ORIGINAL ARTICLE

Differential transcriptional responses underlie dietary induction of intestinal carbohydrase activities in house sparrow nestlings

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Summary

Many species show diet-induced flexibility of activity of intestinal enzymes; however, molecular and genetic mechanisms responsible for such modulation are less known, particularly in altricial birds. The goal of our study was to test whether a diet-induced increase in activity of intestinal maltase and sucrase in house sparrow nestlings is matched with an increase in maltase–glucoamylase (*MG*) and sucrase–isomaltase (*SI*) complex mRNAs respectively. Both enzyme activities were significantly higher in mid-intestine of nestlings fed a medium-starch (MS) diet compared to those fed a starch-free (SF) diet. In contrast to the similar pattern of dietary induction for both enzyme activities, diet MS elevated significantly only the level of *MG* mRNA, but not *SI* mRNA. The coordinated increase in activity of maltase and in *MG* mRNA is consistent with the hypothesis that dietary induction of this enzyme is under transcriptional control. In contrast, the lack of such coordination for changes in activity of sucrase and *SI* mRNA suggests that upregulation of this enzyme may be achieved by post-translational factor(s). We conclude that genetic mechanisms responsible for diet-induced flexibility of digestive enzymes in birds may differ from that observed in mammals.

Introduction

Many birds and mammals are omnivorous, eating a wide variety of foods depending on changing availability or nutritional requirements, and their production of digestive enzymes and nutrient transporters may depend on their substrates in the diet (Karasov et al., 2011). Digestive flexibility (or plasticity) denotes that ability to adaptively modulate the biochemistry of the gut to digest different food types (Sabat et al., 1998). For digestive enzymes, reversible phenotypic plasticity, that is phenotypic flexibility (a term that distinguishes it from irreversible developmental plasticity; Brzęk et al., 2011; Piersma and van Gils, 2011), can provide good correspondence between production of digestive enzymes and changes in diet composition and, hence, concentration of enzyme substrates in the gut (Karasov et al., 2011). Such correspondence is considered adaptive because

putative costs associated with the maintenance of the gut need to be balanced by benefits of gut function (i.e. extraction of nutrients and energy). Flexibility could maximize the digestibility of substrates when they are at high dietary levels and minimize the cost of synthesizing excess enzymes (or devoting limited membrane space to it (Diamond, 1991)) when their substrates are at low levels.

House sparrow (HOSP, *Passer domesticus*) nestlings are a particularly suitable model to study digestive flexibility. In the wild, very young HOSP nestlings are fed mainly on insects, whereas the diet of older nestlings contains more seeds (Anderson, 2006). Gradual increase in the carbohydrate content in diet is accompanied by simultaneous increase in activity of digestive enzymes that digest carbohydrates (Caviedes-Vidal and Karasov, 2001). In captive HOSP nestlings hand-fed with a liquefied diet containing starch, proteins and lipids (mimicking a mix of seeds

and insect consumed by older nestlings), activity of intestinal carbohydrases (maltase and sucrase) was twice higher than in nestlings fed on starch-free (SF) diet containing only protein and lipid (mimicking insects consumed by younger nestlings) (Brzęk et al., 2009; Brzęk et al., 2011). This difference was completely reversible when the diets were switched (Brzęk et al., 2011). The modulation was apparently specific to intestinal carbohydrases because the activity of aminopeptidase N did not differ substantially between the diet groups (Brzęk et al., 2009; Brzęk et al., 2011).

Results of described experiments show that diet composition may induce the activity of intestinal carbohydrases in young HOSP but do not reveal molecular mechanisms responsible for this effect. In rodents, dietary carbohydrate induction of intestinal carbohydrase activities appears to be largely due to an increased mRNA transcription rate (Yasutake et al., 1995; Tanaka et al., 1998; Kishi et al., 1999; Mochizuki et al., 2010). However, to our knowledge, this has never been tested in birds; notwithstanding, their digestive physiology may differ considerably from that of mammals (McWhorter et al., 2009). The goal of this study was to test for the association between inductions of intestinal maltase and sucrase activities and increase in the expression of the maltase–glucoamylase (*MG*) and sucrase–isomaltase (*SI*) genes, by assessing their respective mRNAs levels. We first developed specific primers for HOSP of the genes of interest, *MG* and *SI*, and then used them to perform RT-qPCR on the same tissues used in assays of enzyme activity.

Materials and methods

Intestinal samples analysed in this study represent a subset from birds that were the subjects of an experiment described elsewhere Brzęk et al. (2009). Briefly, 3-day-old (day of hatch = day 0) HOSPs were collected and hand-fed with either of two contrasting diets as in Lepczyk et al. (1998), a starch free diet (hereafter SF, with 0% corn starch, 59.6% casein and 20% corn oil) and medium-starch (hereafter MS, with 25.4% corn starch, 46.2% casein and 8% corn oil). SF diet was intended to mimic the insect-based diet typical of very young HOSP nestlings, whereas MS diet mimicked a mixed insect–seed diet of older nestlings (however, protein content in this diet was still higher than carbohydrate content). Diet assignment in both HOSP nestling groups was maintained during all the trial, although meal size was increased with age (for details, see Brzęk et al., 2009).

Enzyme activities and mRNA levels were measured in intestinal tissue of 12-day-old nestlings, which is a few days before their typical fledging age (15 days). Birds were euthanized with CO_2 and dissected to remove the gastrointestinal tract. Intestines were flushed with ice-cold avian Ringer solution. Pieces of approximately 1 cm were cut from the proximal, medial and distal (relative to the pyloric valve) regions of the intestine and weighed (± 0.1 mg). Each piece was divided longitudinally and those sections weighed. One of those pieces was snap frozen in liquid N_2 and stored at -80 °C to later assess disaccharidase activities. The other piece was submerged in RNAlater and kept overnight at 4 °C and then stored at -80 °C until RNA extraction procedure was performed. Sample size was $n = 4$ nestlings for each diet. All experimental procedures were approved by the University of Wisconsin-Madison ethics committee (permit number RARC A-01269-4-10-06).

Determination of disaccharidases activities

Assays of maltase activity were performed as described in detail elsewhere (Brzęk et al., 2009). In brief, tissues were thawed at 4 °C and homogenized (Omni 5000 homogenizer; Omni International, Waterbury, CT, USA; 20 s) in the homogenization buffer (350 mmol/l mannitol in 1 mmol/l Hepes–KOH, pH 7.0). Gut homogenates were appropriately diluted with the homogenization buffer and incubated with 30 μl of 56 mmol/l maltose in 0.1 mol/l maleate and NaOH buffer, pH 6.5, at 40 °C for 20 min. Then, 400 μl of a stop–develop reagent (GAGO-20 glucose assay kit; Sigma-Aldrich, Saint Louis, MO, USA) was added to each tube, vortexed and incubated at 40 °C for 30 min. Finally, 400 μl of 6 mol/l H_2SO_4 was added to each tube, and the absorbance was read at 540 nm. Sucrase activity was determined in a similar way using sucrose instead of maltose as substrate. Maltase and sucrase activities of each intestinal section were calculated and then expressed as micromoles of substrate hydrolysed per minute per gram wet mass of tissue. We did not express activity per mg protein because in several of our previous works with HOSPs nestlings and adults, we demonstrate that protein content is strongly correlated with small intestine tissue mass and gives similar patterns of intestinal enzyme activities (Lepczyk et al., 1998; Caviedes-Vidal et al., 2000; Caviedes-Vidal and Karasov, 2001). We used homogenates of intestinal tissue, which yield a measure with high repeatability of the activity of brush border membrane (BBM) enzymes (Brzęk et al., 2013). In mice, there is no significant difference between sucrase activity measured in tissue homogenates and in everted sleeves that isolate the

measurement to the BBM (Lee et al., 1998). Thus, the homogenate likely gives a good indication of BBM activity, and there does not seem to be an overestimate due to many other possible intracellular sources of activity. Also, another method involving isolation of BBM vesicles may result in an underestimation of enzymatic capacity because of inefficient recovery during the isolation process (Martínez del Rio, 1990).

Assessment of mRNA levels

We first developed specific primers for HOSPs of the genes of interest, *MG* and *SI*. Reverse-transcription reactions were run on total RNA (see below procedure). PCR products were cloned and sequenced (partial sequences for *MG* and *SI* were published in the GenBank database, Table 1).

Total RNA from intestinal samples (proximal, medial and distal regions) of HOSPs was extracted using PureLink Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA, USA) and quantified using microspectrophotometry (Nano-Drop Technologies, Wilmington, DE, USA). RNA integrity was measured using the Experion System (Bio-Rad Laboratories, Hercules, CA, USA).

Purified RNA was converted to cDNA immediately or stored frozen at −80 °C. First-strand cDNA synthesis was performed using the iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, the reaction was performed with 1.0 μg total RNA in 15 μl RNAse-free water, 4 μl 5X iScript reaction mix and 1 μl iScript reverse transcriptase. Reactions were performed under the following conditions: 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min in a GeneAmp thermocycler (Applied Biosystems, Waltham, MA, USA). Finally, the cDNA was diluted to a final volume of 330 μl and stored at 4 °C.

Table 1 Sequences and GenBank accession numbers of assessed genes in this study

Gene	Accession numbers	5'/3' Primer	Product size (bp)
MG	GQ919053.1	Fw CATTGAGACGGTGTTTCCAG Rv AGGTTGAACTCCAGCATTCC	153
SI	GQ919054.1	Fw TGGAATAGATACCGCCTTCCT Rv CCACTGTGCTTCAGGGAAGT	103
β-Actin	AY045726	Fw TGCGTGACATCAAGGAGAAG Rv CGGATGTCCACATCACACTT	244
GAPDH	AF255390	Fw TGCTGCTCAGAACATTATCCCA Rv TTTCCCACAGCCTTAGCAGCT	54

Fw, forward; Rv, reverse; bp, base pairs.

Under our experimental conditions, we determined that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Wynne et al., 2008) and β-actin (Lorenzen et al., 2001) were suitable reference transcripts to use as a reference RNA, as evaluated by the BestKeeper software program (Pfaffl et al., 2004).

All qPCRs were performed in triplicate 25 μl volumes using iQ SYBRGreen Supermix (Bio-Rad Laboratories). A master mix was prepared for each primer set containing SYBRGreen Supermix and an appropriate volume of each primer to yield a final concentration of 200 nM. Reactions were performed on a Realplex Real-Time PCR Detection system (Eppendorf, Hamburg, Germany) using a 2-step amplification plus melting curve protocol. The reaction conditions were 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s and 55 °C for 45 s; the melt curve protocol began immediately after amplification and consisted of 1 min at 55 °C followed by 80–10 s steps with a 0.5 °C increase in temperature at each step. Threshold values for Cq determination were generated automatically by the Realplex software. Lack of variation in PCR products and the absence of primer–dimers were ascertained from the melt curve profile of the PCR products.

The analysis of Cq data from qPCR runs followed MIQE guidelines (Bustin et al., 2009). Briefly, we first averaged qPCR triplicates for each sample and then means were normalized to reference genes (β-actin and GAPDH) using the following equation: $Cq_{normalized} = Cq_{(gene\ of\ interest)} - [1/n \sum_{i=1} Cq_{(ref\ gene)\ i}]$, relativized according to $N_{Rel} = 2^{(Cq\ mean - Cq)}$. These values represent relative quantities of mRNA and are expressed as arbitrary units. Efficiencies of the qPCR primers of genes of interest were calculated from standard curves (in all cases, we found values between 90% and 100%, data not shown).

Statistical analysis

Results are given as means ± 1 SEM. All tests were carried out using SYSTAT (Wilkinson, 1992). To analyse the effect of diet composition (MS and SF) and intestinal position (proximal, medial and distal) on maltase and sucrase activities, as well as on the level of their mRNA, repeated measures ANOVA (RM-ANOVA) were used. Pearson's correlation coefficients (r) and regression equations were calculated to analyse the relationship between enzyme activities and mRNAs. In all tests, the significance level was set at α < 0.05. To meet RM-ANOVA assumptions, mRNA data were natural log transformed.
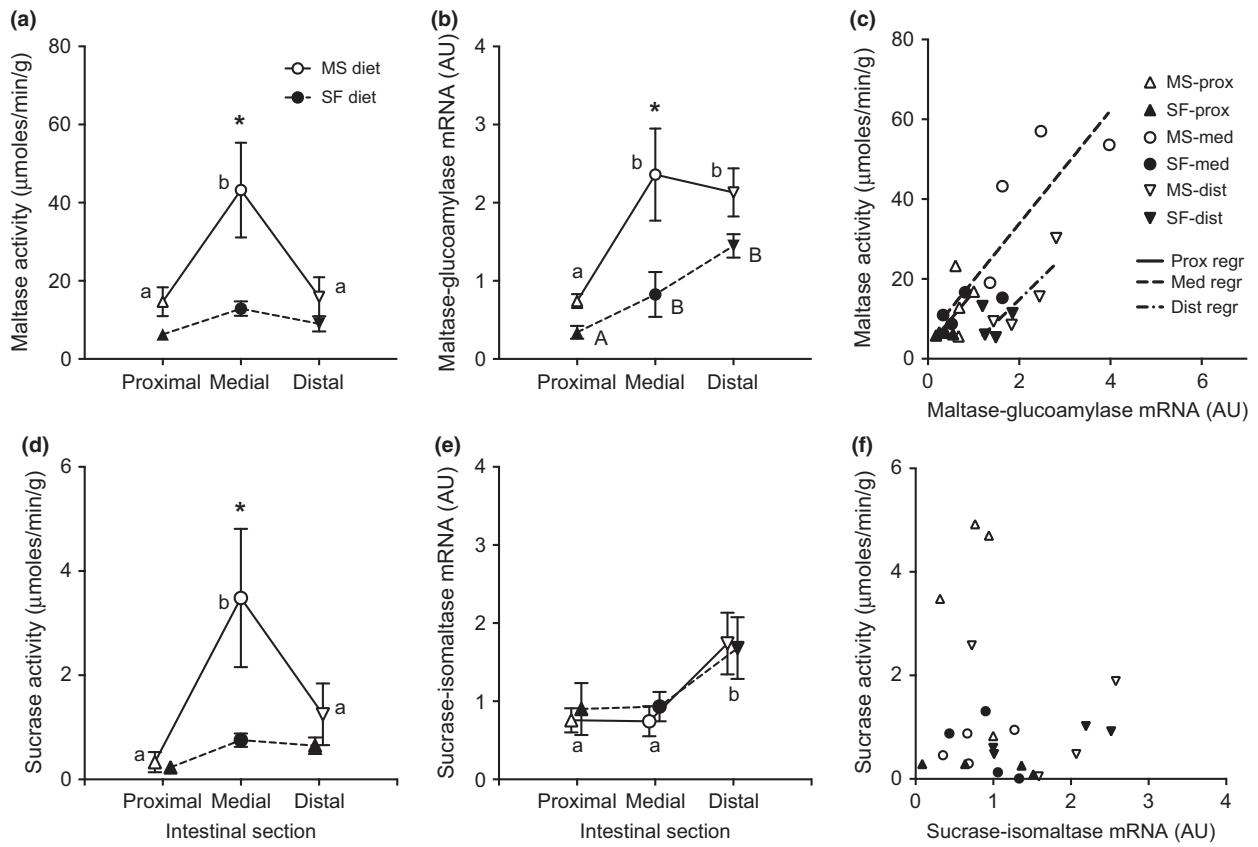
Fig. 1 Intestinal disaccharidase activities and gene expressions (mRNA) of the proximal (upside triangles), medial (circles) and distal (downside triangles) regions of the small intestine of 12 days old house sparrow nestlings fed with either a starch-containing (empty symbols) or a starch-free diet (filled symbols). Panels A–B and D–E show plots for maltase activity and maltase-glucoamylase mRNA and sucrase activity and sucrase-isomaltase mRNA, respectively along the small intestine. Panels C and E show the association between enzyme activities and gene expressions values and linear regressions by intestine sections (proximal: continuous line, medial: dashed line and distal: dash-dotted line). Different Latin characters and asterisks in each plot, represent significant differences ($p < 0.05$) for the intestine position and diet effects, respectively.

Results and discussion

Maltase and sucrase activities varied significantly with both diet and intestinal position [Fig. 1(a, d)], although the effect of diet depended on intestinal position (significant interactions diet x intestinal position; Table 2). The patterns overall were similar for both enzyme activities, as showed by results of the post hoc comparisons (Fig. 1a,d). First, both enzyme activities were significantly higher in nestlings fed the MS diet only at the medial section (maltase p = 0.004; sucrase p = 0.019). Second, in nestlings fed the SF diet, neither enzyme activity varied significantly with intestinal position (p's > 0.7; Fig. 1a,d), whereas in nestlings fed the MS diet, activities of both carbohydrases were significantly higher in the medial intestine than in either the proximal or distal regions (p's < 0.003; Fig. 1a,d). Our results of disaccharidases activities are similar to those reported in rats, where disaccharidases

adaptation to a high-carbohydrate diet was greatest in jejunum, and less marked in the distal portion, where enzyme activity was low. This is probably due to the rapid digestion of starch and absorption of smaller sugars, resulting in little sugar reaching the ileum under normal circumstances (McCarthy et al., 1980). It has been argued that the amount of substrate in the lumen regulates the expression of the enzyme and its activity, at least those that are transcriptionally regulated (Mochizuki et al., 2010; Inoue et al., 2011). One reason that may explain why the medial section of the small intestine has the highest disaccharidase activities compared to the proximal and distal sections when starch is present in the diet is that in the proximal section, the substrates for these enzymes are not readily available because the secretion of pancreatic amylase occurs in the proximal section. Then, products of amylase activity (e.g. maltose, maltotriose and α-dextrins), which in turn are the substrates for the

Table 2 Summary of repeated measures ANOVA results for the effects of diet, position and interaction (diet vs. position) along small intestine on disaccharidases activities and mRNA ($n = 4$ for all treatments and intestinal positions). mRNA data were natural log transformed

	Repeated measures ANOVA		
	Effect of diet	Effect of position	Interaction
Maltase activity	$F_{1,6} = 8.88$; $p = 0.025$	$F_{2,12} = 17.92$; $p = 0.0003$	$F_{2,12} = 8.31$; $p = 0.005$
MG mRNA	$F_{1,6} = 9.87$; $p = 0.020$	$F_{2,12} = 38.31$; $p = 0.0001$	$F_{2,12} = 3.27$; $p = 0.074$
Sucrase activity	$F_{1,6} = 4.57$; $p = 0.076$	$F_{2,12} = 15.91$; $p = 0.0004$	$F_{2,12} = 8.87$; $p = 0.004$
SI mRNA	$F_{1,6} = 0.50$; $p = 0.506$	$F_{2,12} = 10.27$; $p = 0.003$	$F_{2,12} = 0.80$; $p = 0.473$

disaccharidases, may be not present or are in low concentrations and therefore producing a low signal for protein expression. Under this scenario, the medial segment receives the highest amount of substrates to hydrolyse and diminishes towards the end of the intestine. This pattern of disaccharidase substrate availability along the intestine matches the activity pattern of maltase and sucrase and gene expression of MG, in which its expression is compatible with a transcriptional regulation.

Effects of diet and intestinal position on mRNAs differed markedly from patterns observed for carbohydrase activities. First, nestlings fed the MS diet showed higher values of MG mRNA but not SI mRNA, and near-significant interaction between diet composition and intestinal position suggests that MG mRNA was elevated mainly in medial region of the intestine, that is the same region where we observed dietary induction of maltase activity (Table 2; Fig. 1b,e). Second, both mRNAs increased significantly from the proximal to the distal region of the small intestine (Fig. 1b,e).

We found that young HOSP nestlings fed on MS diet showed greater activities of both maltase and sucrase, as well as a higher level of MG mRNA, than birds fed on diet SF. On the other hand, diet did not affect SI mRNA. The increase in maltase due to the diet was highly correlated with an increase in MG mRNA ($r = 0.49$, $p = 0.0001$, Fig. 1c), consistent with a transcriptional regulation of the enzyme expression. Oppositely, the increase in sucrase activity was not associated with an increase in SI mRNA ($r = 0.02$, $p = 0.498$, Fig. 1f), which is inconsistent with transcriptional control.

Our results for maltase and MG mRNA are in agreement with studies in rodents where dietary induction of intestinal carbohydrase activities appears to be largely due to an increased protein transcription rate (Yasutake et al., 1995; Tanaka et al., 1998; Kishi et al., 1999; Mochizuki et al., 2010). In mice, the increase in the jejunal activity of maltase caused by a high-starch diet is regulated transcriptionally by increased acetylation of histones and binding of CREB-binding protein (CREBBP, a coactivator) and

the transcriptional factors caudal type homeobox 2 (CDX2) and HNF1 homeobox (HNF1) in the promoter/enhancer and transcriptional regions of *Mgam* gene (Mochizuki et al., 2010)). Similarly, also dietary induction of sucrase activity is under transcriptional control in rodents. Increase in sucrase activity by high-carbohydrate diet in rats is also associated with increased level of SI mRNA (Yasutake et al., 1995; Inoue et al., 2011) and increased histone acetylation (Inoue et al., 2011), although – unlike the case of dietary induction of maltase – the expression of CDX2 and HNF1 was not affected by dietary manipulation (Inoue et al., 2011). But, a notable finding in our study was that in the nestling HOSPs, the increase in sucrase activity was not associated with an increase in SI mRNA. Presumably, other post-transcriptional changes are responsible for dietary-induced changes in sucrase activity in birds. For example, sucrase activity may increase due to the activation of enzyme stored in enterocytes or may reflect a reduction in the rate of catabolism of the BBM enzyme. Perreault and Beaulieu (1996) hypothesized that the lack of relationship between enzyme activity and mRNA abundance may be related to the fact that SI is synthesized by both crypt and villus cells, although the processing of the cotranslationally glycosylated high mannose precursor is dependent on the state of differentiation of the enterocytes. According to this, possibly there are differences in the proportion of differentiated/undifferentiated enterocytes along small intestine. These differences probably represent a general mechanism for the regulation of the expression of differentiated cell products at the post-translational level.

We also hypothesize that the transcriptional control cannot fully explain the gradient in the activity of the carbohydrases along the intestine in nestling HOSPs. Both mRNAs increased along the small intestine (p's ≤ 0.003; Table 2), with statistically significant differences between proximal and distal regions (Fig. 1b, e). In contrast, the activities of both studied enzymes in MS nestlings did not differ significantly between the proximal and distal regions, but were significantly

higher in medial part of the intestine (Fig. 1a,d). Interestingly, our results for sucrase match very well patterns described in 14-day-old pigeons by Dong et al. (2012a,b). In the pigeons, as in nestling HOSPs, *SI* mRNA increased from the proximal to the distal small intestinal regions, whereas sucrase activity was significantly highest in the midgut. Thus, the observed pattern may represent that the regulation of sucrase along the intestine in birds may be achieved by post-translational factor(s). However, a caveat to this interpretation must be posed. Both studies (this study, Dong et al., 2012b) used intestinal homogenate as starting material to quantify mRNA. Therefore, it is possible that using scraped mucosa or isolated enterocytes, the gene expression patterns can vary, because of a change in the relative contribution of subjacent tissues to intestinal epithelia among segments. Hence, the relationship between the genes of interest and the reference gene expressions may be altered, even though our reference genes were validated (see above *Assessment of mRNA levels* section). A support for this interpretation may be apparent in the different regression lines maltase activity and its mRNA (Fig. 1c), where the slopes are not different ($F_{3,40} = 0.24$, $p = 0.87$), but the intercept of the distal values is significantly lower than those of the proximal and medial sections ($F_{3,43} = 5.14$, $p = 0.004$). In any case, a formal test of the level of equivalency between the use of homogenates and isolated enterocytes is mandatory in future studies.

In conclusion, to our knowledge, this is the first study of gene expressions associated with dietary changes of carbohydrases in passerine (i.e. perching) birds. Our results suggest that the effect of diet composition on gene expression in birds is different from that observed in rodents (such as mice and rats) and therefore offer further evidence that features of digestive physiology differ considerably between two groups of extant endotherms (i.e. birds and mammals; compare McWhorter et al., 2009). Enhanced understanding of mechanisms responsible for digestive modulation should improve predictions about how birds can respond to changes in diet that occur due to habitat alterations or climate change.

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