

## RESEARCH ARTICLE

# Rapid and parallel changes in activity and mRNA of intestinal peptidase to match altered dietary protein levels in juvenile house sparrows (*Passer domesticus*)

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## ABSTRACT

Although dietary flexibility in digestive enzyme activity (i.e. reaction rate) is widespread in vertebrates, mechanisms are poorly understood. When laboratory rats are switched to a higher protein diet, the activities of apical intestinal peptidases increase within 15 h, in some cases by rapid increase in enzyme transcription followed by rapid translation and translocation to the intestine's apical, brush-border membrane (BBM). Focusing on aminopeptidase-N (APN), we studied intestinal digestive enzyme flexibility in birds, relying on activity and mRNA data from the same animals. Our model was nestling house sparrows (*Passer domesticus*), already known to modulate intestinal peptidase activity when switching between lower and higher protein diets. Twenty-four hours after a switch from an adequate, lower protein diet to a higher protein diet, APN activity was increased in both whole intestinal tissue homogenates and in isolated BBM, but not at 12 h post-diet switch. Twenty-four hours after a reverse switch back to the lower protein diet, APN activity was decreased, but not at 12 h post-diet switch. Changes in APN activity in both diet switch experiments were associated with parallel changes in APN mRNA. Although transcriptional changes seem to be an important mechanism underlying dietary modulation of intestinal peptidase in both nestling house sparrows and laboratory rodents, the time course for modulation in nestlings seemed slower (taking approximately twice as long) compared with laboratory rodents. It may be ecologically advantageous if nestlings biochemically restructure their gut in response to a sustained increase in insects and protein intake rather than one or a few lucky insect meals.

**KEY WORDS:** Aminopeptidase-N, Enzyme activity, mRNA, Diet switch, Intestine, Phenotypic flexibility, Transcriptional regulation

## INTRODUCTION

Many omnivorous avian species change diet in response to seasonal or unexpected changes in food availability and types (McWhorter et al., 2009). Different diets may be composed of different levels of substrates that require different particular complements of enzymes

for their breakdown, and the enzymatic capacity of the small intestine is modulated in response to changes in dietary substrate level such as increases in dietary protein or starch (Karasov et al., 2011). This phenotypically flexible response has been called enteroplasticity (Drozdzowski et al., 2009). The hypothesis of evolutionary economic design suggests that there is natural selection for a match between digestive capacity of the vertebrate small intestine and nutrient demand, so that ingested nutrients are not wasted in excreta due to insufficient digestive capacity, and that expenditures building and maintaining the molecular machinery to hydrolyse substrates are not wasted when substrate levels are low (Karasov and Hume, 1997). The intestinal enzyme aminopeptidase-N (EC 3.4.11.2; APN), which is located in the apical, brush-border membrane (BBM) of enterocytes, participates in the final hydrolysis of peptides released during protein breakdown by stomach and pancreatic proteases. About 90% of omnivorous passerines challenged with large increases in dietary protein content increased intestinal peptidase activity (McWhorter et al., 2009), the sole exception being zebra finch (*Taeniopygia guttata*), which are mainly seed specialists (Brzek et al., 2010). Other birds that increase peptidase activity on a higher protein diet include adult pigeons (*Columba livia*) (Ciminari et al., 2005) and juveniles of both mallards (*Anas platyrhynchos*) and northern bobwhites (*Colinus virginianus*) (Rott et al., 2017a). Although the existence of flexibility of peptidase activity has been described in scores of avian and other vertebrate species (Karasov et al., 2011), much less is known about the underlying mechanisms.

In mammals also, intestinal peptidase activity is increased on a higher protein diet, based mainly on studies in laboratory rats (Raul et al., 1987; Sonoyama et al., 1994) and some wild rodents (Wang et al., 2019). In laboratory rats, transition to higher protein diets induced intestinal dipeptidyl peptidase IV (DPP4), its mRNA, and protein abundance in the BBM, measured by immunoblotting (Suzuki et al., 1993, 1995). Those results suggested that dietary protein could influence peptidase expression at the level of gene transcription. Also in laboratory rats, intestinal APN activity is increased on higher protein diets (Raul et al., 1987; Sonoyama et al., 1994), although perhaps not with parallel change in mRNA (Sonoyama et al., 1994). Dietary induction of peptidase in rats can be very rapid, with molecular changes apparent within an hour of a large dietary pulse of peptide (Ahnen et al., 1982; Reisenauer and Gray, 1985) and maximal increase in activity of APN within 15 h of a diet switch (Raul et al., 1987), almost as fast as dietary induction of intestinal carbohydrases (Cézard et al., 1983; Hoffman and Chang, 1993).

In house sparrows [*Passer domesticus* (Linnaeus 1758)], the avian model in this study, a dietary switch from lower to higher dietary protein induced intestinal APN activity in intestinal homogenates of both adults (Caviedes-Vidal et al., 2000) and nestlings (Rott et al., 2017b). The increase was apparently maximal

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in the nestlings 24 h after the diet switch, although the timing was not conclusive because the study lacked a direct comparison of nestlings switched to higher protein with simultaneous controls (nestlings not switched) (Rott et al., 2017b). Also, whether dietary induction of APN is associated with increased APN mRNA is not known. Indeed, the mechanism(s) and time course underlying dietary induction of intestinal peptidases are unknown for any bird, including poultry, as far as we know (Karasov and Douglas, 2013).

In two experiments with nestling house sparrows, we tested the hypothesis that adaptive modulation of intestinal APN activity occurs rapidly, relying on transcriptional control, i.e. regulation of mRNA level in enterocytes followed by translocation of enzyme to the BBM. The nestlings, which naturally experience large changes in dietary carbohydrate and protein content as their parents transition them from consuming insects to seeds (Anderson, 2006), are a good model system for these tests because they can be raised in captivity and fed artificial diets whose composition can be rapidly changed and mimic the different diet chemistries in nature (Rott et al., 2017b).

In experiment 1, which focused on the time course for dietary induction of APN activity and its mRNA, our prediction 1 was that APN activity in both intestinal tissue homogenates and BBM of nestlings would be induced within 24 h by sudden dietary increase in protein (casein). Prediction 2 was that increase in APN activity would be associated with, or preceded by, increased abundance of its mRNA.

Experiment 2 focused on downward modulation of APN activity. Evidence in Rott et al. (2017b) indicated that APN activity in intestinal tissue homogenates declined 24 h after switch from higher to lower protein diet, but it was not conclusive because the study lacked a direct comparison of nestlings switched to lower carbohydrate with simultaneous controls (nestlings not switched). In experiment 2, therefore, prediction 3 was that APN activity in both intestinal tissue homogenates and BBM of nestling house sparrows would decline within 24 h of a sudden dietary decrease in protein, and prediction 4 was that decline in APN activity would be associated with, or preceded by, decreased abundance of its mRNA.

Overall, our study was designed to advance understanding of mechanisms of intestinal enzyme modulation in birds. However, the study also advances ecological knowledge about the ability of wild birds to adjust to changes in their nutritional resources that occur due to human activities such as habitat alterations and climate change.

## MATERIALS AND METHODS

### Study site, bird collection, experimental diets and feeding

Our study site, bird collection and housing, experimental diets and daily feeding routine followed exactly the protocol of Rott et al. (2017b). During summers 2016 and 2017, house sparrow nestlings 3 days post-hatch were collected from nests in the vicinity of University of Wisconsin (UW)-Madison campus. They were immediately housed in environmental chambers under controlled conditions (ambient temperature 35°C, relative humidity 50–55%, 15 h:9 h light:dark regime) and manually fed semi-synthetic diets hourly 15 times per day beginning at 06.00 h. The UW-Madison ethics committee approved the experimental procedures (permit IACUC-A005514).

Experiment 1 in 2017 was designed to study induction of APN. Upon arrival in environmental chambers all nestlings were fed a lower protein, higher starch 'S' diet (26.5% w/w casein, 38% corn starch, 8% corn oil, 10.5% other essential nutrients, 17% non-nutritive bulk; Rott et al., 2017b). The protein content of the lower protein S-diet still contained sufficient levels of essential amino

acids and total protein to support growth in birds (Klasing, 1998). Following 3 days on this diet, birds were randomly assigned to one of two diet groups, one switched at 06:00 h to a higher protein, lower starch 'P' diet (59.5% w/w casein, 5% corn starch, all other components identical to S-diet diet; Rott et al., 2017b), and the rest maintained on S-diet.

Experiment 2 in 2016 was the reverse of experiment 1, designed to study downward modulation of APN. Upon arrival, all nestlings were initially fed the higher protein, lower starch P-diet. Following 3 days on this diet, birds were randomly assigned to one of two diet groups, one switched at 06.00 h to the lower protein, higher starch S-diet and the rest maintained on P-diet.

### Tissue harvest and assays for enzyme activity and mRNA

In both experiments 1 and 2, after a specified time on dietary treatments (maximum 24 h), nestlings were weighed, euthanized with CO<sub>2</sub>, and dissected to remove the intestines, pancreas and liver. At this age, the nestlings were too sexually immature to assign sex by inspection of gonads, and we did not perform a molecular test for sex. The whole intestine was perfused with ice-cold avian Ringer's solution, quickly blotted dry for weighing, and then placed on an iced small steel plate and longitudinally divided into three pieces. One was immediately stored at –80°C for later measurement of intestinal aminopeptidase-N (APN) and sucrase activity in tissue homogenates (Rott et al., 2017b). Enterocytes were isolated from the remaining two-thirds of tissue (MacDonal et al., 2008) and half the isolate was stored in RNAlater Stabilization Solution at –80°C for later measurement of APN and sucrase-isomaltase (SI) mRNAs (see below). The remaining enterocytes were used to isolate BBM (Brun et al., 2020b,c), which were then stored at –80°C for later analyses. We measured APN and maltase activities in isolated BBMs. We used maltose for the BBM measurement because SI hydrolysis rate on maltose (i.e. rate of release of glucose) is much higher than on sucrose, which makes measurements more accurate when using the very small amounts of isolated BBM. Parallel changes in both activities are expected because SI alone accounts for all maltase and sucrase activity in house sparrows (Brun et al., 2020c). Because of low recovery in a large proportion of birds in experiment 1, we measured activity on multiple pools created from BBM of two to three birds from within each treatment group. In experiment 2, recoveries were better and so measures are presented for individual birds. All the analyses on stored tissue and tissue isolates were completed within 2 years or less.

### Assessment of mRNA levels and measurement of RNA

#### RNA extraction

All procedures were carried out using RNase AWAY Surface Decontaminant (Thermo Fisher Scientific) for decontaminating apparatus, bench tops, glassware and plasticware. Total RNA was isolated from frozen tissue using the PureLink RNA Mini Kit (Invitrogen), according to the manufacturer's instructions.

#### RNA quantification and RNA integrity control

Total RNA concentration was quantified with the Epoch Microplate Spectrophotometer (BioTek) measuring the extinction at 260 nm. Additionally, the OD 260:280 ratio (an indication of protein contamination) was evaluated. Only the RNA samples with OD 260:280 ratio between 1.9 and 2.1 were used for analyses. The integrity of RNA samples was also assessed by gel electrophoresis. For genomic DNA removal, a DNase digestion was carried out by treating the total RNA with DNase I (RNase-free) (NEB) according to the manufacturer's instructions.

### Reverse transcription

First-strand cDNA was synthesized by reverse transcribing 0.5 µg of total RNA in a final reaction volume of 20 µl, using iScript cDNA Synthesis Kit (Bio-Rad) on RNA from tissue according to the manufacturer's instructions. A minus reverse transcription control was performed on all samples to check the removal of all the contaminating genomic DNA.

### Primer designing

Primer pairs for APN mRNA expression analysis were designed by PrimerQuest Tool (IDT), the parameters were analysed by OligoAnalyzer Tool (IDT) and Tm calculator (NEB) and were based on RNA or DNA sequences of a related bird, *Taeniopygia guttata* (source: Invitrogen) [primer sequences 5'–3' (forward/reverse) TACCTCCACACCTTCTCCTAC/CGGTCCATGATGC TTGCTGAT]. Primers for SI mRNA and for candidate reference genes *GAPDH* and *EEF1A* were as used previously (Gatica-Sosa et al., 2018).

### Quantitative PCR analysis

Quantitative PCR (qPCR) was performed in the RealPlex ep Gradient S Mastercycler (Eppendorf). The reactions were carried out in twin.tec 96-well real-time PCR plates (Eppendorf) in a final volume of 20 µl. cDNA (5 µl) was added to 15 µl of reaction mix containing 10 µl SYBR Green qPCR Master Mix (Applied Biosystems), 0.5 µl of each primer [20 (µmol l<sup>-1</sup>) µl<sup>-1</sup>] and 4 µl RNase free water. Cycling conditions were as follows: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. After 40 cycles, the specificity of amplicons was verified by melting curve analysis (60 to 95°C). All reactions were run in triplicate and the mean threshold cycle ( $C_t$ ) values were used for further analysis. If one of the three  $C_t$  values deviated from the other two by more than 0.36, it was excluded from further analysis or the experiment was repeated.

### Quantitative PCR efficiency

Standard curves were generated using relative concentration versus the  $C_t$ . The linear correlation coefficient ( $R^2$ ) of all the genes ranged from 0.996 to 1.000. Based on the slopes of the standard curves, the amplification efficiencies ( $E$ ) of the standards are 100% (derived from the formula  $E=10^{-(1/\text{slope})}-1$ ). The unnormalized  $C_t$  values of all the genes in all the samples were within 13.5 to 30.5 cycles, covered by the range of the standard curves. Nuclease-free water was used for the no-template controls.

### Calculation of mRNA abundance

The analysis of  $C_t$  data from qPCR runs followed MIQE guidelines (Bustin et al., 2009). Briefly, we first averaged qPCR technical triplicates for each sample and then means were normalized with NORMA-Gene according to Heckmann et al. (2011), which normalizes the mRNA by calculating mean expression values for each replicate across the studied genes and subsequently calculating a normalization factor that estimates and reduces the systematic bias of a replicate across all genes (Heckmann et al., 2011). These values were relativized by subtracting them from those of the control group (S- and P-diet groups for the APN upmodulation and downmodulation experiments, respectively). The difference was used as the negative exponent of a two-exponential function (Schmittgen and Livak, 2008). These values represent relative quantities of mRNA and can be interpreted as fold time changes in relation to a control (Schmittgen and Livak, 2008). For comparison purposes, we also calculated mRNA abundance by an alternative

method, the comparative  $C_t$  method, using *GAPDH* as the reference gene (chosen among *EEF1A1*, *YWHAZ* and *18S* by geNorm and NormFinder; Andersen et al., 2004; Vandesompele et al., 2002). As an initial test of our procedures we measured APN mRNA in thawed and homogenized tissue samples from the prior study of dietary induction of APN activity in nestlings (Rott et al., 2017b), which used the same diets as in this study. One day after diet switch to higher protein diet, APN activity in medial intestine was increased 35% ( $P=0.003$ ), and APN mRNA was higher by both methods of normalization ( $P\leq 0.001$ ,  $t$ -tests; Table S1).

### Data analysis

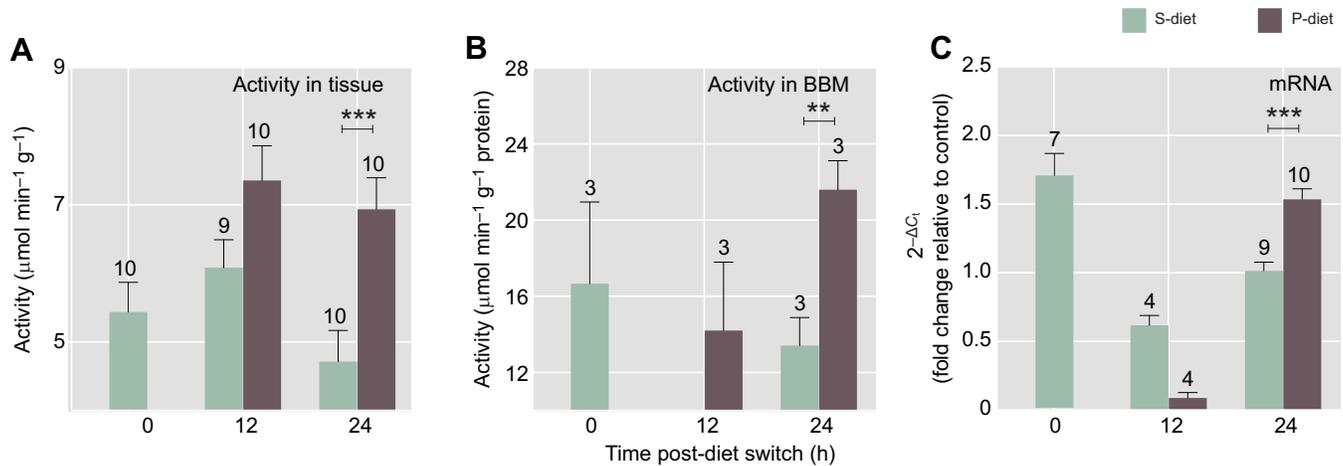
Results are given as means±s.e.m. unless indicated otherwise [ $N$ =number of birds (or pools of birds) per treatment; each treatment was defined according to diet and hours post-diet switch]. Siblings were not included within any single treatment group, with the exception of an error in forming one group in experiment 1; this pair of siblings was only 2% of all birds in this study. Data on mean enzyme activity or mRNA were plotted according to diet group (P or S) and as a function of time since the diet switch (along the  $x$ -axis; time 0 h corresponds to real local time of 06:00 h). In treatment groups in which diet was not changed (referred to as 'controls'), we tested for a circadian rhythm on the mean rates using a one-way ANOVA with time as a factor. Preceding this, we confirmed that data were normally distributed (Lilliefors test) and homoscedastic (Levene's test) (Wilkinson, 2009), but in a few cases we first log<sub>10</sub> transformed the data to make this so. Because of possible diurnal rhythm in APN (Nashiro et al., 1992; Tavakkolizadeh et al., 2005), we used simple two-sample  $t$ -tests to test for an effect of diet at a specified time. Preceding this we tested for homoscedasticity (Levene's test), and if that was lacking then we used the  $t$ -test for separate variances, the Welch's  $t$ -test (Welch, 1947; Wilkinson, 2009). In one instance data were not normally distributed, and there we used the non-parametric Kolmogorov–Smirnov two-sample test. mRNA exponential data were log<sub>2</sub> transformed before tests. For all two-sample tests, after first confirming in the tissue homogenates the earlier evidence of change in APN activity 24 h after diet switches (Rott et al., 2017b), subsequent two-sample tests were one-tailed for the *a priori* predictions for BBM enzyme activity and enterocyte mRNA from the same tissues in the same animals. Significance level was set at  $P<0.05$ .

## RESULTS

### Experiment 1

In experiment 1, controls (nestlings maintained on the lower protein S-diet throughout) had relatively constant APN activity in whole tissues (one-way ANOVA with time as factor;  $F_{2,26}=2.44$ ,  $P=0.11$ ). However, consistent with our first prediction, house sparrow nestlings initially raised for 3 days on lower protein S-diet and then switched to higher protein P-diet had higher APN activity in whole tissue homogenates 24 h post-diet switch than similar-aged controls ( $t_{18}=3.44$ ,  $P=0.003$ , two-tailed) (Fig. 1A). At an earlier time point (12 h post-diet switch), APN activity of nestlings eating higher protein diet was not significantly elevated above that in controls ( $t_{17}=1.96$ ,  $P=0.067$ ). Corresponding to the increased APN activity in tissue homogenates, nestlings that fed for 24 h on higher protein diet had higher APN activity in isolated BBM compared with the controls (lower protein diet group;  $t_4=3.87$ ,  $P=0.009$ ; one-tailed for *a priori* prediction) (Fig. 1B).

In experiment 1, APN mRNA in isolated enterocytes in controls (nestlings maintained on lower protein S-diet throughout) varied significantly with time (one-way ANOVA on log-transformed



**Fig. 1.** APN in homogenates of intestinal tissue, in brush-border membrane (BBM) and in isolated enterocytes in nestling house sparrows in the hours following a diet switch from the lower protein S-diet to the higher protein P-diet (experiment 1). (A) Intestinal tissue (activity); (B) BBM (activity); (C) isolated enterocytes (mRNA). At the beginning of the experiment, time is 0 h for nestlings on S-diet just prior to diet switch, and 'control' birds on this diet at other time points are represented by the left-hand green bar at each time point, whereas the right-hand brown bar represents birds that were diet-switched. Values are means  $\pm$  s.e.m., and numbers above bars are number of birds in each treatment group (or pools of birds in the case of BBM). Controls are not represented at 12 h in B because of loss of samples. Asterisks denote significant differences (\*\*\* $P$ <0.001, \*\* $P$ <0.01) between diet groups at the 24 h time point (no differences earlier; see Results, 'Experiment 1').

values with time as factor,  $F_{2,17}=25.2.9$ ,  $P=0.0001$ ; Fig. 1C). APN mRNA in controls was significantly lower 12 h after switch (coincides with 18:00 h) than either at the beginning (time=0) or 24 h after (06:00 h next day) (*post hoc* Tukey's comparison,  $P$ <0.005), and lower at time=24 than at 0 h ( $P$ <0.002). Consistent with our second prediction, and with our preliminary data on mRNA in whole tissue homogenates from the nestlings from the prior study (Table S1), nestlings that were switched to higher protein P-diet had higher APN mRNA 24 h post-diet switch compared with controls (nestlings that remained eating lower protein S-diet) ( $t_{12}=5.22$ ;  $P=0.0001$ ; one-tailed for *a priori* prediction; Fig. 1C). At an earlier time point (12 h post-diet switch), APN mRNA of nestlings eating higher protein diet was not significantly elevated above that in controls ( $t_{13,04}=2.17$ ,  $P=0.12$ , Welch's test for unequal variances; Fig. 1C).

Overall, when nestlings were switched from lower protein S-diet to higher protein P-diet, increases relative to controls in APN activity were associated with increases relative to controls in APN mRNA. The diet-induced increase in APN activity and mRNA in the P-diet group was specific for APN, based on comparison with other simultaneous measurements in the same birds (Brun et al., 2020a). For example, SI activity and mRNA from nestlings in the higher protein P-diet group 24 h after the diet switch was not higher but instead lower than in same-aged individuals in the S-diet group

(Table 1). Thus, the upward modulation of APN activity and its mRNA in experiment 1 was an enzyme-specific effect.

### Experiment 2

In experiment 2, controls (nestlings maintained on higher protein P-diet throughout) had relatively constant APN activity over 24 h in whole tissues (one-way ANOVA with time as factor,  $F_{2,23}=0.23$ ,  $P>0.7$ ; Fig. 2A) and in isolated BBM ( $F_{2,8}=0.57$ ,  $P>0.5$ ; Fig. 2B). However, consistent with prediction 3 and the earlier finding of reduced APN activity in house sparrow nestlings 24 h after a switch from higher to lower protein diet (Rott et al., 2017b), the nestlings initially raised for 3 days on higher protein P-diet and then switched to lower protein S-diet had lower APN activity in whole tissue homogenates compared with similar-aged controls at 24 h post-diet switch ( $t_{21}=1.76$ ;  $P=0.047$ , one-tailed for *a priori* prediction) (Fig. 2A). At the earlier time point, 12 h post-diet switch, APN activity of nestlings eating lower protein diet was not significantly lower than in controls ( $t_{15}=1.80$ ,  $P=0.092$ ). For APN in BBM, the apparent declines at either 24 h or 12 h post-diet switch were not significant (at 24 h,  $t_{11}=1.259$ ,  $P=0.117$ ; one-tailed for *a priori* prediction; at 12 h,  $t_5=1.282$ ,  $P=0.13$ ), perhaps owing to more limited sampling.

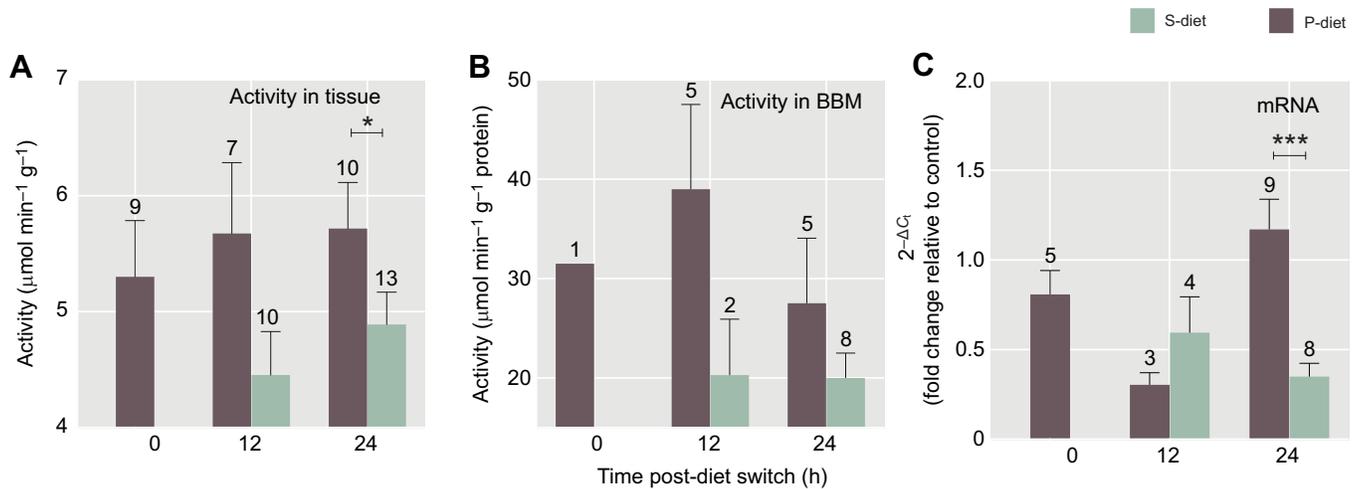
In experiment 2, APN mRNA in isolated enterocytes in controls varied significantly with time (one-way ANOVA with time as

**Table 1.** Tests for effect of diet on sucrase-isomaltase (SI) activity and its mRNA in *Passer domesticus* nestlings 24 h following switch from lower protein, higher starch S-diet to higher protein, lower starch P-diet

	SI activity in tissue homogenates ( $\mu\text{mol min}^{-1} \text{g}^{-1} \text{tissue}$ )			Maltase activity in BBM ( $\mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$ )			SI mRNA in enterocytes (normalized values)		
	Mean	s.d.	$n^*$	Mean	s.d.	$n^\ddagger$	Mean	s.d.	$n^*$
S-diet	1.237	0.533	10	382	154	3	1.807	0.766	7
P-diet	0.759	0.418	10	276	71.7	3	0.428	0.983	7
<i>t</i> -test	$t_{17}=2.23$ $P=0.039^a$			$t_4=1.08$ $P=0.17^b$			$t_{12}=2.93$ $P=0.006^b$		

Data from Brun et al. (2020a). \* $n$ , number of individual birds;  $^\ddagger n$ , number of pools of brush-border membrane (BBM), each made from BBM of two to three birds;  $^a t$ -test was two-tailed.

$^b$ One-tailed *t*-test for *a priori* prediction following demonstrated difference in activity in tissues.



**Fig. 2.** APN in homogenates of intestinal tissue, in BBM, and in isolated enterocytes of nestling house sparrows in the hours following a diet switch from the higher protein P-diet to the lower protein S-diet (experiment 2). (A) Intestinal tissue (activity); (B) BBM (activity); (C) isolated enterocytes (mRNA). At the beginning of the experiment, time is 0 h for nestlings on P-diet just prior to diet switch, and 'control' birds on this diet at other time points are represented by the left-hand brown bar at each time point, whereas the right-hand green bar represents birds that were diet-switched. Values are means  $\pm$  s.e.m., and numbers above bars are number of birds in each treatment group. Asterisks denote significant differences (\*\*\* $P$ <0.001, \* $P$ <0.05) between diet groups at the 24 h time point (no differences earlier; see Results, 'Experiment 2').

factor,  $F_{2,13}=25.0$ ,  $P=0.0001$ ; Fig. 2C). APN mRNA in controls was significantly lower 12 h after switch (coincides with 18:00 h) than either at the beginning (time=0) or 24 h after (*post hoc* Tukey's comparison,  $P$ <0.00015). Consistent with our prediction 4 and with the finding for APN activity in tissues, nestlings that were switched to lower protein S-diet had lower APN mRNA 24 h post-diet switch compared with controls (*t*-test,  $P=0.0001$ , one-tailed for *a priori* prediction). APN mRNA did not differ by diet group at 12 h post-diet switch ( $t_4=0.23$ ;  $P>0.4$ ; Fig. 2C).

Overall, when nestlings were switched from higher protein P-diet to lower protein S-diet, decreases relative to controls in APN activity (Fig. 2A) were associated with decreases relative to controls in APN mRNA (Fig. 2C). In experiment 2, the diet-induced decrease in APN activity and mRNA in the S-diet group was specific for APN, based on comparison with other simultaneous measurements in the same birds (Brun et al., 2020a). For example, SI activity and its mRNA from nestlings in the lower protein S-diet group 24 h after the diet switch were not lower but were higher than in same-aged individuals in the P-diet group (Table 2). Thus, the downward modulation of APN activity and its mRNA was an enzyme-specific effect.

### Experiments 1 and 2 combined

Experiments 1 and 2 individually found associations between APN activity in tissue homogenates and BBM and mRNA, as expected.

As a final test of these expected associations, we regressed APN activity in tissue homogenates (the *y*-value) against both APN activity in BBM and APN mRNA (the *x*-values), using data for nestlings that were fed on their respective diets for at least 24 h, in whom adjustment to diet was fairly complete. APN activity in tissue homogenates was positively correlated with APN activity in the isolated BBM [slope by model II regression= $0.15\pm 0.039$  ( $P$ <0.05), intercept= $2.11\pm 0.93$  ( $P$ <0.05)], with no difference by experiment ( $F_{2,21}=1.37$ ,  $P>0.2$  by *F*-test recommended by Motulsky and Ransnas, 1987). Although this correlation (Fig. 3A) is not strong, it remained significant ( $P$ <0.05) even when the single highest value of BBM APN activity ( $x=52.5$ ,  $y=7.8$ ) was removed. APN activity in tissue homogenates was positively correlated with mRNA in isolated enterocytes (model II regression; Fig. 3B), but with a difference by experiment ( $F_{2,44}=5.39$ ,  $P$ <0.01). For experiment 1, the intercept was  $4.74\pm 0.42$  ( $P$ <0.05) and the slope was  $3.36\pm 0.70$  ( $P$ <0.05). For experiment 2, the intercept was  $5.51\pm 0.37$  ( $P$ <0.05) and the slope was  $1.10\pm 0.29$  ( $P$ <0.05).

### DISCUSSION

In an omnivorous animal, enterocytes might be exposed daily to different nutrients that vary according to the animal's nutrient intake, and enteroplasticity helps to ensure an appropriate match between variation in dietary nutrient load and the intestine's capacity to

**Table 2.** Tests for effect of diet on SI activity and mRNA in *P. domesticus* nestlings 24 h following switch from higher protein, lower starch P-diet to lower protein, higher starch S-diet

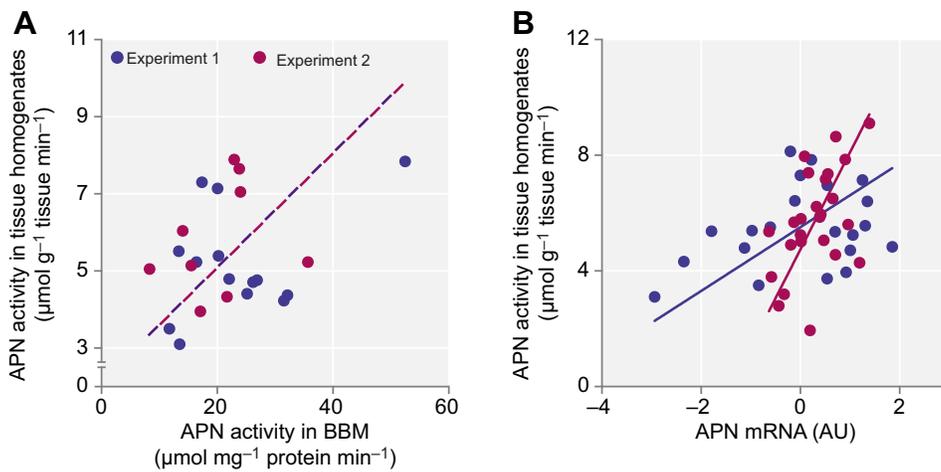
	SI activity in tissue homogenates ( $\mu\text{mol min}^{-1} \text{g}^{-1}$ tissue)			SI activity in BBM ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein)			SI mRNA in enterocytes (normalized values)		
	Mean	s.d.	<i>n</i>	Mean	s.d.	<i>n</i>	Mean	s.d.	<i>n</i>
S-diet	1.071	0.363	13	850	271	7	1.276	1.896	10
P-diet	0.392	0.149	10	442	189	7	0.039	0.405	9
<i>t</i> -test	$t_{17}=6.11$ $P<0.001^a$			$t_{12}=3.26$ $P=0.003^b$			$t_{10}=2.01$ $P=0.036^{b,c}$		

Data from Brun et al. (2020a).

<sup>a</sup>Two-tailed *t*-test.

<sup>b</sup>One-tailed test for a *a priori* prediction following demonstrated difference in activity in tissues.

<sup>c</sup>SI mRNA was not normally distributed: non-parametric Kolmogorov–Smirnov test,  $P=0.034$ .



**Fig. 3. Activity of APN in whole intestine homogenates regressed (model II regression) against APN activity in BBM isolates and APN mRNA in isolated enterocytes.** (A) BBM isolates; (B) isolated enterocytes. Each point is the intestine of an individual nestling colored according to whether it was from experiment 1 (red) or experiment 2 (blue). All these nestlings had fed on their respective diets for at least 24 h. A single, dashed, red/blue line was fitted to all the data in A because the correlation did not differ by experiment ( $P > 0.2$ ; see text). Separate lines are shown in B because the pattern did differ by experiment ( $P < 0.01$ ; see Results, 'Experiments 1 and 2 combined'). AU, arbitrary units.

process nutrients (Drozdowski et al., 2009). Our study in nestling house sparrows considerably advances our knowledge about the time course and cellular basis for adjustment to variation in dietary protein, as discussed in the sections that follow. Although both juveniles and adults of other birds are also known to increase peptidase activity on higher protein diets (McWhorter et al., 2009), this is the first study in birds of the underlying mechanisms and time course.

#### Cellular basis for modulation of APN

The dietary-induced upward and downward modulation of APN activity in intestinal tissue homogenates, and in APN mRNA in isolated enterocytes, were specific for that enzyme. For example, 24 h after nestlings were switched from lower protein, higher starch S-diet to higher protein, lower starch P-diet, APN activity in tissues and mRNA in enterocytes increased, whereas in the same tissues, activity and mRNA of SI declined (Table 1). Analogously, the diet switch in experiment 2 was followed by a decline in APN activity in tissues and in APN mRNA, whereas in the same tissues activity and mRNA of SI increased (Table 2). Nestlings consuming the P- and S-diets did not differ in intestine mass or length (Rott et al., 2017b), so non-specific effects on enzyme activities per gram of tissue due to changes in intestine gross morphology are not apparent. The elevation of APN activity 24 h after switch to higher protein P-diet was parallel and even similar in magnitude in tissue homogenates and in isolated BBM (respectively, +50 and +60%; cf. Fig. 1A and B). Overall, in both experiments, the two activity measures in tissue homogenates and in isolated BBM were positively correlated (Fig. 3A), which is consistent with the idea, but not proof, that the locational source of the increased activity in whole tissues is the BBM. Thus, our data in total are consistent with the overall hypothesis that a major mechanism underlying the matching of intestinal APN activity to dietary protein level is transcriptional control, i.e. regulation of mRNA level in enterocytes followed by translocation of synthesized enzyme to the BBM. We came to the same conclusion in our parallel study of the matching of intestinal sucrose and maltase activity to dietary starch level, undertaken in the same nestling house sparrows (Brun et al., 2020a).

Rodents also modulate intestinal APN activity in positive association with changes in dietary protein (Raul et al., 1987; Sonoyama et al., 1994; Wang et al., 2019). However, a possible difference with the nestling house sparrow is that parallel change in APN mRNA was not demonstrated in laboratory rats in the one study of which we are aware (Sonoyama et al., 1994), which led those authors to conclude that translational and/or post-translational

regulation for the expression of intestinal APN is necessary for the response to changes in dietary protein. Additional study of this issue in both rodents and birds seems warranted. Also, the signals and mechanisms controlling activation of transcription are generally unknown. Intestinal transporter and enzyme activities, including SI, are known to be influenced by levels of their substrates and by hormones; we are unaware of any demonstrations of the involvement of nerves in enteroplasticity (Drozdowski et al., 2009; Sanderson and Naik, 2000). Although the direct links between hormone or substrate level and controllers of gene expression have not been described, there is evidence in rats that in dietary adaptation of maltase glucoamylase (MGAM) its mRNA transcription is regulated through histone acetylation and binding of several co-factors in the promoter/enhancer and transcriptional regions of *MGAM* (Mochizuki et al., 2010). The extent to which underlying mechanisms are similar in birds will be exciting to explore.

#### Time course for modulation of intestinal APN

In our study of intestinal enzymes and their mRNA we have identified both technical and biological issues that confound the study of their temporal changes in growing and developing animals. Study of diet-induced changes over the course of a single day is confounded if there are diet-independent changes during the day, for example as in a diurnal rhythm even in animals on constant diet (Nashiro et al., 1992; Tavakkolizadeh et al., 2005). Study of diet-induced changes over several days is confounded if there are diet-independent developmental changes over the time interval. We have found examples of both in our studies with nestling house sparrows. There is evidence of a diet-independent diurnal rhythm in APN mRNA (Figs 1C and 2C) and also for the intestinal enzyme SI mRNA (Brun et al., 2020a). Over periods of just 3 days that bracket nestling ages of this study, intestine mass and activities of APN and SI increased in nestlings held on constant diet (Brzek et al., 2009). Also in that study, the levels of putative reference genes (*GAPDH*, *EEF1A1*) in enterocytes changed in nestlings on constant diet over 3 days (C. Gatica-Sosa and E.C.-V., unpublished observations). Such a change in reference genes has the potential to alter the representation of relative mRNA over 3 days (or perhaps less), even if it was constant in an absolute sense. For these reasons, we routinely (i) tested for an effect of time by one-way ANOVA in our controls (nestlings not diet-switched), which we found for APN mRNA (Figs 1C and 2C), and (ii) favored simple two-sample *t*-tests to test for an effect of diet at specified times, and (iii) eschew making comparisons of mRNA over a time span of several days.

Under our general hypothesis of transcriptional control of dietary modulation of digestible enzyme activity, how might one interpret our observations that mRNA appears to decline between the hours of 06:00 h and 18:00 h and then increase between 18:00 h and 06:00 h, with no parallel change in enzyme activity (Figs 1 and 2)? Let us assume that the measured mRNA and enzyme levels reflect levels in pools that are subject to continuous mRNA and enzyme synthesis and degradation (Hoffman and Chang, 1993). For rats, Broyart et al. (1990) found that a dietary pulse of sucrose increased the SI mRNA transcription and accumulation within 1.5 h, but there was then a 1.5 h lag between detection of accumulated mRNA and increase in SI activity. They interpreted the lag to reflect a processing time that included translocation of enzyme from intracellular sites of synthesis to the BBM. If this scenario applied to nestlings, one might predict a cycle in enzyme activity out of phase by at least ~2 h with the mRNA cycle. However, we did not systematically measure enzyme activity between 12 and 24 h post-diet switch. Besides factors that might make putative diurnal rhythms in mRNA and enzyme levels asynchronous, other factors might make them differ in the magnitude of change. For example, if enzymes, once synthesized, do not degrade as rapidly as mRNA, then their pool sizes might not fluctuate to the same extent as those of mRNA (Hoffman and Chang, 1993). Thus, although we have evidence of a diurnal rhythm in APN mRNA (and also in mRNA of SI; Brun et al., 2020a), our sampling was not fine scale enough either temporally or in terms of synthesis/degradation to interpret the functional significance of the observed rhythm in mRNA in terms of intestinal enzyme activity.

Although the house sparrow and laboratory rat seem to share some mechanisms of dietary induction of intestinal enzymes, the time course for modulation seems slower in nestlings compared with laboratory rats. As discussed above, after a large pulse of a new nutrient (e.g. peptide or carbohydrate) some of the intracellular mechanistic events occur so fast in rats that significant increases in intestinal activity and mRNA of APN and SI are induced maximally within 6–15 h by their respective dietary substrates and they remain at that level for at least the subsequent 48 h (Cézard et al., 1983; Hoffman and Chang, 1993; Raul et al., 1987). We failed to find in nestlings compelling evidence for induction of activity or mRNA 12 h post-diet shift for either APN (Fig. 1) or for SI (Brun et al., 2020a), although both were induced by 24 h post-diet switch (Figs 1 and 2; see also Rott et al., 2017b) and activity remained elevated for at least the subsequent 48 h, based on the prior study by Rott et al. (2017b). Thus, nestlings seemed slower (i.e. took approximately twice as long) compared with laboratory rats in increasing mRNA and activity of APN and SI following an increase in their respective dietary substrate. This may be understandable considering differences in ecology and evolution.

Energy is probably more limiting for wild animals than for *ad libitum*-fed laboratory rats, and there are plausible biosynthetic energy costs associated with dietary modulation of intestinal enzymes. Redesign of the intestine's hydrolytic capacity based on a single large pulse (i.e. meal) of a new substrate might be a costly mistake for wild animals surviving on sometimes limiting resources. In the case of nestling house sparrows that increasingly rely on carbohydrate-rich plant material during development (Anderson, 2006), there might be selection against those that immediately alter enzyme activity towards protein shortly after just one or a few lucky insect meals. However, a more sustained increase in insects and protein intake during a sudden peak in arthropod abundance might merit a biochemical restructuring of the gut for greater reliance and possibly faster growth on a protein-rich diet

(Rott et al., 2017b). As it is, the response time of nestling house sparrows allows them to track daily changes in resource availability with fine-tuned changes in digestive performance. As we argued elsewhere (Brun et al., 2020a), their response time for change is 'fast but not too fast', in a sense similar to an adage about intestinal digestive capacity ('enough but not too much'; Diamond, 1991). Future studies might test other wild species in comparison with domesticated species for similar mechanisms of enzymatic modulation but somewhat slower response times.

In summary, although mechanisms underlying dietary induction of intestinal APN seem similar in nestling house sparrows and laboratory rodents, the time course for modulation in nestlings seemed half as fast compared with laboratory rodents. Besides their importance for advancing understanding of intestinal enzyme modulation, additional studies of this sort are also important ecologically for understanding the ability of animals to adjust to changes in their food resources including those due to human activities such as climate change and habitat alterations.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: A.B., M.E.M., W.H.K., E.C.-V.; Methodology: A.B., M.E.M.; Formal analysis: W.H.K., E.C.-V.; Writing - original draft: W.H.K., E.C.-V.; Writing - review & editing: A.B., M.E.M., W.H.K., E.C.-V.; Supervision: W.H.K., E.C.-V.; Funding acquisition: W.H.K., E.C.-V.

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#### References

- Ahnen, D. J., Santiago, N. A., Cezard, J. P. and Gray, G. M. (1982). Intestinal aminooligopeptidase: *in vivo* synthesis on intracellular membranes of rat jejunum. *J. Biol. Chem.* **257**, 12129–12135.
- Andersen, C. L., Jensen, J. L. and Ørntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **64**, 5245–5250. doi:10.1158/0008-5472.CAN-04-0496
- Anderson, T. R. (2006). *Biology of the Ubiquitous House Sparrow*. Oxford: Oxford University Press.
- Broyart, J.-P., Hugot, J.-P., Perret, C. and Porteau, A. (1990). Molecular cloning and characterization of a rat intestinal sucrose-isomaltase cDNA. Regulation of sucrose-isomaltase gene expression by sucrose feeding. *Biochim. Biophys. Acta (BBA) Gene Struct. Expr.* **1087**, 61–67. doi:10.1016/0167-4781(90)90121-H
- Brun, A., Magallanes, M. E., Barrett-Wilt, G. A., Karasov, W. H. and Caviedes-Vidal, E. (2020a). Dietary adaptation to high starch involves increased relative abundance of sucrose-isomaltase and its mRNA in nestling house sparrows. *Am. J. Physiol.* (in press). doi:10.1152/ajpregu.00181.2020
- Brun, A., Magallanes, M. E., Martínez del Río, C., Barrett-Wilt, G. A., Karasov, W. H. and Caviedes-Vidal, E. (2020b). A fast and accurate method to identify and

- quantify enzymes in brush-border membranes: *in situ* hydrolysis followed by nano LC-MS/MS. *Methods Protoc.* **3**, 15. doi:10.3390/mps3010015
- Brun, A., Mendez-Aranda, D., Magallanes, M. E., Karasov, W. H., Martínez del Río, C., Baldwin, M. W. and Caviedes-Vidal, E.** (2020c). Duplications and functional convergence of intestinal carbohydrate-digesting enzymes. *Mol. Biol. Evol.* **37**, 1657-1666. doi:10.1093/molbev/msaa034
- Brzek, P., Kohl, K., Caviedes-Vidal, E. and Karasov, W. H.** (2009). Developmental adjustments of house sparrow (*Passer domesticus*) nestlings to diet composition. *J. Exp. Biol.* **212**, 1284-1293. doi:10.1242/jeb.023911
- Brzek, P., Lessner, K. M., Caviedes-Vidal, E. and Karasov, W. H.** (2010). Low plasticity in digestive physiology constrains feeding ecology in diet specialist, zebra finch (*Taeniopygia guttata*). *J. Exp. Biol.* **213**, 798-807. doi:10.1242/jeb.037259
- Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L. et al.** (2009). The MIQE guidelines: Minimum Information for publication of Quantitative real-time PCR Experiments. *Clin. Chem.* **55**, 611-622. doi:10.1373/clinchem.2008.112797
- Caviedes-Vidal, E., Afik, D., Martínez del Río, C. and Karasov, W. H.** (2000). Dietary modulation of intestinal enzymes of the house sparrow (*Passer domesticus*): testing an adaptive hypothesis. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **125**, 11-24. doi:10.1016/S1095-6433(99)00163-4
- Cézard, J. P., Broyart, J. P., Cuisinier-Gleizes, P. and Mathieu, H.** (1983). Sucrase-isomaltase regulation by dietary sucrose in the rat. *Gastroenterology* **84**, 18-25. doi:10.1016/S0016-5085(83)80161-9
- Ciminari, M. E., Valle Moyano, G. D., Chediack, J. G. and Caviedes-Vidal, E.** (2005). Feral pigeons in urban environments: dietary flexibility and enzymatic digestion? *Rev. Chil. Hist. Nat.* **78**, 267-279. doi:10.4067/S0716-078X2005000200011
- Diamond, J.** (1991). Evolutionary design of intestinal nutrient absorption: enough but not too much. *News Physiol. Sci.* **6**, 92-96. doi:10.1152/physiologyonline.1991.6.2.92
- Drozdowski, L. A., Clandinin, M. T. and Thomson, A. B. R.** (2009). Morphological, kinetic, membrane biochemical and genetic aspects of intestinal enteroplasticity. *World J. Gastroenterol.* **15**, 774-787. doi:10.3748/wjg.15.774
- Gatica-Sosa, C., Brzek, P., Magallanes, M., Karasov, W. H. and Caviedes-Vidal, E.** (2018). Intestinal alpha-glycosidase transcriptional responses during development and diet adjustment in altricial birds. *J. Exp. Biol.* **221**, jeb171827. doi:10.1242/jeb.171827
- Heckmann, L.-H., Sørensen, P. B., Krogh, P. H. and Sørensen, J. G.** (2011). NORMA-Gene: a simple and robust method for qPCR normalization based on target gene data. *BMC Bioinform.* **12**, 250. doi:10.1186/1471-2105-12-250
- Hoffman, L. R. and Chang, E. B.** (1993). Regional expression and regulation of intestinal sucrase-isomaltase. *J. Nutr. Biochem.* **4**, 130-142. doi:10.1016/0955-2863(93)90041-T
- Karasov, W. H. and Douglas, A. E.** (2013). Comparative digestive physiology. *Compr. Physiol.* **3**, 741-783. doi:10.1002/cphy.c110054
- Karasov, W. H. and Hume, I. D.** (1997). Vertebrate gastrointestinal system. In *Handbook of Comparative Physiology* (ed. W. Dantzler), pp. 409-480. Bethesda, MD: American Physiological Society.
- Karasov, W. H., Martínez del Río, C. and Caviedes-Vidal, E.** (2011). Ecological physiology of diet and digestive systems. *Annu. Rev. Physiol.* **73**, 69-93. doi:10.1146/annurev-physiol-012110-142152
- Klasing, K.** (1998). *Comparative Avian Nutrition*. CAB Publishing.
- MacDonal, O., Chediack, J. G. and Caviedes-Vidal, E.** (2008). Isolation of epithelial cells, villi and crypts from small intestine of pigeons (*Columba livia*). *Biocell* **32**, 219-227. doi:10.32604/biocell.2008.32.219
- McWhorter, T. J., Caviedes-Vidal, E. and Karasov, W. H.** (2009). The integration of digestion and osmoregulation in the avian gut. *Biol. Rev.* **84**, 533-565. doi:10.1111/j.1469-185X.2009.00086.x
- Mochizuki, K., Honma, K., Shimada, M. and Goda, T.** (2010). The regulation of jejunal induction of the maltase-glucoamylase gene by a high-starch/low-fat diet in mice. *Mol. Nutr. Food Res.* **54**, 1445-1451. doi:10.1002/mnfr.200900467
- Motulsky, H. J. and Ransnas, L. A.** (1987). Fitting curves to data using nonlinear regression: a practical and nonmathematical review. *FASEB J.* **1**, 365-374. doi:10.1096/fasebj.1.5.3315805
- Nashiro, K., Murakami, K. and Mimura, G.** (1992). Diurnal-variation and increase of disaccharidase activity in diabetic rats. *J. Nutr. Sci. Vitaminol.* **38**, 265-276. doi:10.3177/jnsv.38.265
- Raul, F., Goda, T., Gossé, F. and Koldovský, O.** (1987). Short-term effect of a high-protein/low-carbohydrate diet on aminopeptidase in adult rat jejunoileum. *Biochem. J.* **247**, 401-405. doi:10.1042/bj2470401
- Reisenaue, A. M. and Gray, G. M.** (1985). Abrupt induction of a membrane digestive enzyme by its intraintestinal substrate. *Science* **227**, 70-72. doi:10.1126/science.3838079
- Rott, K. H., Caviedes-Vidal, E. and Karasov, W. H.** (2017a). Activity of intestinal enzymes responds to multiple dietary signals in birds. *Integr. Comp. Biol.* **57**, E391.
- Rott, K. H., Caviedes-Vidal, E. and Karasov, W. H.** (2017b). Intestinal digestive enzyme modulation in house sparrow nestlings occurs within 24 h of a change in diet composition. *J. Exp. Biol.* **220**, 2733-2742. doi:10.1242/jeb.157511
- Sanderson, I. R. and Naik, S.** (2000). Dietary regulation of intestinal gene expression. *Annu. Rev. Nutr.* **20**, 311-338. doi:10.1146/annurev.nutr.20.1.311
- Schmittgen, T. D. and Livak, K. J.** (2008). Analyzing real-time PCR data by the comparative  $C_T$  method. *Nat. Protoc.* **3**, 1101-1108. doi:10.1038/nprot.2008.73
- Sonoyama, K., Kiriya, S. and Niki, R.** (1994). Effect of dietary protein level on intestinal aminopeptidase activity and mRNA level in rats. *J. Nutr. Biochem.* **5**, 291-297. doi:10.1016/0955-2863(94)90034-5
- Suzuki, Y., Erickson, R. H., Sedlmayer, A., Chang, S. K., Ikehara, Y. and Kim, Y. S.** (1993). Dietary regulation of rat intestinal angiotensin-converting enzyme and dipeptidyl peptidase IV. *Am. J. Physiol.* **264**, G1153-G1159. doi:10.1152/ajpki.1993.264.6.G1153
- Suzuki, Y., Erickson, R. H., Yoon, B.-C. and Kim, Y. S.** (1995). Transcriptional regulation of rat intestinal angiotensin-converting enzyme and dipeptidyl peptidase IV by a high proline diet. *Nutr. Res.* **15**, 571-579. doi:10.1016/0271-5317(95)E0002-Y
- Tavakkolizadeh, A., Ramsanahie, A., Levitsky, L. L., Zinner, M. J., Whang, E. E., Ashley, S. W. and Rhoads, D. B.** (2005). Differential role of vagus nerve in maintaining diurnal gene expression rhythms in the proximal small intestine. *J. Surg. Res.* **129**, 73-78. doi:10.1016/j.jss.2005.05.023
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F.** (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, research0034.1. doi:10.1186/gb-2002-3-7-research0034
- Wang, L., Caviedes-Vidal, E. and Karasov, W. H.** (2019). Diet composition modulates intestinal hydrolytic enzymes in white-footed mice (*Peromyscus leucopus*). *J. Mammal.* **100**, 1512-1521. doi:10.1093/jmammal/gyz110
- Welch, B. L.** (1947). The generalization of 'Student's' problem when several different population variances are involved. *Biometrika* **34**, 28-35. doi:10.2307/2332510
- Wilkinson, L.** (2009). *Systat for Windows: Statistics, Version 13*. Evanston, IL: Systat, Inc.