Intestinal α –glycosidase transcriptional responses during development and diet adjustment in altricial birds

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SUMMARY STATEMENT

Increased α –glycosidase transcription doesn't occur as house sparrow and zebra finch nestlings age from hatch to adulthood, but does occur when nestlings of the former species adjust to higher starch diet.

ABSTRACT

We describe developmental changes in maltasic activity and its mRNA through adulthood, and in response to increase in dietary starch. We studied house sparrows (HOSP; *Passer domesticus* L.), which undergo a natural switch from insects to starch-containing seed diet during development, and zebra finch (ZEBF; *Taeniopygia guttata* V.), which have a relatively fixed starchy-seed diet during development. In ZEBF, in whom maltasic activity increased with age but not with dietary starch, α –glycosidase (AG) mRNA was not affected by either age or dietary starch level. In HOSP nestlings, in whom maltasic activity increased with age and with added starch, AG mRNA was higher on diet with added starch but did not increase with age. These results are consistent with the idea that the apparent programmed developmental increase in maltasic activity is not mainly under transcriptional control of AG mRNA, whereas induction of maltasic activity by increased dietary starch is.

INTRODUCTION

Major changes in intestinal enzymes occur during development in vertebrates, but only in mammals are associated transcriptional responses well described, based on studies in about a dozen species (Karasov and Douglas, 2013). A fundamental question is whether the patterns in mammals are general for vertebrates. In developing eutherian mammals, activity increases markedly for starch digesting α -glycosidases (sucrase-isomaltase (SI) and maltase-glucoamylase (MG)), and decreases after weaning for milk disaccharide-digesting lactase-phlorizin hydrolase, in most cases accompanied by parallel changes in the expression of their genes (Galand 1989, Karasov and Douglas 2013). A variety of studies have shown that these changes often occur in the absence of specific signals from either the gastrointestinal (GI) tract lumen or circulation, though levels of some hormones and growth factor(s) are involved in maturation and growth. Earlier or later inclusion of specific substrates in the diet may advance or delay a change in an enzyme's activity and expression of its mRNA (Karasov and Douglas, 2013). The picture that

emerges is of a putative genetic program that rules developmental changes in expression of intestinal α -glycosidases (Galand 1989) that can be modified by environmental factors such as a change in diet composition. In humans, the maltasic activity derives from two α -glycosidases in the intestinal apical-membrane ("brush-border" membrane, bbm): maltase glucoamylase (MG, Enzyme Commission number (EC) 3.2.1.20 and 3.2.1.3, encoded by a gene *MGAM*) and sucrase isomaltase (SI, EC 3.2.148 and 3.2.1.10 encoded by a gene *SI*) (Nichols et al., 2003). In rats, addition of carbohydrate to the diet induces increases in both maltasic and sucrasic activity, and these are accompanied by increases in the expression of the genes *MGAM* and *SI* (Karasov and Douglas, 2013).

Changes in intestinal α -glycosidases and their mRNA during posthatch development in birds has barely been studied. Changes in α -glycosidase mRNA in chickens (*Gallus gallus domesticus*; Galliformes) were described only for the first 7 d post hatch (Sklan et al., 2003; Uni et al., 1999) and without any changes in diet composition. In this paper we describe developmental changes in maltasic activity and its mRNA through adulthood, and in response to modification of dietary starch, in two altricial species in a different avian order, Passeriformes, than chickens. We studied two models of altricial development: house sparrows (HOSP; *Passer domesticus*) (Brzek et al., 2009; Caviedes-Vidal and Karasov, 2001), which undergo a natural switch from low to high starch diet (i.e., from >80% insect diet to 80% seed diet (Anderson, 2006)) during development, and zebra finch (ZEBF; *Taeniopygia guttata*) (Brzek et al., 2010), which have a relatively fixed starchy seed diet during development (Zann, 1996). Using mainly samples of tissue in which maltasic activity had previously been characterized in nestling and adult ZEBF (Brzek et al., 2010) and in nestling HOSP (Brzek et al., 2009; Brzek et al., 2011), as well as in samples from a new experiment with adult HOSP, we test several predictions about gene expression that arise from the general patterns seen during mammalian development and from the more limited information available on birds.

In chickens, changes in sucrasic activity immediately before and after hatch seemed correlated with changes in α -glycosidase mRNA (AG mRNA) (Karasov and Douglas, 2013; Sklan et al., 2003; Uni et al., 1999; Uni et al., 2003), suggesting that developmental variation in sucrasic activity is transcriptionally controlled. Note that for birds, we refer to the mRNA as AG mRNA because, to our knowledge, none of the cloned avian α-glycosidases has been expressed and had its expression product characterized for activity. Based on patterns in mammals and the

chicken, we predicted (#1) that posthatch increases in maltasic activities, which occur in both HOSP (Brzek et al., 2009; Caviedes-Vidal and Karasov, 2001) and ZEBF (Brzek et al., 2010), would be matched by increases in AG mRNA.

Inclusion of extra carbohydrate in the diet induces disaccharidase activity in HOSP nestlings and fledglings (Brzek et al., 2009, 2011), but not in HOSP adults (Caviedes-Vidal et al., 2000) or in ZEBF (Brzek et al., 2010). Considering that in rats diet-induced increases in activity of SI and MG are accompanied by increases in expression of their genes (above), we predicted (#2) that inclusion of extra carbohydrate in diets would increase levels of AG mRNA in young HOSP but not in ZEBF or in adult HOSP.

MATERIALS AND METHODS

Collection, feeding, and maintenance of birds

We used adult and nestling HOSP collected in the wild and then maintained in captivity, and captive, colony-raised adult and nestling ZEBF. Because all the methods on collection, feeding and maintenance were developed and described in previous studies with HOSP nestlings (Brzek et al., 2009; Brzek et al., 2011), HOSP adults (Caviedes-Vidal et al., 2000) and nestling and adult ZEBF (Brzek et al., 2010), we do not repeat them here in detail. All experimental procedures were approved by the University of Wisconsin-Madison Animal Care and Use Committee.

Experimental diets and feedings

Synthetic diets used in these studies were fed in liquefied form to nestlings and in powdered form to post-fledge birds. The diets were composed of the same ingredients but differ in their amounts of corn-starch (carbohydrate), casein (protein), and corn oil (lipid) (Table 1). For each species, there are at least two diets, nearly isocaloric, that have names corresponding to their relative amounts of starch, and for clarity we have retained the names of the diets that were used in the respective source studies. Although there is a 0 starch diet for HOSP, ZEBF nestlings and adults require some starch in their diets (Brzek et al., 2010), but for both species the higher starch diet contained an additional 0.21-0.25 g starch/g diet dry mass. Nestlings from the same clutch were randomly assigned to different diets. Nestlings were syringe-fed hourly 15 times per day with meal sizes calculated using age-specific energy requirements for each species. Post-fledging birds were provided with powdered diet and drinking water *ad libitum*. In general, food

intake of young birds and adults of both species did not differ by diet, and neither did body masses differ by diet at the time tissues were harvested (Brzek et al., 2009, 2010, 2011; Caviedes-Vidal et al., 2000).

Experimental schedules

Nestling ZEBF. Performance of ZEBF nestlings was tested at three time points (days posthatch, dph; hatch day = 0) during their development: (i) the phase of rapid development of the gastrointestinal tract, which occurs at 5 dph corresponding to about 28% of the days to fledging (18 days) (Brzek et al., 2010); (ii) the day of the peak in intestine mass, which occurs at 8 dph corresponding to about 44% of the days to fledging; and (iii) the time of acquisition of adult body mass, which occurs at 15 dph corresponding to about 83% of the days to fledging. Previously unpublished data supporting these designations can be seen in the Fig. S1.

Nestling HOSP. Performance of HOSP nestlings, which fledge at about 14 dph (faster than ZEBF) was tested at three time points during their development that corresponded to the same three developmental phases used in ZEBF (Brzek et al., 2009; Caviedes-Vidal and Karasov, 2001): (i) the phase of rapid development of the gastrointestinal tract, which occurs at 4 dph corresponding to about 28% of the days to fledging; (ii) the day of the peak in intestine mass, which occurs at 6 dph corresponding to about 43% of the days to fledging; and (iii) the time of acquisition of adult body mass, which occurs at 12 dph corresponding to about 85% of the days to fledging.

Postfledging ZEBF and HOSP. Adult ZEBF were raised on the diets for 15-21 d. Fledgling HOSP were raised on their respective diets from 3 dph to 30 dph. Adult HOSP were fed for 1 d natural seeds mixed 50:50 with their respective powdered synthetic diet, and then 14-15 d on pure synthetic diet.

Harvesting and storing of tissues

Birds were killed with CO_2, weighed (± 0.1 g), and dissected to remove the small intestine. We do not report here on other tissues harvested, which is reported elsewhere (Brzek et al., 2009; Brzek et al., 2011; Brzek et al., 2010; Caviedes-Vidal et al., 2000). Small intestine (between pyloric sphincter and vestigial cecae) was flushed with ice-cold Ringer solution, weighed (± 0.1 mg), cut into three sections corresponding to proximal, middle, and distal regions, and

immediately preserved in liquid N_2 and stored at -80 ^{o}C (for enzyme assays) or in RNALater. Although maltasic activity was originally measured and reported for all three regions in both HOSP (Brzek et al., 2009; Brzek et al., 2011) and ZEBF (Brzek et al., 2010), gene expression was measured and reported, along with maltasic activity, only in the middle section because this is a region where maltasic activity is routinely high and where a previous study of both HOSP (Gatica-Sosa et al., 2016) and ZEBF (unpublished data – see Fig. S2) showed that gene expression was highest. The single exception was that in adult HOSP we used proximal intestine, where activity was highest, because of loss of some midgut samples.

Measurement of RNA

Total RNA from intestinal samples was extracted using PureLink Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA, USA), and was quantified using microspectrophotometry (Nano-Drop Technologies, Wilmington, DE, USA). RNA integrity was measured in all samples using the Experion System (Bio-Rad Laboratories, Hercules, CA, USA) except in HOSP adults fed on starch and starch-free diets, whose RNA integrity was assessed in agarose gel stained with GelRed (Biotium Inc., Hayward, CA, USA). Purified RNA was converted to cDNA immediately, or after storage frozen at -80 °C, using the iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). For AG mRNA we developed specific primers based on published sequences for the gene of interest, *MGAM* (Gatica-Sosa et al., 2016). GenBank accession numbers for the genes of interest are EU855810.1 for ZEBF and GQ919053.1 for HOSP, and for the reference genes, *ß-actin* and *GAPDH*, (AY045726) and (AF255390) respectively. Suitability of GAPDH and *ß-actin* as reference genes was evaluated using BestKeeper software (Pfaffl et al., 2004). Real-time PCR to quantify mRNA levels relative to the two reference genes was performed using iQ SYBRGreen Supermix (Bio-Rad Laboratories , Hercules, CA, USA) as described previously (Gatica-Sosa et al., 2016).

Data management

All data. Results are given as means ± 1 s.e.m. (*n*= number of birds per treatment; each treatment defined according to age and diet). Maltasic activities and AG mRNA levels as a function of age and formulated diet (Table 1) were compared using two-way-ANOVA with interaction followed

by post-hoc Student's LSD comparisons. Data from hatchling HOSP, which had unspecified wild diets, were compared with data from HOSP 4 dph eating formulated diet using a *t*-test. Correlations between maltasic activity and AG mRNA were tested using least squares linear regression. For all these analyses, ANOVA assumptions regarding normality and homoscedasticity were tested using Levene and Shapiro Wilk tests, respectively. When normality was not met, a log10 transformation was used. All tests were carried out using JMP PRO, version 13 (SAS Institute Inc., Cary, NC, 1989-2007). In all cases, the significance level was set at $P < 0.05$. During initial planning of experiments, we sought a power (i.e., 1-β) of 0.8. Considering the anticipated variance and differences by age and diet in our measurements of enzyme activity and mRNA (Gatica-Sosa et al. 2016), we planned for a minimum of at least 4 individuals for each combination of age and diet and exceeded this minimum for both HOSP and ZEBF.

RESULTS AND DISCUSSION

In ZEBF nestlings, maltasic activity increased with age ($F_{3,26}$=10.9, P<0.001) but was not affected by dietary starch level ($F_{1,26}$=0.4, P>0.5; Fig. 1A; Table S1). As predicted, AG mRNA was not affected by dietary starch level ($F_{1,27}$=0.3, P>0.6), but contrary to our prediction it did not increase with age ($F_{3,27}$=1.8, P=0.18; Fig. 1B). In HOSP nestlings, maltasic activity increased with age out to 30 dph ($F_{4,42}$=26.9, P<0.001), and was higher in those raised continuously on the diet with added starch ($F_{1,42}$=22.7, P<0.001; Fig. 1C). As predicted, AG mRNA in HOSP was higher in those raised continuously on the diet with added starch ($F_{1,38}$=17.0, P=0.002; Fig. 1D). However, contrary to our prediction, in HOSP AG mRNA did not increase with age on both diets (diet*age interaction $F_{4,38}$=5.4, P=0.0016; Fig. 1D) as maltasic activity had (cf. 0 starch diet in Fig. 1C,D).

The most distinctive feature in HOSP and ZEBF regarding development of intestinal maltasic capacity is that mass-specific maltasic activity continued to increase with age (Fig. 1A,C). In HOSP, the mass-specific activity continued to rise to a value at 30 dph of more than ten times the value at hatch (Fig. 1C; see also (Caviedes-Vidal and Karasov, 2001)). In contrast, in chickens and in poultry, mass-specific maltasic activity rises to about four times the value at hatch very quickly, and then remains relatively constant thereafter (Karasov et al., 2002). The intestines of avian species generally increase in size until about half of the adult weight is

reached (Karasov et al., 2002). The overall maltasic capacity over the full intestine length, which is the product of intestinal mass and mass-specific maltasic activity, increases with age mainly due to the continued increase in intestinal size, but in HOSP, also due to the increase in mass-specific activity. Avian altricial species have faster postnatal growth rates than similar-sized precocial species. Altricial species' continually increasing enzymatic capacities, achieved through higher mass-specific activity and/or intestine mass, may be a prerequisite for supporting their higher growth rate.

Contrary to our prediction, increases in mass-specific maltasic activity with age were not associated with increases in its mRNA in HOSP or ZEBF (Fig. 1B,D). The explanation is not as simple as a lack of transcriptional flexibility, in light of the obvious flexibility in response to level of dietary starch (Fig. 1D). Some other possible explanations include changes in small intestine structure such as (i) increases with age in microvillous surface area per mg or per cm^2 nominal intestinal area and (ii) increase in the proportion of cells on the villi that are mature and hence exhibiting high expression, and/or changes in enzyme dynamics such as (iii) changes in the relationship of enzyme synthesis rate in relation to degradation rate. Relatively larger amounts of the enzyme per unit apical membrane, measured by Western blot for example, would be apparent in the case of the latter two explanations but not the first.

As we predicted, increased mass-specific maltasic activity was associated with increase in its mRNA in nestling and fledgling HOSP fed diet with increased starch (Fig. 1C,D). Although the diets also had different lipid contents (Table 1), which has been shown to alter disaccharidase activity in HOSP (Brzek et al., 2013), other studies with HOSP have confirmed that induction of maltasic activity by increased dietary starch occurs even when dietary lipid is held constant, and as quickly as 24 h after a diet switch (Brzek et al., 2013; Rott et al., 2017). The correlation of induced maltasic activity with increase in its mRNA by the starch diet ($F_{1,22}$=47.7, P<0.0001; Fig. 2A) was in contrast to the lack of correlation between these values for birds fed the starch free diet ($F_{1,22}$=0.399, P=0.53; Fig. 2B). These patterns are consistent with the hypothesis that dietary induction of this enzyme is under transcriptional control, which is consistent with similar findings in mammals (Karasov and Douglas, 2013). In young HOSP the inducing effect of dietary starch on maltasic activity and its mRNA is entirely reversible (Brzek et al., 2011; Karasov, 2011), reflecting flexibility in both transcription and activity. But, the inducing effect of increased dietary starch on activity and mRNA was not apparent in either

adult HOSP (Fig. 1D; see also (Caviedes-Vidal et al., 2000)) or in nestling or adult ZEBF (Fig. 1B). An explanation might be a total lack of transcriptional flexibility, perhaps due to a loss of some key components of the transcription activation signals associated with increased dietary starch. Resolution of this awaits the characterization of the transcriptional promoter(s) of the AG genes in birds.

Differences among species or even cohorts within species in transcriptional and enzymatic flexibility in response to increased dietary starch are expected from an ecological and evolutionary perspective. In nature, HOSP nestlings transition from a diet of mainly arthropods, which are high in protein and low in carbohydrates, to a diet of mainly seeds, many of which are high in carbohydrates and low in protein (Rott et al., 2017). Flexibility in maltasic activity and AG mRNA maximizes the digestibility of starch when it is at high dietary levels and minimizes the cost of synthesizing excess enzyme when starch is at low levels (Rott et al., 2017). In contrast, the regulatory capacity for reversible digestive flexibility is arguably wasteful in specialist feeders. This may explain why HOSP adults, which essentially are specialists on nonanimal foods, did not modulate maltasic activity or AG mRNA when fed diets with different amounts of starch (Fig. 1). Likewise, ZEBF are diet specialists that consume only carbohydrate-rich seeds beginning at hatching (Brzek et al., 2010), and neither nestlings nor adults modulated maltasic activity or AG mRNA when fed diets with different amounts of starch. Hence, intestinal α –glycosidase and its mRNA are stimulated by dietary carbohydrate in nestling HOSP but not in adult HOSP or in ZEBF.

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COMPETING INTERESTS

No competing interests declared.

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Table 1. Composition (% of dry mass) of diets used in experiments

	Zebra finch		House sparrow			
	Nestlings and adults		Nestlings and fledglings (30d-old)		Adults	
	Medium starch (MS) [+]	High starch (HS) [+]	0 starch[#]	+ starch[#]	0 starch	+ starch
Corn starch	25.4	46.2	0	25.4	0	25.4
Casein (protein)	46.2	25.4	59.6	46.2	59.6	34.2
Corn oil	8.0	8.0	20	8.0	20	20
Alphacel non-nutritive bulk	4.9	4.9	4.9	4.9	4.9	4.9
Silica sand	5.0	5.0	5.0	5.0	5.0	5.0
Amino acids, vitamins, mineral salt, etc.*	10.5	10.5	10.5	10.5	10.5	10.5
Energy, kJ/g[&]	16.1	16.0	19.0	16.1	19.0	18.8

*Content as described by Lepczyk et al., 1998.

[+]diet as named and described in Brzek et al., 2010

[#]diet as named and described in Brzek et al., 2009

[&]Energy content, approximate value calculated

Figures

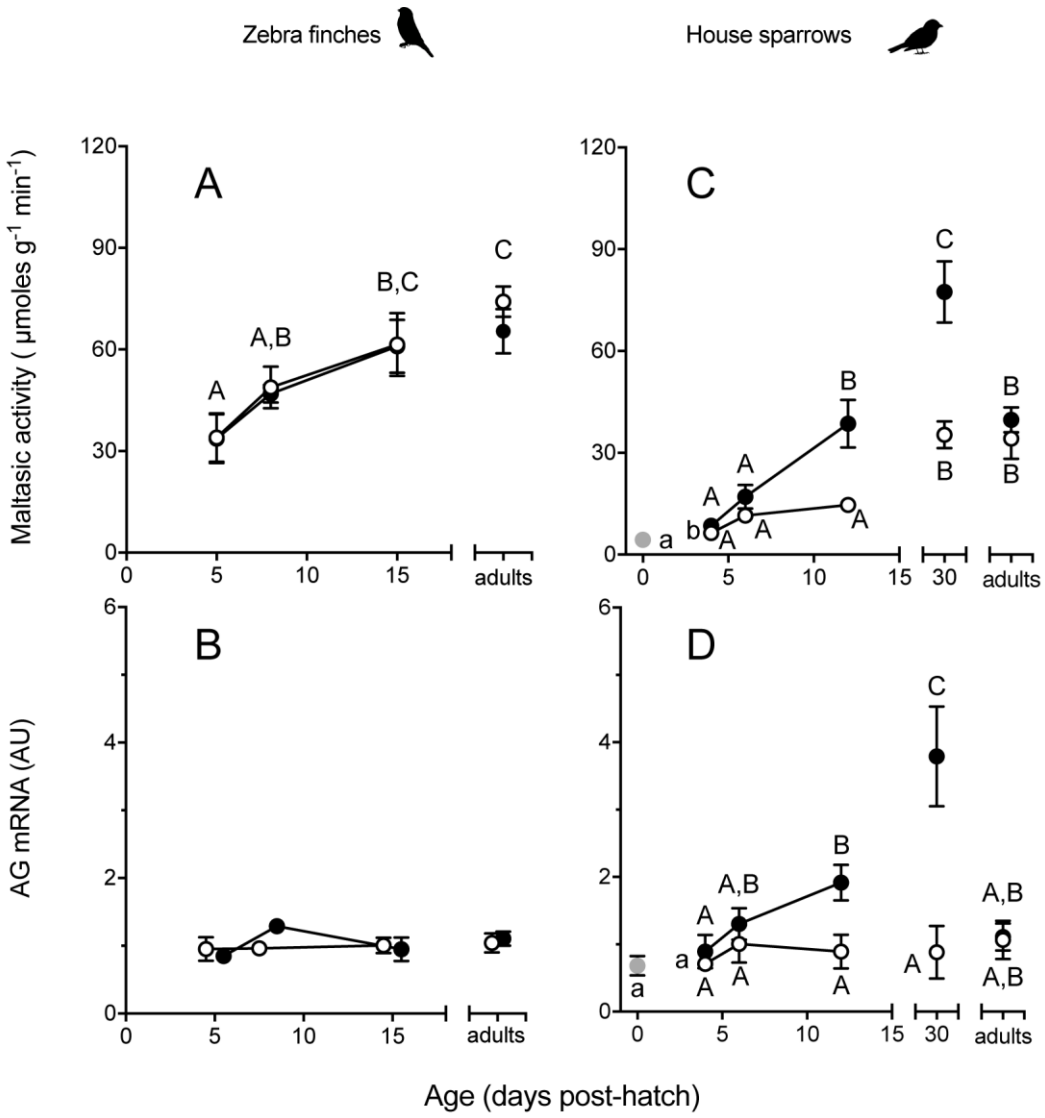


FIGURE 1. Intestinal maltasic activity and α –glycosidase (AG) mRNA as a function of age in zebra finches (ZEBF) and house sparrows (HOSP) raised on diets with differing starch contents. (A) ZEBF maltasic activity on medium starch (MS) diet (unfilled circles) and high starch (HS) diet (filled circles). (B) ZEBF AG mRNA on the two diets (symbols as in A). (C) HOSP maltasic activity on zero (0) starch diet (unfilled circles) and starch-containing (+starch) diet (filled circles). (D) HOSP AG mRNA on the two diets (symbols as in C). Values are means±s.e.m. Within each plot, different characters represent significant differences ($P < 0.05$)

for the age and diet effect. In all the HOSP plots, the left-most circle colored in grey represents values for nestlings removed from nests in the wild on the day of hatch. Those HOSP hatchlings differed in maltasic activity from 4dph nestlings fed formulated diet ($t_{(7.89)}$=2.62, P =0.031; signified by different lower case letters) but they did not differ in AG mRNA ($t_{(7.22)}$=0.63, P>0.5; signified by same lower case letter). All other values within a plot that share the same upper case letters do not differ significantly by Student's LSD tests). Number of individuals used in each group by species, assay, diet and age were as follows: ZEBF: four individuals of 5, 8, 15 dph for both diets (i.e., HS and MS) for mRNA and maltasic activity, but 3 were measured for maltase in the 5 dph MS group, and 5 and 6 adults were assayed for both mRNA level and maltase activity for MS and HS, respectively; HOSP: four individuals of 4, 6, 12, 30 dph and adults for both diets (i.e., SF and MS diets) were assayed for mRNA and maltase activity, but 8 SF fed 12 dph nestlings for maltase, and 4 and 10 hatchlings for mRNA and maltase were assayed, respectively.

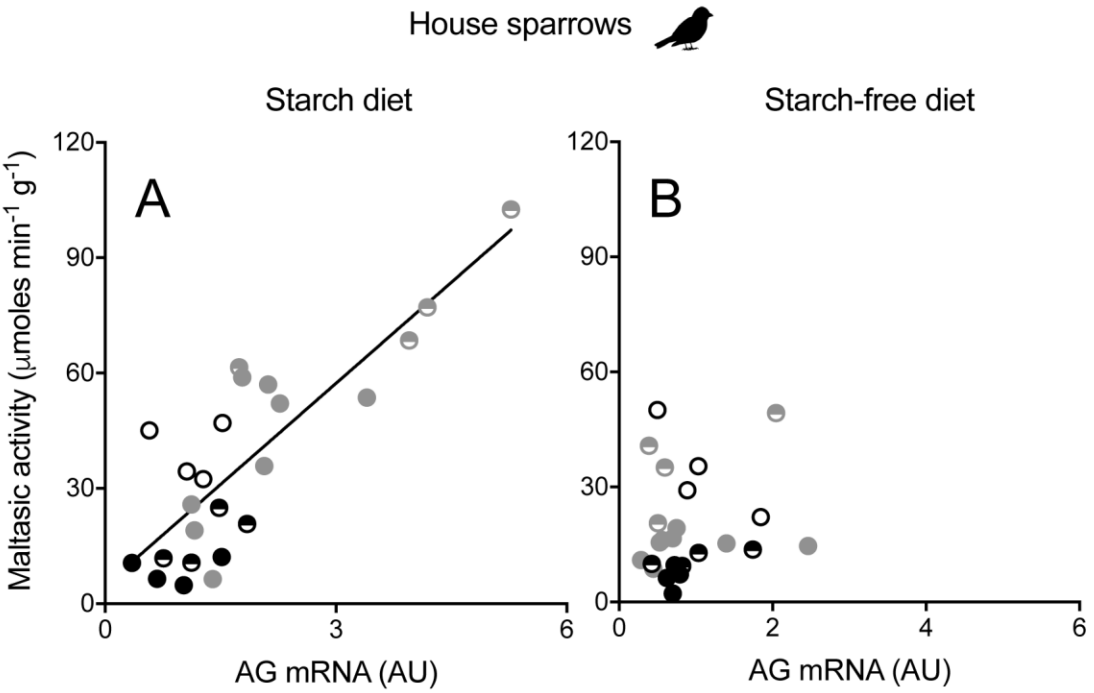
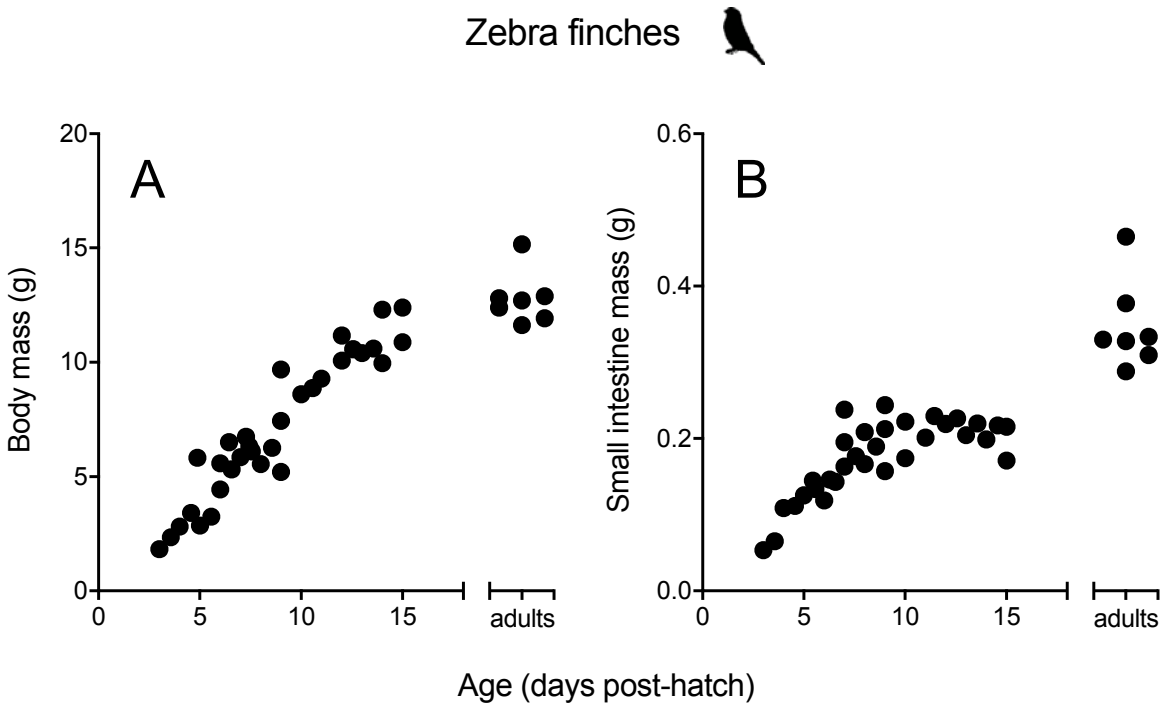


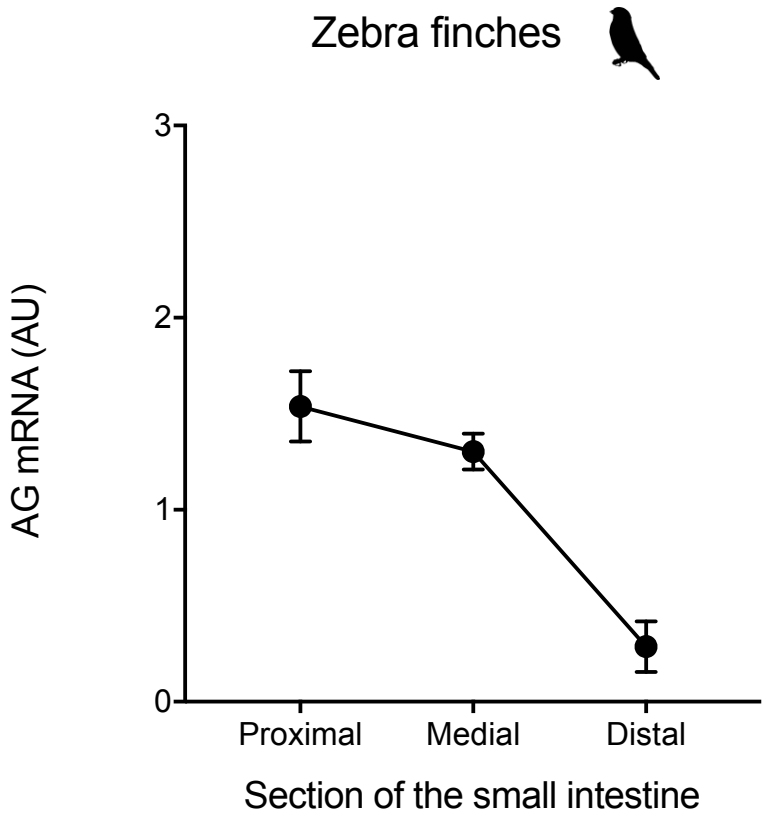
FIGURE 2. Correlation of maltasic activity and AG mRNA. Maltasic activity in HOSP nestlings and adults fed starch-containing diet was correlated to an increase in its AG mRNA ($F_{1,22}$=47.7, P<0.0001; equation: y=17.52 (±2.54) · x + 4.77 (±5.53), r^2=0.68; Fig. 2A), while this correlation was not apparent for birds fed a starch free diet ($F_{1,22}$=0.399, P=0.53, r^2=0.02 Fig. 2B). Black filled, half black filled, gray filled, half gray filled and black empty circles represent 4, 6, 12, 30 dph HOSP nestlings and adults, respectively.

Supplementary Table 1. Results of two-way ANOVAs to evaluate the effect of diet, age and their interaction on maltasic activity and AG mRNA of ZEBF and HOSP, respectively.

Measurement					
Maltase activity					
	Species				
	ZEBF (n=34)	Source of Variation	*df*	*F*	*P-value*
		Age	3	10.88	0.0001
		Diet	1	0.36	0.5564
		Interaction	3	0.21	0.8923
		Error	26		
		Total	33		
	HOSP (n=52)	Source of Variation	*df*	*F*	*P-value*
		Age	4	26.91	0.0001
		Diet	1	22.74	0.0001
		Interaction	4	5.38	0.0014
		Error	42		
		Total	51		
AG mRNA level					
	Species				
	ZEBF (n=35)	Source of Variation	*df*	*F*	*P-value*
		Age	3	1.75	0.18
		Diet	1	0.25	0.62
		Interaction	3	1.37	0.27
		Error	27		
		Total	34		
	HOSP (n=48)	Source of Variation	*df*	*F*	*P-value*
		Age	4	5.36	0.0016
		Diet	1	17.01	0.0002
		Interaction	4	5.48	0.0014
		Error	38		
		Total	47		



Supplementary Figure 1. Body mass (A) and small intestine mass (B) in zebra finch nestlings as a function of age (days post hatch) and as compared to adults. In this preliminary study, the birds received daily a mixture of seeds and bird food and fresh water *ad libitum*, and every other day a specially prepared egg food to support growth and reproduction. Each point is an individual bird.



Supplementary Figure 2. Amount of intestinal AG mRNA as a function of intestinal position in adult Zebra finches. AG mRNA differed with intestinal position ($F_{2,13.9}$=22.49, P<0.0001) and was significantly lower in distal intestine (P<0.05) than in either of the more proximal regions, which did not differ from each other (P=0.18). Data for 11 birds eating either diets MS or HS were pooled because AG mRNA did not differ as a function of diet (P=0.17).