Afik

et al. 1995), rufous-collared sparrow (*Zonotrichia capensis*), common diuca finch (*Diuca diuca*; Sabat et al. 1998), pine warbler (*Dendroica pinus*; Levey et al. 1999), and house sparrow (*Passer domesticus*; Caviedes-Vidal et al. 2000). However, in almost all of the above studies (with the exception of Levey et al. 1999), experimental birds did not modulate the activity of carbohydrase enzymes (usually maltase activity) in relation to varying carbohydrate content of diet. Moreover, another study found that different species fed identical diets show significantly different activities of sucrase, suggesting an important genotypic component to the expression of this enzyme (Martinez del Rio et al. 1988).

Thus, studies so far in adult passerine birds suggest low intraspecific phenotypic flexibility but possible interspecific variation in activities of carbohydrate-hydrolyzing enzymes. Although predictions of the adaptive modulation hypothesis have been confirmed in many groups of animals (e.g., Kapoor et al. 1975; Schondube et al. 2001), we are aware of only one such comparative study in altricial birds, which found higher activity of maltase and sucrase in nectarivorous and omnivorous than in insectivorous species (Martinez del Rio 1990). However, birds are particularly interesting subjects for such analyses because requirements of flight ability are likely to put strong pressure on their digestive physiology (e.g., need for mass savings; cf. Caviedes-Vidal et al. 2007). The aim of this study was to carry out a more detailed analysis of interspecific variation of digestive enzymes in passerine birds. We focused on species with different compositions of their typical diets and analyzed key digestive enzymes involved in different steps of nutrient digestion and use (pancreatic and intestinal enzymes digesting proteins and carbohydrates, and one hepatic enzyme related to postabsorptive protein metabolism). We predicted that if evolutionary shifts in diet composition trigger relevant, simultaneous changes in activities of digestive enzymes, then mass-specific activities of studied enzymes should be correlated positively with the content of their relevant substrates in species' diets.

Material and Methods

Sample Collecting

Target species for our analysis (Table 1) were selected on the basis of published information on their diet and ease of capture. During July 2008, birds were captured using mist nets in state wildlife areas and private land properties in Dane County, Wisconsin, under U.S. Fish and Wildlife Service permit MB699480-0 and with permission from the Wisconsin Department of Natural Resources or property owners. All experimental procedures were approved by the University of Wisconsin–Madison Animal Care and Use Committee (permit RARC A01347-0-04-08). Individuals of all nontarget species were carefully released. Birds of the target species were euthanized in the field by thoracic compression. This method relies on quick and sustained pressure on the bird's chest to inhibit ventilation; it is considered painless and is recommended by the American Veterinary Medical Association (AVMA) for euthanizing small birds under field conditions (AVMA 2007 Guidelines on Euthanasia). Abdominal cavities of euthanized birds were immediately opened, and intestines, pancreas, and liver were dissected and stored in liquid nitrogen. Before freezing, intestines were perfused and rinsed with cold avian Ringer solution and cut into three sections of similar length, corresponding to proximal, middle, and distal regions (following Martinez del Rio 1990). Tissues were brought back to the laboratory and stored at −80°C until analysis.

Determination of Diet Characteristics

Information on the average whole-year percentage of seed, plant, insect, and fruit material in each species' diet was obtained from the Birds of North America (specific sources are listed in Table 1). We used the average nutrient contents of these food types (Wyatt 1967; Englyst 1981; Robbins 1993; Karasov and Martinez del Rio 2007) to estimate percent dietary starch, sucrose, and protein for each species. The conventional nitrogen to protein conversion factor 6.25 was used for seed, plant, and insect material, while 5.64 was used for conversion of fruit (Levey et al. 2000).

Pancreatic Enzyme Assays

Frozen pancreas samples were crushed into several smaller pieces. In order to account for possible spatial heterogeneity in enzyme activities, four to five pieces from different parts of the pancreas were pooled for each enzyme assay. Assays followed procedures used in previous studies (Caviedes-Vidal and Karasov 2001).

Activity of amylase was measured by a modification of the 3,5-dinitrosalicylate method (Dahlqvist 1962; Hjorth 1979). Several pieces of pancreas were thawed and homogenized for 30 s in 10 mL/g tissue of 50 mM Tris/HCl buffer (pH 6.9, containing 3 mM taurocholic acid, 0.27% [w/v] Triton X-100, 1 mM benzamidine, and 2 mM hydrocinnamic acid). Appropriately diluted 100-μL aliquots were incubated with 100 μL of 2% potato starch (Sigma S2630) at 40°C for 3 min. The reaction was terminated by the addition of 200 μL dinitrosalicylate reagent. Blank samples contained exactly the same ingredients, but dinitrosalicylate was added before the substrate and then handled in the same way as other samples. The tubes were immersed in boiling water for 10 min and cooled with tap water. Two milliliters of distilled water were added to each tube, and the absorbance was read at 530 nm.

For analysis of pancreatic trypsin and chymotrypsin, several pieces of pancreatic tissue were homogenized for 30 s using 10 mL/g tissue of 50 mM Tris/HCl buffer (pH 8.2, containing 3 mM taurocholic acid and 0.27% [w/v] Triton X-100). To activate zymogens, homogenate samples were incubated with 0.3% enterokinase (Sigma E0632) for 40 min at 25°C in 50 mM Tris/HCl buffer (pH 8.2) containing 20 mM $CaCl_2$. Preliminary data indicated that this treatment gave reproducible maximal activation of the proteolytic zymogens (K. D. Kohl, personal observation). Samples were centrifuged for 15 min at

Table 1: Species used in this study; number of individuals used (*n*); type of dietary preference; content of starch, sucrose, and protein in their diet; and source of information about their food composition

Name				Content in Diet (%)			
Common	Scientific	*n*	Dietary Preference	Starch	Sucrose	Protein	Source
House finch	*Carpodacus mexicanus*	3	Granivore	66	.8	13	Hill 1993
House sparrow	*Passer domesticus*	9	Granivore, occasionally insectivore	77	Trace	14	Lowther and Cink 2006
Northern cardinal	*Cardinalis cardinalis*	3	Omnivore	37	1.4	23	Halkin and Linville 1999
American robin	*Turdus migratorius*	3	Omnivore	2	3.2	26	Sallabanks and James 1999
Black-capped chickadee	*Poecile atricapillus*	4	Insectivore, occasionally plant material	15	.8	40	Smith 1993
Barn swallow	*Hirundo rustica*	6	Insectivore	5	Trace	54	Brown and Bromberger-Brown 1999

Note. See "Material and Methods" for details of determination of diet characteristics.

20,000 *g* at 4°C to remove a white suspension that sometimes appears in solution (E. Caviedes-Vidal, personal observation). Aliquots of 16 μL supernatant mixed with 144 μL distilled water were assayed to measure trypsin activity, using 800 μL 1 mM DL-BAPNA (benzoyl-arginine-p-nitroanilide) solution as substrate at pH 8.2 for 10 min at 40°C. The reaction was terminated by adding 160 μL of 30% acetic acid. Blank samples contained exactly the same ingredients, but acetic acid was added before the substrate and then handled in the same way as other samples. The liberated amount of p-nitroaniline was estimated by reading the absorbance at 410 nm (Erlanger et al. 1961) and using a p-nitroaniline standard curve. Chymotrypsin activities were also measured by the amount of p-nitroaniline released by hydrolysis, using 160 μL of homogenate supernatant and 800 μL of 1 mM GPNA (N-glutaryl-l-phenylalaninep-nitroanilide) solution at pH 7.6 and 40°C. The reaction was terminated with 160 μL of 30% acetic acid solution. The absorbance of the mixture was measured at 410 nm (Erlanger et al. 1966).

Intestinal Enzyme Assays

We measured the activity of membrane-bound enzymes in whole-tissue homogenates rather than in mucosal samples or isolated brush border preparations to avoid underestimation of activity, as previously reported (Martinez del Rio 1990).

We assayed disaccharidase (maltase, sucrase) activity using a modification of the colorimetric method developed by Dahlqvist (1984). Assays are described in detail elsewhere (Martinez del Rio 1990; Fassbinder-Orth and Karasov 2006). Briefly, tissues were thawed at 4°C and homogenized in 350 mM mannitol in 1 mM N-2-hydroxyethylpiperazine-N′-2-ethanosulfonic acid (Hepes)-KOH, pH 7.0. Intestinal homogenates (30 μL) diluted with 350 mM mannitol in 1 mM Hepes-KOH were incubated with 30 μL of 56 mM maltose or 56 mM sucrose in 0.1 M maleate and NaOH buffer, pH 6.5, at 40°C for 20 min. Next, 400 μL of a stop-develop reagent (GAGO-20 glucose assay kit; Sigma Aldrich, St. Louis) was added to each tube, vortexed, and incubated at 40°C for 30 min. Last, 400 μL of 12 N H_2SO_4 was added to each tube, and the absorbance was read at 540 nm.

We used L-alanine-p-nitroanilide as a substrate for aminopeptidase-N. To start the reaction, we added 10 μL of the homogenate to 1 mL of assay mix (2.0 mM L-alanine-p-nitroanilide in 1 part of 0.2 M NaH_2PO_4/Na_2HPO_4 buffer number 1, pH 7, and 1 part of deionized H_2O) previously warmed to 40°C. The reaction solution was incubated for 20 min at 40°C and then terminated with 3 mL of ice-cold 2 N acetic acid, and absorbance was measured at 384 nm.

Alanine Aminotransferase Assay

Frozen liver samples were crushed into several small pieces, and four to five pieces from different parts of the liver were pooled for enzyme assays to account for possible spatial heterogeneity in alanine aminotransferase (ALT) activity. Enzyme activity was measured following a method that monitors the rate of NADH oxidation via the lactic dehydrogenase reaction (Segal and Matsuzawa 1970). Several pieces of liver tissue were weighed and homogenized for 30 s using a 1 : 9 dilution with 9 parts 0.14 M KCl solution. Homogenate was centrifuged for 30 min at 14,000 *g* at 5°C. The supernatant was diluted 1 : 4 with 0.14 M KCl, and 25 μL was added to 1.5 mL of a reaction mixture with concentrations of the following reagents: 0.1 M KPO_4 buffer, pH 7.3, 4.1 mM α-ketoglutarate, 17 mM L-alanine, 2.5 units lactic dehydrogenase, and 0.17 mM NADH.

The reaction mixture was placed in a water-jacketed cuvette held at 40°C. The change in absorbance at 340 nm was recorded and used to calculate units of ALT (1 unit = formation of 0.55 μmol of pyruvate per minute; Segal and Matsuzawa 1970).

Protein Content

ALT activity was normalized to hepatic protein content, which was measured with an advanced protein assay reagent (Bio-Chemika 57697). A standard curve for protein was created using varying concentrations of bovine serum albumin.

Determination of pH Optima

We tested for differences in the pH optima of maltase, sucrase, and aminopeptidase-N in the medial portion of intestine of three randomly selected birds of each species. The above assays

were performed using the homogenates and a 0.05 M maleate: NaOH buffer system with increasing pH in increments of 0.5, ranging from 5 to 7.5 for the disaccharidases, and a 0.1 M sodium phosphate buffer with pH ranging from 5.5 to 8.5 for aminopeptidase-N. For each individual, we calculated relative mass-specific enzyme activities, expressed as mass-specific activity at a particular pH divided by maximal mass-specific activity at the pH that was found to be optimal in the sample from this individual bird. Average relative mass-specific activities for all target species are shown in the appendix in the online edition of *Physiological and Biochemical Zoology*. Because the pH optimum for a given enzyme in a given species sometimes differed from the assay pH (which was 6.5 for maltase and 7 for aminopeptidase-N), we repeated our analysis of the relationship between dietary substrate content and enzyme activity using pH-corrected data. This was done by dividing the experimentally measured enzyme activity at assay pH for each individual in each species by the respective species' mean relative enzyme activity at assay pH.

Statistical Analyses

Activities of intestinal enzymes per gram of tissue were calculated for the proximal, middle, and distal segments of intestine and then used to calculate whole-intestine mass-specific activity by weighting values according to the masses of each segment. Summed activities of intestinal enzymes were calculated by multiplying segmental mass-specific activities by the masses of each intestinal segment and summing for the whole intestine. For pancreatic and hepatic enzymes, we calculated only their mass-specific activities and not summed enzyme activities because we did not have accurate masses of whole organs.

Single-factor ANOVA was conducted to check whether our methods could detect interspecific differences in whole-intestine mass-specific enzyme activities. For interspecies comparisons, phylogeny must be taken into account to avoid overestimating the number of degrees of freedom in the statistical analyses, which occurs because species are phylogenetically related and thus not entirely independent units (Garland et al. 1992). We applied the independent contrast method, which is a standard method to test for correlated evolution of continuous traits (Garland et al. 1992). This method takes into account the differences between species in measured variables as well as phylogenetic tree branch lengths. We used a phylogeny proposed by Barker et al. (2002), using the nuclear RAG-1 and *c-mos* genes (Fig. 1). Phylogenetically independent contrasts (PICs) were conducted using phylogenetic comparative methods of COMPARE (ver. 4.6b; http://compare.bio.indiana.edu/; Martins 2004). We then carried out two sets of linear regression analyses using PIC values, in each case forced through the origin, as recommended by Garland et al. (1992): (1) simple regression between mass-specific activity of enzymes and estimated dietary content of appropriate substrate (protein, starch, sucrose); with this analysis, we checked whether mass-specific activity of enzymes is matched to diet composition; (2) multiple regression, with either intestine mass or summed activity of intestinal enzymes as the dependent variable, and body mass and estimated dietary content of appropriate substrate as independent variables; with this latter analysis, we explored the potential link between intestinal mass and diet composition, which might adjust summed enzyme activity to diet composition even in the absence of changes in mass-specific activity of enzymes.

All analyses of regressions presented in "Results" are based on PIC analysis, and their sample size was $n = 5$. All tests were carried out using SYSTAT (Wilkinson 1992) and SAS software with a significance level set at $\alpha = 0.05$.

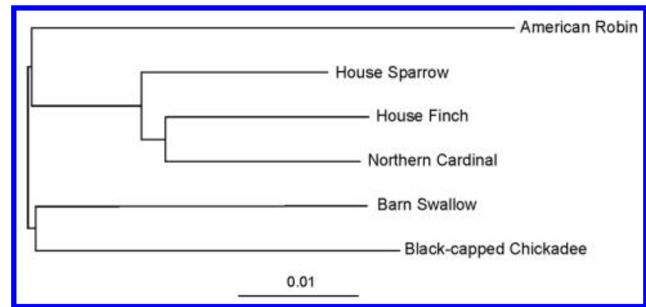

Figure 1. Phylogenetic tree of species used in this analysis, based on maximum likelihood tree of Barker et al. (2002). Branch lengths are proportional to expected changes per individual nucleotide site; unit represents 0.01 changes.

Results

Interspecific Variation in Mass-Specific Enzyme Activities

Mass-specific activity of all analyzed enzymes showed significant variation between species (data not corrected for phylogeny; ANOVA, $P < 0.05$ for all enzymes). There was a significant positive correlation between contrasts in mass-specific activity of enzymes involved in digestion of starch and contrasts in estimated dietary starch content (amylase: $r^2 = 0.937$, $P = 0.002$; Fig. 2*B*; maltase: $r^2 = 0.908$, $P = 0.003$; Fig. 2*D*). Interestingly, contrasts in mass-specific activity of sucrase were also correlated with contrasts in dietary starch content ($r^2 = 0.807$, $P = 0.015$; Fig. 2*F*) but not in dietary sucrose ($r^2 = 0.281$, $P = 0.28$).

Enzymes related to protein digestion did not show significant relationships between contrasts in their mass-specific activity and contrasts in estimated dietary protein content (trypsin: $r^2 = 0.069$, $P = 0.62$; Fig. 3*B*; chymotrypsin: $r^2 = 0.205$, $P = 0.37$; Fig. 3*D*; aminopeptidase-N: $r^2 = 0.053$, $P = 0.66$; Fig. 3*F*). Similarly, contrasts in mass-specific activity of ALT, a hepatic enzyme involved in protein catabolism, did not correlate with contrasts in dietary protein content ($r^2 = 0.146$, $P = 0.45$; Fig. 3*H*).

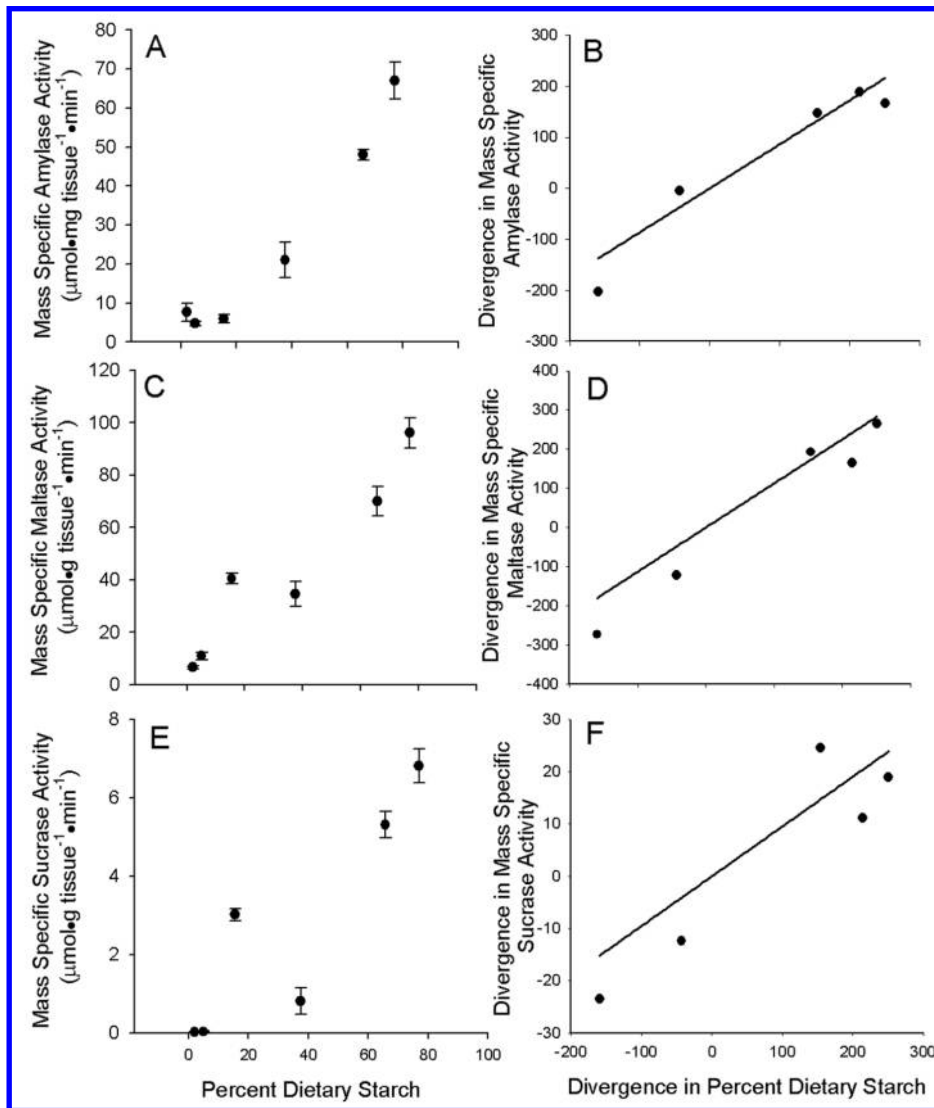

Figure 2. Mass-specific activity of carbohydrases—amylase (*A*, *B*), maltase (*C*, *D*), and sucrase (*E*, *F*)—plotted against estimated dietary starch content (%). In the left column, means ± SEM for each species are shown, which represent the following species (from left to right along the *X*-axes): American robin, barn swallow, black-capped chickadee, northern cardinal, house finch, and house sparrow (the order of species is not the same as in Fig. 3). In the right column, phylogenetically independent contrasts for species-specific average values are shown.

Interspecific Variation in Summed Enzyme Activities

There was a highly significant positive correlation between contrasts in intestine mass and contrasts in body mass ($r^2 = 0.966$, $P = 0.0004$). However, contrasts in intestine mass were not correlated with contrasts in estimated dietary content of starch, sucrose, and protein, when contrasts in body mass were controlled for ($P > 0.25$ for all comparisons). Contrasts in summed activities of maltase and sucrase were positively correlated with contrasts in dietary starch content ($r^2 = 0.818$, $P = 0.038$ and $r^2 = 0.860$, $P = 0.025$, respectively; contrasts in body mass were controlled for). However, there was no correlation between contrasts in summed activity of aminopeptidase-N and estimated dietary protein content ($P = 0.72$) or contrasts in summed activity of sucrase and in dietary sucrose content ($P = 0.31$).

pH Optima

Disaccharidase pH optima showed little variation between species (see appendix). Maltase pH optima ranged from 5.5 to 6.5, which is the same range as found by Martinez del Rio (1990). Sucrase pH optimum was not analyzed for the barn swallow or American robin, since these species had sucrase activities <0.05 μmol/g tissue/min. However, for all species analyzed, the sucrase pH optimum was 5.5. Aminopeptidase-N optima showed wider interspecific variation, from 6.5 in the American

robin and northern cardinal to 8.0 in the black-capped chickadee.

To investigate whether experimental pH influenced the relationships between dietary substrate and enzyme activities, we repeated our analyses of correlation between these traits using pH-optimum-adjusted mass-specific activities. This correction did not change our findings. Regressions of PICs still showed a significant correlation between contrasts in estimated dietary starch and in mass-specific maltase activity ($r^2 = 0.890$, $P = 0.005$) and a lack of relationship between contrasts in dietary protein and in mass-specific aminopeptidase-N activity ($r^2 = 0.038$, $P = 0.71$). We did not correct sucrase activity, since optima could not be measured for all species. However, because the relationship between pH and sucrase activity was similar for all species with detectable activity of this enzyme (see appendix), interspecific variation in pH optimum is unlikely to affect our conclusions.

Discussion

Diet Composition and Interspecific Variation in Enzyme Activity

The bird species we studied had variable diets, ranging from insectivores, to omnivores, to seed specialists (granivores). We hypothesized, in accordance with the adaptive modulation hypothesis, that different contents of starch and protein in consumed diets should select for different levels of mass-specific activities of digestive enzymes, adjusting them to species-specific diet type. Indeed, we found that differences in estimated dietary starch content were positively correlated to changes in mass-specific activities of enzymes involved in digestion of carbohydrates (amylase, maltase, and sucrase). The strength and significance of these correlations are remarkable because our analysis is based on only six species. On the other hand, there was no correlation between differences in estimated dietary protein content and mass-specific activities of enzymes involved in digestion and processing of protein (trypsin, chymotrypsin, aminopeptidase-N, ALT). Finally, we found no evidence that intestinal mass is related to diet composition; therefore, interspecific differences in summed activities of intestinal enzymes mainly reflect changes in mass-specific activities of enzymes. We emphasize that almost all previous laboratory experiments carried out on adult passerine birds have shown a lack of phenotypic flexibility of intestinal disaccharidases (see "Introduction"). Therefore, interspecific variation of enzyme activities observed in our study in wild birds likely reflects genetically based differences (i.e., differences in constitutive enzyme levels) rather than a flexible phenotypic match to the composition of consumed diet.

We did not directly quantify protein and carbohydrate content in the diets of the studied species but relied on published data. Therefore, it might be argued that real diet composition, particularly protein content, in studied populations differed among species less than we assumed. If so, some plasticity of enzyme activity adjusting it to local feeding conditions might result in the lack of correlation between activities of proteases and assumed dietary protein content. However, this is rather unlikely, since dietary habits of some species in our study are so different (e.g., barn swallow and house finch) that it is reasonable to assume they differed greatly in amount of consumed protein. Moreover, we were able to detect significant interspecies differences in activity of proteases that were not related to diet composition. We also observed considerable interspecific variation in pH optimum of aminopeptidase-N. However, correction of activity of this enzyme measured at pH 7 to activity under species-specific, optimal pH did not change our conclusions; therefore, we conclude that the lack of interspecific correlation between mass-specific activity of proteases and diet composition represents a genuine pattern (or lack thereof).

An interesting observation is that the mass-specific activity of sucrase was correlated with estimated dietary content of starch rather than sucrose, its direct substrate. This could be due to the fact that measured sucrase activity reflects the activity of the enzyme sucrase-isomaltase, which hydrolyzes both sucrose and maltose (Hunziker et al. 1986). Therefore, correlation between dietary starch content and sucrase activity may suggest that this enzyme plays a relatively more important role in the digestion of maltose (a product of starch digestion) than sucrose. This hypothesis seems probable for the species analyzed in this study because starch content in their diet is usually much higher than sucrose content (Table 1). However, this might not be true in, for example, nectarivorous birds, which consume food rich in sucrose and poor in starch (e.g., hummingbirds; cf. Schondube and Martinez del Rio 2004). Moreover, sucrase-isomaltase is involved in the digestion of branched starch chains (amylopectin; Nichols et al. 2003) and thus might be particularly important when species' diets include significant content of branched starch.

Why Do Activities of Proteases Not Show Interspecific Matching to Diet Composition?

We found significant positive relationships between carbohydrases and dietary starch but not between proteases/peptidases and dietary protein. Another phylogenetically informed comparative analysis in minnows (fishes) yielded a similar result: a significant positive relationship between activity of carbohydrases and dietary carbohydrate content but not between activity of proteases and dietary proteins (German et al. 2010). Similarly, evolutionary diet changes in phyllostomid bats seem to be accompanied by changes in activity of intestinal disaccharidases but not aminopeptidase-N (Schondube et al. 2001). This difference is perhaps expected because all animals, regardless of diet, need protein, and so there should not be strong selection for a low protein processing capability in animals. Unlike the case for sucrase and maltase activity, which can be immeasurably low or absent in species that do not ingest those substrates (see Fig. 2), even animals with low protein intakes must maintain some protease activity to absorb an adequate amount of nitrogen and essential amino acids. Indeed, Hofer and Schiemer (1981) argued that it would be advantageous for herbivores with relatively low protein intake but relatively rapid

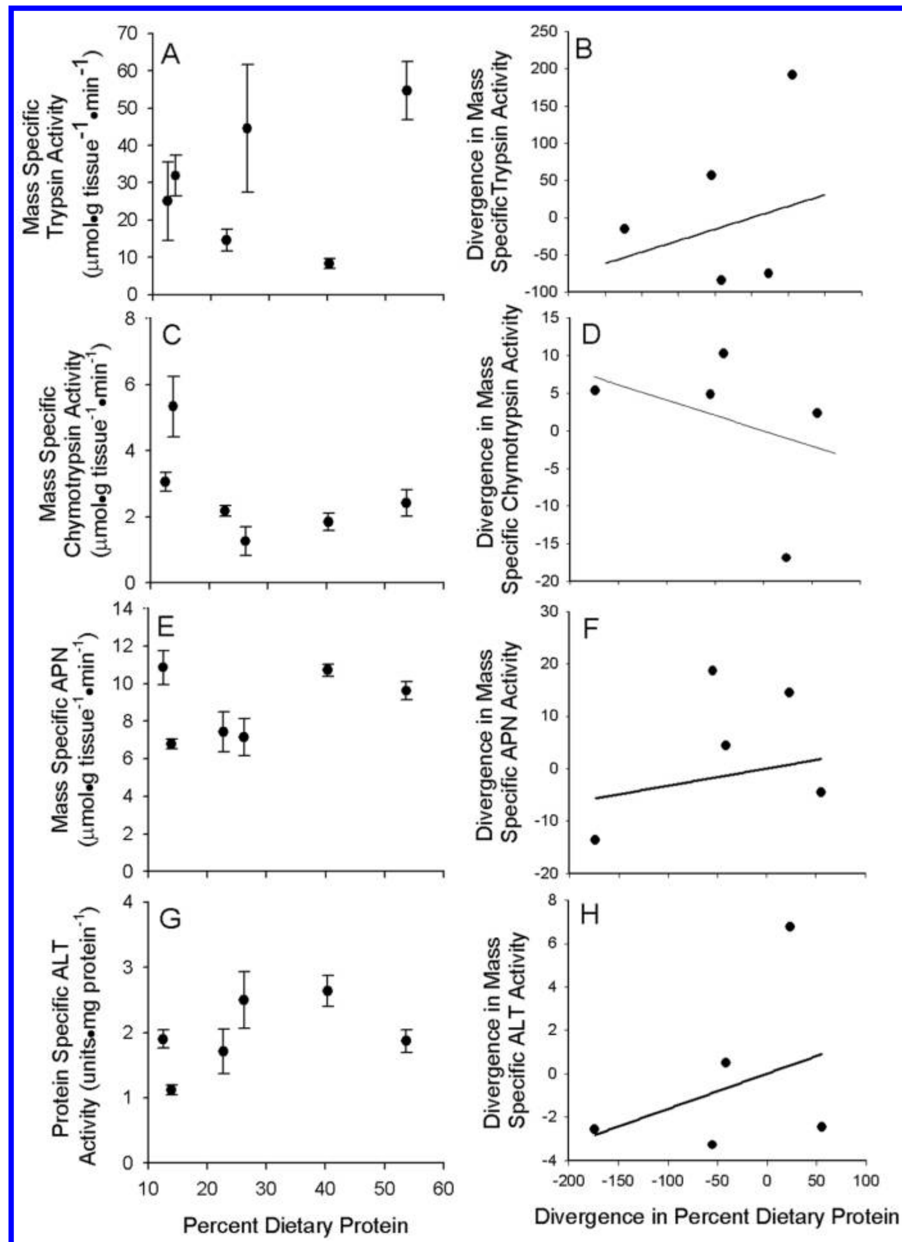

Figure 3. Mass-specific activity of proteases—trypsin (*A*, *B*), chymotrypsin (*C*, *D*), aminopeptidase-N (*APN*; *E*, *F*), and alanine aminotransferase (*ALT*; *G*, *H*)—plotted against estimated dietary protein content (%). In the left column, means ± SEM for each species are shown, which represent the following species (from left to right along the *X*-axes): house finch, house sparrow, northern cardinal, American robin, black-capped chickadee, and barn swallow (the order of species is not the same as in Fig. 2). In the right column, phylogenetically independent contrasts for species-specific average values are shown.

gut throughput to have compensatorily higher biochemical capacity to process proteins and recover them rather than excrete them. Thus, the ranges of dietary protein contents (e.g., 13%–40% in Table 1) and protease capacities across species with different dietary habits are relatively smaller than the ranges of dietary carbohydrate contents (e.g., 2%–77% in Table 1) and carbohydrase capacities across species. The smaller ranges may require larger sample sizes to demonstrate statistically significant correlations between proteases and dietary protein across species. In contrast, laboratory experiments showed that within many avian species, increases in dietary protein content induce increases in intestinal aminopeptidase-N activity (Afik et al. 1995; Martinez del Rio et al. 1995; Sabat et al. 1998; Caviedes-Vidal et al. 2000). Thus, the bigger mystery, for which we have no ready answer, is why within omnivorous passerine species analogous modulation of carbohydrase activity in relation to dietary carbohydrate is rarely observed, at least in adults. In other words, combined results of cited laboratory experiments

and this study suggest that in adult passerines, the activity of proteases is matched to diet composition on an intraspecific level and the activity of carbohydrases on an interspecific level. Studies involving the costs and benefits of modulation and/or the effects of age and phylogeny may be needed to make sense of these patterns.

In summary, our study supported the predictions of the adaptive modulation hypothesis in adult passerine birds for enzymes related to the digestion of carbohydrates but not for those involved in the digestion and postabsorptive processing of proteins. It is unclear whether the pattern of adaptive modulation is generalizable, since other groups of birds show different levels of flexibility of their digestive enzymes. For example, members of the Galloanserae clade probably possess a capacity for intraspecific modulation of disaccharidase activity when adults (Ciminari et al. 2004), whereas pigeons, which are more closely related to passerines, do not possess such a capacity (Ciminari et al. 2005). Also, young passerine birds may show higher flexibility of carbohydrases than adults, and presence of this flexibility may be related to their feeding ecology (significant flexibility occurs in young house sparrows with variable diet but is absent in young zebra finches, which are diet specialists; Brzęk et al. 2009, 2010). Therefore, a broader analysis of variation in flexibility of digestive enzymes—which would include effects of phylogeny, developmental state, and feeding habits—may extend our knowledge about physiological constraints in the evolution and ecology of feeding in birds. Similarly, it is interesting to test how general is the pattern of higher impact of evolutionary changes in diet composition on activity of carbohydrases than proteases, which was observed in passerines (this study), bats (Schondube et al. 2001), and fishes (German et al. 2010).

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