Intestinal digestive enzyme modulation mechanism in omnivorous birds and mammals

By

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Dissertation Abstract

The diet of an animal shapes its role in ecological communities and drives evolution, and conversely, its digestive performance is a reflection of its ecology and evolutionary history. Thus, advances in our knowledge in digestive mechanisms improve our ability to predict how animals respond to alterations in their nutritional environment, including those caused by anthropogenic climate and land use change. The guiding hypothesis in digestive ecology is the adaptive modulation hypothesis, which suggests that digestive enzyme activities are matched to their respective dietary substrate level so that ingested nutrients are not wasted in excreta due to insufficient digestive capacity, and so membrane space or expenditures building/maintaining the intestinal hydrolytic machinery are not wasted when substrate levels are low. On the other hand, dietary specialists may have lost such modulatory abilities, instead expressing a narrower range of digestive enzyme activity levels that is consistent to their limited variation in dietary macronutrients. While the adaptive modulation hypothesis is generally supported among fish, rodents, bats, and songbirds, important gaps in the knowledge remain in the certain clades and the molecular mechanisms underpinning changes in the digestive enzyme profiles. It is also relatively rare that the principles highlighted by adaptive modulation are utilized in order to understand niche partitioning in competing species whose populations are endangered.

In Chapter 1, we tested the predictions based on the adaptive modulation hypothesis in juvenile northern bobwhites (*Colinus virginianus*; a New World quail species, order Galloanserae) and juvenile and adult domestic chickens (*Gallus gallus domesticus*; order Galloanserae) addressing the knowledge gaps in avian clades, individual age, and the effect of lipid on disaccharidase activities. Unlike what is known in omnivorous mammals, not all intestinal digestive hydrolases appear to be modulated adaptively by birds, and few experimental

studies have tested this ability outside of the order Passeriformes (songbirds). We also tested whether the ability to modulate digestive hydrolases decreases with maturity in chickens as was found in house sparrows (*Passer domesticus*; a songbird species), the only avian species studied to date on the effect of age in relation to digestive hydrolase modulation. We found that small intestinal α-glucosidase (AG; maltase and sucrase) activities were induced by dietary starch in both juvenile and adult chickens but not in northern bobwhites; aminopeptidase-N (APN) activities were induced by dietary protein in both bobwhites and juvenile but not adult chickens; AG activities were suppressed by an increase in dietary lipid in both bobwhites and juvenile but not adult chickens; and APN activities were not suppressed by high dietary lipid in any birds. Our work updated the adaptive modulation abilities in relation to phylogeny in birds. The overall finding suggests that all avian omnivores studied to date adaptively modulate at least one hydrolase, but which type of hydrolase is modulated may be partly influenced by clade (i.e., evolutionary history, constrained by ancestry). We further proposed that adaptive modulation is more critical in growing birds and less so in mature adults and that the suppressive effect of dietary lipid on AG activities may be part of adaptive modulation.

In Chapter 2, we investigated using the same chicken individuals in Chapter 1 the molecular mechanisms by which this modulation of AG activities and sugar transporters occur, particularly whether coordinated gene expression explains the digestive flexibility we observe at the whole-organism level. We tested the hypothesis that the modulation mechanisms of small intestinal α-glucosidases and sugar transporters would be co-modulated by their dietary carbohydrate signals similarly to in laboratory rodents and controlled primarily at the transcriptional level as suggested in rodents and house sparrow. This study represents the first investigation on the molecular mechanisms of digestive hydrolases modulation where different

age groups are included and dietary lipid was varied to test for the mechanisms by which AG suppression occurs under a high lipid diet. We employed an RNA-Seq approach with pre-selected genes of interest and a series of linear modeling to test our *a priori* predictions based on reported modulation mechanism. We found that avian α-glucosidase and sugar transporter transcript abundance are comodulated adaptively similarly to in rodents and that this modulation decreases in magnitude through maturity. Consistent with the hypothesis that AG activity suppression by dietary lipid would be adaptive, we found AG transcript abundance suppressed by high dietary lipid. As predicted, maltase-glucoamylase (*MGAM*) transcript abundance was positively correlated with the whole tissue-level maltase activity. However, the correlation between sucrase-isomaltase (*SI*) transcript abundance and sucrase activity varied across dietary groups from positive to negative, primarily affected by the lipid content in the diet. We conclude that chickens share general similarities in the modulation mechanisms of AG gene expression by diet with rodents, either representing a high degree of conservation or convergent evolution in these mechanisms. Our results also highlight the impact of high dietary lipid in multiple molecular mechanisms in the expression pathways of AG, including post-translational processes. These findings advance fundamental knowledge on the digestive response of animals consuming diets high in both carbohydrates and lipids. One potential application is the improved understanding of digestive response in urban wildlife because this combination of macronutrients is common in modern human refuse.

In Chapter 3, we applied the principles developed under adaptive modulation in order to understand the digestive mechanisms enabling two competing carnivores, the fisher (*Pekania pennanti*) and the marten (*Martes americana* or *M. caurina*) to coexist through dietary niche partitioning. Fishers and martens are closely-related and competing carnivores in forests across

North America, but in the Pacific Northwest, where both species are endangered, fishers consume insects, fungi, and plants as ~50% of their diet. We investigated whether fishers and martens have the ability to digest these atypical foods by sampling both species from two regions: the Pacific Northwest, where only fishers consume a range of non-meat diet items in addition to vertebrate prey and the Great Lakes Region, where both species are predominantly meat-eaters. We first demonstrated using zymography-based proteomics that both fishers and martens produce functional trehalase, the enzyme required for digesting trehalose, the principal carbohydrate in fungi and insects. We then tested the hypotheses that fishers and martens would show species-level difference in diet and digestive hydrolase profiles reflecting adaptations to occupying different dietary niches. Consistent with previous studies, fishers and martens in the Great Lakes Region as well as martens in the Pacific Northwest were primarily meat eaters, whereas fishers in the Pacific Northwest predominantly consumed fungi and insects, as revealed by stable isotope analyses. As predicted, we found that the investment in trehalase and maltase, relative to APN, was higher in fishers compared to martens. This species-level difference was consistent between regions; fishers in the Pacific Northwest did not differ in their investment in trehalase or maltase compared to fishers in the Great Lakes. These findings suggest that while both fishers and martens possess some ability for diet and digestive flexibility, but the greater digestive spare capacity of fishers for non-meat digestion may explain why fishers, despite being the dominant competitor, switch diets more readily than martens when the two species co-occur. The ability of carnivoran mammals to digest and utilize non-meat food items as a source of nutrients is poorly studied, and this case study highlights the importance of investigating digestive ecology to better understand their niche partitioning for their conservation.

Table of Contents

Macronutrient Signals for Adaptive Modulation of Intestinal Digestive Enzymes in Two Omnivorous Galliformes

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Abstract

According to the adaptive modulation hypothesis, digestive enzyme activities are matched to their respective dietary substrate level so that ingested nutrients are not wasted in excreta due to insufficient digestive capacity, and so membrane space or expenditures building/maintaining the intestinal hydrolytic machinery are not wasted when substrate levels are low. We tested predictions in juvenile northern bobwhites (*Colinus virginianus*) and juvenile and adult domestic chickens (*Gallus gallus domesticus*) by feeding them on diets varying in starch, protein, and lipid composition for 7-9 d (bobwhites) or 15 d (chickens). Birds were euthanized, intestinal tissue harvested, and enzyme activities measured in tissue homogenates from proximal, medial and distal small intestine. We found that (1) α-glucosidase (AG; maltase and sucrase) activities were induced by dietary starch in both juvenile and adult chickens but not in northern bobwhites; (2) aminopeptidase-N (APN) activities were induced by dietary protein in both bobwhites and juvenile but not adult chickens; (3) AG activities were suppressed by an increase in dietary lipid in both bobwhites and juvenile but not adult chickens; and (4) APN activities were not suppressed by high dietary lipid in any birds. We review findings from 35 analogous trials in 16 avian species. 100% of avian omnivores modulate at least one enzyme in response to change in dietary substrate level. AG induction by dietary carbohydrate occurs in more members of Galloanserae than in Neoaves, and all members of Neoaves tested so far increase APN activity on high dietary protein, whereas fewer of the Galloanserae do.

1. Introduction

The ability to modulate digestive enzymes is an important physiological attribute of an animal that may facilitate or restrict diet switching, and thus has major implications for whole-organism performance, ecology, and evolution (Karasov et al., 2011). It has been hypothesized that activities of digestive enzymes would be matched to their respective substrate level so that ingested nutrients are not wasted in excreta due to insufficient digestive capacity, and so that membrane space and/or expenditures building and maintaining the intestinal machinery to hydrolyze and absorb substrates are not wasted when substrate levels are low (adaptive modulation hypothesis; Diamond and Hammond, 1992; Karasov and Diamond, 1988). The hypothesis also suggests that natural selection would confer omnivores (i.e. dietary generalists) the ability to reversibly modulate digestive enzyme profiles to match their current diet (digestive flexibility), whereas for dietary specialists such regulatory mechanism would be lost due to the energetic and material costs of maintaining such a mechanism (Diamond and Hammond, 1992; Karasov and Diamond, 1988). In agreement with this hypothesis, many studies have found for omnivores positive correlation between dietary content of macronutrients and their corresponding digestive enzymes within and across species of various taxa (reviewed in (Karasov and Douglas, 2013; Karasov et al., 2011). Rodents, for example, appear to match levels of intestinal α-glucosidases (AGs; sucrase-isomaltase [SI] and maltase glucoamylase [MGAM]) to dietary carbohydrate level, and levels of intestinal aminopeptidase-N (APN) to dietary protein levels (Wang et al., 2019).

Most birds studied to date (14 out of 15 species; listed in (McWhorter et al., 2009) and (Kohl et al., 2017a)) have shown the ability to adaptively modulate at least one small intestinal digestive enzyme activity to match the composition of macronutrient levels in their diet. The sole

exception is the zebra finch (*Taeniopygia guttata*) which has little digestive flexibility in response to dietary manipulation, reflecting their natural diet throughout life that consists primarily of carbohydrate-rich seeds and their metabolism that cannot be sustained by diets low in starch or high in protein (Brzek et al., 2010). Other birds studied show varying degrees of omnivory (e.g., omnivorous throughout life, switch diets ontogenetically or seasonally, and/or metabolically capable of thriving on diets that vary wildly on macronutrient composition). However, not all intestinal enzymes appear to be adaptively modulated as in omnivorous rodents. For example, most adults of the order Passeriformes, the best represented clade in digestive flexibility studies (8 species), exhibit APN modulation by dietary protein but lack the ability to modulate intestinal AG (maltase and sucrase) activities in relation to dietary starch level (McWhorter et al., 2009). Limited work on Galloanserae (waterfowl, quails, and chickens) suggested the reverse pattern in this clade; 5 out of 6 species may have the mechanisms to modulate intestinal AG in relation to dietary starch, but not APN activity in relation to dietary protein (Kohl et al., 2017a; McWhorter et al., 2009). It has been further speculated that these apparent differences could be associated with phylogeny or due to the digestive contribution of cecal APN activity (Ciminari et al., 2014; Kohl et al., 2017a; McWhorter et al., 2009). Many Galloanserae retain well-developed ceca that function as fermentative digestive chambers, whereas Passeriformes and Columbiformes, members of Neoaves, do not (DeGolier et al., 1999; Hunt et al., 2019).

There are fewer studies in birds that consider how variation in dietary lipid levels influences intestinal enzymes. In wild and laboratory rodents (Dudley et al., 1994; Goda and Takase, 1994; Honma et al., 2007; Mochizuki et al., 2010b; Wang et al., 2019) and fish species (Cahu et al., 2000), high dietary lipid suppressed intestinal activity of AGs. This effect was

tested for, and found, in only two Passeriformes species (Brzek et al., 2013; Caviedes-Vidal et al., 2000; Levey et al., 1999) and not tested at all in Galloanserae (Karasov et al., 2011). Integrated analysis of digestive strategy using reactor models suggest how these effects of elevated dietary lipid on intestinal enzymes might be interpreted within the adaptive modulation hypothesis. The overall breakdown of dietary substrates are a product of hydrolysis rate and digesta retention time (Karasov and Douglas, 2013). In vertebrates fed higher dietary lipid, the high fat concentration suppresses gastric motility and overall digesta flow rate (Karasov and Hume, 1997), and it seems plausible that an economical compensatory response might be to lower enzyme levels so that membrane space and/or expenditures building and maintaining them are not wasted when overall enzymatic capacity has been increased by longer time to achieve breakdown. In this integrated view, enzyme capacity can be traded-off against retention time, and one predicts lower enzymatic activity on diets with high lipid content.

We also know relatively little about whether the ability to modulate intestinal digestive enzymes changes with age in birds. The sole omnivorous avian species studied in both juveniles and adults is the house sparrow (*Passer domesticus*; Passeriformes), and nestling sparrows have been shown to modulate both AGs and APN and do so rapidly – within 24 h of a diet switch (Brun et al., 2021a; Brun et al., 2021b; Rott et al., 2017). However, in house sparrows, the mechanisms for AG modulation are apparently lost over the course of maturation, because adults do not modulate either maltase or sucrase activity in relation to changing dietary carbohydrate level (Gatica-Sosa et al., 2018). This partial loss of modulation capability may reflect the shift from a mixed insect/plant material diet of juveniles toward specialization on a mainly plant dominated diet in adult sparrows (Martin et al., 1951), but it may also reflect the greater importance for nestlings to optimize digestion to remain on their developmental schedule in the

face of uncertainty in the quantity and quality of food (Brzek et al., 2011). We currently lack information on the age-related change in digestive enzyme modulation patterns in any other bird.

In this study we advance knowledge about digestive enzyme modulation, and its possible variation among avian clades, with new experiments with members of Galloanserae that add a new species and test for the first time for change in modulation with age in any species of Galloanserae. We tested juvenile northern bobwhites (*Colinus virginianus*), for the first time, and juvenile domestic chickens (*Gallus gallus domesticus*) for how their intestinal maltase, sucrase, and APN activities vary in response to variation in dietary starch, protein, and lipid. Specifically, would their modulation pattern conform to predictions of the general adaptive modulation hypothesis, showing adaptively increased AGs and APN on diets with higher levels of their respective substrates, and showing suppression of activities on high lipid diet? We also used adult chickens for comparison with the juveniles to test for an effect of age on diet induced intestinal enzyme modulation for the first time in Galloanserae. With our new data, we present a summary of adaptive intestinal digestive enzyme modulation patterns of all birds thus far studied, linked to a time-scaled cladogram.

2. Material & methods

2.1. Ethics statement

All animal procedures were approved by a University of Wisconsin (UW)–Madison Animal Care and Use Committee (ACUC, protocol #A005855 and #A005855-A2), and the feeding and care were overseen by the UW–Madison Research Animal Resource Center (RARC).

2.2. Experimental diets

2.2.1. Northern bobwhites

The three experimental diets, P (high protein), S (high starch), and L (high lipid), were composed of the same ingredients but differed by design in their proportions of carbohydrate (corn starch; Sigma #S4126), protein (casein; Sigma #C3400), and lipid (corn oil; MP Biomedicals #C7920; Table 1). Diets also included a constant amount of specific essential amino acids, vitamins and minerals (content described by (Lepczyk et al., 1998)) and a variable amount of "inert" ingredients (alphacel non-nutritive bulk + silica sand) used to make the calculated gross energy content similar across diets (15.4 ± 0.4 kJ/g dry mass). The diets that contained lower amounts of protein (C and L) still contained at least 3–5% more protein than is required for growth in bobwhite chicks (Klasing, 1998).

2.2.2. *Domestic chickens*

The three experimental diets (Table 2) were prepared similarly to the bobwhite diet, but we made two modifications. First, two of the three diet groups were similarly higher in two of the three macronutrients than one other diet: PL (high in protein and lipid), SP (high in starch and protein), and SL (high in starch and lipid). This design (see (Brzek et al., 2013) was chosen to maximize our ability to detect the suppressive effect on a digestive enzyme (e.g., AGs) of a non-substrate macronutrient (e.g., lipid). Second, we chose the PL diet to be starch-free in order to maximize our ability to detect modulation of AG activities, knowing that domestic chickens can thrive on such a diet (Biviano et al., 1993).

2.3. Animals, husbandry & experimental feeding

2.3.1. Northern bobwhites

Thirty-six day-old northern bobwhite quail chicks were purchased and shipped from Purely Poultry (Freemont, WI) to UW–Madison and housed in the campus Poultry Research Laboratory (PRL). Each was randomly assigned to one of three poultry battery cages (35 cm × 100 cm × 30 cm) under constant environmental conditions (room temperature 25.0 ± 0.5 °C, with additional cage heating for hatchlings providing a gradient 25.0–35.0 °C; relative humidity 45–65%; 24 h light) where they were provided with *ad libitum* water and food. Food (pre-2016 UW Chick Diet) was prepared by the UW College of Agriculture and Life Sciences Feed Mill and contained 57.6% corn, 35% soybean meal, 3.0% corn oil by dry mass (21.9 % crude protein, 2.6 % crude fiber, and 2.5% ether extract). Birds were raised for the next 18 days to acclimatize them and verify good health. Under surveillance of an RARC veterinarian they were treated once during that period with the antibiotic amprolium. Then, they began a 9 day period during which they were transitioned to one of three experimental diets (Table 1) by mixing in a gradually increasing proportion of the respective experimental diet into their feed (3 days each on 25% new:75% original, 50:50, and 75:25). Birds were on 100% experimental diet (*ad libitum* food and water) for 7–9 d (S diet, 7.4 ± 0.7 d; P diet, 7.4 ± 0.7 d; L diet, 7.5 ± 0.8 d); this could not be done on the same day due to personnel and time constraints.

2.3.2. Domestic chickens

One-day old domestic chickens (Barred Plymouth Rock) were purchased from Meyer Hatchery, Polk, OH (day 2 on arrival). Chickens for the juvenile study were purchased in 6 different lots and for the adult study in 3 different lots, because of constraints on number of personnel available to process all animals at a single time. Juveniles were kept under similar conditions as the northern bobwhites for acclimatization. Owing to the different years the study was conducted,

the standard UW Chick Diet had a slightly different composition from the one fed to bobwhites: 57.40% corn, 34.94% soybean meal, and 3.00% corn oil (21.9% crude protein, 2.6% crude fiber, and 2.6% ether extract). For the study on juveniles, birds on day 4 were randomly assigned to one of the three diets (cages) and a 50:50 mix of original and experimental diet was fed. From day 5, juveniles were fed 100% experimental diet (*ad libitum* food and water) for 15 d.

For the study on adults, we raised the juveniles on the UW Chick Diet in the battery cages as described above until ~45 days, at which point birds were moved to an indoor floor pen (240 cm × 340 cm × 250 cm). As before, they were provided *ad libitum* water and food (UW All Mash: ; 55.29%, 17.97% soybean meal, 7.19% wheat midds, 4.99% alfalfa meal, and 3.99% corn oil; 15.2% crude protein, 3.7% crude fiber, and 2.6% ether extract) and raised under temperature 22.5 ± 0.5 °C, relative humidity 45–65%, and 16:8 h light:dark. At age 211 d birds were transitioned onto their randomly assigned experimental diet (Table 2) over a three-day period (25% new: 75% original, 50:50, and 75:25 each day) and then were on 100% experimental diet (*ad libitum* food and water) for 15 d.

2.4. Harvest of intestinal tissue

Northern bobwhites were collected at the final age of 70–72 d, chicken juveniles at 20 d, and chicken adults at 230 d. Bird were weighed (± 0.1 g), tarsus length was measured (± 0.1 mm), and euthanized via CO_2 asphyxiation followed by rapid cardiac compression or cervical dislocation. The gut below the gizzard was immediately dissected out and placed in ice-cold avian Ringer in bobwhites (Caviedes-Vidal et al., 2000; Caviedes-Vidal and Karasov, 1996) or phosphate buttered saline (PBS; Sigma #P5368) in chickens (Iji et al., 2001). After separating the small intestine from the ceca and colon, the small intestine was divided into thirds of

approximately equal length, proximal, medial, and distal. Each of the sections was sampled in an identical manner. Digesta was removed distally by profusion of avian Ringer (bobwhites) or PBS (chickens) and collected flash frozen in liquid nitrogen. Intestinal sections were blotted dry, weighed (± 0.0001 g), opened longitudinally, and measured for length (± 0.1 cm) and largest and smallest width (± 0.01 mm). In chickens, an RNA sample was collected from the center of each section in RNAlater (Invetrogen #AM7021; quenched for 24 h at 4 °C and stored at -80 °C). The remaining intestine was cut longitudinally in half, and one of those halves were processed for enterocyte isolation (Brun et al., 2020a; Mac Donal et al., 2008). The other half was flash frozen and stored at -80 °C for later measurement of intestinal enzyme activity in tissue homogenates.

2.5. Digestive enzyme assays

We assayed for maltase, sucrase and APN activity of whole intestinal tissue as previously described (Brzek et al., 2009; Brzek et al., 2011; Brzek et al., 2013). Small intestine samples were thawed on ice, weighed, and homogenized in ice-cold mannitol buffer (1:11 w/v, 300 mM mannitol in in 1 mM Hepes-KOH, pH 7.0).

Assays for maltase and sucrase activities were identical except for the substrate and dilution of the homogenate (homogenate diluted 1:250 for maltase and 1:10 for sucrase in mannitol buffer). This diluted homogenate (30 µL) was incubated with 30 µL of 56 mM maltose (or sucrose) in 0.1 M maleate-NaOH buffer (pH 7.0) at 40 °C for 20 min. Then, a stop-develop reagent (400 µL; Sigma #GAGO20-1KT for glucose assay) was added, vortexed, and incubated at 40 °C for 30 min. Finally, 12N H_2SO_4 was added and the absorbance was read at 540 nm using a BioTek Epoc microplate reader.

For the APN assay, homogenate (2.5 µL) was added to 250 µL of 2 mM L-alanine-*p*-

nitroanilide (Sigma #A9325; diluted in 0.1 M NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0) and incubated for 20 min at 40 °C. The reaction was stopped by adding 750 µL of 4N acetic acid, and the absorbance was read at 380 nm.

2.6. Statistical analyses.

We conducted all statistical analyses using R v. 4.0.3 (R Core Team, 2024) via RStudio platform v. 1.4.1106. Tests were performed 2-tailed with critical $\alpha = 0.05$. In juvenile and adult chickens, lot was included as a potential confounding variable but only retained as necessary based on Akaike's information criterion with small sample correction (AICc, MuMIn v. 1.43.17; Bartoń, 2022). Model assumptions were verified using residual plots, and no final model showed multicollinearity issues (variance inflation factor < 10). We present all results as least square mean ± s.e.m. unless otherwise specified.

2.6.1. Body and intestinal morphometrics

Body mass (g), tarsus length (mm), and mass (g), length (cm), and nominal area (cm^2) of the whole small intestine were compared across diet for bobwhites and then separately for chicken age groups (juveniles and adults) using linear models. Post hoc Tukey tests (lsmeans v. 2.30-0; Lenth and Hervé, 2018) were used to determine group-level differences.

We then tested whether intestinal wet mass per unit length ($g\ cm^{-1}$), intestinal wet mass per unit nominal surface area ($g\ cm^{-2}$), and intestinal nominal surface area per unit length ($cm^2\ cm^{-1}$) differed by diet in relation to intestinal position using linear mixed models (lmer in lmerTest v. 3.1-3; Kuznetsova et al., 2020) in order to choose a metric for enzyme activity normalization. Intestinal position, diet, and the interaction between position and diet were

modeled as fixed effects and bird ID as a random effect (equivalent to repeated measures analysis of variance). The interaction term was removed in all comparisons based on AICc; thus we report the main effects of diet and post hoc Tukey results (lsmeans).

2.6.2. Mass-specific digestive enzyme activities by intestinal position

We chose to normalize our intestine mass-specific digestive enzyme activity data by intestinal mass based on our morphometrics findings (see Results). For bobwhites, we fitted a linear mixed model (lmer) with fixed effects as intestinal position, diet, and the interaction between intestinal position and diet, and bird ID as a random effect predicting each of the 3 digestive enzyme activities. We anticipated the position×diet interaction (Ciminari et al., 2014; Kohl et al., 2017a); hence, this term was retained in the final model. Significance of each term was tested using type III analysis of variance (ANOVA) with Satterthwaite's method (*F* statistic). Post hoc Tukey tests on the effect of diet were performed at each intestinal position (lsmeans).

For chickens we fitted a single model that included both age groups in order to test the effect of age on mass-specific AG and APN activities in relation to diet (unconfounded by tissue size difference). We fitted a generalized linear mixed model (glmer in lme4 v. 1.1-25; Bates et al., 2020) with gamma error structure and identity link. Fixed effects were specified as age (juvenile or adult), intestinal position, diet, and all 2-way and 3-way interactions and bird ID as a random effect. GlmerControl was set as optimizer = 'optimx' and optCtrl = list(method='nlminb' (optimx v. 2021-10.12; Nash, 2021). We retained all interaction terms throughout because these terms may reflect modulation mechanisms that change in relation to age and/or position (Brzek et al., 2013; Caviedes-Vidal et al., 2000; Ciminari et al., 2014; Kohl et al., 2017a). Significance of terms was tested using type III Wald χ^2 test, and post hoc Tukey tests on the effect of diet were

performed at each intestinal position within an age group (lsmeans).

2.6.3. Summed enzyme activity across the whole small intestine

We estimated the summed enzyme activity of whole intestine (μmol min^{-1}) for each individual by multiplying the wet mass-specific enzyme activity (μmol min^{-1} g^{-1}) at each intestinal position by the wet mass (g) of the respective intestinal position (proximal, medial, or distal) and then summing the three products. This estimate of whole intestinal summed enzyme activity was compared using linear models (lm of lme4) run separately on bobwhites, chicken chicks, and chicken adults. Body mass was included in all initial models as a potential confounder but was only retained when there was AICc support. Post hoc Tukey tests on diet were conducted using lsmeans.

2.6.4 Integration of findings with comparable findings on other avian species

We searched the literature for analogous studies in other avian species. Findings on 16 species were arranged in a temporally informed phylogeny based on (Kumar et al., 2017). Proportions of species exhibiting traits were compared across taxonomic using Fisher's Exact Test.

3. Results

3.1. Diet effect on body and intestinal morphometrics

3.1.1. Diet effect on body and intestinal morphometrics of northern bobwhites

Diet had no effect on either body mass, tarsus length, or small intestine length of juvenile bobwhites (Table 3). Diet affected both whole small intestine mass and nominal surface area, with larger sizes in S group than P group or L group.

Small intestinal morphometric measures, wet mass per unit length (Fig. 1a), wet mass per unit area (Fig. 1b), and area per unit length (Fig. 1c) all differed by intestinal position (respectively, $F_{2, 69} = 39.16$, $P < 0.0001$, $F_{2, 46} = 25.74$, $P < 0.0001$, $F_{2, 72} = 26.05$, $P < 0.0001$) and by diet ($F_{2, 69} = 5.10$, $P = 0.008$, $F_{2, 23} = 5.79$, $P = 0.009$, $F_{2, 72} = 3.27$, $P = 0.04$). Post hoc tests showed intestinal mass per length, mass per area and area per unit length was highest in the S group.

3.1.2. Diet effect on body and intestinal morphometrics of juvenile and adult chickens

Diet had no effect on body mass of juvenile or adult chickens (Table 4). Diet affected tarsus length of juveniles ($F_{2, 34} = 3.37$, $P = 0.046$), with longer tarsi in SL than SP and neither group differing from PL. Adult tarsus length did not vary by diet.

Whole small intestine mass differed by diet in juveniles with SL group greater than SP group, but no other group differences (post hoc Tukey tests; Table 4). Juvenile intestine length showed the same pattern. Intestinal nominal area also differed in juveniles, with SL group larger than both PL and SP groups. In adults, diet did not affect small intestine mass, length, or nominal area.

Small intestinal morphometric measures in chickens, wet mass per unit length (Fig. 2a, b), wet mass per unit area (Fig. 2c, d), and area per unit length (Fig. 2e, f) all differed by intestinal position in both juveniles and adults, but in most cases did not differ by diet. In only two instances were effects of diet on these measures isolated in post hoc comparisons. Intestinal mass per unit length in juveniles (Fig. 2a) varied by intestinal position ($F_{1, 83} = 774$, $P < 0.0001$) and diet ($F_{2, 34} = 5.81$, $P = 0.007$), and post hoc Tukey test on diet showed group SL to be higher than group SP. Likewise, intestinal mass per unit area in juveniles (Fig. 2c) varied by intestinal

position ($F_{1, 83} = 379$, $P < 0.0001$) and diet ($F_{2, 39} = 4.23$, $P = 0.02$), and post hoc Tukey on diet showed group SL to be higher than group SP.

Taken together, diet-driven difference on intestinal morphometrics occurred primarily in northern bobwhites (S group higher than either P or L groups) and chicken chicks (SL higher than SP group), and this effect was largely consistent across all intestinal positions. Because these examples all involve the measures of mass per unit length or per unit area of intestine, we concluded that intestinal mass was the best measure for use in normalizing digestive enzyme activity per unit of intestine.

3.2. Digestive enzyme activities in northern bobwhites

3.2.1. Northern bobwhites, activities per gram wet intestine by intestinal position

Small intestinal mass specific digestive enzyme activities of juvenile northern bobwhites varied by diet and/or intestinal position, and no enzyme activity showed significant interaction effect between diet and intestinal position (Fig. 3, Table 5).

Contrary to Prediction #1, neither maltase nor sucrase activity were higher on the higher starch S diet compared to the lower starch P diet (Fig. 3a, b).

Consistent with Prediction #2, APN activity was higher on the higher protein P diet compared to the lower protein S diet, primarily in the medial small intestine (Fig. 3c).

Consistent with Prediction #3, AG activities were lower on the higher lipid diet L compared to the lower lipid P diet, particularly in the medial small intestine (Fig. 3a, b).

Contrary to Prediction #4, APN activity was not lower on the higher lipid L diet than lower lipid S diet (Fig. 3c).

3.2.2 Northern bobwhites, summed enzyme activity of whole small intestine

Here we tested for dietary modulation of the summed intestinal maltase, sucrase and APN activities of the whole small intestine (μmol min^{-1}) as calculated in section 2.6.3. Summed AG and APN activities varied with diet for AGs (for maltase $F_{2, 24} = 27.17$, $P < 0.0001$; for sucrase $F_{2, 24} = 28.04$, $P = 0.0004$; Fig. 4a, b) but not for APN ($F_{2, 23} = 1.74$, $P = 0.2$, Fig. 4c).

Contrary to Prediction #1, summed sucrase activity was not higher in northern bobwhites fed the higher starch S diet than the lower starch P diet (Fig. 4b), which was consistent with the finding at the tissue specific level (Fig. 3b in section 3.2.1). However, summed maltase activity was higher in group S than group P, even though modulation was not shown at the mass specific level (Fig. 3a). The explanation is that the intestines of bobwhites on the S diet were about 24% heavier than on the P diet (Fig. 1a, Table 3) which, in combination with slightly but not significantly higher activity per gram intestine, led to apparently higher summed maltase activity.

For Prediction #2, summed APN activity did not differ with diet ($F_{2, 34} = 0.80$, $P = 0.46$; Fig. 4c), even though modulation was shown at the mass specific level (see section 3.2.1). The same heavier bobwhite intestine on the S diet likely explains this. The difference in intestine mass was counter to, and obscured, the higher APN activity that was measurable at the tissue level.

Consistent with Prediction #3 for AGs, summed activity of both AGs was lower in the higher lipid L group compared to the lower lipid P group (Fig. 4a, b), which was also the case for tissue specific activities (Fig. 3a, b).

Contrary to Prediction #4 for APN, summed activity was not lower on the higher lipid L diet than lower lipid S diet (Fig. 4c), which was consistent with the finding at the tissue specific level (Fig. 3c).

3.3 Digestive enzymes in juvenile and adult chickens

3.3.1. Juvenile and adult chickens, activities per gram protein by intestinal position

In chickens, intestinal mass specific digestive enzyme activities varied by diet, and those effects depended on intestinal position, age group, and/or interactions among these 3 predictor variables (Fig. 5, Table 6).

Consistent with Prediction #1, AG activities in juvenile chickens were higher on the higher starch SL diet compared to the zero starch PL diet, especially in the medial small intestine (Fig. 5a, c). In adults, however, Prediction #1 was upheld only for maltase activity; for sucrase activity the values for SL diet were not higher than for PL diet (Fig. 5b, d). In Discussion we explain how different modulation responses for the two AGs are plausible in chickens.

Consistent with Prediction #2, APN activity in juveniles was higher on the higher protein diet PL compared to the lower protein SL diet (Fig. 5e). In adults, however, Prediction #2 was soundly rejected because APN activity did not differ among diets at any position (Fig. 5f).

Consistent with Prediction #3, AG activities in juveniles were lower on the higher lipid diet SL compared to the lower lipid SP diet (Fig. 5a, c), but in adults Prediction #3 for AGs was soundly rejected because activity of neither maltase nor sucrase was lower on the higher lipid diet SL compared to the lower lipid SP diet (Fig. 5b, d).

Prediction #4 did not hold for APN activity in either juveniles or adults, because it was not suppressed on the higher lipid PL diet in comparison to the lower lipid SP diet (Fig. 5e, f).

3.3.2 Juvenile and adult chickens, summed enzyme activity of whole small intestine

Summed AG and APN summed activities varied with diet in juveniles (for maltase $F_{2, 38} = 7.0$, P

= 0.003; for sucrase $F_{2, 33} = 13.5$, $P < 0.0001$; for APN $F_{2, 33} = 3.9$, $P = 0.003$; Fig. 6a, c, e). For adult chickens, summed AG activities varied with diet (for maltase $F_{2, 21} = 14.7$, $P < 0.0001$; for sucrase $F_{2, 21} = 7.8$, $P = 0.003$; Fig. 6b, d), but summed APN activity did not ($F_{2, 21} = 1.1$, $P = 0.36$; Fig. 6f).

Consistent with Prediction #1, summed AG activities in juveniles were higher on the higher starch SL diet compared to the zero starch PL diet (Fig. 6a, c). In adults, Prediction #1 was upheld for summed maltase activity, but summed sucrase activity was not elevated on the higher starch SL diet compared to the zero starch PL diet (Fig. 6a). The patterns matched what would be expected based on the findings at the tissue specific level (Fig. 5a–d).

Consistent with Prediction #2, APN summed activity in juveniles was higher on the higher protein diets PL compared to the lower protein SL diet (Fig. 6c). In adults, however, Prediction #2 was rejected because summed APN activity did not differ by diet (Fig. 6f). These findings were consistent with the finding at the tissue specific level (Fig. 5e, f).

Contrary to Prediction #3 for AGs, summed AG activities in juvenile chickens was not lower on the higher lipid SL diet compared to the lower lipid SP diet (Fig. 6e) even though that was the case at the mass specific level (Fig. 5a, c). The 35% suppression of AG activities in juvenile chickens consuming SL diet in comparison to SP diet (Fig. 5a, c) is obscured in the estimate of summed AG activity by the 30% heavier intestine in the chicks fed the SL diet in comparison to the SP diet (Fig. 2c, Table 4). In adults, Prediction #3 was rejected because neither summed maltase nor summed sucrase activity was lower on the higher lipid SL diet compared to the lower lipid SP diet (Fig. 6b, d), similarly to the tissue specific findings (Fig. 5b, d).

Contrary to Prediction #4 for APN, neither juveniles nor adults showed summed APN activity lowered on the higher lipid PL diet in comparison to the lower lipid SP diet (Fig. 6e, f),

which was also the case in the tissue specific results (Fig. 5e, f).

Discussion

4.1 Study overview

With careful attention to diet and statistical design, we tested in two galliform species more than two dozen specific predictions related to the adaptive modulation hypothesis, as applied to intestinal hydrolytic enzymes (Table 7). We discuss mainly mass-specific hydrolase activities because the focus of the study was whether mechanisms are present for nutrient-specific modulation of these hydrolases and use the intestinal morphometrics data to interpret summed activities across the whole small intestine. It has been hypothesized that nonspecific (e.g., small intestinal morphology) changes may occur as part of adaptive modulation (Biviano et al., 1993; Martínez del Rio et al., 1995). However, reported changes in intestinal morphology with diet change appear inconsistent even among similarly designed studies with chickens (e.g., our study vs. Biviano et al., 1993; Ciminari et al., 2014). Also, most analogous avian studies did not find such diet-related changes in morphology (e.g., Brzęk et al., 2013; Kohl et al., 2017b). These conditions make this hypothesis difficult to test within the scope of the current study.

In relation to our predictions (Tables 1, 2), findings, for the most part, indicate that (#1) AG activity is induced by high dietary starch level, as predicted, in juveniles of chickens but not northern bobwhites, (#2) APN activity is induced by high dietary protein, as predicted, in juveniles of both chickens and northern bobwhites, and (#3) in juveniles of both species high dietary lipid suppresses AG activities as predicted, but not APN activity. Our findings with adult chickens suggest their intestinal enzymes are much less responsive to the dietary manipulations of starch, protein and lipid. Thus our results overall do not support the notion of a common

pattern of modulation of intestinal enzymes within Gallanserae, but that patterns of modulation can differ according to both species and age within species.

In only three instances (marked with asterisks in Table 7), the findings based on tissue-level activity measures were not in agreement with those for activity over the whole intestine, which is estimated as the product of activity per g intestine and intestine mass. As explained in Results, all three of those discrepancies seem explainable when considering the effects diets had, in some cases, on whole intestine mass.

In the following sections we discuss in more detail our results in relation to the three major predictions from the adaptive modulation hypothesis. We also review findings from 33 analogous trials in 15 other avian species.

4.2. Induction of α-glucosidase activities by dietary starch

We found strong evidence of AG induction by starch in juvenile chickens. The induction in mass specific AG activities was 80–90 % in medial intestine in the SL group compared to the PL group (Fig. 5a, c), and when summed across the whole intestine represented a 40–60% increase (Fig. 6a, c).

In adult chickens fed higher starch SL diet compared to the zero starch PL diet we found higher maltase (by 70%) and sucrase (by 65%) activities (Fig. 5b, d), though the difference was not significant for sucrase ($P = 0.13$). Lower power to detect a difference might be the explanation, because the experiment with adults had smaller sample size than that for the juveniles (respectively, $n = 8$ and $n = 14$). Alternatively, it is plausible that maltase and sucrase activities could show different modulation patterns in chickens. This is because chickens have two distinct enzymes that hydrolyze small oligomers of glucose: sucrase-isomaltase (SI), which

hydrolyzes both maltose and sucrose, and a co-ortholog called avian-derived alpha α-glucosidase (ADAG), which seems functionally convergent with mammalian MGAM and hydrolyzes maltose but not sucrose (Brun et al., 2020b).

Our evidence of AG induction by high dietary starch is consistent with the findings of previous work in juvenile chickens, where the magnitudes of induction ranged from 30% – 100% (Biviano et al., 1993; Ciminari et al., 2014; Siddons, 1972). The range in magnitude of effect may be attributable to the different composition of diets used. One of those studies also found induction in maltase but not sucrase activity (Ciminari et al., 2014).

The mechanisms underlying the induction of AG activity by dietary starch in Galloanserae have not been studied (but see ontogenetic studies by (Sklan et al., 2003; Uni et al., 1999; Uni et al., 2003). However, in laboratory rodents (Honma et al., 2007; Mochizuki et al., 2010a) and in house sparrow nestlings (Brun et al., 2021a) the induction is known to be primarily controlled transcriptionally. Specifically, laboratory mice on a high-starch diet showed acetylation of histones H3 and H4 with increased binding of transcriptional factors Cdx2 (caudal-type homeobox transcription factor 2) and HNF1 (hepatocyte nuclear factor 1) to the promotor/enhancer regions of the AG genes *SI* (sucrase-isomaltase) and *MGAM* (maltase glucoamylase), resulting in increased mRNA levels of these genes (Honma et al., 2007; Mochizuki et al., 2010a). A similar scenario was recently suggested in house sparrows nestlings: *SI* mRNA expression, sucrase-isomaltase relative abundance on the intestinal brush border membrane, and sucrase and maltase activity all increased within 24 h of a switch from a low-starch to a high-starch diet (Brun et al., 2021a). We hope to test for similar changes in an upcoming study of transcriptomic and proteomic changes in tissues from the juvenile and adult chickens reported on here.

In contrast to chickens, northern bobwhites did not exhibit induction of intestinal mass-specific maltase or sucrase activity on the high-starch S diet compared to the low-starch P diet (Figure 3a, 3b). An earlier study found bobwhites also do not up-regulate intestinal glucose transport activity when fed higher starch diet (Karasov et al., 1996). Taken together, it appears that northern bobwhites lack the mechanism(s) for specifically inducing AG or glucose transporter activities (e.g., increase in enzyme or transporter abundance) following higher dietary starch signal.

4.3. Induction of aminopeptidase-N activity by dietary protein

We found strong evidence of APN induction by elevated dietary protein in juvenile chickens. The increase in APN activity in those fed higher protein diet PL compared to lower protein diet SL, ~38%, was smaller than the level of induction of AG by dietary starch (above) (Fig. 5e). Ours is the first study to report APN induction by protein in chickens, as the only other study that tested APN modulation did not find evidence for it (Ciminari et al., 2014), possibly owing to insufficient statistical power (n = 6–7 per diet, compared to our n = 14).

We also found evidence for APN induction in the northern bobwhites, where APN activity/g tissue was ~40% higher in those fed higher protein diet P in comparison to lower protein diet S (Fig. 3c). As discussed above, the ~24% heavier intestine mass in the diet S group obscured the difference in APN at the whole-intestine level.

The molecular mechanisms underlying APN induction by protein in Galloanserae have not been studied. In house sparrow nestlings APN induction appears to be transcriptionally controlled (Brun et al., 2021b). A switch from a low protein diet to a high protein diet resulted in an increase in APN transcript abundance and APN activity at the brush border membrane as well

as in whole tissue homogenates within 24 h (Brun et al., 2021b), similarly to what was found for AGs (Brun et al., 2021a). We plan to test the hypothesis of transcriptional control of APN in chicken juveniles and adults in the future.

In contrast to juveniles of chickens and northern bobwhites, adult chickens did not exhibit induction of mass-specific APN activity on higher protein diets (PL, or even SP) in comparison to the lower protein diet (SL; Fig. 5f). It will be interesting to learn whether some molecular mechanism(s) underlying APN induction on higher dietary protein in chicken juveniles are to some degree "silenced" as part of the maturation process in chickens.

4.4. Suppression of intestinal hydrolases by high dietary lipid

Higher dietary lipid suppressed activity of AGs in juveniles of both northern bobwhites (Fig. 3a, b) and chickens (Fig. 5a, b) by 30–35%. As mentioned above, a comparable decline in intestine mass in the chickens, but not the bobwhites, obscured this effect when viewed at the whole intestine level (Fig. 6a, c). There are no comparable data in any prior study on either species.

The molecular basis of this suppression has been studied in rodents. Downregulation of the *SI* gene transcription by high dietary lipid is suggested in laboratory mice (Honma et al., 2007). Furthermore, during the post-translational processing of SI in rats, high dietary lipid may reduce the level of unsialylated galactose on the glycosylated chain of SI and thereby may suppress the transport of SI onto the brush border membrane (Mochizuki et al., 2010b). However, these studies in rats did not hold starch levels constant, though the ratio of starch/lipid in the diet varied. However, in house sparrows, elevated dietary lipid with starch held constant was also found to suppress AG activity (Fig. 7; Supplementary Table S1) and reduce SI mRNA (ECV, unpublished data). It will be interesting to test for impacts on AG mRNA also in the juvenile

chickens. Also, insofar as high dietary lipid did not suppress AG activity in the adult chicken (Fig. 5f), it will be interesting to learn whether some molecular mechanism(s) underlying this effect are to some degree "silenced" as part of the maturation process in chickens.

We suggested that the suppressive effect of dietary lipid on AG activity might be adaptive because lipid is more energy-dense than starch and a high-lipid diet suppresses gastric motility and overall digesta flow rate (Karasov and Hume, 1997), and hence the animal may not need to invest as much in the production of AGs when the diet is rich in lipid. There is evidence that the lipid effect on intestinal AG activity depends on the type of lipid. For example, the suppressive effect may only occur when the diet is rich in unsaturated fats like corn oil (Dudley et al., 1994) and when chain length of triacylglycerol (triglyceride) is long (Takase and Goda, 1990). This would seem to argue against the prediction proposed based on an adaptive modulation hypothesis. Also, the suppressive effect of lipid on intestinal enzymes seems absent, so far, in the case of APN (Fig. 7, Supplementary Table S1). But, analogous to what has been argued previously in regard to dietary modulation of amino acid transport (Karasov et al., 1987), whereas breakdown of carbohydrate might be adaptively repressed under some conditions, it might be more detrimental to do so for protein breakdown because that yields essential nitrogen and some essential amino acids. Plausibly, different regulatory patterns regarding AGs and peptidases can sometimes be understood as a compromise among conflicting constraints imposed by protein's multiple roles as a source of energy, nitrogen, and essential amino acids. Future transcriptomic and proteomic studies of intestinal tissue that allows global profiling of mRNA and brush border proteins may shed light into the molecular mechanisms of the lipid effect on intestinal hydrolases and its biological significance.

4.5 Comparative, evolutionary, and ecological aspects of dietary modulation of intestinal hydrolases

Our study raises to 16 the number of species that have been tested for modulation of intestinal hydrolases by changes in levels of dietary macronutrients (total of 35 trials; Fig. 7; Supplementary Table S1). Ninety-four percent of species tested have been shown to modulate at least one small intestinal digestive enzyme. The single exception, the zebra finch, is considered a specialist on seeds from hatch to adulthood (Brzek et al., 2010), and neither juvenile or adult elevated AGs on higher dietary starch, nor did they elevate APN on higher dietary protein. Perhaps the intestinal hydrolytic needs of zebra finch eating a relatively constant diet are always satisfied by a single, constitutively expressed level of intestinal hydrolases. Arguably, a regulatory system, like that which underlies adaptive modulation of intestinal enzymes, is not selected for unless the benefits it provides outweigh its costs (Buddington et al., 1991). More studies of avian specialist species are needed to test this hypothesis.

Among avian omnivores, a curious pattern identified earlier (McWhorter et al., 2009) was that members of Neoaves (including passeriform and columbiform birds) seem not to modulate their levels of intestinal AGs, but do modulate intestinal peptidases, in response to the respective dietary substrate concentration. The pattern seemed opposite among members of Galloanserae (including galliform and anseriform birds). Our review of a now larger number of tests provides some support for these patterns; yet, they are certainly not absolute. Some members of Neoaves, house sparrow juveniles in particular, do increase AG activity on high dietary carbohydrate, and some members of Galloanserae, northern bobwhite juveniles in particular, do not (Fig. 7; Supplementary Table S1). In this particular comparison we excluded the Pine warbler (*Setophaga pinus*) because varied dietary lipid levels in high- and low-starch

diets confounds the straightforward test in that study for induction of AGs by dietary carbohydrate (Levey et al., 1999). Overall, AG induction by dietary carbohydrate occurs in more members of Galloanserae (86% of 7 species) than in Neoaves (14% of 7 species; $P = 0.03$ by Fisher's Exact Test). All members of Neoaves tested so far increase APN activity on high dietary protein (100% of 8 species), whereas fewer of the Galloanserae do (57% of 7 species; $P = 0.08$).

The question remains, however, about the meaning of these apparent differences, but we think this is beyond the scope of the current study. However, for the future, what new data might be collected and be most illuminating? As more data on modulation of intestinal enzymes are accumulated, future analyses should test for patterns in a phylogenetically informed manner. The presence or absence of a digestively functioning cecum is highly correlated with the phylogenetic pattern (DeGolier et al., 1999; Hunt et al., 2019; McWhorter et al., 2009), but inclusion of qualitative and quantitative data on diet (e.g., % cell wall material) and cecal structure (e.g., relative lengths of ceca) and functioning (e.g., % of N recycled by gut microbiome, % carbohydrate broken down) might help explain variation within the Galloanserase regarding modulation of intestinal digestive enzymes.

Our findings with adult chickens suggest their intestinal enzymes are much less responsive to the dietary manipulations of starch, protein and lipid than juvenile chickens (Table 7). Similarly, in house sparrows the induction of AG by dietary starch occurs only in the juveniles and not the adults, although suppression of AG activity by dietary lipid occurs in both (Fig. 7, Suppl Table S1). In the house sparrow, it was proposed (Brzek et al., 2011) that the partial loss of modulation capability may relate to the shift from a mixed insect/plant material diet of juveniles toward specialization on a mainly plant dominated diet in adults, but it might

also reflect the greater importance for nestlings to optimize digestion to remain on their developmental schedule in the face of uncertainty in the quantity and quality of food. These explanations might apply also to the chicken, because it is common among wild galliforms that the juveniles are more insectivorous than the adults (Klasing, 2005). Also noteworthy is that our results on adult chickens represent the first characterization of modulation pattern in any adult Galloanserae species, with the remainder of tests on juveniles at varying ages and/or stages of development. In contrast, modulation patterns of Neoaves are only known in adults except house sparrows and the specialist zebra finches whose nestlings were also studied. It is unclear, therefore, whether the modulation patterns often found in Galloanserae represent their mature digestive "phenotype," or that the juveniles of omnivorous Neoaves in general actually possess the mechanisms for modulating AGs like in house sparrows. Certainly, more comparisons of juveniles and adults in other species are necessary to understand the biological significance of age differences in intestinal enzyme modulation.

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Table 1. Composition of experimental northern bobwhite diets (% dry mass). P = high-protein; S = high-starch; L = high-lipid. Contrasts among these diets test the 4 major predictions on intestinal α-glucosidase (AG) and aminopeptidase-N (APN) activities[2]

Diet	Corn starch	Milk casein	Corn oil	Inert[1]	Essential[1]	Energy (kJ/g)
P	5	59.5	8	17	10	14.8
S	38	26.5	8	17	10	14.8
L	5	26.5	25	33	10	15.5

[1]Contents as described in Lepczyk et al. 1998.

[2]**Prediction #1** – for AGs, S > P; tests for induction of AGs by starch, with lipid held constant

[2]**Prediction #2** – for APN, P > S; tests for induction of APN by protein, with lipid held constant

[2]**Prediction #3** – for AGs, L < P; tests for suppression of AGs by lipid, with starch held constant

[2]**Prediction #4** – for APN, L < S; tests for suppression of APN by lipid, with protein held constant

Table 2. Composition of experimental chicken diets (% dry mass). PL = high protein; SP = high starch; SL = high lipid. Contrasts among these diets test the 4 major predictions on intestinal α-glucosidase (AG) and aminopeptidase-N (APN) activities [3]

Diet[1]	Corn starch	Milk casein	Corn oil	Inert[2]	Essential[2]	Energy (kJ/g)
PL	0	42	16	32	10	13.8
SP	34	42	4	10	10	15.2
SL	34	16	16	24	10	15.2

[1]All 3 diets meet nutritional requirement of chickens (National Research Council Subcommittee on Poultry Nutrition. (1994)); chickens are known to thrive on diets that contain substrate levels like these or even higher than proposed here (Biviano et al. 1993; Myers and Klasing 1999; Ciminari et al. 2014).

[2]Contents as described in (Lepczyk et al. 1998).

[3]**Prediction #1** – for AGs, SL > PL; tests for induction of AGs by starch, with lipid held constant

[3]**Prediction #2** – for APN, PL > SL; tests for induction of APN by protein, with lipid held constant

[3]**Prediction #3** – for AGs, SL < SP; tests for suppression of AGs by lipid, with starch held constant

[3]**Prediction #4** – for APN, PL < SP; tests for suppression of APN, by lipid with protein held constant

Table 3. Indices of body size and small intestine size in juvenile northern bobwhites raised 15 days on three diets (n = 8 to 10 per diet)

Diet[1]	Body mass	Tarsus length	Small intestine		
	(g)	(mm)	Length (cm)	Mass (g)	Nominal area (cm^2)
P	96.9 ± 2.9	31.3 ± 0.5	38.0 ± 1.0	2.27^{b} ± 0.09	30.9^{b} ± 1.0
S	100.6 ± 3.1	31.5 ± 0.6	40.0 ± 1.1	2.82^{a} ± 0.09	35.6^{a} ± 1.0
L	89.8 ± 3.3	31.1 ± 0.6	36.8 ± 1.0	2.13^{b} ± 0.10	31.1^{b} ± 1.1
F-test[2] for diet effect	$F_{2, 24} = 2.99$	$F_{2, 24} = 0.14$	$F_{2, 24} = 2.13$	$F_{2, 24} = 15.24$	$F_{2, 24} = 6.96$
P-value	$P = 0.07$	$P = 0.87$	$P = 0.14$	$P < 0.0001$	$P = 0.004$

[1]Diets described in Table 1

[2]Type III ANOVA on linear models; subscripts for F correspond to degrees of freedom. Values are least square means, and those with different superscripted letters differed significantly in post hoc comparison test

Table 4. Indices of body size and small intestine size in juvenile (n = 14 per diet) and adult chickens (n = 8 per diet) raised 15 days on three diets.

Age, Diet[1]	Body mass	Tarsus length	Small intestine		
	(g)	(mm)	Length (cm)	Mass (g)	Nominal area (cm^2)
Juvenile, PL	98.0 ± 6.0	30.5^{ab} ± 0.8	65.2^{ab} ± 1.5	5.61^{ab} ± 0.27	59.5^{b} ± 1.7
Juvenile, SP	99.0 ± 6.1	31.8^{b} ± 0.7	63.4^{b} ± 1.5	5.06^{b} ± 0.27	57.4^{b} ± 1.7
Juvenile, SL	112.0 ± 6.0	33.3^{a} ± 0.8	69.2^{a} ± 1.5	6.52^{a} ± 0.27	65.6^{a} ± 1.7
F-test[2] for diet effect	$F_{2, 34}$ = 1.68	$F_{2, 34}$ = 3.37	$F_{2, 34}$ =4.14	$F_{2, 34}$ = 7.62	$F_{2, 34}$ = 6.20
P-value	P = 0.20	P = 0.046	P = 0.02	P = 0.002	P = 0.005
Adult, PL	2667.1 ± 101.6	105.4 ± 1.8	111.8 ± 2.5	27.70 ± 1.79	180.8 ± 7.8
Adult, SP	2672.2 ± 101.6	104.5 ± 1.8	117.8 ± 2.5	30.50 ± 1.79	195.5 ± 7.8
Adult, SL	2779.5 ± 101.6	103.7 ± 1.8	113.8 ± 2.5	30.84 ± 1.79	192.1 ± 7.8
F-test[2] for diet effect	$F_{2, 21}$ = 0.38	$F_{2, 21}$ = 0.24	$F_{2, 21}$ = 1.46	$F_{2, 21}$ = 0.98	$F_{2, 21}$ = 0.98
P-value	P = 0.69	P = 0.79	P = 0.25	P = 0.41	P = 0.39

[1]Diets described in Table 2

[2]Type III ANOVA on linear models; subscripts for F correspond to degrees of freedom. Values are least square means, and those with different superscripted letters differed significantly in post hoc comparison test.

Table 5. Type III ANOVA comparing dietary effect on intestinal enzyme activity per gram of wet intestine (μmol min^{-1} g^{-1} tissue) in relation to intestinal position in juvenile northern bobwhites. Each diet group was represented by 8–10 individuals. Tests were performed using linear mixed model (lmer) with bird ID as a random effect.

	Maltase			**Sucrase**			**APN**		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Position	2, 48	9.96	0.0002	2, 48	13.51	<0.0001	2, 48	0.26	0.780
Diet	2, 24	10.65	0.0005	2, 24	7.98	0.0021	2, 24	5.52	0.011
Position×diet	4, 48	1.35	0.265	4, 48	2.40	0.0633	4, 48	0.95	0.446

Table 6. Type III ANOVA comparing dietary effect on intestinal enzyme activity per gram of wet intestine (μmol min^{-1} g^{-1} tissue) in relation to intestinal position in chickens. Each diet-age group was represented by $n = 14$ juveniles and $n = 8$ adults. Tests were performed using generalized linear mixed models (glmer) with bird individual specified as a pseudoreplicated random effect.

	Maltase			**Sucrase**			**APN**		
	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*
Age	1	8.09	0.004	1	12.09	0.0005	1	7.27	0.0070
Position	2	12.58	0.0019	2	50.12	<0.0001	2	4.27	0.118
Diet	2	39.98	<0.0001	2	36.93	<0.0001	2	7.89	0.0193
Age×position	2	5.66	0.059	2	10.71	0.004	2	8.40	0.0150
Age×diet	2	0.93	0.625	2	0.91	0.064	2	0.47	0.789
Position×diet	4	36.24	<0.0001	4	76.08	<0.0001	4	1.60	0.810
Age×position×diet	4	10.35	0.035	4	24.35	<0.0001	4	3.28	0.512

Table 7. Overview of predicted and observed patterns of dietary modulation of enzyme activity. More detailed explanation of the specific tests and predictions can be found in sections 1 and 2.2.1.

		Prediction #1	Prediction #2	Prediction #3	Prediction #4
Juv. bobwhites	Specific test	For AGs, S > P	For APN, P > S	For AGs, L < P	For APN, L < S
	Finding at tissue level	No	Yes	Yes	No
	Finding for whole intestine	Maltase – Yes* Sucrase – No	No*	Yes	No
Juv. chickens	Specific test	For AGs, SL > PL	For APN, PL > SL	For AGs, SL < SP	For APN, PL < SP
	Finding at tissue level	Yes	Yes	Yes	No
	Finding for whole intestine	Yes	Yes	No*	No
Ad. chickens	Specific test	For AGs, SL > PL	For APN, PL > SL	For AGs, SL < SP	For APN, PL < SP
	Finding at tissue level	Maltase – Yes Sucrase – No	No	No	No
	Finding for whole intestine	Maltase – Yes Sucrase – No	No	No	No

*This finding, which conflicts with a finding at the tissue level, is possibly confounded by a diet effect on size of the intestine (see Results)

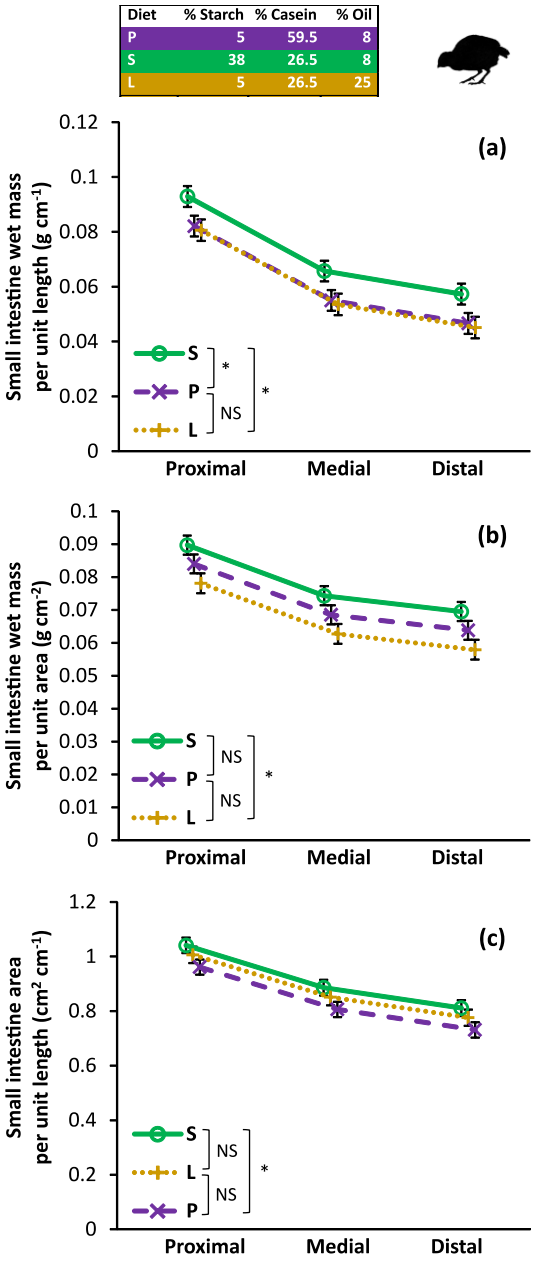

Figure 1. Small intestine morphometrics of juvenile (70–72 d old) northern bobwhites fed experimental diets (see table legend in upper left) 7–9 d. S = high starch; P = high protein; L = high lipid. Sample sizes are $n = 9$ for S, $n = 9$ for P, and $n = 8$ for L diet. Data are least square means ± s.e.m. Significant group-level effects are denoted with an asterisk on the legend (at the lower left within each panel), while NS indicates non-significance.

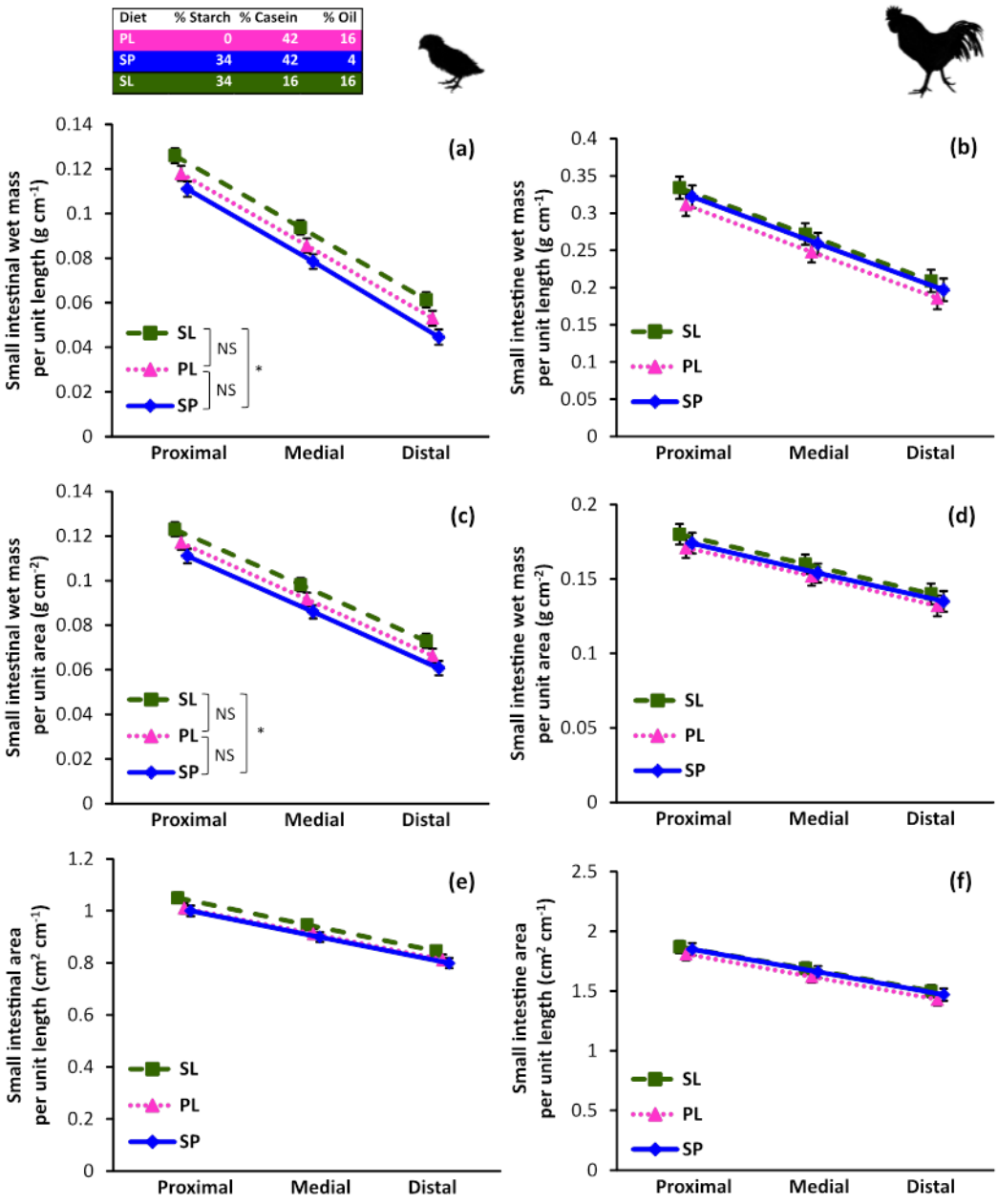

Figure 2. Small intestine morphometrics of juvenile (panels a, c, e; 20 d old; *n* = 14 per diet) and adult (b, d, f; 230 d old; *n* = 8 per diet) chickens that were fed experimental diets (table legend) 15 d. PL = high protein; SP = high starch; SL = high lipid. Data are least square means ± s.e.m. Significant group-level effects are denoted with an asterisk on the legend (within each panel), while NS indicates non-significance.

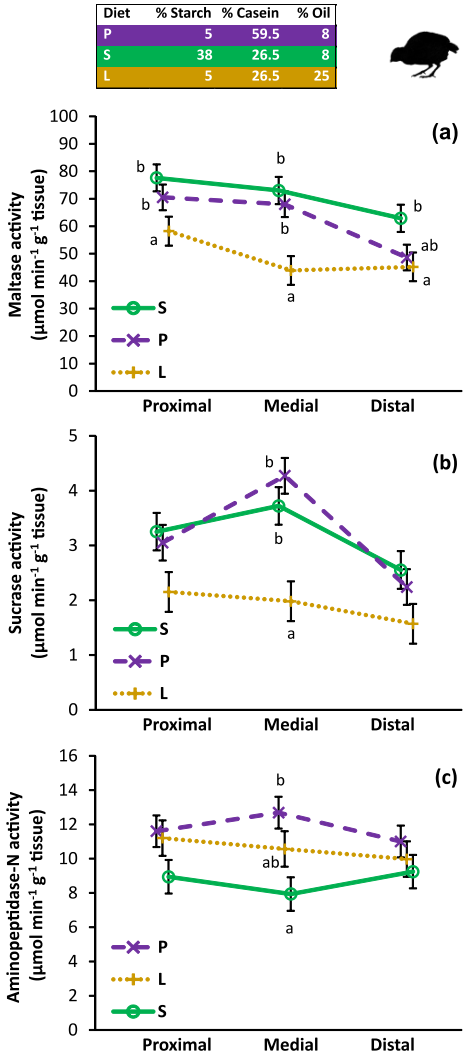

Figure 3. Diet (table legend) effect on digestive enzyme activities in relation to small intestinal position in juvenile (70–72 d old) northern bobwhites fed 7–9 d. S = high starch; P = high protein; L = high lipid. Sample sizes are n = 9 for S, n = 10 for P, and n = 8 for L diet. Data are least square means ± s.e.m. Asterisk indicates significant main effect of diet, and NS indicates non-significance (post hoc Tukey test). Specific predictions tested using post hoc comparisons:

1) induction of α-glucosidases (AGs) by starch: S > P in panels (a, b)

2) induction of aminopeptidase-N (APN) by protein: P > S in (c)

3) suppression of AGs by lipid: L < P in (a, b)

4) suppression of APN by lipid L < S in (c)

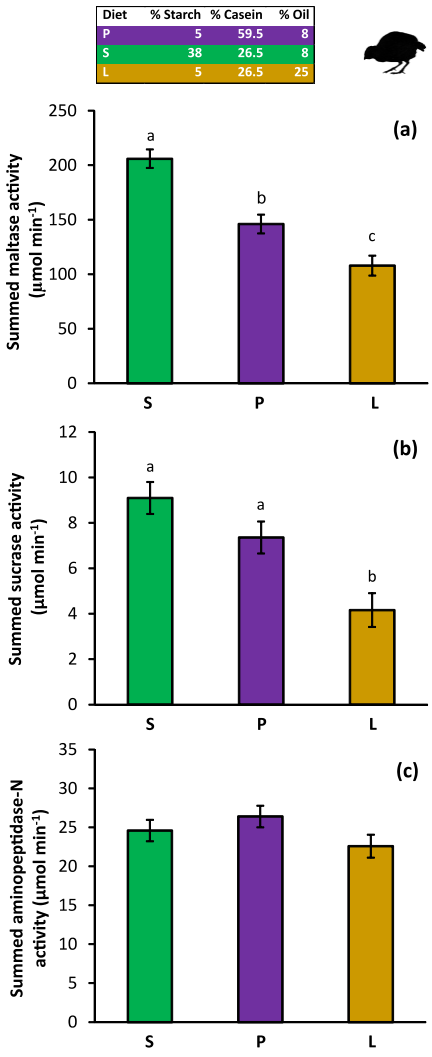

Figure 4. Diet (table legend) effect on summed enzyme activity across the whole small intestine in juvenile (70–72 d old) northern bobwhites fed 7–9 d. S = high-starch; P = high-protein; L = high-lipid. Sample sizes are $n = 9$ for S, $n = 9$ for P, and $n = 8$ for L diet. Data are least square means ± s.e.m. Different letters at the top of each bar indicate significant difference by diet. Specific predictions tested using post hoc comparisons:

1) induction of α-glucosidases (AGs) by starch: S > P in (a, b)

2) induction of aminopeptidase-N (APN) by protein: P > S in (c)

3) suppression of AGs by lipid: L < P in (a, b)

4) suppression of APN by lipid L < S in (c).

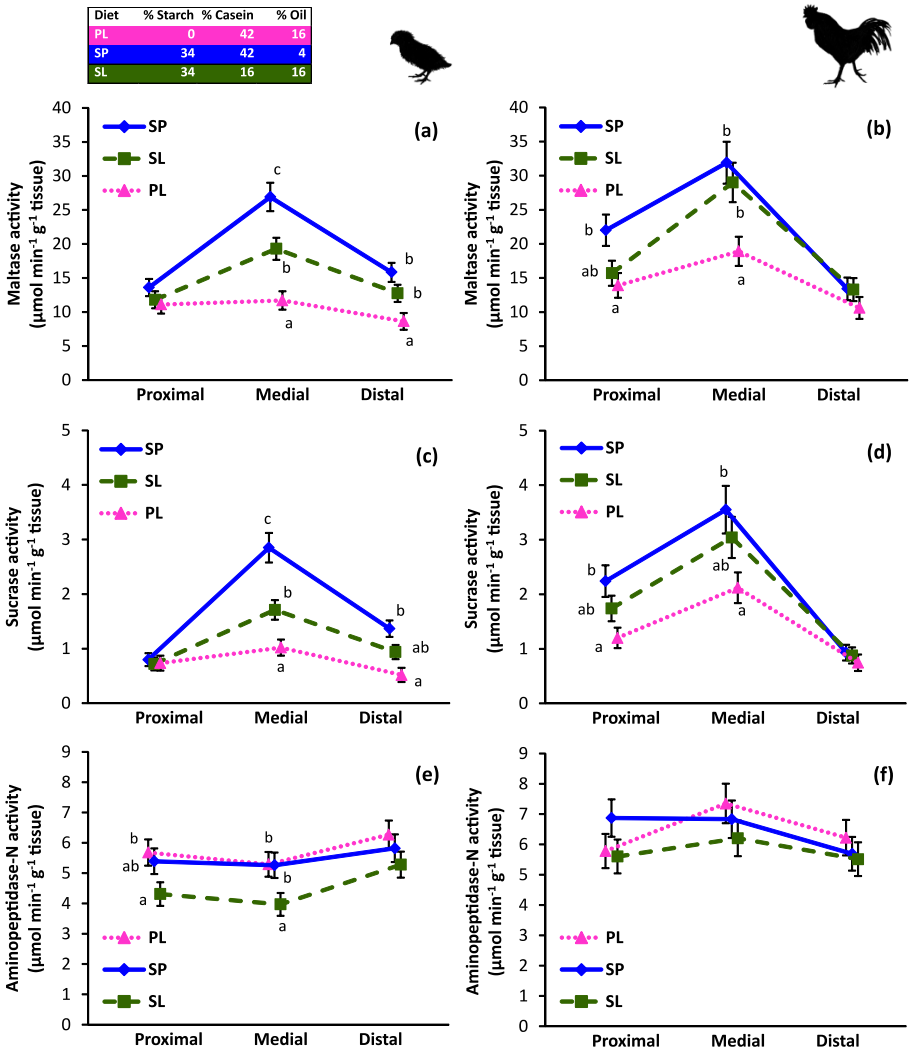

Figure 5. Diet (table legend) effect on digestive enzyme activities in relation to small intestinal position in juvenile (a, c, e; 20 d old; n = 14 per diet) and adult (b, d, f; 230 d old; n = 8 per diet) chickens fed 15 d. PL = high protein; SP = high starch; SL = high lipid. Data are least square means ± s.e.m. Different letters at the means indicate significant differences at that position based on post hoc Tukey test. Specific predictions tested:

1) induction of α-glucosidases (AGs) by starch: SL > PL in (a–d)

2) induction of aminopeptidase-N (APN) by protein: PL > SL in (e, f)

3) suppression of AGs by lipid: SL < SP in (a–d)

4) suppression of APN by lipid PL < SP in (c)

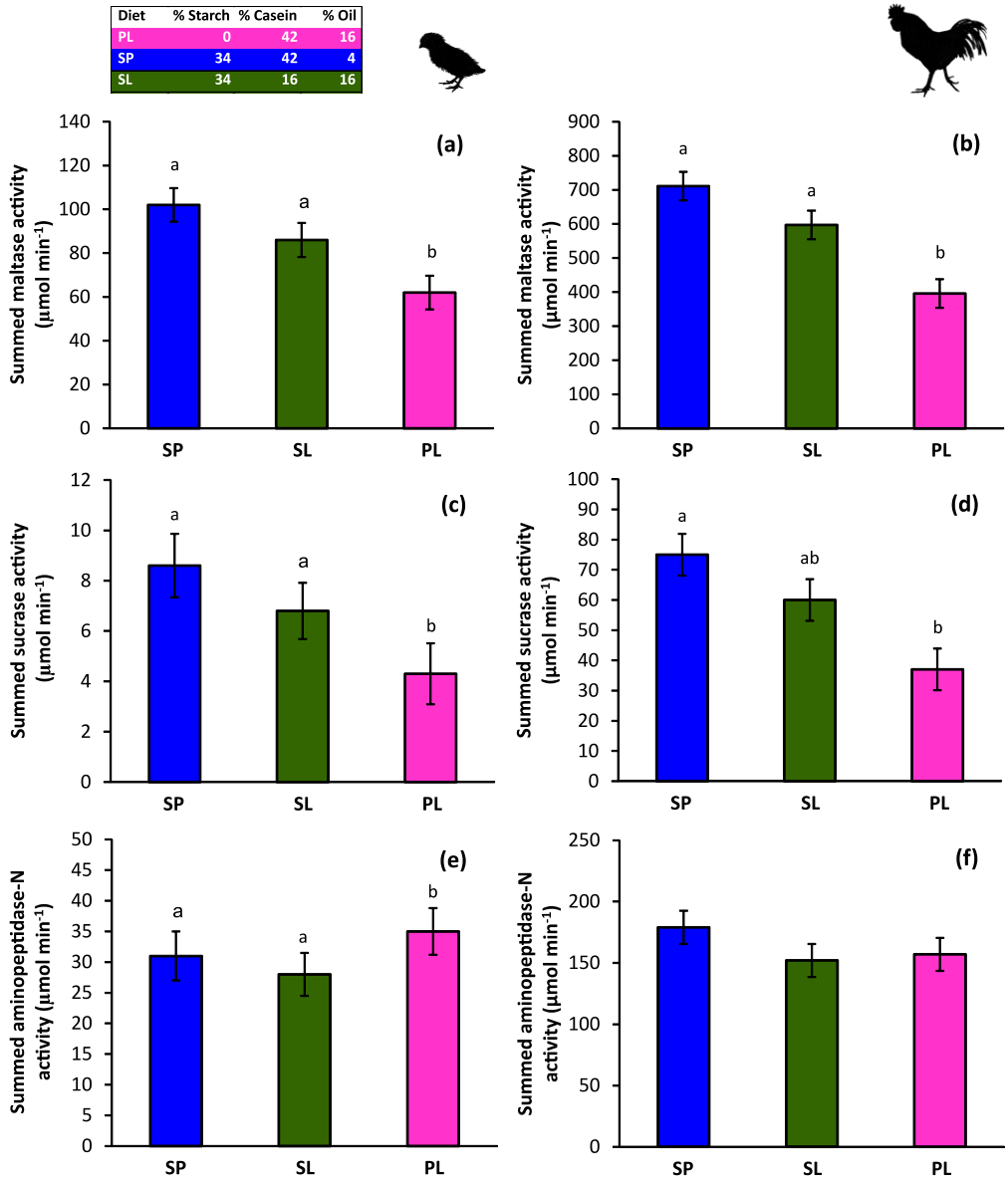

Figure 6. Diet (table legend) effect on summed enzyme activity across the whole small intestine in chicken juvenile (a, c, e; 20 d; *n* = 14 per diet) and adult (b, d, f; 230 d; *n* = 8 per diet) chickens fed 15 d. PL = high protein; SP = high starch; SL = high lipid. Data are least square means ± s.e.m. Different letters at the top of each bar indicate significant difference by diet. Specific predictions tested: 1) induction of α-glucosidases (AGs) by starch: SL > PL in (a –d); 2) induction of aminopeptidase-N (APN) by protein: PL > SL in (e, f); 3) suppression of AGs by lipid: SL < SP in (a–d); and 4) suppression of APN by lipid PL < SP in (c).

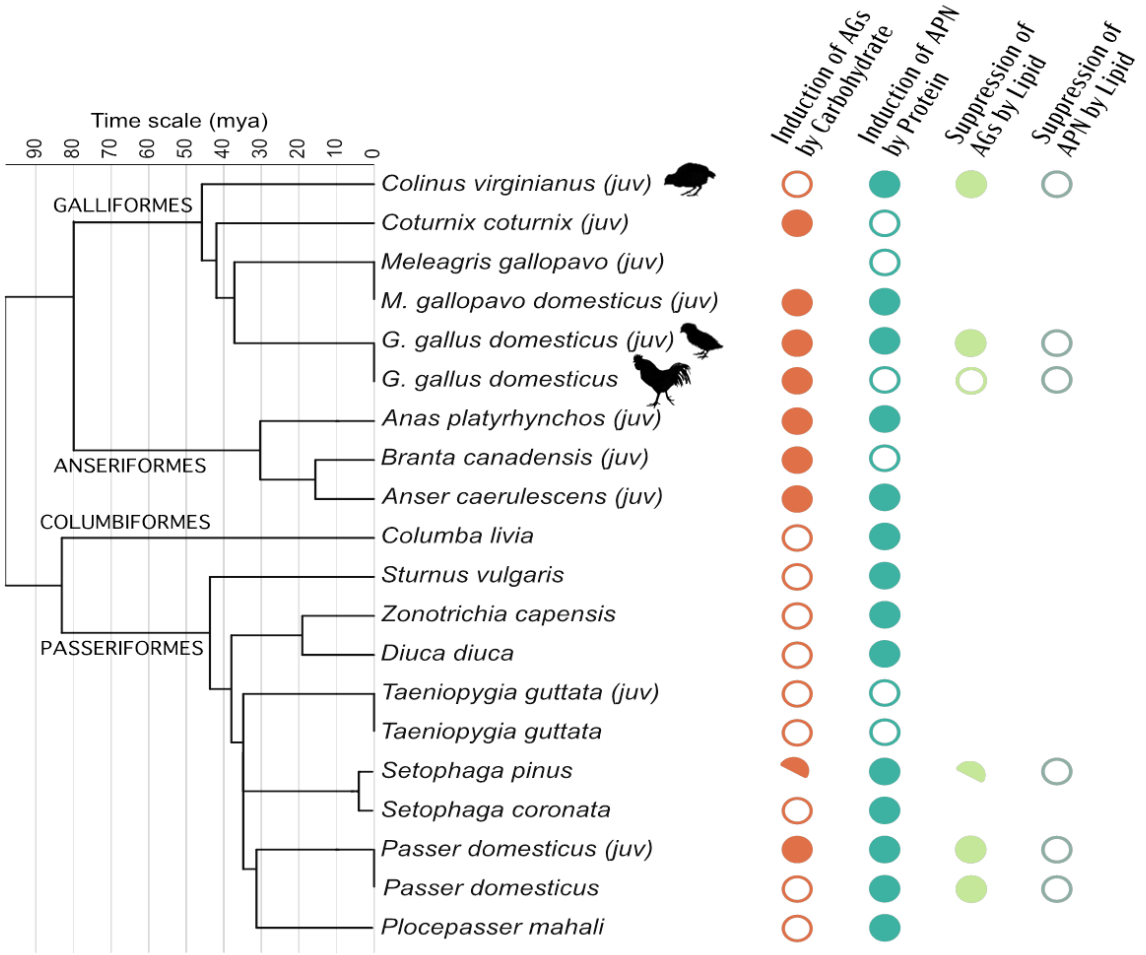

Figure 7. Intestinal enzyme activity response to altered dietary signal mapped onto phylogeny of 16 avian species. Each of the four columns describes a prediction for α-glucosidases (AGs) or aminopeptidase-N (APN). Each row represents the species tested. A circle in a column corresponds to a test that was performed; absence of circle corresponds to no test for that particular prediction for that particular species. A filled circle corresponds to a positive result ("Yes"), and an unfilled circle corresponds to a negative result ("No"). A circle half-filled corresponds to an ambiguous result in a test. Adults were tested unless "(juv)" is beside the species name, in which case juveniles were tested. Pictograms indicate results from the current study.

Alpha-glucosidase and sugar transporter gene expression modulation and coexpression in response to dietary starch and lipid signals in the small intestinal epithelial cells of juvenile and adult chickens

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Abstract

Digestive flexibility allows omnivorous animals to modulate activities of digestive hydrolases and/or nutrient transporters to match the dietary nutrient level, and laboratory rodents show a comodulated unit of α-glucosidase and sugar transported genes that are comodulated by dietary starch signal. However, such comodulation is yet to be fully described in birds, and we also do not know whether the pattern of suppressed α-glucosidase activity by dietary lipid and the decline in its modulation through maturity occurs at the level of transcription. We tested the hypothesis that the modulation mechanisms of small intestinal α-glucosidases and sugar transporters of juveniles and adult chickens by their dietary macronutrient levels are similar to what is known in house sparrows (*Passer domesticus*) and laboratory rodents. We employed an RNA-Seq approach with pre-selected genes of interest and a series of linear modeling to test our *a priori* predictions based on reported modulation mechanism. We found that avian α-glucosidase and sugar transporter gene expressions are comodulated adaptively similarly to in rodents, that these modulations include suppressions by dietary lipid, and that transcriptional-level modulation decreases in magnitude through maturity. Tissue-level maltase-glucoamylase (*MGAM*) transcript abundance was positively correlated with the whole tissue-level maltase activity. However, the relationship between sucrase-isomaltase (*SI*) transcript abundance and sucrase activity varied across dietary groups from positive to negative, primarily affected by high lipid in the diet. Due to this discrepancy in transcription of *SI* gene and SI activity we further explored the possibility of genes involved in post-translational processes of SI activity change (glycosylation) and protein expression onto the cell membrane also being correlated with α-glucosidase activity. We found that SI-glycosylating β-1,4-galactosyltransferase (*B4GALT5*) expression was modulated by diet and was correlated with sucrase activity only on a diet high in

starch and protein. Transcript abundances of α-gustducin (*GNAT3*) and TRPM5, known to be involved in the rapid exocytosis of vesicles carrying sugar transporters through taste reception, were correlated with whole tissue α-glucosidase activity. Our study highlights the impact of high dietary lipid in many of the proposed mechanisms in the adaptive modulation of *α*-glucosidases and modulation features that seem unique to birds or similar to rodent models in birds.

Introduction

Digestive flexibility allows animals to reversibly modulate digestive enzyme and/or nutrient transporter profiles to match the nutrient levels in their diet and hence is likely adaptive for an omnivore (Ferraris and Diamond 1997, Karasov and Hume 1997, Myers and Klasing 1999, Brzęk et al. 2009, Brzęk et al. 2011). This view is generally supported in omnivorous birds (reviewed by (Oguchi et al. 2022)) as well as in wild small mammals (Wang et al. 2019)) and laboratory rodents (Goda and Takase 1994, Honma et al. 2007, Mochizuki et al. 2010a). In some cases, likely underlying molecular mechanisms have been described. For example, in laboratory rodents dietary starch induces intestinal *α*-glucosidase (maltase and sucrase) activities by upmodulation of transcription of *α*-glucosidase genes through transcription factors caudal-related homeobox 2 (CDX2) and hepatocyte nuclear factor 1 (HNF1) binding to the promotor/enhancer regions and through acetylation of nearby H3 and H4 histones (Mochizuki et al. 2010a). A similar increase in transcription of *α*-glucosidase genes and *α*-glucosidase protein expressions on the brush border (apical) membrane of small intestinal enterocytes, leading to increased *α*-glucosidase (maltase and sucrase) activities has been reported in house sparrow (*Passer domesticus*) nestlings fed a high-starch vs. low-starch diet (Gatica-Sosa et al. 2018, Brun et al.

2021) and domestic chickens (*Gallus gallus domesticus*) supplemented with microencapsulated botanicals and organic acids (Toschi et al. 2024).

Based on the hypothesis of economical design (Karasov and Diamond 1988, Karasov and Douglas 2013), one might predict matches achieved not only between dietary nutrient loads and capacities for hydrolysis and absorption, but also matches, or at least proportionalities, between steps along such a pathway so that energy and material involved in the entire pathway is not wasted as excess. Indeed, Sullivan et al. (2021) recently described in mice a transcriptionally comodulated unit of small intestinal α-glucosidases (*MGAM* and *SI*) and sugar transporters (*SLC5A1*, *SLC2A2*, and *SLC2A5*) as the "Carbohydrate Transcriptional Program (CTP)" that responds positively to increased dietary starch content. Limited evidence suggests that domestic turkeys (*Meleagris gallopavo gallopavo*; but not their wild counterparts) (Suvarna et al. 2005) and domestic chickens (Barekatain et al. 2019, Toschi et al. 2024) (but see (Barfull et al. 2002)) modulate in synchrony both α-glucosidase activity and transporter-mediated glucose absorption in the small intestine. A focused study in birds investigating possible coordinated gene expression (constituting a unit akin to the reported rodent CTP) is needed.

Complicating the picture of adaptive modulation of α-glucosidases, however, is the suppressive effect of high dietary lipid, a nonsubstrate, on tissue-level maltase and sucrase activity. This lipid effect has been commonly observed in avian and rodent studies testing for it (Caviedes-Vidal et al. 2000, Honma et al. 2007, Mochizuki et al. 2010a, Mochizuki et al. 2010b, Brzęk et al. 2013, Wang et al. 2019, Oguchi et al. 2022), but its adaptive significance has been unclear (Karasov et al. 2011, Brzęk et al. 2013, Karasov and Douglas 2013). In Oguchi et al. (2022) we proposed the hypothesis that this response may be indeed economical and adaptive. This is because the overall breakdown of dietary substrates are a product of hydrolysis rate and

digesta retention time (Karasov and Douglas 2013), and an increase in dietary lipid suppresses gastric motility and extends overall digesta retention time (Karasov and Hume 1997). That is, lowering hydrolase levels could save brush border membrane space and/or energy and material for enzyme production without compromising the overall enzymatic capacity of the intestine when longer breakdown time is allowed under a high-lipid diet. If α-glucosidase and sugar transporter gene expression levels are suppressed by increased dietary lipid our hypothesis would be supported.

Another curious observation in the only two species of birds (house sparrow: (Caviedes-Vidal et al. 2000, Brzęk et al. 2013) and domestic chicken: (Oguchi et al. 2022)) whose α-glucosidase activities have been measured in both juveniles and adults is that the adaptive induction by dietary starch seems to decrease through maturity. House sparrow adults show complete lack of α-glucosidase induction by starch whereas the suppressive effect of lipid is retained (Caviedes-Vidal et al. 2000), and in chicken adults, a general weakening of both effects are found compared to juveniles (Oguchi et al. 2022). We do not know whether this decrease is reflective of changes in gene expression levels, which would be suggestive of an adaptive decrease in modulation.

In regards to the molecular mechanisms underpinning the change in α-glucosidase and/or sugar transporter activities or gene expressions, the recentSullivan et al. (2021) described the upmodulating the transcription of α-glucosidase and sugar transporter genes (the CTP) through Notch signaling-mediated suppression of interlukin-22. Although the detailed mechanisms are yet to be explored, intestinal epithelial cells exposed to high dietary starch upmodulates the expression of the Jagged-2 (*JAG2*) Notch ligand for the Notch receptor of γδ T lymphocytes in the lamina propria (Sullivan et al. 2021). These activated T cells then suppress interleukin-22

production by innate lymphoid cells, and this suppression of interleukin-22 allows increased transcription of the CTP genes (Sullivan et al. 2021). In birds, however, little is known apart from the finding that in house sparrows gene *SI* (for sucrase-isomaltase) is largely under transcriptional control (Gatica-Sosa et al. 2018, Brun et al. 2021). Several other pathways have been identified in laboratory rodents in addition to the aforementioned involvement of transcription factors and histone acetylation (Mochizuki et al. 2010a). One, possibly specific to sucrase-isomaltase, is the post-translational glycosylation by β-1,4-galactosyltransferases of SI that increases its transport onto the brush border membrane (Mochizuki et al. 2010b). Another is a rapid pathway where mature SLC2A2 (GLUT2, facilitated sugar transporter) proteins are expressed onto the brush border membrane through the exocytosis of vesicles containing SLC2A2 (Kellett et al. 2008). This mechanism is initiated by the activation of G-protein coupled sweet receptors (T1R2/T1R3) activating α-gustducin (G-protein), which in turn activates a kinase cascade responsible for vesicle exocytosis (Kellett et al. 2008). Concordantly, sodium-dependent glucose transporter SLC5A1 (SGLT1) symports Na^+ and glucose into the cell, causing enterocyte membrane to depolarize, which triggers voltage-gated Ca^{2+} ion channels to open, which then activates TRPM5 (non-specific cation channel) to cause further depolarization, leading to exocytosis of vesicles with SLC2A2 (Sprous and Palmer 2010).

In the present study on we tested the hypothesis that the modulation mechanisms of small intestinal α-glucosidases and sugar transporters of juveniles and adult chickens are similar to what is known in house sparrows and rodents. We employed RNA-Seq, pre-selected genes of interest, and a series of linear modeling to test our *a priori* predictions that 1) avian α-glucosidase and sugar transporter gene expressions are comodulated adaptively akin to rodent CTP by dietary signals, 2) these modulations include suppressions by dietary lipid, 3)

transcriptional-level modulation decreases in magnitude through maturity, 4) Notch ligand JAG2 expression increases for Notch-mediated suppression of interleukin-22 (involved in upmodulating the putative CTP genes) in birds similarly to what is known in rodents (Sullivan et al. 2021), and 5) tissue-level α-glucosidase activities are largely under transcriptional control. As SI expression produced unexpected results under prediction 4, we additionally explored two post translational processes. One was whether the transcription of SI-glycosylating enzyme (β-1,4-galactosyltransferase) is correlated with SI and sucrase activity, and the other was whether the expression of gustducin and TRPM5 may be correlated with α-glucosidase activity (surrogate for the putative CTP proteins expressed on the brush border membrane). The individuals we studied are a subset of juvenile and adult chickens whose tissue-level maltase and sucrase activities are well-characterized (Oguchi et al. 2022). From our findings a picture is emerging that the effect of high dietary lipid has an effect on many putative modulatory mechanisms of α-glucosidases and that the "avian solution" of adjusting to varied diets may have unique features yet may share similarities to rodents with respect to comodulation and putative mechanisms for gene and protein expressions.

Methods

Ethics statement

All animal work was approved by the University of Wisconsin (UW)–Madison Animal Care and Use Committee under Protocol #A005855-A2, and the care of animals was overseen by the UW–Madison Research Animal Resources Center.

Domestic chicken diets

Experimental diets were as described in our previous work on digestive hydrolase activities (Oguchi et al. 2022). Briefly, two of the three diets were designed to be similarly higher in two of the three macronutrients than one other diet: PL (high in protein and lipid), SP (high in starch and protein), and SL (high in starch and lipid; Table 1). This design was chosen to maximize our ability to test predictions 1 and 2. We designed the PL diet to be starch-free in order to maximize our ability to test prediction 1, knowing that domestic chickens can thrive on such a diet (Biviano et al. 1993).

Domestic chickens and harvest of intestinal tissue

One-day old domestic chickens (Barred Plymouth Rock from Meyer Hatchery, Polk , OH were raised until age 20 d (juveniles) or 230 d (adults), with their last 15 d of their life on 100% experimental diet (ad libitum food and water; see (Oguchi et al. 2022) for detail on rearing conditions).

Birds were euthanized via CO_2 asphyxiation. The gut below the gizzard was immediately dissected out and placed in ice-cold phosphate buttered saline (PBS; Sigma #P5368) (Iji et al. 2001), and the small intestine was divided into thirds of approximately equal length, proximal, medial, and distal. Intestinal sections were cleaned with PBS, blotted dry, weighed (± 0.0001 g), opened longitudinally, and measured for length (± 0.1 cm) and largest and smallest width (± 0.01 mm). RNA sample was collected from the center of each section and stored in RNAlater (Invetrogen #AM7021; quenched for 24 h at 4 °C prior to storing at -80 °C). The remaining intestine was cut longitudinally in half, and one of those halves was processed for enterocyte isolation (Mac Donal et al. 2008, Brun et al. 2020a) from each gut region. The other half was flash frozen and stored at -80 °C for later measurement of intestinal enzyme activity in tissue

homogenates (as reported in (Oguchi et al. 2022)). We used only the RNA sample from the medial section (jejunum) in the RNA-Seq analysis after learning that the modulation of α-glucosidases occurs most prominently in the medial section (Oguchi et al. 2022).

Samples and preparation

The transcriptomes of $n = 5–6$ individuals per diet per age group were sequenced. These individuals are a subset of birds used in (Oguchi et al. 2022) that yielded, following the preparation procedure, the highest RNA quality (see RNA integrity, below). RNAlater-stored (-80 °C after quenching) whole intestinal samples from the center of the medial intestine were used as the starting material. The center of the small intestine would be most likely to yield diet group differences in digestive α-glucosidases and nutrient transporters because key α-glucosidase activities showed that the largest response to diet occurred in the medial intestine (Oguchi et al. 2022).

In order to isolate intestinal epithelial cells (enrich enterocytes) we used a chelation method of Mac Donal et al. (2008) modified following Belluoccio et al. (2013). Specifically, the chelating reagent was made by dissolving solid EDTA (10% final concentration) in RNAlater titrated to pH = 9.2 (with NaOH to help dissolving EDTA). This EDTA-RNAlater solution was then re-titrated to pH = 5.2 using HCl (Belluoccio et al. 2013). Samples were exposed to 1 h of this chelating reagent on ice (4 °C), and cells were isolated by vigorous shaking for 1 min at the end. The intestinal muscle and serosal tissue was removed and cells were pelleted by 500 $\times g$ centrifugation, and then cleaned by re-suspending them in PBS and washing them twice with 500 $\times g$ centrifugation.

Isolation and purification of RNA from the epithelial cells were performed using PureLink RNA Mini Kit (Invetrogen #12183018A) as per the manual method of the manufacturer's protocol. RNA integrity was checked using Agilent RNA6000 (RNA integrity number range: 7.5–9.5 in sequenced samples), cDNA library was prepared for Illimina TruSeq Stranded mRNA (poly-A enrichment), and high-throughput RNA sequencing (2×151 bp) was performed using the Illumina NovSeq 6000 platform at the University of Wisconsin–Madison Biotechnology Center.

Quality control and read mapping

We followed the overall approach described by (Sandholt et al. 2021). We used the graphical user interface platform of Galaxy (usegalay.org) to perform RNA-Seq data read mapping. Quality of raw reads was checked with FastQC Galaxy ver. 0.74, and paired-end reads were trimmed for Illumina Universal Adapters (TruSeq3-PE) using TrimGalore Galaxy ver. 0.6.7 (github.com/FelixKrueger/ TrimGalore) with default settings (Magallanes-Alba et al. 2023). After checking quality with FastQC again, trimmed reads were read mapped to the broiler chicken (*G. gallus*) reference genome (GCF_016699485.2_bGalGal1.mat.broiler.GRCg7b) using HISAT2 Galaxy ver. 2.2.1 (Kim et al. 2019, Perlas et al. 2021, Magallanes-Alba et al. 2023) with the assistance by the chicken gene transfer format (GTF) file. One adult of diet group PL failed at read mapping due to very high sequence duplication level (30% of sequences having >10k sequence duplication) and was removed from the study. We accepted the default setting for most options (Park et al. 2017, Perlas et al. 2021), but specified the strandedness information of the reads as reverse for Illumina (--rna-strandness --RF). Mapped reads were annotated and counted with featureCounts Galaxy ver. 2.0.3 (Liao et al. 2014, Magallanes-Alba et al. 2023) using the

GRCg7b chicken general feature format (GFF) file. Outputs of featreCounts from individuals were compiled using Multi-Join (Galaxy ver. 9.3).

Filtering and normalization

From this point forward all analyses were performed on R ver. 4.4.1 (R Core Team 2024) through RStudio ver. 2024.12.0+467. We used limma ver. 3.60.3 (Ritchie et al. 2015) to filter, normalize, and transform the raw gene read counts. A group vector with diet and age combined into a single factor (Diet.Age) was assigned to the dataset using function DGEList. Then, function filterByExpr was used to filter out lowly-expressed genes. Raw read counts were then normalized across samples based on the library size of individuals via trimmed mean of M-values (TMM; function normLibSizes) to express transcript abundance of each gene as counts per million reads (CPM). We finally applied voom transformation using function voomWithQualityWeights to express relative transcript abundance as $\log_2$CPM.

Genes of interest

We selected from the literature a consortium of intestinal α-glucosidases and sugar transporters that are functionally linked, other proteins that have been reported or proposed to contribute to sugar absorption, and proteins reported to play a part in the modulation mechanism of α-glucosidases and sugar transporters in rodents (Table 2). We focused our *a priori* expectations regarding these genes and tested these predictions through a series of linear models (see below).

Our preliminary analysis of differential expression using all transcripts was unable to detect most genes of interest as significant (see Supplementary Information). This was likely

owing to the subtle differences in gene expression among diet groups, which would correspond to only 20–50% difference in the protein (digestive enzyme) activity between them.

Statistical analyses overview

In our statistical tests results are reported two-tailed with critical $\alpha = 0.05$ unless specified otherwise. Model reduction (removal of non-significant covariates) was first performed using R package lmtest ver 0.9.40 function stepAIC followed by sequential removal based on *P*-values. Graphing relied on R package ggplot2 ver. 3.5.1 (Wickham 2016) and 3D plots on scatterplot3d ver. 0.3.44 (Ligges and Mächler 2003). Model assumptions were confirmed to have been met by residual and quantile-quantile plots.

Testing for the diet effect and coexpression of α-glucosidases and sugar transporters

Diet effect on α-glucosidase activities and transcript abundance of α-glucosidases and sugar transporters

Our earlier study (Oguchi et al. 2022) using a larger sample size ($n = 14$ juveniles and $n = 8$ adults per diet group) showed that maltase activity was induced at the whole tissue level due to dietary starch (Table 1; SL > PL) in both juveniles and adults and suppressed by dietary lipid (SL < SP) only in juveniles. Similarly, sucrase activity was induced by dietary starch (SL > PL) and suppressed by lipid (SL < SP) in juveniles, and although neither effect reached significance in adults the combined effect was (SP > SL) (Oguchi et al. 2022). We first tested whether these differences were detectable in the subset of individuals used in this study. We then tested whether these same patterns occurred at the level of gene expression of α-glucosidases and sugar transporters.

Maltase sucrase activities and transcript abundance of *MGAM, SI, SLC5A1* (SGLT1: sodium-glucose transporter 1), *SLC2A2* (GLUT2: glucose transporter 2), and *SLC2A5* (GLUT5: glucose transporter 5) were each predicted using generalized linear model (GLM) with gamma error structure for maltase and sucrase activity and using simple linear model (LM) for transcript abundance, both using R package MASS ver. 7.3.61 (Venables and Ripley 2013). Age, diet, and their interaction were specified as the predictors. The interaction between age and diet was expected based on the findings of enzymatic activity (Oguchi et al. 2022); thus, we performed no model reduction. Likelihood ratio χ^2 test results from type III analysis of deviance ("ANOVA") of the final model are reported for maltase and sucrase activity and F test results from type III ANOVA for transcript abundance. Diet group comparisons in each age group were done using R package lsmeans ver. 2.30.0 as a *post hoc* Tukey test (Lenth 2016) to test our *a priori* predictions of the inductive effect of starch (SL > PL) and suppressive effect of lipid (SL < SP) as observed in α-glucosidase activities (Oguchi et al. 2022).

Coexpression of α-glucosidases and sugar transporters

Because *MGAM* transcript abundance matched closely with the maltase activity of homogenates in relation to diet and age and correlated well with the maltase activity across diets (i.e., better than *SI* did with sucrase activity), we chose to use *MGAM* as the predictor for identifying concordantly expressed genes that may constitute a putative CTP (sensu Sullivan et al. 2021). Abundance of *SI*, *SLC5A1*, *SLC2A2*, and *SLC2A5* were each fitted with an LM with the main predictor of *MGAM*. The exploratory full model had covariates Age and Diet to be sequentially reduced ($P > 0.05$) toward a minimal model only containing *MGAM*. Lastly, we predicted *SLC2A5* with *SI* using the same method above because *SLC2A5* is a specialized fructose

transporter and *SI* is the only brush border α-glucosidase that produces fructose (due to hydrolysis of sucrase into glucose and fructose) (Karasov and Douglas 2013). Type III ANOVA of the final model and the significance of the slope of *MGAM* (or *SI*) are reported.

Testing for the modulation of the Notch ligand JAG2 transcriptions

We tested whether the signaling mechanism for upmodulating the gene expression of the recently described mouse CTP (Sullivan et al. 2021) was also at work in chickens. In this pathway intestinal epithelial cells under a high starch diet increase the expression of jagged canonical Notch ligand 2 (*JAG2*), which signals to γδ T cells through contact (Notch signaling) (Sullivan et al. 2021). The Notch-signaled γδ T cells then suppress the production of interleukin-22, which has a suppressive effect on the CTP) in other cells (Sullivan et al. 2021). We acknowledge that our ability to fully test this mechanism is limited because our samples (enriched epithelial cells) would have depleted leucocytes that reside in the lamina propria. However, we expected *JAG2* expression to be upmodulated in chickens with the pattern of SP ≥ SL > PL with comodulation between *JAG2* and *MGAM*, as was found in rodents (Sullivan et al. 2021). We tested these predictions by modeling *JAG2* with age * diet being the predictor, and then with *MGAM* (with age * diet as possible covariates) using LM.

Correlation between transcript abundance and corresponding enzymatic activity

We tested whether changes in the transcript abundance of *MGAM*, and *SI* predicted their respective changes in digestive activity (maltase and sucrase) levels measured in whole tissue homogenates (μmol min^{-1} g^{-1} of wet tissue). The gene *MGAM* codes for the avian-derived α-glucosidase (analogous to the mammalian maltase glucoamylase) which is the main contributor

of intestinal maltase activity (Brun et al. 2020b). The gene *SI* codes for the sucrase-isomaltase which is the main contributor of intestinal sucrase activity (Brun et al. 2020b). The α-glucosidase activities (μmol min^{-1} g^{-1} tissue) of the individuals were obtained as part of our earlier study (Oguchi et al. 2022). We fitted gamma GLM with identity link to predict maltase and sucrase activity by their respective transcript abundance (*MGAM* and *SI*). For the correlation between maltase activity and *MGAM*, age and the interaction between age and transcript abundance were also included as potential covariates because adults had significantly higher α-glucosidase activities (Oguchi et al. 2022). For sucrase activity and *SI* it was apparent that diet had a mediating effect on this relationship; thus, diet and the interaction of diet × transcript abundance were also included in the initial model. Both models were reduced (with $P > 0.05$) toward a minimal model with only transcript abundance. The slope of *MGAM* or *SI* was tested by a *t*-test and the significance of *MGAM* or *SI* in the predictive model was tested by Likelihood ratio χ^2 test results from type III analysis of deviance. We additionally performed the same modeling on maltase and sucrase activities normalized to protein content (μmol min^{-1} g^{-1} of protein) to test whether the age-related change are attributable to intestinal morphological change.

Transcription modulation of β-1,4-galactosyltransferase V (B4GALT5)

β-1,4-galactosyltransferases of the Golgi apparatus are known in rats to increase the levels of unsialylated galactose on the glycosylated chain of SI to enhance its transport onto the brush border membrane (Mochizuki et al. 2010b). It has been reported that this level of glycosylation of SI was reduced when feeding rats a high fat/carbohydrate ratio diet, possibly due to the suppressive effect of lipid on β-1,4-galactosyltransferases (Mochizuki et al. 2010b). We tested the inductive effect of starch and the suppressive effect of lipid on the transcription of β-1,4-

galactosyltransferase V (*B4GALT5*), which shows high activities in the small intestine (Furukawa and Sato 1999). We modeled *B4GALT5* with age * diet as predictors, and then tested the correlation between *B4GALT5* and SI (with age, diet, and their two-way interactions with *SI* as possible covariates) as the predictor using LM. We then tested the correlation between sucrase activity and *B4GALT5* by predictors and covariates of Age + Diet + SI + B4GALT5 + Diet:SI + Age:B4GALT5 + Diet:B4GALT5 + SI:B4GALT5 as the initial model with sequential model reduction.

Transcription modulation of the sweet receptor-mediated and TRPM5-mediated pathways for protein expression

A sweet receptor-mediated upmodulation of the exocytosis of vesicle with sugar transporters (SLC2A2, and hence their expression on the brush border membrane surface) has been described in mammals. Sweet receptor (T1R2/T1R3) of the enterocyte activates the G-protein subunit α-gustducin (*GNAT3*), which in turn activates a kinase cascade responsible for exocytosis of vesicles carrying sugar transporters (SLC2A2) (Kellett et al. 2008, Sprous and Palmer 2010, Shirazi-Beechey et al. 2011). Birds, however, lack the specialized sweet taste receptor, and instead, the umami receptor (T1R1/T1R3) is responsible for sweet sensing (Roura et al. 2013, Baldwin et al. 2014). Nevertheless, α-gustducin activation occurs in chickens sensing sweetness, so we tested for the evidence of *GNAT3* modulation. We modeled *GNAT3* with age * diet being the predictor, and then with *MGAM* (with age, diet, and their two-way interactions with *MGAM* as possible covariates) as the predictor using LM. Finally, given that α-gustducin is involved in signal transduction for the vesicle exocytosis of some members of the putative CTP (SLC2A2), we tested the correlation between maltase activity and *GNAT3* by predictors and covariates of +

Age + Diet + GNAT3 + MGAM + Age:GNAT3 + Diet:GNAT3, as the initial model with sequential model reduction.

A parallel pathway leading to the exocytosis of vesicles carrying sugar transporters (SLC2A2) involves depolarization of the enterocyte cell membrane assisted by TRPM5 (transient receptor potential cation channel subfamily M member 5), a nonspecific cation channel on the brush border membrane (Kellett et al. 2008, Sprous and Palmer 2010). In enterocytes, this Ca^{2+}-activated cation channel is activated following depolarization of the membrane due to the influx of Na^{+} via SLC5A1 (SGLT1) and subsequent Ca^{2+} influx via voltage-gated Ca^{2+} channels (Sprous and Palmer 2010). We modeled *TRPM5* using the same methods described for *GNAT3*.

We are aware that a similar mechanism in the specialized enteroendocrine (taste receptor) cells of the intestinal epithelium in rodents has been reported to secrete paracrine hormones GLP1 (glucagon-like peptide 1) and GIP (gastric inhibitory polypeptide) to target enterocytes, upmodulating SLC5A1 and SLC2A2 expression on the brush border (Egan and Margolskee 2008). However, we did not test this mechanism further because the genes of the receptors for these hormones (*GLP1R* and *GIPR*) had 0 reads in most individuals.

Results

SP>SL>PL in α-glucosidase activities and transcripts of α-glucosidases and sugar transporters

Wet mass-specific small intestinal maltase activity of individuals included in this study varied by diet ($\chi^2 = 14.86$, $df = 2$, $P = 0.0005$); age was marginally nonsignificant ($\chi^2 = 3.51$, $df = 1$, $P = 0.06$), and the interaction between age and diet was also nonsignificant ($\chi^2 = 0.47$, $df = 2$, $P = 0.79$). Sucrase activity also varied by diet ($\chi^2 = 37.03$, $df = 2$, $P < 0.0001$); age was a significant predictor ($\chi^2 = 7.76$, $df = 1$, $P = 0.005$), whereas age × diet was not ($\chi^2 = 2.09$, $df = 2$, $P = 0.35$).

One-tailed post hoc Tukey tests failed to show maltase induction by starch (SL > PL) or suppression by lipid (SL < SP) in juveniles unlike with the larger sample size of (Oguchi et al. 2022), although the combined effect of SP > PL was significant (Figure S5A). We also failed to detect SL > PL in the adults unlike in (Oguchi et al. 2022), but SP > PL was similarly detected (Figure S5B). We also failed to demonstrate SL > PL in sucrase activity of juveniles unlike in (Oguchi et al. 2022), but their lipid effect (SL < SP) was apparent (Figure S5C). Only adult sucrase activity showed the combined effect of induction by starch and suppression by lipid (SP > PL; Figure S5D) similarly to (Oguchi et al. 2022).

As expected, we found that α-glucosidases *MGAM* and *SI* and transporters *SLC5A1* and *SLC2A2* varied by diet, and those effects depended on the interaction between age and diet in *MGAM* and *SLC2A2* (Table 3). Contrary to our expectations, we did not find these effects in *SLC2A5* (Table 3).

We expected evidence for the induction by starch (SL > PL) in one-tailed post hoc Tukey tests. We found this pattern in juvenile *MGAM* (Figure 1A), juvenile and adult *SLC5A1* (Figure 1E, 1F), and juvenile *SLC2A2* (Figure 1G). No other comparison group showed SL > PL (Figure 2).

We expected evidence for the suppression by lipid (SL < SP) in one-tailed post hoc Tukey tests. We found this pattern in juvenile *MGAM* (Figure 1A), juvenile *SI* (Figure 1C), juvenile *SLC5A1* (Figure 1E), and juvenile *SLC2A2* (Figure 1G). No other comparison group showed SL < SP (Figure 1). Additionally, the combined effect of induction by starch and suppression by lipid (SP > PL) was found to be significant in adult *SLC2A2* (Figure 1H).

Genes comodulated as a putative carbohydrate transcriptional program

As expected, a comodulated unit of α-glucosidases and sugar transporters (putative CTP) was observed. *MGAM* expression was positively correlated with the expressions of *SI* ($\chi^2 = 41.54$, $df = 1$, $P < 0.0001$; slope: $t = 6.10$, $P < 0.0001$; Figure 2A), *SLC5A1* ($\chi^2 = 53.40$, $df = 1$, $P < 0.0001$; slope: $t = 6.86$, $P < 0.0001$; Figure 2B), *SLC2A2* ($\chi^2 = 9.91$, $df = 1$, $P = 0.002$; slope: $t = 3.57$, $P = 0.001$; Figure 2C), and *SLC2A5* ($\chi^2 = 5.98$, $df = 1$, $P = 0.014$; slope: $t = 2.07$, $P = 0.047$; Figure 2D). Similarly, the specialized fructose transporter *SLC2A5* was positively correlated with fructose-producing *SI* ($\chi^2 = 25.15$, $df = 1$, $P < 0.0001$; slope: $t = 4.56$, $P < 0.0001$; Figure 2E). Additionally, diet had an effect on the relationship between *SLC2A2* and *MGAM* ($\chi^2 = 6.84$, $df = 1$, $P = 0.03$; Figure 2C).

Modulation of JAG2 Notch ligand

JAG2 was not affected by age, diet, or their interaction (Table 3, Figure 3A and 3B). The response of *MGAM* on *JAG2* was diet dependent ($F_{2, 29} = 3.50$, $P = 0.04$), with the main effect of JAG2 nonsignificant ($F_{2, 29} = 0.08$, $P = 0.78$) with the main effect of diet significant ($F_{2, 29} = 8.49$, $P = 0.001$). Generalized linear hypothesis tests revealed that the slope was only positive under diet SP and was nonsignificant under diets SL and PL (Figure 3C).

Correlations between digestive α-glucosidase transcript abundance and activity

As predicted, we found that transcript abundances of *MGAM* correlated positively with maltase activity in whole intestinal tissue homogenates of the same individuals ($\chi^2 = 11.28$, $P = 0.0007$; Figure 4A) with age being a significant covariate ($\chi^2 = 12.26$, $P = 0.0004$; lower activities in juveniles than in adults). Normalized for protein mass of the small intestinal homogenates as the

measure of maltase activity the only significant effect was *MGAM* itself ($\chi^2 = 7.17$, $P = 0.007$; Figure 4B).

Contrary to our prediction, however, the relationship between *SI* and tissue-level sucrase activity varied among diet groups (diet × SI interaction: $\chi^2 = 13.65$, $P = 0.001$). Additionally, the main effects of *SI* ($\chi^2 = 12.42$, $P = 0.0004$), age ($\chi^2 = 30.84$, $P < 0.0001$) and diet ($\chi^2 = 11.43$, $P = 0.003$) were also significant. Generalized linear hypothesis tests revealed that the slope was only positive under diet SP, nonsignificant under diets SL, and negative under diet PL (Figure 4C). Normalized for protein mass of the small intestinal homogenates as the measure of sucrase activity, we again found significant diet × SI interaction ($\chi^2 = 10.43$, $P = 0.005$), the main effect of SI ($\chi^2 = 5.00$, $P = 0.03$), and the main effect of diet ($\chi^2 = 8.99$, $P = 0.01$), with the slopes varied by diet similarly to the findings of the wet mass-specific sucrase activities (generalized linear hypothesis tests; Figure 4D).

Transcription modulation of β-1,4-galactosyltransferase (B4GALT5)

We found significant age, diet, and age × diet interaction effects on *B4GALT5* (Table 3). *Post hoc* analyses showed SL had lower *B4GALT5* transcript levels compared to PL or SL in juveniles (Figure 5A) but not in adults (Figure 5B).

SI transcript abundance was positively correlated with *B4GALT5* transcript abundance ($F_{1, 30} = 8.85$, $P = 0.006$), with age ($F_{1, 30} = 18.88$, $P = 0.0001$) and diet ($F_{2, 30} = 6.36$, $P = 0.005$) as significant covariates (Figure 5C). Post hoc analyses showed that SL < PL = SP in *B4GALT5* across all levels of *SI* (Figure 5C).

Predicting tissue-level sucrase activity with *B4GALT5* and *SI*, we found that *SI* was nonsiginificant in the presence of *B4GALT5* in the model and thus was removed. In the final

model, we found significant diet × B4GALT5 interaction (χ^2 = 7.25, P = 0.03) with no significant main effect (Figure 5D). Generalized linear hypothesis tests revealed that the slope is positive for SP only and nonsignificant for SL and PL.

Transcription modulation of GNAT3-mediated and TRPM5-mediated pathways for the expression of α-glucosidases

GNAT3 was not modulated by diet, nor was age × diet significant, and age was the only important predictor of *GNAT3* (higher in adults: t = 2.39, P = 0.02; Table 3). *GNAT3* was not correlated with *MGAM* either (χ^2 = 0.52, df = 1, P = 0.47; slope: t = -0.74, P = 0.47), with diet (χ^2 = 32.53, df = 2, P < 0.0001) remaining as the only important predictor in the correlation model.

On the other hand, when predicting tissue-level maltase activity with both *MGAM* and *GNAT3* in the same model, *MGAM* was dropped during model reduction and only *GNAT3* (χ^2 = 12.68, df = 1, P = 0.0003) and diet (χ^2 = 25.80, df = 2, P < 0.0001) remained as significant in the final model (Figure 6A).

TRPM5 was not directly related to diet, age or their interaction (Table 3). *TRPM5* was not correlated significantly with *MGAM* either (χ^2 = 0.18, df = 1, P = 0.67; slope: t = -0.42, P = 0.68), and diet (χ^2 = 33.73, df = 2, P < 0.0001) was the only term remaining in the correlation model.

Similarly to what we found in *GNAT3*, maltase activity was positively correlated with *TRPM5* (χ^2 = 9.05, df = 1, P = 0.002) with diet being a significant covariate (χ^2 = 12.80, df = 1, P = 0.002), and *MGAM* was removed as nonsiginificant from this model during the reduction process (Figure 6B).

Discussion

We tested the hypothesis that juveniles and adult chickens modulate the expression of α-glucosidase and sugar transporter genes according to their dietary macronutrient signals similarly to what is known in house sparrows and laboratory rodents by evaluating a series of *a priori* predictions. As expected, in most regression analyses we found the starch-free PL group to cluster in the lower left quadrant and the group SP (high in starch and low in lipid) cluster in the upper right quadrant, suggesting that increased starch and decreased lipid in the diet have a positive effect on the level of expressions of genes involved in carbohydrate digestion and absorption. We discuss each major finding in relation to these predictions.

A comodulated unit of α-glucosidases and sugar transporters responded to dietary signals of starch and lipid in chickens

We found evidence of transcriptional control of *MGAM* and *SI* by diet starch and lipid signals. Our earlier study (Oguchi et al. 2022) on the whole-tissue level α-glucosidase activities using a larger sample size (n = 14 juveniles and n = 8 adults per diet group) showed clear evidence of maltase and sucrase activity being induced by starch (PL < SL) in juveniles and maltase in adults and both being suppressed by lipid in juveniles (SL < SP). In the subset of samples (n = 6 juveniles and n = 4–7 adults per diet) used for this RNA-Seq study, we were only able to observe significant suppression by lipid in juvenile sucrase, although the combined trend (PL < SP) was always significant (Figure S5). The transcript abundance of *MGAM* and *SI* showed a consistent pattern to this (PL < SP) in all comparison groups (Figure 2A–D), and starch induction of *MGAM* and lipid suppression of *MGAM* and *SI* were apparent in juveniles (Figure 2A, B). These findings suggest that starch and lipid signals affect the small intestinal α-glucosidase gene

expression, which in turn affect the digestive enzyme activity as found in Oguchi et al. (2022), similarly to what we know in nestling house sparrow *SI* (Gatica-Sosa et al. 2018, Brun et al. 2021). Additionally, *MGAM* appears to be more sensitive to these dietary signals than *SI* in chickens, a pattern also seen in the whole tissue-level enzymatic activity (Oguchi et al. 2022).

We also found that sugar transporter gene expressions were modulated similarly to α-glucosidases. Juveniles showed starch induction (PL < SL) and lipid suppression (SL < SP) in sugar transporter gene expression of *SLC5A1* (SGLT1, sodium-dependent glucose transporter; Figure 2E) and *SLC2A2* (GLUT2, facilitated glucose transporter; Figure 2G). In adults *SLC5A1* was similarly induced by starch (Figure 2F), and the overall (combined) effect of starch and lipid (PL < SP) was found in both these glucose transporters (Figure 2F, H). Previous studies on chickens were mixed in their ability to detect *SLC5A1* modulation in relation to diet nutrient compositions (Barfull et al. 2002, Barekatain et al. 2019, Toschi et al. 2024). Our study, using semisynthetic diets that only differ in the composition of starch, protein, and lipid conclusively shows that the transcriptional modulation mechanisms exist for *SLC5A1* and *SLC2A2* that is responsive to dietary starch.

We further found that *MGAM*, *SI*, *SLC5A1*, *SLC2A2*, and *SLC2A5*, plausibly constitute a CTP in chickens (Figure 3), as was described in mice (Sullivan et al. 2021). An additional diet effect was found in *SLC2A2* (Figure 3C), which suggests that a further fine-tuning mechanism responsive to dietary starch and/or lipid signal may be present for *SLC2A2*. Age was only significant in the relationship between *SLC2A5* and *SI* (see later on age effects).

SLC2A5 uniquely showed no signs of induction or suppression (Figure 2I, J), even though it was comodulated with *MGAM* (Figure 3D). *SLC2A5* may not be modulated strongly because it is a specialized fructose transporter (Karasov and Douglas 2013) and our diet did not

contain fructose or sucrose (which is hydrolyzed to glucose and fructose). Indeed, the comodulation of *SLC2A5* with *SI* appears stronger than that with *MGAM*. If so, the modulation mechanism of the putative chicken CTP may be capable of differentiating glucose and fructose, and have an independent signal for glucose-absorbing and fructose-absorbing pathways within the program.

The putative chicken carbohydrate transcriptional program was suppressed by dietary lipid

Our study provides the strongest evidence yet in any animal that the gene expression of the putative CTP is suppressed by high dietary lipid. This lipid effect has been shown on α-glucosidase activity in fish (Cahu et al. 2000), rodents (Dudley et al. 1994, Goda and Takase 1994, Honma et al. 2007, Mochizuki et al. 2010b, Wang et al. 2019), and birds (Levey et al. 1999, Caviedes-Vidal et al. 2000, Brzęk et al. 2013, Oguchi et al. 2022), but its adaptive significance has been uncertain (Karasov et al. 2011, Wang et al. 2019). In our prior study of chicken intestinal hydrolase activity (which includes the same individuals in here) (Oguchi et al. 2022), we proposed the notion that the suppressive effect of lipid on intestinal α-glucosidase activities may be adaptive because lipid is more energy-dense than starch and increases digesta retention time (Karasov and Hume 1997), and therefore the animal may afford to reduce the expression of α-glucosidases and compensate for that with longer reaction (digesta retention) time. The reduction of the putative CTP by lipid, as evident in juvenile chickens (Figure 2A, C, E, G), is evidence supporting this notion that this lipid effect is part of the adaptive modulation of digestive hydrolases and transporters. Furthermore, many natural diets of omnivorous birds (e.g., fruits and seeds) are generally only high in either carbohydrates or lipids (Herrera 1984, White 1989, Herrera 1998, Karasov and Martínez del Rio 2007, Oguchi et al. 2017). Therefore,

an adaptive antagonistic modulation of the gut for starch vs. lipid may also have an ecological evolutionary significance. In that regard, it is plausible that consuming unnatural (i.e., anthropogenic) food that is high in both starch and lipid may not be detrimental to urban birds at the level of digestion and absorption. In turn, the adaptive suppression of the putative CTP by dietary lipid may in fact be part of the "toolkit" that urban-adapted omnivorous birds employ to thrive on such a diet (see (Basile et al. 2021)).

Modulation of the putative CTP was reduced through maturity

Our study is the first to test the effect of age on the putative carbohydrate transcription program in any animal species. As was found in the tissue-level intestinal α-glucosidase activities with a larger sample size (Oguchi et al. 2022), we observed an almost universal decline in modulation of α-glucosidase gene expressions from juveniles (Figure 2A, C, E, G) to adults (Figure 2B, D, F, H). Correlating other carbohydrate transcriptional program genes with *MGAM* did not recover age as a significant covariate predictor (Figure 3A–D); only *SLC2A5* vs. *SI* had age being significant (Figure 3E). This observation, as well as the lack of age effect in the results testing for modulation mechanisms (see later), suggests that the underlying mechanisms of the carbohydrate transcriptional program remains largely consistent with age, but simply becomes less responsive to dietary signals. Regardless, this decrease of modulation with age seems unapparent in laboratory mice because mouse individuals used by Sullivan et al. (2021) were close to physical maturity (7–12 weeks old) and yet showed pronounced induction of the CTP by starch. Perhaps some aspect of the adult chicken's natural history is either increasing the cost of modulation and/or reducing the cost of lack of modulation. Reduction in modulation could conceivably be adaptive if adults tend to face day-to-day variation in macronutrient levels (i.e.,

variation through time occurs too fast for modulation to be a worthwhile investment), or adults may be able to afford not modulating these proteins thanks to longer digesta retention time or having a larger gut (Karasov and Hume 1997).

JAG2 Notch ligand expression was correlated with MGAM only under diet that is low in lipid and high in starch and protein (diet SP)

The induction mechanism of the putative CTP as proposed by Sullivan et al. (2021) is that interleukin-22 is suppressive for the putative CTP, and that release of interleukin-22 from innate lymphoid cells of the lamina propria are suppressed by γδ T cells that became activated due to its Notch receptor binding to the JAG2 ligand of enterocytes. Our RNA-Seq data were unfortunately limited to the intestinal epithelial cells, which did not allow us to test for the change in interleukine-22 production by the lamina propria lymphoid cells. However, Sullivan et al. (2021) presented evidence that high luminal sugar induces the expression of the *JAG2* gene, and thus we expected to find the pattern SP ≥ SL > PL in *JAG2* and comodulation of *JAG2* and *MGAM*.

Contrary to our expectations, we found no significant direct effect of diet on *JAG2* transcript abundance (Figue 4A and 4B). Furthermore, *JAG2* showed a positive correlation with *MGAM* only in diet SP, and in the two high-lipid diets (PL and SL) the correlation was absent (Figure 4C). It is important to note that our ability to effectively test the involvement of this interlukin-22 suppression pathway of the putative CTP modulation is limited by our diet design (even if we could measure IL-22). This is because IL-22 suppression of the CTP is thought to occur when dietary protein content is increased (Sullivan et al. 2021), and unfortunately, we traded off protein with lipid in our diet groups SP vs. SL (in order to test the suppression of the CTP by lipid; Table 1). Nevertheless, it appears that JAG2 signaling of enterocytes to γδ T cells

is overridden by some other suppressive signal that is driven by high lipid content in the diet. Further experiments using 3 diets, each high in only one macronutrient (i.e., high starch, high protein, and high lipid), will be needed to further clarify the effect of JAG2-Notch signaling. Additionally, we note that the relative expression level of *JAG2* is very small compared to *MGAM* (Figure 4C), suggesting that a small change in *JAG2* expression could potentially induce a large change in *MGAM* expression when dietary lipid is low.

MGAM transcript abundance is positively correlated with whole intestinal maltase activity, but the relationship between SI transcript abundance and sucrase activity is lipid- and protein-dependent

We found that *MGAM* abundance was positively correlated with the whole tissue-level maltase activity of the same individuals regardless of age (Figure 5A and 5B), supporting the prediction that transcriptional control is important in the diet-driven modulation of intestinal α-glucosidase activities. This finding is consistent with what is known in house sparrows (Gatica-Sosa et al. 2018, Brun et al. 2021) and rodents (Honma et al. 2007, Mochizuki et al. 2010a).

However, contrary to our expectation, the relationship between *SI* transcript abundance and sucrase activity was found to be diet-specific (Figure 5C and 5D). Specifically, only under low-lipid diet with high starch and protein (diet SP) did we find the expected positive correlation between *SI* and tissue-level sucrase activity (Figure 5C and 5D). Compared to this SP diet, in the SL diet starch was held constant (high) but protein was traded off with lipid (Table 1), and in this SL group we found no correlation between *SI* and sucrase activity (Figure 5C and 5D). Furthermore, compared to the SL diet, in the PL diet lipid was held constant (high) and starch was traded off with protein (Table 1), and in this PL group we found a negative correlation

between *SI* and sucrase activity (Figure 5C and 5D). These general relationships appear to be consistent across age groups.

Our results suggest that the transcriptional control of sucrase activity is counteracted by signals of dietary lipid and further dietary protein under high lipid. Such a "decoupling" of α-glucosidase transcript abundance and tissue-level activity is very unlike what is known in house sparrows and rodents, and to the best of our knowledge, has never been reported before. This could be due to these earlier tests (Honma et al. 2007, Mochizuki et al. 2010a, Gatica-Sosa et al. 2018, Brun et al. 2021) not including a high lipid diet in their experiments, and we face difficulty understanding the mechanisms behind or the adaptive significance of this pattern. However, it is clear that the suppressive effect of dietary lipid on sucrase is not only limited to the suppression of the transcription of the putative CTP but extends to post transcriptional control of sucrase-isomaltase (SI). We need more studies investigating the dietary conditions and mechanisms that may result in this kind of "decoupling" between α-glucosidase transcription and enzymatic activity, particularly by having high lipid experimental diets.

Additionally, we found that wet tissue mass-specific maltase (Figure 5A) and sucrase (Figure 5B) activities were higher in adults compared to juveniles given the transcript abundance. Our earlier study that included the same individuals reported here found that whole intestinal wet mass per unit nominal area (measured in the scale of g cm^{-1}) was indeed greater in adults compared to juveniles (Oguchi et al. 2022). It is also known that intestinal villi development begins pre-hatch (by 15 days of incubation) (Uni et al. 2003) and continues through at least 84 days post-hatch (Mitjans et al. 1997) in chickens. Thus, the age difference we observed here plausibly reflects increased absorptive enterocyte abundance and brush border membrane area per unit nominal surface area in adults compared to juveniles. We found support for this

interpretation when we correlated the abundance of *MGAM* and *SI* with protein mass-specific maltase and sucrase activities (Figure 5B and 5D). Here, the age effect was no longer present, and increased protein concentration in the small intestinal tissue is consistent with increased villus density and/or length.

Transcription of the glycosylating enzyme β-1,4-galactosyltransferase V (B4GALT5)was affected by dietary lipid and comodulated with SI and tissue-level sucrase activity

Because we found that dietary lipid impacts the relationship between *SI* transcript abundance and SI activity, we explored the possibility of post-translational mechanisms being modulated due to dietary signals. One mechanism known in rodents that impacts whole intestinal sucrase activity post translationally is the addition of unsialylated galactose on the glycosylated chain of SI in the Golgi apparatus by β-1,4-galactosyltransferases (Mochizuki et al. 2010b). Increased glycosylation is thought to enhance the transport and exocytosis of mature SI onto the brush border membrane, and a high fat/carbohydrate ratio diet suppresses the activity of β-1,4-galactosyltransferases in laboratory rats, and it has been speculated that dietary lipid may suppress the activity of these enzymes (Mochizuki et al. 2010b). Our diet design (Table 1) was suited for differentiating the effect of the induction by starch vs. suppression by lipid on β-1,4-galactosyltransferase V gene (*B4GALT5*) transcription.

As expected, we found that dietary lipid has suppressive effect on *B4GALT5* transcription (SL < SP) but only in juvenile chickens (Figure 6A) and not in adults (Figure 6B). We also found the unexpected pattern of SL < PL in juvenile chickens (Figure 6A). Thus, in juvenile chickens *B4GALT5* transcription is suppressed only when their diet is high in both lipid and starch. This

pattern at least partially explains the lack of correlation between SI transcript abundance and tissue-level sucrase activity (Figure 5C and 5D).

We also found a positive correlation between *SI* and *B4GALT5* with diet (SL < PL = SP) and age (juveniles > adults) being important covariates (Figure 6C). This finding further supports the view that the suppression of tissue-level sucrase activity by dietary lipid may be adaptive in nature. Moreover, when, diet, *B4GALT5*, and their interaction was added as predictors to the model correlating *SI* against tissue-level sucrase activity, *SI* was dropped as nonsignificant, leaving age and the interaction between diet and *B4GALT5* as more strongly correlated with sucrase activity than *SI*. While we still do not understand the mechanisms behind the modulation of *B4GALT5* transcription, our results suggest that the tissue-level sucrase activity is under the strong influence of β-1,4-galactosyltransferases, which are in turn, modulated by dietary lipid levels.

Alpha-gustducin (GNAT3) and TRPM5 transcript abundance were correlated with α-glucosidase activity but not with α-glucosidase transcript abundance

We explored the possibility of gene expression modulation of GNAT3 and TRPM5 as an additional post-translational mechanism for the putative CTP proteins being expressed on the cell membrane. We did not find evidence of *GNAT3* or *TRPM5* being modulated by diet, but we found that the transcript abundance of these genes was correlated with tissue-level α-glucosidase activity, a proxy for α-glucosidase abundance on the brush border membrane. Indeed, when *GNAT3* or *TRPM5* (with diet being a covariate) was added to the model correlating MGAM with tissue-level maltase activity, *MGAM* was dropped as nonsignificant and *GNAT3* (Figure 7A) or *TRPM5* (Figure 7B) remained as more predictive of maltase activity than *MGAM*. These findings

again suggest the strong influence of post-transcriptional process in the diet-driven modulation α-glucosidase activity at the whole tissue level. The mechanisms of α-gustducin and TRPM5 leading to enterocyte membrane depolarization and exocytosis of vesicles containing sugar transporters have been tested only for SLC2A2 (GLUT2) in rodents (Kellett et al. 2008, Sprous and Palmer 2010). However, it is reasonable to expect that the protein expression of α-glucosidases is modulated by a similar mechanism to SLC2A2 (possibly sharing the same mechanism, e.g., expressed in the same vesicle being exocytosed), given their comodulation of transcription (Figure 3). An alternative explanation is that the upmodulation of protein expression of α-glucosidases on the brush border relies on a different mechanism which concordantly occurs with α-gustducin- and TRPM5-mediated expression of SLC2A2. We are in favor of the former explanation (shared mechanism) on the basis of parsimony, but further testing will be needed.

Whichever the case may be, our data suggests that *GNAT3* and *TRPM5* are modulated independently of *MGAM* or *SI* and hence the putative CTP, as evidenced by a lack of comodulation between *MGAM* or *SI* and *GNAT3* or *TRPM5*. This finding is consistent with that of Sullivan et al. (Sullivan et al. 2021), who showed that direct sensing of glucose by epithelial cells is insufficient to induce a robust upmodulation of gene expression of the CTP.

The signaling cascades leading to the exocytosis of vesicles with sugar transporters and (supposedly) α-glucosidases occur rapidly using pre-existing α-gustducin (GNAT3) and TRPM5 proteins already expressed (Kellett et al. 2008, Sprous and Palmer 2010, Roura et al. 2013). As such, it may seem curious that the gene expressions of *GNAT3* and *TRPM5* are positively correlated with a proxy of α-glucosidase abundance (i.e., whole tissue-level activity). It is conceivable that increasing α-gustducin and TRPM5 allows a more rapid activation of

downstream pathways leading to exocytosis or that these two proteins are relatively short-lived and continuous replacement is necessary when they are heavily used due to continuous activation of the exocytosis pathways.

Evolutionary perspectives: Chickens show apparent similarities in mechanisms for diet-driven modulation of intestinal α-glucosidases and sugar transporters to rodents

Taken together, our study highlighted that a putative CTP akin to rodents seem to exist in chickens, and that the induction mechanisms of the putative CTP also appears similar to those shown in rodents, i.e., the involvement of both gustducin- and TRPM5-mediated protein expression on the brush border membrane and the Notch-mediated activation of γδ T cells in allowing increased gene expression of the putative CTP.

Birds have a number of unique features in their digestion and absorption (summarized in (McWhorter et al. 2009)), including the apparent reduction of diet-driven modulation of α-glucosidase and sugar transporters through maturity (Figure 1) (Karasov 2017). Recent studies using genomics tools have revealed that functionally similar digestive and absorptive mechanisms that bird share with mammals can in fact be a result of convergent evolution (as in the case of the avian-derived α-glucosidase vs. mammalian maltase-glucoamylase, both called "MGAM" by convention (Brun et al. 2020b)) or re-evolving a novel function for a protein to compensate for a function lost through evolution (as in the case of umami taste receptor taking the role of sweet sensing (Roura et al. 2013, Baldwin et al. 2014, Toda et al. 2021). Considering that bird ancestors have evolved for at least 50 million years as hypercarnivores (meat-eating theropod dinosaurs) and later evolved omnivory (Rezende et al. 2020), these patterns of convergent or re-evolution may not be surprising. Our findings on modulatory mechanisms beg

the question whether these apparent similarities between chickens and rodents are homologous or that they represent a product of convergent and/or re-evolution. Future studies taking a similar approach to (Baldwin et al. 2014, Brun et al. 2020b, Toda et al. 2021) on the genes and proteins involved in the modulation mechanisms proposed here will shed new light on the evolutionary pattern of the intestinal adjustment to dietary signals among vertebrates.

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Tables and Figures

Table 1. Composition of experimental diets (% dry mass) used in isolated small intestinal epithelial tissue RNA-Seq study of chickens. PL = high in starch and protein; SL = high in starch and lipid; SP = high in starch and protein. With this diet design, PL < SL shows the induction of intestinal α-glucosidase and sugar transporter gene expression, and SL < SP the suppression of these genes by lipid. See {Oguchi, 2022 #791} for details.

Diet[1]	Corn starch	Milk casein	Corn oil	Inert[2]	Essential[2]	Energy (kJ/g)
PL	0	42	16	32	10	13.8
SL	34	16	16	24	10	15.2
SP	34	42	4	10	10	15.2

[1]All 3 diets meet nutritional requirement of chickens {National Research Council Subcommittee on Poultry Nutrition, 1994 #654}; chickens are known to thrive on diets that contain substrate levels like these or even higher than proposed here {Ciminari, 2014 #644;Myers, 1999 #481;Biviano, 1993 #482}.

[2]Contents as described in {Lepczyk, 1998 #541}

Table 2. Genes of interest in the present study of gene expression modulation underpinning the adaptive response of small intestine epithelial cells to dietary starch and lipid.

Gene	Protein function	Reference
MGAM	Avian-derived α-glucosidase (convergently evolved analog of mammalian maltase-glucoamylase) on brush border. Maltase activity.	{Brun, 2020 #730;Sullivan, 2021 #827}
SI	Sucrase-isomaltase on bush border. Sucrase activity and some maltase activity.	{Brun, 2020 #730;Sullivan, 2021 #827}
SLC5A1	Solute carrier family 5 member 1 (sodium/glucose cotransporter 1 [SGLT1]) on brush border. Sodium-dependent glucose uptake.	{Karasov, 2013 #472;Sullivan, 2021 #827}
SLC2A2	Solute carrier family 2 member 2 (glucose transporter 2 [GLUT2] on brush border (at least in rodents) and basolateral. Facilitated glucose (and fructose) uptake.	{Karasov, 2013 #472;Sullivan, 2021 #827}
SLC2A5	Solute carrier family 2 member 5 (glucose transporter 5 [GLUT5]) on brush border. Facilitated fructose uptake.	{Karasov, 2013 #472;Sullivan, 2021 #827}
JAG2	Jagged canonical Notch ligand 2. Basolateral ligand for contact-dependent Notch signaling for γδ T cells. Expression known to increase in intestinal epithelial cells in response to high dietary starch. Notch-signaled γδ T	{Sullivan, 2021 #827}

	cells suppress interleukin-22 production in other cells.	
B4GALT5	β-1,4-galactosyltransferase (β-1,4-GalT) V. One of seven subtypes of β-1,4-GalT which are suggested to increase SI activity through glycosylation. β-1,4-GalT V likely plays an important role in the small intestine as its abundance there is higher than other β-1,4-GalT subtypes.	{Mochizuki, 2010 #692;Asano, 1997 #864;Furukawa, 1999 #865}
GNAT3	Guanine nucleotide-binding protein G(t) subunit α-3 (α-gustducin). Part of the G-protein that is activated by sweet taste receptors (T1R2/T1R3 in mammals and T1R1/T1R3 [umami receptor] in birds). Activates kinase cascade responsible for exocytosis of sugar transporter-carrying vesicles.	{Roura, 2013 #843;Baldwin, 2014 #842;Kellett, 2008 #844}
TRPM5	Transient receptor potential cation channel subfamily M member 5. Ca^{2+}-activated nonspecific cation channel involved in depolarization of cell membrane which triggers exocytosis of sugar transporter-carrying vesicles.	{Kellett, 2008 #844;Sprous, 2010 #841}

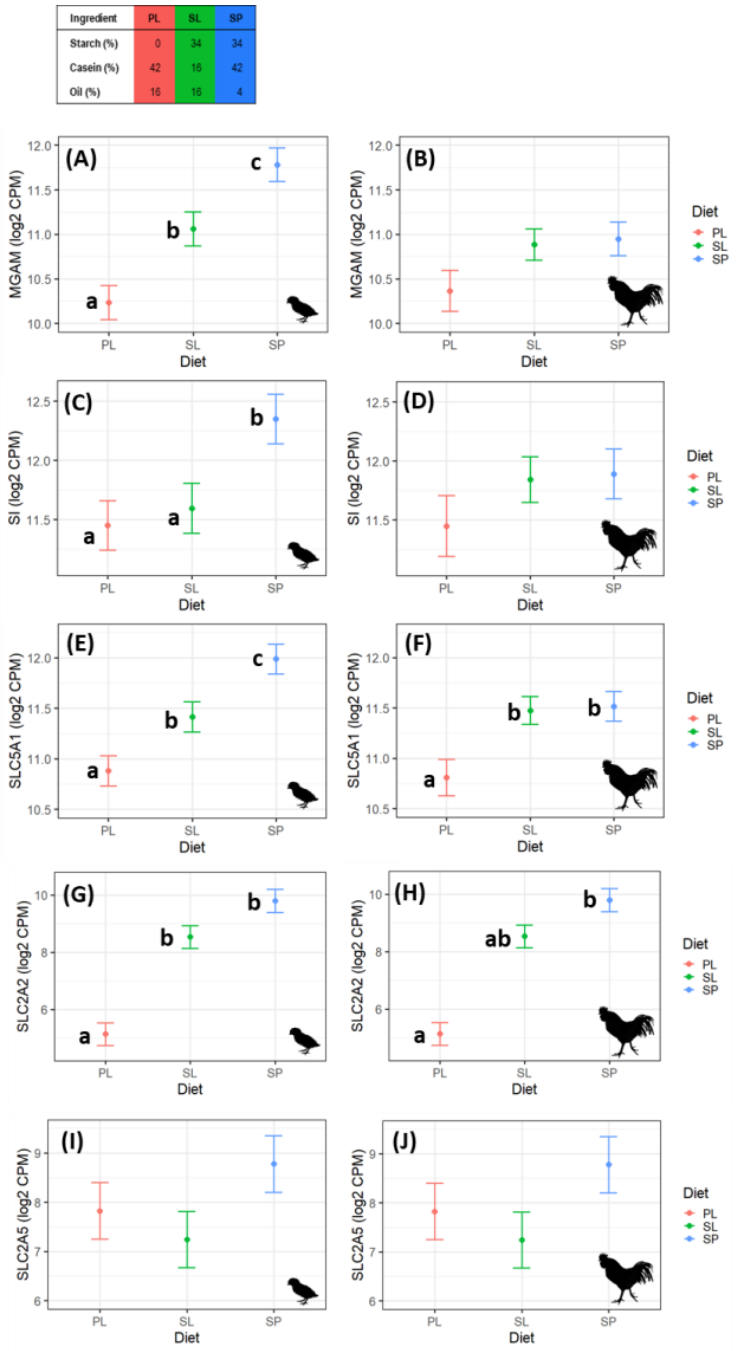

Figure 1. Diet effect on the small intestinal epithelial cells' transcript abundance of α-glucosidase (*MGAM* and *SI*) and sugar transporters (*SLC5A1*, *SLC2A2*, and *SLC2A5*) in the small intestine epithelial cells of juvenile (A, C, E, G, I; 20 d old; $n = 6$ per diet) and adult (B, D, F, H, J; 230 d old; $n = 4–7$ per diet) chickens. Data represent least square means ± s.e.m., and different letters at the means indicate significant differences based on one-tailed post hoc Tukey tests.

Table 3. Diet effect on the small intestinal epithelial cells' transcript abundance of the genes of interest in relation to age in chickens. Values are from type III ANOVA on linear models. Each diet-age group was comprised of $n = 6$ juveniles and $n = 4–7$ adults.

Gene	Age		Diet		Age × diet	
	$F_{1, 29}$	P	$F_{2, 29}$	P	$F_{2, 29}$	P
MGAM	0.20	0.66	16.77	**<0.0001**	3.14	0.06
SI	0.0001	0.99	5.26	**0.01**	1.48	0.24
SLC5A1	0.09	0.77	13.92	**<0.0001**	1.75	0.19
SLC2A2	3.09	0.09	36.22	**<0.0001**	7.39	**0.003**
SLC2A5	1.38	0.25	1.83	0.18	0.66	0.52
JAG2	0.62	0.43	2.58	0.09	2.24	0.12
B4GALT5	5.50	**0.03**	12.13	**0.0001**	4.16	**0.03**
GNAT3	12.60	**0.001**	2.73	0.08	0.07	0.93
TRPM5	2.16	0.15	0.34	0.72	0.08	0.92

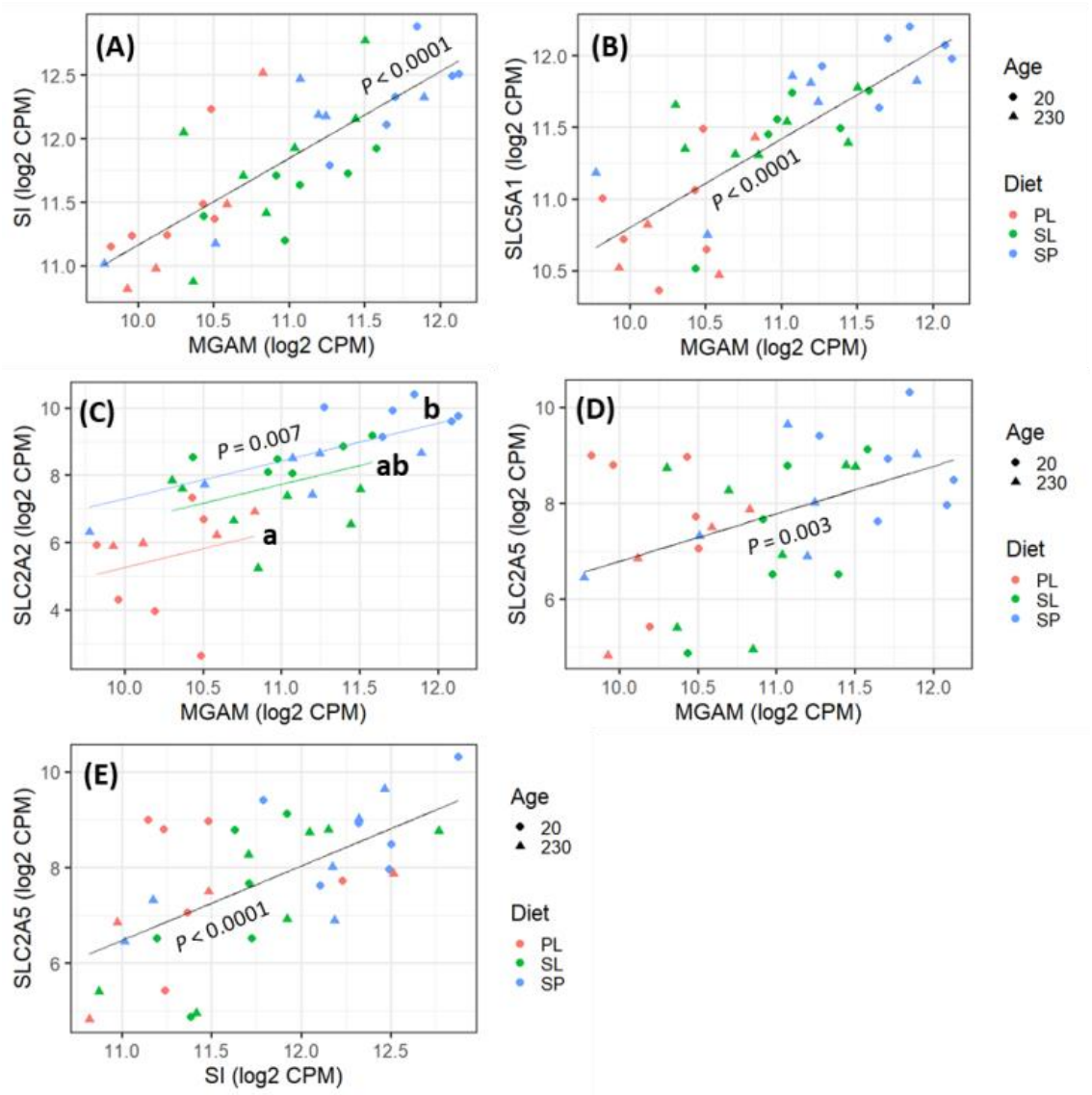

Figure 2. Correlating the transcript abundance of *SI* (A), *SLC5A1* (B), *SLC2A2* (C), and *SLC2A5* (D) with that of *MGAM* and *SLC2A5* with *SI* (E) in the small intestine epithelial cells of chicken individuals. Diet-specific regression lines (with different letters assigned to significantly different diet groups) wherever significant. PL = high in protein and lipid, SL = high in starch and lipid, and SP = high in starch and protein. *P*-values are those of the slope.

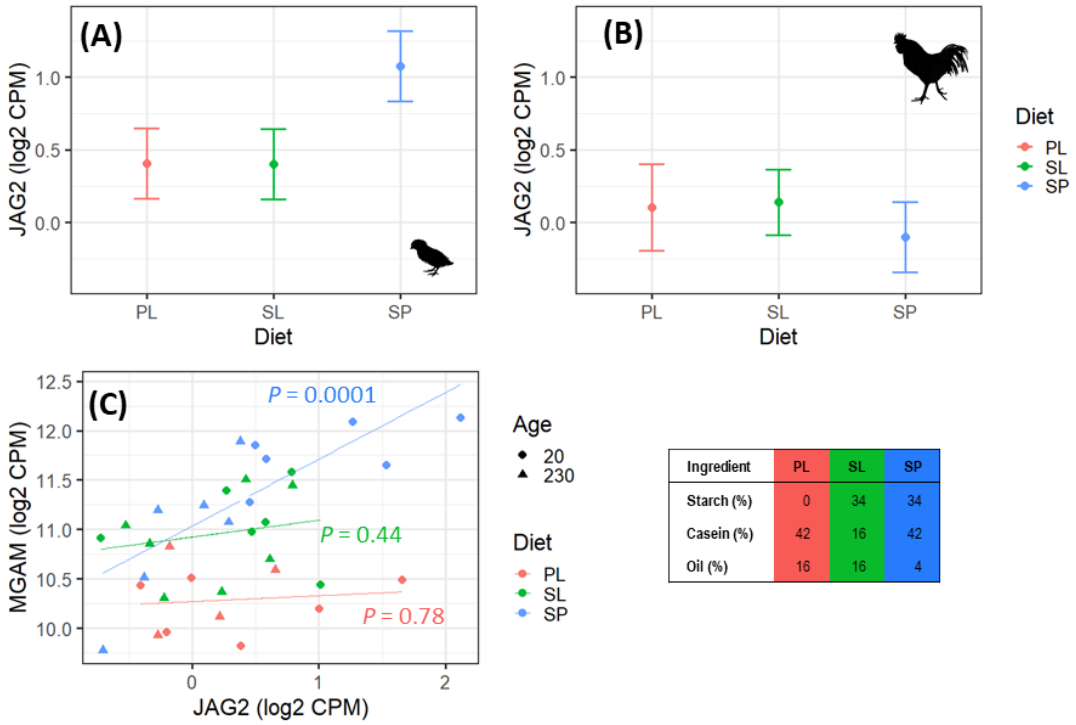

Figure 3. Diet effect on the small intestinal epithelial cells' transcript abundance of Notch-signaling JAG2 gene in the small intestine epithelial cells of juvenile (A; n = 6 per diet) and adult (B; 230 d old; n = 4–7 per diet) chickens and the correlation between *MGAM* and *JAG2*. Data in A and B represent least square means ± s.e.m., and different letters at the means indicate significant differences based on one-tailed post hoc Tukey tests. Diet effects in the correlation in C are expressed as different colors, and *P*-values are those of the slope.

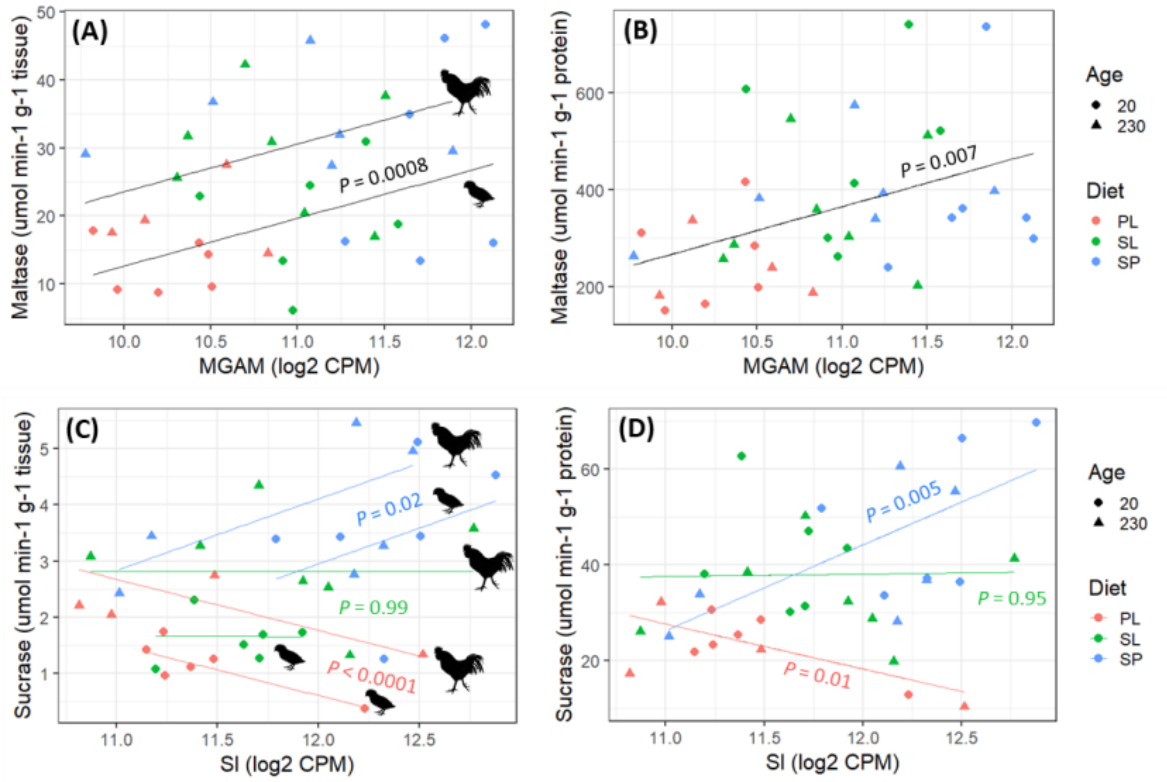

Figure 4. Effect of *MGAM* transcript abundance in the small intestine epithelial cells on maltase activity per g of wet tissue (A) and per g of proein (B) and *SI* transcript abundance on sucrase activity per g of wet tissue (C) and per g of protein (D) in the same chicken individuals. Age and diet effects are respectively denoted as pictograms and color. PL = high in protein and lipid, SL = high in starch and lipid, and SP = high in starch and protein. *P*-values are those of the slope.

Ingredient	PL	SL	SP
Starch (%)	0	34	34
Casein (%)	42	16	42
Oil (%)	16	16	4

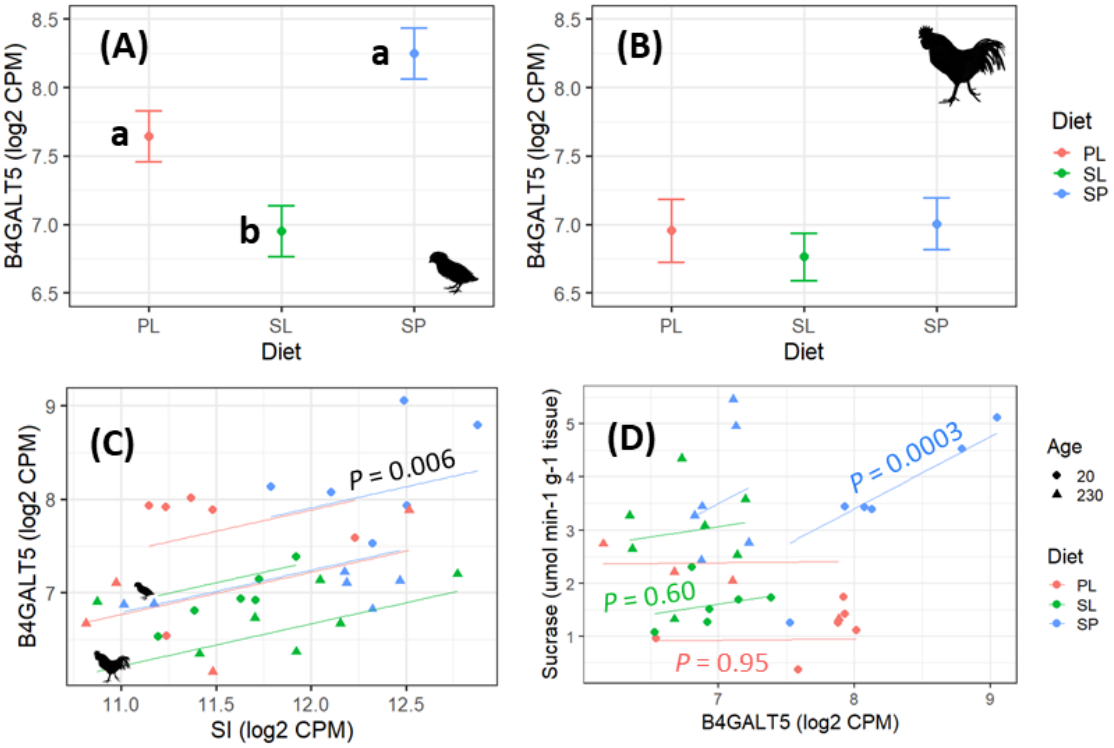

Figure 5. Diet effect on the small intestinal epithelial cells' transcript abundance of protein-glycosylating enzyme β-1,4-galactosyltransferase 5 (*B4GALT5*) gene in the small intestine epithelial cells of juvenile (A; $n = 6$ per diet) and adult (B; 230 d old; $n = 4–7$ per diet) chickens, the correlation between *B4GALT5* and *SI* (C), and the correlation between wet tissue-level Sucrase activity with *B4GALT5*. Data in A and B represent least square means ± s.e.m., and different letters at the means indicate significant differences based on one-tailed post hoc Tukey tests. Diet effect in the correlation in C and D are expressed as different colors, and *P*-values are those of the slope.

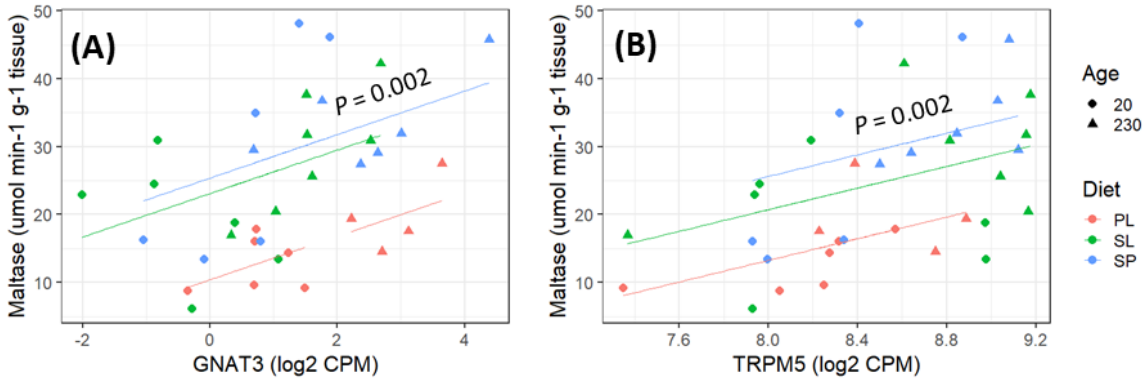

Figure 6. Correlating tissue-level maltase activity with the transcript abundance of α-gustducin (GNAT3; A) and TRPM5 (B; both of which involved in the exocytosis of vesicles containing digestive hydrolases and/or transporters onto the brush border membrane) in the chicken small intestine epithelial cells. Diet-specific regression lines are provided with different colors. PL = high in protein and lipid, SL = high in starch and lipid, and SP = high in starch and protein. *P*-values are those of the slope.

Supplementary Information

Supplementary Tables and Figures

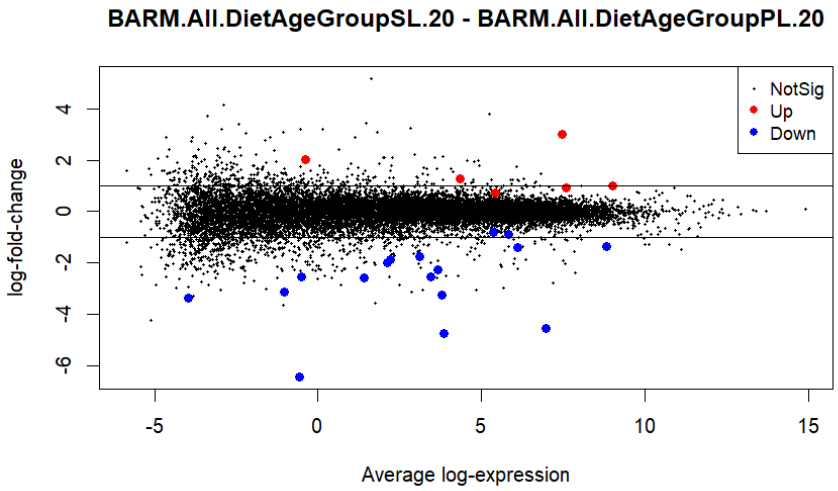

Figure S1. Differentially expressed genes (Benjamini-Hochberg adjusted $P < 0.1$) comparing diet groups PL and SL (testing for induction by starch) in juvenile chickens. The 6 upmodulated and 17 downmodulated genes by starch are highlighted.

Table S1. Differentially expressed genes (6 upmodulated and 17 downmodulated; Benjamini-Hochberg adjusted $P < 0.1$) comparing diet groups PL and SL (testing for induction by starch) in juvenile chickens. Log-2 fold change (logFC), Average expression (AveExpr), *t*-statistic (t), raw *P*-value (P.Value), Benjamini-Hochberg adusted P-value based on (adj.P.Val), and Bayes log-odds of differential expression (B) were provided by TopTable of limma Voom. *SLC2A2* (bold) was the only induced gene detectable among the genes of interest.

	logFC	AveExpr	t	P.Value	adj.P.Val	B
FBLN1	-2.24687	3.653653	-5.72142	2.13E-06	0.033311	4.624286
SEBOX	-6.4429	-0.57864	-5.28519	7.78E-06	0.041783	1.780002
CNDP2	-1.37136	8.832117	-5.1209	1.27E-05	0.041783	3.184222
SLC13A2	-4.53958	6.957803	-5.10106	1.34E-05	0.041783	3.146051
SLC2A2	**2.984411**	**7.4807**	**5.069668**	**1.47E-05**	**0.041783**	**3.079686**
LRRN4	-3.1199	-1.04624	-5.03158	1.65E-05	0.041783	1.825979
GLDC	-3.2381	3.79283	-4.98829	1.87E-05	0.041783	2.810402
FAM53A	0.709262	5.441136	4.850904	2.81E-05	0.051492	2.480815
CYP4A22	0.99344	8.998129	4.773493	3.53E-05	0.051492	2.267266
A2ML1	-2.56188	1.418617	-4.77189	3.55E-05	0.051492	1.642328
LOC418170	0.923412	7.582878	4.748291	3.80E-05	0.051492	2.199317
APRT	1.262291	4.343862	4.734589	3.96E-05	0.051492	2.166542
DDO	-2.54975	3.43943	-4.62882	5.40E-05	0.064836	1.848323
CNDP1	-1.86864	2.225787	-4.56116	6.58E-05	0.070504	1.340887
PRKG2	-1.38139	6.102635	-4.54521	6.90E-05	0.070504	1.645552
KIF12	-2.54271	-0.51765	-4.5104	7.63E-05	0.070504	0.552659

LRFN5	-4.72298	3.842631	-4.50846	7.68E-05	0.070504	1.270308
NRBP2	-1.97974	2.111079	-4.48538	8.21E-05	0.071224	1.257958
TLL2	-1.7354	3.111788	-4.39668	0.000106	0.087358	1.239077
ATP7A	-0.82114	5.350137	-4.36323	0.000117	0.091457	1.152995
LOC121111296	2.022039	-0.37114	4.337628	0.000126	0.093817	0.432866
LOC101749127	-3.36843	-3.96573	-4.29903	0.000141	0.096306	-1.185
CAPN8	-0.89686	5.820485	-4.29718	0.000142	0.096306	0.975127

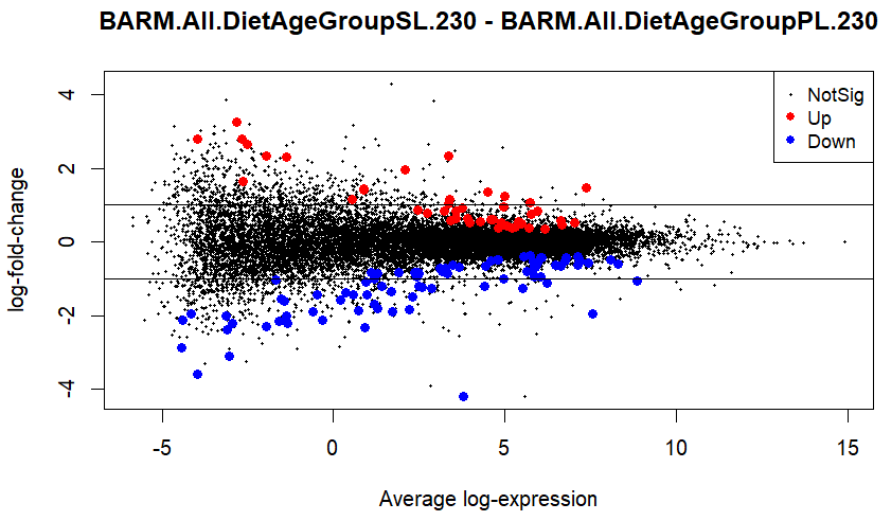

Figure S2. Differentially expressed genes (Benjamini-Hochberg adjusted $P < 0.1$) comparing diet groups PL and SL (testing for induction by starch) in adult chickens. The 46 upmodulated and 83 downmodulated genes by starch are highlighted.

Table S2. Differentially expressed genes (46 upmodulated and 83 downmodulated; Benjamini-Hochberg adjusted $P < 0.1$) comparing diet groups PL and SL (testing for induction by starch) in adult chickens. Log-2 fold change (logFC), Average expression (AveExpr), *t*-statistic (t), raw *P*-value (P.Value), Benjamini-Hochberg adusted P-value based on (adj.P.Val), and Bayes log-odds of differential expression (B) were provided by TopTable of limma Voom. None of the genes of interest was detectable as differentially expressed.

	logFC	AveExpr	t	P.Value	adj.P.Val	B
RFX3	-0.88406	2.472816	-6.11129	6.74E-07	0.00765	5.966898
GLDC	-4.19013	3.79283	-5.98435	9.80E-07	0.00765	5.61996
JARID2	-0.58482	5.951438	-5.58607	3.19E-06	0.016584	4.395649
LOC101751160	-1.24857	2.875676	-5.36624	6.12E-06	0.023873	3.887422
HAL	-1.69789	1.187739	-5.20705	9.81E-06	0.025084	3.378566
UTRN	-0.64923	6.643765	-5.1972	1.01E-05	0.025084	3.283177
FHAD1	-1.21305	1.403041	-5.13334	1.22E-05	0.025084	3.204355
DENND1B	-0.59473	5.922386	-5.11565	1.29E-05	0.025084	3.039762
MTCL1	-1.2731	5.521122	-4.98522	1.89E-05	0.032794	2.690159
SLC39A11	0.834464	5.958745	4.882932	2.56E-05	0.03992	2.375647
THBS1	2.339976	3.363984	4.83677	2.93E-05	0.041582	2.406918
STK38L	-1.10451	6.24313	-4.79317	3.33E-05	0.04334	2.142128
LOC121108597	-1.42324	0.979247	-4.75911	3.68E-05	0.044224	2.211149
KITLG	1.95695	2.083111	4.69446	4.45E-05	0.0465	2.050265
SLC2A9	-1.21204	4.407417	-4.69335	4.47E-05	0.0465	1.936502
SERPINI1	-1.43751	0.582391	-4.6566	4.98E-05	0.047055	1.915528

MRPS14	0.615466	4.667519	4.628853	5.40E-05	0.047055	1.713822
GLTP	0.927219	3.744509	4.627154	5.43E-05	0.047055	1.800928
CMAS	0.962566	4.972873	4.540954	6.98E-05	0.053508	1.44155
CDO1	-1.8954	1.715059	-4.53291	7.15E-05	0.053508	1.59989
LOC776593	-3.11287	-3.05349	-4.51669	7.49E-05	0.053508	1.257994
IPCEF1	-0.71386	3.271898	-4.50762	7.70E-05	0.053508	1.498916
GCAT	-1.8669	0.741535	-4.49939	7.88E-05	0.053508	1.531819
BCKDHB	-1.48613	2.31978	-4.41308	0.000101	0.063812	1.208204
DFNB59	-1.57896	0.212347	-4.39417	0.000107	0.063812	1.132887
ALDH8A1	-1.22095	2.580006	-4.37012	0.000115	0.063812	1.160734
TDH	-1.35297	1.682622	-4.35834	0.000119	0.063812	1.137715
LOC107054112	-2.22588	-1.34781	-4.34937	0.000122	0.063812	0.997262
ZBTB42	-0.6994	3.676467	-4.34508	0.000123	0.063812	0.992821
LOC417372	0.857929	2.471922	4.335003	0.000127	0.063812	1.095212
PTTG1IP2	0.488551	5.479167	4.327875	0.00013	0.063812	0.816527
LOC124417663	-2.37753	-3.09198	-4.31548	0.000135	0.063812	0.807707
SIGMAR1	0.649595	3.909231	4.314648	0.000135	0.063812	0.90313
BRSK1	-0.82835	1.917995	-4.28246	0.000148	0.067792	0.946057
LOC107054940	-1.82987	2.206797	-4.27339	0.000152	0.067792	0.90097
BLOC1S1	0.829851	3.241845	4.243569	0.000166	0.068391	0.803339
LOC112532493	-2.14815	-1.5869	-4.23274	0.000171	0.068391	0.402465
CCDC167	0.639037	3.560275	4.231123	0.000172	0.068391	0.736961
REEP5	0.570567	6.640125	4.225165	0.000175	0.068391	0.525458

FBXO5	-0.9443	2.425788	-4.22409	0.000175	0.068391	0.779459
TMEM243	-1.01093	4.952899	-4.21316	0.000181	0.068857	0.510839
RHCE	-0.82253	1.102823	-4.19654	0.00019	0.070515	0.742312
LOC112531487	-2.32281	0.905925	-4.17818	0.0002	0.072612	0.673467
PCF11	-0.50856	7.17672	-4.16899	0.000205	0.07286	0.397756
LOC770653	0.787042	2.747852	4.149744	0.000217	0.073054	0.596998
MCMDC2	-1.00759	1.185884	-4.14519	0.00022	0.073054	0.609955
DGKD	-0.63882	7.130405	-4.14511	0.00022	0.073054	0.329104
S100A6	1.469836	7.366454	4.136812	0.000225	0.073147	0.310651
LOC121107742	-0.83382	2.353015	-4.12841	0.000231	0.073147	0.532854
LOC107054686	-2.29543	-1.97173	-4.1064	0.000246	0.073147	0.442914
SMG1	-0.51362	6.768204	-4.10413	0.000247	0.073147	0.19996
LOC101747667	-1.55178	-1.52654	-4.09391	0.000255	0.073147	0.339447
LOC101749127	-3.5944	-3.96573	-4.0938	0.000255	0.073147	-0.6733
CD276	0.578391	3.402104	4.082879	0.000263	0.073147	0.349313
ZXDC	-0.48487	4.762269	-4.07534	0.000269	0.073147	0.142063
SLC6A2	2.334845	-1.96458	4.072077	0.000271	0.073147	0.40907
TNRC6A	-0.43294	6.77646	-4.07072	0.000272	0.073147	0.108957
TSPAN18	-1.42719	-0.48858	-4.06631	0.000276	0.073147	0.31772
FER	-0.48156	4.812428	-4.06528	0.000276	0.073147	0.110279
CTH	-0.87063	3.339849	-4.05758	0.000283	0.073529	0.124183
ACMSD	-1.21942	2.492312	-4.04367	0.000294	0.075254	0.318846
LOC112530182	-2.01732	-1.36585	-4.03776	0.000299	0.075298	0.142594

SPCS1	0.391486	4.81871	4.02786	0.000308	0.075506	0.032993
YBX3	0.55518	5.4002	4.02039	0.000314	0.075506	-0.03204
ARID1B	-0.39523	5.548089	-4.0161	0.000318	0.075506	-0.0555
LOC421108	-2.20675	-2.94189	-4.01486	0.000319	0.075506	-0.16916
GSN	1.058529	5.741497	4.004678	0.000329	0.076569	-0.0649
FAM43A	1.643082	-2.6494	3.999456	0.000334	0.076573	-0.55909
IQGAP2	-0.48813	8.094872	-3.97338	0.000359	0.081272	-0.08616
SAP18	0.355316	6.171783	3.968058	0.000365	0.081331	-0.18699
LOC121111987	-1.61514	-1.42634	-3.95653	0.000377	0.082552	0.085132
FAXDC2	-1.94244	7.566544	-3.95289	0.000381	0.082552	-0.17696
LOC107053576	2.790509	-3.96483	3.94289	0.000392	0.083762	-1.07384
GAL3ST1	2.787125	-2.65971	3.935324	0.0004	0.083886	-0.72184
MYLK3	-0.70897	3.115897	-3.9287	0.000408	0.083886	-0.0766
NFATC2	-0.6609	4.444795	-3.92816	0.000408	0.083886	-0.24419
S100A11	1.144298	0.527379	3.914156	0.000425	0.084791	-0.03503
NFKBIZ	-0.9411	6.041222	-3.90556	0.000435	0.084791	-0.34922
DPF3	-0.52396	4.588336	-3.8996	0.000443	0.084791	-0.31021
TNRC6B	-0.41678	6.036348	-3.89489	0.000449	0.084791	-0.38548
DNAJB2	0.829616	3.577561	3.890758	0.000454	0.084791	-0.18489
RBFOX1	-2.11995	-4.39813	-3.8892	0.000456	0.084791	-0.56217
CRTAP	2.315558	-1.37529	3.887955	0.000458	0.084791	-0.43604
EFTUD2	0.381028	5.71394	3.884887	0.000462	0.084791	-0.40861
TTYH3	-0.62413	6.476802	-3.88448	0.000462	0.084791	-0.39582

CCNJ	-0.84628	2.502645	-3.88015	0.000468	0.084791	-0.12428
RBM6	-0.35843	5.746551	-3.87656	0.000473	0.084791	-0.43383
BTBD8	-0.67859	5.896664	-3.86372	0.00049	0.086919	-0.46753
TMEM128	0.504505	5.392235	3.859433	0.000496	0.086987	-0.47034
AASS	-2.13862	-0.33412	-3.84853	0.000511	0.088706	-0.14991
C1GALT1C1	0.437845	5.052803	3.839572	0.000524	0.089971	-0.48561
LOC121107923	-1.8981	-0.58665	-3.83177	0.000536	0.090454	-0.22901
LOC121111166	-0.83851	2.453985	-3.82791	0.000542	0.090454	-0.26209
UPB1	-1.38205	0.35955	-3.82614	0.000545	0.090454	-0.20959
PTPN12	-0.53744	5.839582	-3.80529	0.000577	0.092056	-0.62518
SH3TC2	-1.8089	1.286289	-3.80229	0.000582	0.092056	-0.26804
AES	0.600515	4.586602	3.80194	0.000583	0.092056	-0.55042
NPDC1	1.041237	3.35376	3.795101	0.000594	0.092056	-0.36671
SSR3	0.478278	6.676765	3.792887	0.000598	0.092056	-0.65254
LOC121109515	-2.87691	-4.44922	-3.79109	0.000601	0.092056	-1.25826
PITPNM3	-0.62703	3.473239	-3.79067	0.000602	0.092056	-0.49827
TINAGL1	1.446346	0.871758	3.784263	0.000613	0.092056	-0.31858
SCAMP2	0.518348	7.027626	3.779632	0.000621	0.092056	-0.67224
LOC121107592	-2.02028	-3.13683	-3.77698	0.000625	0.092056	-0.73154
SH3BP5	-0.81175	3.161609	-3.77679	0.000626	0.092056	-0.51775
LOC421415	-1.09906	0.937436	-3.77011	0.000637	0.092056	-0.33707
SAT1	-1.07092	8.865229	-3.77005	0.000637	0.092056	-0.55076
NDUFB4	0.523443	4.912928	3.76774	0.000642	0.092056	-0.66096

SLC19A1	0.744064	5.752208	3.764763	0.000647	0.092056	-0.72014
TIMM23B	0.371555	5.209178	3.76385	0.000649	0.092056	-0.7069
ERBIN	-0.39678	7.126361	-3.75814	0.000659	0.092083	-0.71409
STRIP1	0.414934	5.30955	3.756516	0.000662	0.092083	-0.73456
REEP2	-0.74578	5.820754	-3.75412	0.000666	0.092083	-0.75948
NPFFR2	-1.01945	-1.6821	-3.74501	0.000684	0.09363	-0.48695
DUSP3	1.256287	5.009159	3.737549	0.000698	0.094036	-0.71787
PHKA2	1.15649	3.389112	3.735421	0.000702	0.094036	-0.52722
PIN1	0.511995	3.967653	3.729309	0.000714	0.094036	-0.66305
LOC107050649	0.541046	4.295523	3.727134	0.000719	0.094036	-0.71437
FABP3	1.36977	4.500358	3.725447	0.000722	0.094036	-0.72577
LOC121111287	-1.94672	-4.14765	-3.72509	0.000723	0.094036	-0.82078
FSHR	3.244669	-2.82973	3.714082	0.000745	0.095914	-1.46916
HECTD4	-0.56817	7.44999	-3.71207	0.00075	0.095914	-0.82782
BRWD1	-0.44182	6.078674	-3.70638	0.000762	0.096087	-0.88791
CORO2A	-0.61274	8.291775	-3.70559	0.000763	0.096087	-0.79406
GUCY1A3	2.643873	-2.52472	3.699814	0.000776	0.096417	-1.13435
ABCA1	-0.93532	5.869936	-3.69861	0.000778	0.096417	-0.90104
MPP3	-0.87245	1.276571	-3.6897	0.000798	0.098058	-0.54278
LOC101749809	-0.79068	5.641177	-3.68277	0.000813	0.099187	-0.94833
LOC101751749	1.412391	0.883127	3.677766	0.000825	0.099796	-0.56853

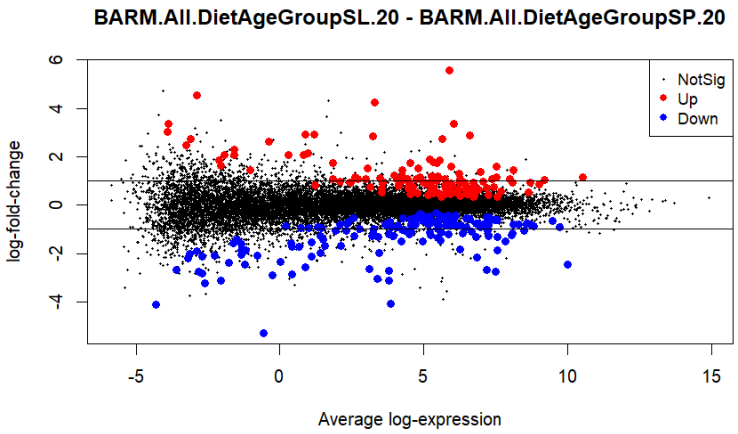

Figure S3. Differentially expressed genes (Benjamini-Hochberg adjusted $P < 0.1$) comparing diet groups SL and SP (testing for suppressive effect of lipid) in juvenile chickens. The 139 upmodulated and 158 downmodulated (suppressed) genes by lipid are highlighted.

Table S3. Differentially expressed genes (139 upmodulated and 158 downmodulated; Benjamini-Hochberg adjusted $P < 0.1$) comparing diet groups SP and SL (testing for suppression by lipid) in juvenile chickens. Log-2 fold change (logFC), Average expression (AveExpr), *t*-statistic (t), raw *P*-value (P.Value), Benjamini-Hochberg adusted P-value based on (adj.P.Val), and Bayes log-odds of differential expression (B) were provided by TopTable of limma Voom. *B4GALT5* (bold) was the only suppressed gene detectable among the genes of interest.

	logFC	AveExpr	t	P.Value	adj.P.Val	B
BBOX1	5.574147	5.897747	10.2034	8.91E-12	1.39E-07	16.01414
GSTT1L	3.340961	6.044314	7.07435	4.06E-08	0.000317	8.559864
HMGCS2	4.242429	3.282967	6.656817	1.36E-07	0.000708	7.34542
USP40	1.504154	4.812682	6.465361	2.38E-07	0.000929	6.972577
PTAFR	1.728408	1.830869	5.779272	1.80E-06	0.005143	4.806038
HBP1	0.840942	6.458667	5.747214	1.98E-06	0.005143	4.946567
VNN2	2.722004	5.634939	5.68144	2.40E-06	0.00527	4.778243
LOC121111296	2.629461	-0.37114	5.591394	3.14E-06	0.00527	3.596824
USP12	-1.21663	4.508662	-5.55654	3.48E-06	0.00527	4.418821
STK38L	1.285817	6.24313	5.540828	3.64E-06	0.00527	4.362438
ABHD3	2.879284	6.600394	5.529457	3.77E-06	0.00527	4.3457
FBP1	-1.1807	8.079994	-5.50519	4.05E-06	0.00527	4.281405
GLI1	2.731034	-3.09631	5.378062	5.91E-06	0.006777	1.357657
PRKCD	-0.80676	7.265458	-5.36839	6.08E-06	0.006777	3.885055
KCNJ5	2.070739	0.309848	5.339784	6.62E-06	0.006886	3.136479
FOXK2	0.495378	5.335752	5.193945	1.02E-05	0.009946	3.384375

AKR1D1	-3.03175	3.400824	-5.05453	1.54E-05	0.013311	3.02584
HEXB	-0.79401	7.158014	-5.03924	1.61E-05	0.013311	2.961187
NCEH1	1.75181	5.446149	5.037465	1.62E-05	0.013311	2.940758
HDAC7	-0.82165	5.953894	-4.98812	1.87E-05	0.014559	2.796936
TIMP3	-1.14973	6.35667	-4.96523	2.01E-05	0.014559	2.735467
B4GALT5	**-1.28027**	**7.265381**	**-4.94492**	**2.13E-05**	**0.014559**	**2.702362**
ACAD9	1.028687	9.191217	4.939047	2.17E-05	0.014559	2.721553
FAM129B	-1.82459	6.275849	-4.92808	2.24E-05	0.014559	2.641816
GLDC	-3.1009	3.79283	-4.86883	2.67E-05	0.016646	2.531486
LOC121110217	-3.1317	-2.05863	-4.81845	3.09E-05	0.016777	1.621568
RD3	-2.72845	3.78282	-4.81668	3.11E-05	0.016777	2.389748
RGS9BP	-1.27872	1.407228	-4.80014	3.26E-05	0.016777	2.200961
SLC5A3	1.737295	4.530192	4.797148	3.29E-05	0.016777	2.312777
LOC112530948	-2.3878	-1.78917	-4.79699	3.29E-05	0.016777	0.90997
DSEL	-2.76318	7.490014	-4.77564	3.51E-05	0.016777	2.230044
CADM1	-0.84667	6.125397	-4.7692	3.57E-05	0.016777	2.180458
FAM89A	-1.0778	4.561443	-4.76399	3.63E-05	0.016777	2.217934
HS3ST1	1.003184	5.307965	4.761771	3.65E-05	0.016777	2.165392
SLC7A10	-3.21643	-2.61326	-4.74529	3.84E-05	0.017107	1.024149
ACSS1A	-2.16425	6.855402	-4.72875	4.03E-05	0.017461	2.084649
LOC416146	0.811272	5.997257	4.707237	4.29E-05	0.017868	2.002663
TSKU	-1.06877	7.207103	-4.7025	4.35E-05	0.017868	2.021299
VDR	-0.90215	6.981558	-4.65631	4.98E-05	0.019492	1.877457

LARGE2	-0.86973	5.532378	-4.65539	4.99E-05	0.019492	1.866757
TAT	-1.53279	1.084003	-4.59804	5.91E-05	0.021929	1.666902
ANKRD27	-0.64631	4.972605	-4.59059	6.04E-05	0.021929	1.701483
CPNE2	-0.90211	6.484004	-4.59052	6.04E-05	0.021929	1.685066
PNPO	0.870375	4.527595	4.571944	6.38E-05	0.022283	1.682946
LOC121109149	2.290879	-1.59863	4.567788	6.46E-05	0.022283	0.301084
LOC769232	1.170262	5.270412	4.561983	6.57E-05	0.022283	1.619037
ABCA1	-1.1996	5.869936	-4.5261	7.29E-05	0.023351	1.506605
PANK1	0.944719	6.250202	4.518596	7.45E-05	0.023351	1.482902
SAMD4A	-0.80561	5.154103	-4.51678	7.49E-05	0.023351	1.494209
GPR55	1.112383	3.487129	4.510928	7.62E-05	0.023351	1.568147
CORO2A	-0.7986	8.291775	-4.49751	7.93E-05	0.023351	1.488138
CCR2	1.065025	4.069838	4.493872	8.01E-05	0.023351	1.497505
ADRA2A	-1.35967	5.491529	-4.49116	8.07E-05	0.023351	1.416912
HECTD1	0.645258	6.9188	4.491071	8.08E-05	0.023351	1.41423
DNM1	-1.13386	5.600968	-4.47595	8.44E-05	0.02396	1.374058
FGB	2.828289	3.236174	4.456277	8.94E-05	0.024856	1.422544
CHST4	-1.47061	5.578165	-4.4487	9.14E-05	0.024856	1.297562
TBC1D30	-0.67826	5.124622	-4.44487	9.24E-05	0.024856	1.293585
SLC43A2	-1.24906	8.056749	-4.43446	9.53E-05	0.024856	1.313738
LOC121107630	-1.85557	-1.18969	-4.4332	9.56E-05	0.024856	0.582879
ENPP7	-2.45942	10.00192	-4.4278	9.71E-05	0.024856	1.341193
PGRMC2	0.997437	5.462412	4.413078	0.000101	0.025525	1.187855

TEK	-1.55846	0.398077	-4.38417	0.00011	0.027205	0.905352
TBL2	-0.62453	5.53694	-4.38022	0.000112	0.027205	1.097331
SPTSSB	-1.26598	3.214009	-4.3715	0.000114	0.027473	1.198272
DDX52	0.910514	5.064626	4.365664	0.000116	0.027519	1.07071
SLC2A9	-1.07546	4.407417	-4.33266	0.000128	0.02921	1.010456
KIFC2	-1.49997	4.206303	-4.32634	0.00013	0.02921	1.049253
SNX8	-1.00117	4.261642	-4.32053	0.000133	0.02921	1.009906
OSBP2	-0.90695	4.071078	-4.32018	0.000133	0.02921	1.017465
DZIP1	-1.09811	4.416088	-4.31991	0.000133	0.02921	1.022633
RRBP1	-0.82034	7.241992	-4.31042	0.000137	0.029607	0.941198
GPR137B	1.214067	3.984289	4.301719	0.00014	0.029663	0.957337
ATP6V1H	-0.6694	4.722055	-4.30031	0.000141	0.029663	0.920606
SLC29A1	-0.99647	2.776889	-4.28287	0.000148	0.030286	0.966747
SLC35A5	0.942927	6.429241	4.28045	0.000149	0.030286	0.828015
RRM2B	-0.5155	6.071564	-4.27938	0.000149	0.030286	0.816436
SEBOX	-5.29582	-0.57864	-4.27244	0.000152	0.030504	0.341193
CHKA	1.60516	7.535445	4.260356	0.000158	0.030632	0.81109
SCN3B	1.903456	5.209813	4.259432	0.000158	0.030632	0.813436
CAAP1	0.680213	6.206104	4.257922	0.000159	0.030632	0.756936
LOC107052962	-1.68511	2.106943	-4.24427	0.000165	0.03134	0.802937
UNC13B	-0.68802	3.806498	-4.2409	0.000167	0.03134	0.831262
MAOB	-1.48898	7.835762	-4.23741	0.000169	0.03134	0.731376
CAPN8	-0.84805	5.820485	-4.19368	0.000191	0.035131	0.591198

GPER1	1.074825	2.99473	4.160881	0.00021	0.03801	0.6497
LIMS1	-0.47607	5.179672	-4.15587	0.000213	0.03801	0.505651
FHIP2A	0.79444	5.748389	4.153107	0.000215	0.03801	0.47073
ECI2	0.758054	7.432144	4.148239	0.000218	0.03801	0.502739
ABCC10	-0.80608	7.066661	-4.14639	0.000219	0.03801	0.473202
CYP2D6	-0.91425	9.718704	-4.13167	0.000229	0.038596	0.539247
LOC107054293	-2.10983	1.097366	-4.12529	0.000233	0.038596	0.551427
SLC16A9	-0.75933	6.824568	-4.12264	0.000235	0.038596	0.402486
IFNAR1	0.626826	5.247799	4.120372	0.000236	0.038596	0.386491
MLXIPL	-1.24022	5.242238	-4.1186	0.000237	0.038596	0.408123
ETNPPL	-2.66859	7.190439	-4.11857	0.000237	0.038596	0.416331
ALDH2	-0.74525	8.618996	-4.10936	0.000244	0.03864	0.429897
FHL3	-0.58112	4.599535	-4.10917	0.000244	0.03864	0.406148
TLR21	-0.83373	4.093116	-4.10703	0.000245	0.03864	0.443147
STXBP1	-0.69427	5.438624	-4.09945	0.000251	0.03864	0.338666
LONRF2	0.660355	5.759848	4.098424	0.000251	0.03864	0.321263
NWD2	-2.56635	0.883702	-4.09701	0.000252	0.03864	0.179649
GLB1	0.930716	6.337675	4.090393	0.000257	0.038908	0.309926
CCR8	1.063567	1.836572	4.086869	0.00026	0.038908	0.436529
TA3	-0.57009	4.548357	-4.0816	0.000264	0.038908	0.348839
LOC124417228	1.621125	-2.06406	4.080153	0.000265	0.038908	-0.59916
HIST1H2B5L	0.922935	2.4412	4.077866	0.000267	0.038908	0.43726
SYAP1	0.561439	6.165352	4.074427	0.000269	0.038929	0.255878

LOC107054880	-1.32776	6.809229	-4.0556	0.000284	0.040704	0.223012
FAM83C	-0.77118	2.341667	-4.04328	0.000294	0.041732	0.345438
UHRF1BP1L	0.685193	5.265504	4.04049	0.000297	0.041732	0.177531
JADE2	0.985438	4.148913	4.030231	0.000306	0.042588	0.243078
RAB18	0.526157	6.576129	4.023984	0.000311	0.042758	0.126087
ESAM	-2.81194	-2.71043	-4.02263	0.000312	0.042758	-1.0728
UNC93A	-0.76936	6.869219	-4.01491	0.000319	0.043329	0.113717
LOC124417710	-2.88738	-0.2729	-4.00041	0.000333	0.044765	-0.18584
NR1H4	-0.63777	5.20232	-3.99212	0.000341	0.045442	0.0454
CD151	-0.59971	7.47114	-3.9865	0.000346	0.045723	0.06469
ITSN1	0.539535	6.102312	3.983993	0.000349	0.045723	0.012334
CTC1	0.732323	3.150377	3.975946	0.000357	0.046391	0.165339
LOC107052974	-1.40918	-1.50439	-3.9495	0.000384	0.049596	-0.2791
VKORC1L1	1.086321	6.249882	3.944612	0.00039	0.049756	-0.0773
PEX11A	1.856585	5.536034	3.942584	0.000392	0.049756	-0.08239
CD38	0.982999	3.569772	3.933505	0.000402	0.050641	0.015296
HSD17B12	0.935376	6.738778	3.913651	0.000425	0.05307	-0.1581
APPL2	-0.85281	6.298535	-3.90943	0.000431	0.05307	-0.17958
HCCS	-0.47747	5.772095	-3.90851	0.000432	0.05307	-0.18772
ECE1	0.896231	5.163556	3.904438	0.000437	0.053152	-0.17523
ONECUT1	-1.73241	0.422724	-3.90244	0.000439	0.053152	-0.06548
LOC121109899	3.019375	-3.90463	3.889471	0.000456	0.05379	-1.5165
LOC101749468	-2.68464	-3.59364	-3.88772	0.000458	0.05379	-1.79635

CPT1A	1.373761	6.960243	3.886156	0.00046	0.05379	-0.21394
LARP4B	0.694225	7.174889	3.880266	0.000468	0.05379	-0.22487
VEGFA	-0.8242	4.556918	-3.87901	0.000469	0.05379	-0.22964
MAPK8IP1	-1.968	1.417966	-3.87901	0.000469	0.05379	-0.13347
MPPED2	-0.95209	1.430018	-3.87771	0.000471	0.05379	-0.12709
TMEM50B	0.446513	6.757163	3.874058	0.000476	0.05379	-0.27027
FAM117B	0.770228	5.825537	3.872148	0.000478	0.05379	-0.28591
LRFN5	-4.05384	3.842631	-3.87179	0.000479	0.05379	-0.10115
LOC101749147	-2.33911	0.026883	-3.86843	0.000483	0.053913	-0.36079
SEC22C	1.085717	7.365985	3.858639	0.000497	0.054459	-0.25956
TMEM243	0.961355	4.952899	3.856103	0.000501	0.054459	-0.30592
LOC121110204	2.063312	-1.93618	3.856092	0.000501	0.054459	-0.92402
C12orf65	0.538371	3.541362	3.854871	0.000502	0.054459	-0.16007
SORD	-0.75162	8.588788	-3.8454	0.000516	0.055171	-0.27375
LOC107048990	0.622485	6.446433	3.845362	0.000516	0.055171	-0.35667
SLC16A6	-1.09096	6.657129	-3.84266	0.00052	0.055215	-0.3387
CD8A	1.062331	4.45001	3.839581	0.000524	0.055318	-0.26549
RCAN1	-0.61194	5.147968	-3.83407	0.000533	0.055807	-0.36876
FAM107A	-0.81332	4.703727	-3.83079	0.000538	0.055873	-0.32848
TRBV6-5	1.192326	4.26622	3.828904	0.00054	0.055873	-0.28188
ACSBG2	-0.76599	5.197115	-3.82587	0.000545	0.055982	-0.36599
PGS1	0.399994	5.157289	3.823387	0.000549	0.056005	-0.39755
BTG1	0.514058	8.650777	3.819172	0.000555	0.056214	-0.33477

IRX6	-4.10487	-4.29929	-3.81744	0.000558	0.056214	-1.76666
MYBPC1	3.375608	-3.88307	3.808124	0.000573	0.056982	-1.17549
SLC13A3	-2.08352	-0.78383	-3.80596	0.000576	0.056982	-0.61473
MRPS6	1.180226	5.486193	3.805783	0.000577	0.056982	-0.44953
CA9	-1.05314	4.383349	-3.80323	0.000581	0.057032	-0.38633
PRKX	0.7594	5.274675	3.795321	0.000594	0.057947	-0.45007
KLHL25	-0.7765	3.831682	-3.79068	0.000602	0.058341	-0.37894
DPYSL2	-0.69023	5.75947	-3.78777	0.000607	0.058456	-0.50191
CREBRF	1.073972	4.743179	3.775898	0.000627	0.059602	-0.45736
VAV2	-0.83872	5.760994	-3.77511	0.000628	0.059602	-0.53099
OMA1	-0.51054	4.758144	-3.77349	0.000631	0.059602	-0.47644
SLCO3A1	1.141917	4.855109	3.772137	0.000634	0.059602	-0.52877
DENND1B	0.453363	5.922386	3.767059	0.000643	0.060093	-0.56579
SPSB3	0.531718	4.576912	3.764022	0.000648	0.060246	-0.49059
TMEM218	1.867635	-2.11629	3.758424	0.000659	0.060641	-1.26234
MNT	0.798109	4.130526	3.757454	0.00066	0.060641	-0.46733
FBXO32	1.58755	5.946252	3.753636	0.000667	0.060933	-0.57912
PLA2G10L	-0.79102	3.709549	-3.74492	0.000684	0.062074	-0.45285
NUS1	0.513075	6.505347	3.741802	0.00069	0.062254	-0.62094
HADHB	1.077971	7.996429	3.736447	0.0007	0.062591	-0.53973
HERPUD2	0.431659	7.240123	3.735754	0.000702	0.062591	-0.62348
PLOD2	-0.70178	6.003081	-3.73273	0.000708	0.062763	-0.65056
ITK	0.74959	4.620705	3.729085	0.000715	0.063047	-0.59578

LOC121107327	-1.85061	-1.34954	-3.72672	0.00072	0.063107	-0.76468
LOC418114	-1.59355	-1.35057	-3.72189	0.000729	0.063364	-0.83815
PLCB4	-0.79625	3.80589	-3.72126	0.000731	0.063364	-0.52587
USP54	-0.42693	5.857177	-3.71613	0.000741	0.063921	-0.69685
KRT80	-1.31351	3.41543	-3.71199	0.00075	0.064303	-0.49889
SLC7A9	-1.06705	8.477034	-3.71006	0.000754	0.064303	-0.63051
LPL	-1.48887	4.963526	-3.70774	0.000759	0.064367	-0.61327
TMPPE	0.959269	7.0646	3.6955	0.000785	0.065621	-0.71432
SLAIN2	-0.43519	6.114835	-3.69511	0.000786	0.065621	-0.75201
TBCA	0.496638	5.505755	3.693555	0.000789	0.065621	-0.75186
RBFOX3	-2.07595	-2.27461	-3.69308	0.00079	0.065621	-1.21404
LOC417937	-0.79572	8.126191	-3.68938	0.000798	0.065951	-0.68594
GGT7	-2.05814	-1.33647	-3.67978	0.00082	0.067377	-1.13107
PDGFB	-0.96206	0.991047	-3.67409	0.000833	0.068093	-0.63741
BEND4	2.930668	1.201855	3.66861	0.000846	0.068777	-0.61352
FAM174B	-0.43419	5.540843	-3.66544	0.000853	0.068806	-0.81919
ELMO3	0.514324	6.764738	3.664724	0.000855	0.068806	-0.81429
COL25A1	-2.76288	-2.82466	-3.65987	0.000867	0.069186	-1.5377
TBC1D23	0.668322	6.576545	3.657645	0.000872	0.069186	-0.84038
CBLB	0.495903	5.877641	3.655895	0.000876	0.069186	-0.85529
ZNF704	-0.89251	3.879751	-3.65383	0.000881	0.069186	-0.7002
HADHA	0.94739	8.709117	3.653418	0.000882	0.069186	-0.72476
ATP2A3	-0.61572	7.570559	-3.65176	0.000886	0.069186	-0.81784

TGIF1	1.52634	3.031137	3.642152	0.00091	0.0707	-0.64679
CABCOCO1	2.4586	-3.26879	3.637404	0.000922	0.071186	-1.52146
VWA5A2	-2.1213	-2.69145	-3.63476	0.000929	0.071186	-1.16915
PUM1	0.382181	6.765565	3.634325	0.00093	0.071186	-0.89857
HMGXB4	-0.5459	4.293332	-3.62314	0.000959	0.072815	-0.83831
RETSAT	1.436805	8.123576	3.622623	0.000961	0.072815	-0.82238
DHRS7	0.919691	8.083706	3.616359	0.000978	0.073729	-0.86513
TOR3A	1.052882	4.280488	3.61287	0.000987	0.074085	-0.87137
CDR2L	-0.8956	1.305405	-3.60719	0.001003	0.074513	-0.75429
SERPINE2	1.138844	7.540498	3.606762	0.001004	0.074513	-0.92224
LIN7C	0.336077	6.729233	3.604895	0.001009	0.074513	-0.9704
LOC107053813	-1.70001	0.673698	-3.60389	0.001012	0.074513	-0.82301
P2RX1	-1.12747	5.221793	-3.59172	0.001046	0.076694	-1.00208
CYP2AC2	-1.16009	3.245513	-3.58634	0.001062	0.077437	-0.81178
DGKH	0.788821	4.566895	3.582442	0.001073	0.077437	-0.98781
DDO	-1.96202	3.43943	-3.58243	0.001074	0.077437	-0.85177
KAT14	-0.3316	5.411973	-3.58148	0.001076	0.077437	-1.02992
FGFBP1	1.783859	5.349016	3.579328	0.001083	0.077539	-1.00506
GDPD2	-0.96607	5.478224	-3.57753	0.001088	0.077568	-1.04286
NFATC3	0.342696	7.545721	3.569752	0.001112	0.078832	-1.03701
MOSPD1	0.514874	6.112878	3.568359	0.001116	0.078832	-1.08113
RGS16	-1.67556	1.551162	-3.56651	0.001122	0.078876	-0.83642
GPX4	1.091256	5.732974	3.562076	0.001135	0.079487	-1.09285

FFAR4	-1.28384	2.590667	-3.55229	0.001166	0.080745	-0.86194
DOCK2	0.784125	4.475676	3.55172	0.001168	0.080745	-1.03469
SLC39A8	2.908478	0.891385	3.551502	0.001169	0.080745	-0.87743
LOC121110159	-1.05184	2.316173	-3.54738	0.001182	0.081304	-0.87768
LOC107052763	2.056653	0.830234	3.540997	0.001203	0.082379	-0.99225
ABCD3	0.865415	9.023633	3.537973	0.001213	0.082702	-1.01192
DPP4	-0.66848	9.473236	-3.536	0.00122	0.082789	-1.00711
ARL5A	0.76155	6.793502	3.52716	0.00125	0.084454	-1.1564
MYCL	2.132533	0.987126	3.523549	0.001262	0.084749	-0.93092
LOC776275	-0.78257	2.085515	-3.52274	0.001265	0.084749	-0.93016
LOC107052686	-1.99849	-3.14589	-3.51832	0.00128	0.085414	-1.66418
CAPN9	-0.72825	5.386651	-3.513	0.001299	0.086288	-1.19934
USP2	-1.27285	3.955373	-3.51138	0.001305	0.086288	-1.11579
MPHOSPH9	0.923658	2.446291	3.509952	0.00131	0.086288	-0.96129
CRCP	1.432123	4.251673	3.506777	0.001321	0.086673	-1.166
SLA	1.072529	2.691073	3.505252	0.001327	0.086673	-0.98776
DLG3	-0.58601	6.600371	-3.50174	0.00134	0.086846	-1.24338
LOC107053690	-2.86524	0.421007	-3.50061	0.001344	0.086846	-1.08701
IQSEC1	0.591553	5.910316	3.498526	0.001351	0.086846	-1.26184
TRUB1	0.690402	4.504282	3.498455	0.001352	0.086846	-1.2154
C2CD3	-0.54131	2.566973	-3.49524	0.001364	0.087253	-1.02307
RBP2	1.135552	10.51519	3.492335	0.001375	0.087558	-1.05651
IFNAR2	0.44439	6.510218	3.489832	0.001384	0.087558	-1.27671

DCUN1D1	0.407711	6.76288	3.489487	0.001385	0.087558	-1.25972
TRIM23	0.67375	3.556129	3.484107	0.001406	0.08818	-1.10097
RAB7L1	0.502378	4.763189	3.48165	0.001415	0.08818	-1.26456
SUSD2	-1.31756	7.572648	-3.48154	0.001416	0.08818	-1.23757
RNH1	0.669765	4.459682	3.477634	0.001431	0.08818	-1.2401
ASAH2	-0.79039	8.698294	-3.47747	0.001431	0.08818	-1.19276
P2RX4	-0.49083	7.272309	-3.47729	0.001432	0.08818	-1.27416
SDC1	-0.79537	5.868826	-3.47571	0.001438	0.08818	-1.30394
PLCB1	-0.79862	3.65746	-3.47521	0.00144	0.08818	-1.12832
DFNA5	-2.64526	3.107589	-3.4722	0.001452	0.088241	-1.05262
RASGEF1C	0.506001	6.715764	3.472092	0.001453	0.088241	-1.31555
CAMK1	-0.69845	2.360988	-3.46564	0.001478	0.089203	-1.07087
GPR146	1.167017	2.614146	3.463862	0.001486	0.089203	-1.09222
KCNV1	-1.91037	-2.90026	-3.46385	0.001486	0.089203	-1.90146
APLN	-2.18989	-3.18836	-3.45742	0.001512	0.089745	-1.94494
LOC101750362	-1.06111	1.935455	-3.45676	0.001515	0.089745	-1.08554
MOB1A	0.419591	5.666957	3.456582	0.001515	0.089745	-1.36702
AK2	0.571274	7.688474	3.456022	0.001518	0.089745	-1.30214
CNDP2	-0.87172	8.832117	-3.44548	0.001562	0.092008	-1.26774
LOC124417089	0.842661	4.131914	3.442467	0.001575	0.092229	-1.23563
N4BP1	0.648375	6.489256	3.440635	0.001582	0.092229	-1.40306
FBXO40	-0.85995	0.193868	-3.43952	0.001587	0.092229	-1.18113
MFSD4A	-0.97402	3.255503	-3.43908	0.001589	0.092229	-1.19129

JMJD7	-0.95599	0.716227	-3.43597	0.001603	0.092474	-1.19784
RALGPS2	0.939668	3.559221	3.435377	0.001605	0.092474	-1.25238
OXR1	0.45205	4.536535	3.432554	0.001618	0.092653	-1.34434
SMAD2	-0.34377	4.865054	-3.43149	0.001622	0.092653	-1.37191
LOC121107312	0.965828	2.079608	3.430508	0.001627	0.092653	-1.15238
C11orf24	0.886101	5.939303	3.429267	0.001632	0.092653	-1.43506
TOMM20	0.432484	6.676237	3.42628	0.001645	0.093069	-1.43514
TBC1D15	0.402598	5.645818	3.423194	0.001659	0.093513	-1.4477
CTIF	-0.54047	5.158656	-3.4203	0.001672	0.093911	-1.4378
FUT8	-0.49315	7.182445	-3.40777	0.00173	0.096608	-1.46082
MTFR1	0.394995	6.25205	3.407205	0.001733	0.096608	-1.49028
SUCO	0.844812	5.956989	3.405648	0.00174	0.09667	-1.49117
TTYH3	-0.55862	6.476802	-3.39846	0.001774	0.097517	-1.50911
FAXDC2	-1.88058	7.566544	-3.39729	0.00178	0.097517	-1.41667
COG3	0.352116	5.602667	3.39492	0.001791	0.097517	-1.51983
PEX3	0.716881	5.328337	3.394799	0.001792	0.097517	-1.5131
LOC101750931	-1.56737	-1.63194	-3.39443	0.001794	0.097517	-1.66891
LOC107054262	-1.41507	1.510283	-3.39428	0.001794	0.097517	-1.23069
KLHL20	-0.4373	4.964565	-3.39333	0.001799	0.097517	-1.49684
NTAN1	-0.44252	4.901466	-3.39101	0.00181	0.097792	-1.47943
IFNG	2.084916	-1.5999	3.38734	0.001828	0.098342	-1.61855
PTPN12	0.495521	5.839582	3.386378	0.001833	0.098342	-1.54626
LOC101749173	1.441574	-1.02219	3.379885	0.001865	0.09945	-1.52237

G6PC	-1.21563	3.77388	-3.37938	0.001868	0.09945	-1.51476
KCNAB2	0.82214	1.219078	3.378433	0.001873	0.09945	-1.27297
LOC124417388	-2.43909	-1.20778	-3.37593	0.001886	0.099602	-1.39045
LOC107054976	4.531749	-2.89459	3.375355	0.001888	0.099602	-1.89619
RBSN	-0.4381	6.108237	-3.37387	0.001896	0.099664	-1.57539

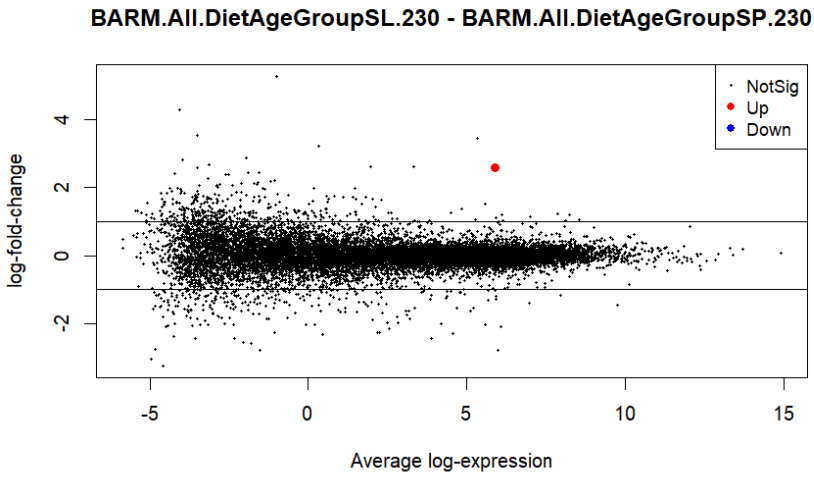

Figure S4. Differentially expressed gene (Benjamini-Hochberg adjusted $P < 0.1$) comparing diet groups SL and SP (testing for suppressive effect of lipid) in adult chickens. The 1 upmodulated gene by lipid are highlighted.

Table S4. Differentially expressed gene (1 upmodulated and 0 downmodulated; Benjamini-Hochberg adjusted $P < 0.1$) comparing diet groups SP and SL (testing for suppression by lipid) in adult chickens. Log-2 fold change (logFC), Average expression (AveExpr), *t*-statistic (t), raw *P*-value (P.Value), Benjamini-Hochberg adusted P-value based on (adj.P.Val), and Bayes log-odds of differential expression (B) were provided by TopTable of limma Voom. *B4GALT5* (bold) was the only suppressed gene detectable among the genes of interest.

	logFC	AveExpr	t	P.Value	adj.P.Val	B
BBOX1	2.575309	5.897747	6.007947	9.14E-07	0.01427	5.218471

Ingredient	PL	SL	SP
Starch (%)	0	34	34
Casein (%)	42	16	42
Oil (%)	16	16	4

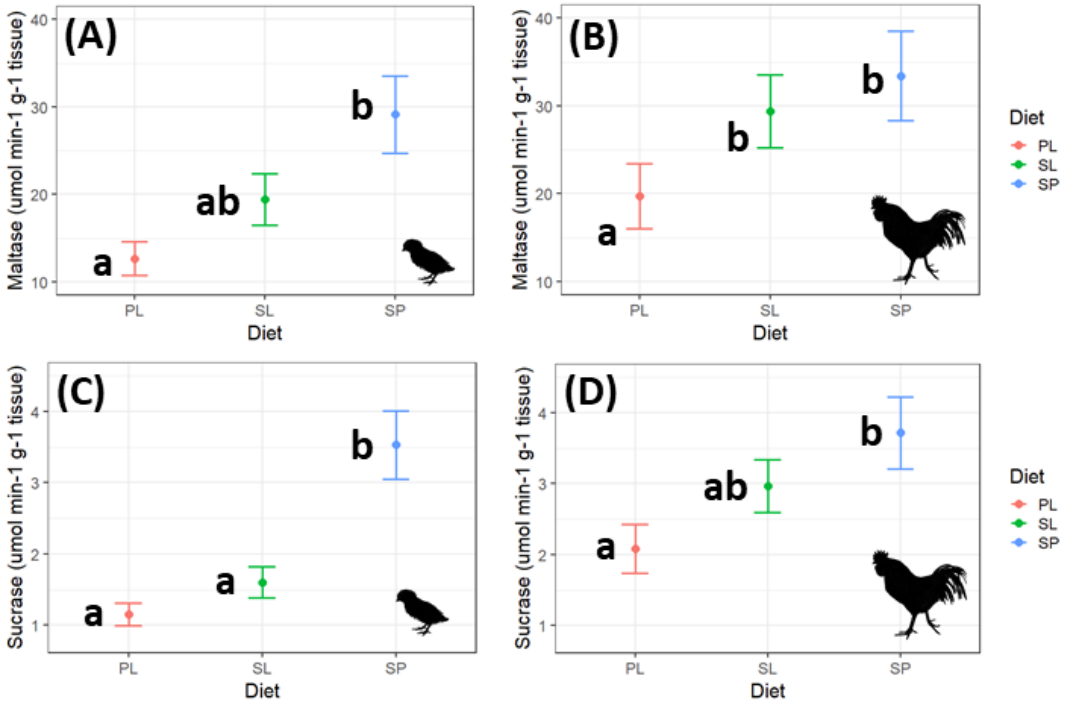

Figure S5. Diet effect on the wet mass-specific small intestinal α-glucosidase (maltase and sucrase) activities of the small intestine of juvenile (A, C; 20 d old; n = 6 per diet) and adult (B, D; 230 d old; n = 4–7 per diet) chickens. Data represent least square means ± s.e.m., and different letters at the means indicate significant differences based on one-tailed post hoc Tukey tests. These tests were performed one-tailed because we expected SL > PL as the inductive effect by dietary starch and SL < SP as the suppressive effect by dietary lipid.

Supplementary Text

Differential expression analysis methods

We performed differential expression analysis using limma Voom functions makeContrasts and contrastFits for detection of genes induced by dietary starch when lipid is held constant (SL – PL; upmodulated = induced) and genes suppressed by increase in dietary starch when starch is held constant (SL – SP; downmodulated = suppressed) in juveniles and adults. Significance was set as $P < 0.1$ for Benjamini-Hochberg adjusted *P*-values as recommended {Benjamini, 1995 #748}.

Differential expression analysis results

Overall, differential expression analyses were unable to detect most of our genes of interest as significantly different in either age group for the inductive effect of starch and suppressive effect of lipid. Among the 6 genes induced by starch, *SLC2A2* was the only gene of interest in juveniles (Figure S1; Table S1), and in adults none of the genes of interest were among the 46 genes induced by starch (Fisgure S2; Table S2). In juveniles, only *B4GALT5* of the genes of interest was among the 158 downmodulated genes detected due to increased lipid in the diet, and no gene was downmodulated by lipid in adults.

Physiology and the co-occurrence of competing carnivores: The diet and digestive enzymes of fishers (*Pekania pennanti*) and martens (*Martes americana* and *Martes caurina*) in the forests of the Great Lakes and the Pacific Northwest

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Abstract

The order Carnivora largely consists of predators of vertebrates, yet many of them retain the ability to consume foods other than meat. The digestive capabilities of such omnivory among carnivorans remains understudied. Fishers (*Pekania pennanti*) and martens (*Martes americana* and *M. caurina*) – two related and competing carnivores in forests across North America – are generally regarded as carnivorous, but some populations consume plants, insects, and fungi. Here, we investigated whether fishers and martens have the capacity to digest these atypical foods by sampling both species from two regions: the Pacific Northwest, where fishers consume a range of diet items and the Great Lakes region, where both species are predominantly meat-eaters. We first demonstrated using zymography-based proteomics that both fishers and martens produce functional trehalase, the enzyme required for digesting trehalose (the principal carbohydrate in fungi and insects). We then tested the hypothesis that fishers and martens would show species-level difference in digestive hydrolase profiles reflecting adaptations to occupying different dietary niches. We estimated individual diet via the analysis of stable isotopes and activities of trehalase, maltase, sucrase, and aminopeptidase-N (APN) in whole intestinal tissue. We found that fishers and martens in the Great Lakes as well as martens in the Pacific Northwest were primarily meat eaters; however, fishers in the Pacific Northwest predominantly consumed fungi and insects. As predicted, we found that the investment in trehalase and maltase, relative to APN, was higher in fishers compared to martens. This species-level difference was consistent between regions; fishers in the Pacific Northwest did not differ in their investment in trehalase or maltase compared to fishers in the Great Lakes. However, indices of diet and digestive hydrolase specialization suggested regional differences, with greater hydrolase specialization in the Pacific Northwest in both species. These findings suggest that while both fishers and martens possess

some ability for diet and digestive flexibility, fishers constitutively produce higher trehalase and maltase activities in their small intestine than martens, which would allow them to have greater spare capacity to digest non-meat dietary items. This difference in digestive capacity may explain why fishers, despite being the dominant competitor, switch diets more readily than martens when they co-occur.

Introduction

What an animal eats drives its ecology and evolution (Hutchinson 1959). Consisting of nearly 300 extant species, the order Carnivora (Class Mammalia) is united by its foraging strategy of predation and consumption of vertebrate meat. These species feature unique adaptations that include carnassial teeth to sheer flesh, short and simple gastrointestinal tracts, and digestive enzymes to break down and digest protein and lipids (Buskirk 2023). Some species of carnivora have, however, evolved to consume other items like insects, fungi, and plant matter (Flynn 1998, Buskirk 2023). Certain lineages, notably the family Ursidae (bears), have evolved to become predominantly omnivorous (e.g., American black bear (*Ursus americanus*) (Landers et al. 1979, Kirby et al. 2017, McLaren et al. 2021, Smith et al. 2025)) or even herbivorous (e.g., giant pandas (*Ailuropoda melaleuca*) (Zhang et al. 2007, Williams et al. 2016, Hu et al. 2017, Wei et al. 2017, Vallittu et al. 2021)).

Few studies have investigated digestive adaptations in other carnivorans that would enable them to consume non-meat items. This is particularly relevant given that digestion of plant, fungal, or insect material require different digestive enzymes compared to meat due to their differences in substrate composition (Supplementary Information Table S1). The digestion of protein in meat typically involves the digestive enzyme aminopeptidase-N (APN) expressed

on the small intestinal brush border membrane of enterocytes, which breaks down peptides produced by gastric and pancreatic proteases (Afik et al. 1995, Karasov and Hume 1997). On the other hand, plant matter is rich in carbohydrates including the refractory cellulose (Table S1). Of the carbohydrates, starch is digested via pancreatic amylase and α-glucosidases (maltase-glucoamylase and sucrase-isomaltase) on the intestinal brush border (Karasov et al. 2011, Brun et al. 2020b). Previous research has shown that the domestication of canids coincided with reliance of dietary starch, leading to the selection on pancreatic amylase (AMY2B) and intestinal glucosidase (MGAM) genes (Axelsson et al. 2013, Pontremoli et al. 2015, Ollivier et al. 2016). While digestion of plant material by carnivores is recognized, it is less understood for other dietary items like fungi and insects. Fungi and invertebrates can be protein-rich, but contain the refractory carbohydrate chitin and the disaccharide trehalose (Table S1), a major energy reserve in fungi and the principal blood sugar in invertebrates (Thompson 2003, Karasov and Martínez del Rio 2007). The hydrolysis of trehalose relies on the brush border enzyme trehalase (Karasov and Martínez del Rio 2007). While some carnivores have retained intact and putatively functional trehalase gene, loss of function of a gene due to mutations (i.e., psuedogenization) appears common in lineages that do not consume fungi or insects (Jiao et al. 2019) as little trehalose is found in plant matter (Grennan 2007) and none in vertebrate meat (Argüelles 2014).

The physiological principle of economical design predicts that the evolution of diet and digestive enzyme activity should be matched to optimize energy and nutrient gain from their diet, and limitations of the brush border membrane surface space may also necessitate such a match (Karasov 1992, Karasov and Hume 1997, Caviedes-Vidal et al. 2000). For example, a close match between diet and intestinal digestive enzyme profiles (trehalase, maltase, sucrase, and APN activities) was demonstrated across 14 species of chiropterans that radiated from a common

insectivorous ancestor to consume nectar, fruit, and blood (Schondube et al. 2001). Economical design also predicts that omnivores are capable of modulating their digestive enzyme profiles to match their current diet and have space capacities in their enzyme profile (producing digestive hydrolases in excess) in preparation for a future diet shift (Karasov et al. 2011). Both modulation and spare capacity have been shown in the omnivorous domestic chickens which would modulate maltase and sucrase activities but also express these enzyme activities under a carbohydrate-free diet (Biviano et al. 1993, Oguchi et al. 2022). As we gain a greater appreciation of the degree of omnivory among carnivorans (Smith et al. 2022, Buskirk 2023), exploring the match between diet and digestive enzymes in carnivorans will advance our knowledge in their nutritional niche and provide physiological mechanisms underpinning their foraging strategies and interspecific interactions.

Fishers (*Pekania pennanti*) and martens (*Martes* spp.) are small carnivores that co-occur across temperate and boreal forests of North America. Their relative body sizes difference (Fishers being 2-5x larger in mass) combined with large spatiotemporal and dietary overlap leads to particularly strong competitive interactions between these two carnivorans (Palomares and Caro 1999, Donadio and Buskirk 2006, Pauli et al. 2022). As some populations of martens in the Great Lakes region and both species in the Pacific Northwest are endangered, understanding their competition and any niche partitioning between fishers and martens has become a priority for conservation (Pauli et al. 2022, Smith et al. 2022, Smith et al. 2023). In the Great Lakes, fishers and martens are both predominantly carnivorous while consuming different vertebrate prey: fishers primarily consume larger and more varied prey species, while martens typically consume small bodied rodent prey (Manlick et al. 2017, Kirby et al. 2018, Smith et al. 2023). However, fishers in the Pacific Northwest also consume large quantities of fungi and insects and

comparatively smaller amounts of meat than in the Great Lakes (Table S2) (Smith et al. 2022, Kuntze et al. 2024, Smith et al. 2024). This observation begs the question on the means by which fishers utilize trehalose of fungi and insects as a source of nutrition (Smith et al. 2022). It is conceivable that fishers produce endogenous trehalase given the short gut retention time (<10 hours (De Cuyper et al. 2020)) and that functional trehalase seems ancestral to mammals and to Carnivora (Jiao et al. 2019). However, it is difficult to predict whether fishers (or martens) produce endogenous trehalase because trehalase loss (pseudogenization) is common and widespread among non-insectivorous carnivorans (Jiao et al. 2019).

Here, we investigated whether fishers and martens were endogenously expressing functional trehalase on the brush border membrane. We then tested the hypothesis that there would be species-level difference in digestive hydrolase profiles of trehalase, maltase, sucrase, and aminopeptidase-N (APN) activities reflecting adaptations through evolutionary history to different dietary niches between fishers and martens. Specifically, we predicted that aminopeptidase-N would be similar across both species and populations because all diets contain protein (as in bats of Schondube et al. 2001) and all individuals would still be carnivorous to varying extent. We further predicted that fishers, the more omnivorous of the two, would exhibit higher trehalase and maltase activities than martens, given that fungi and insects contain both trehalose and animal starch, but not sucrase, given that sucrose is specific to plants (Table S1). For fishers, we tested two competing hypotheses on their mode of digestive flexibility: adaptive modulation and constitutive expression (phylogenetic inertia) (Karasov et al. 2011). If fishers have individual foraging strategies and modulate their digestive enzyme profiles to match the diet, we would observe higher trehalase and maltase activity in fishers of the Pacific Northwest compared to fishers of the Great Lakes (adaptive modulation) (Karasov et al. 2011).

Alternatively, if fishers have adapted to an omnivorous lifestyle in their ancestry and the cost of evolving adaptive modulation exceeds that of the cost of maintaining digestive spare capacity for fungi and insects (i.e., producing digestive enzymes in excess of the current diet for prospective diet switch) (Karasov et al. 2011), then we would observe no regional difference in trehalase or maltase activity. In order to test these hypotheses we estimated the diets of the fisher and marten individuals through stable isotope analyses and measured the small intestinal trehalase, maltase, sucrase, and APN activities of the same individuals.

Methods

Sampling locations

We sampled fishers (n = 12) and American martens (n = 13) trapped from northern Minnesota (Great Lakes region) during the 2021 and 2022 winter trapping season. Specifically, we collected the gastrointestinal tract and hair (fur) from frozen carcasses for analysis. In addition, we sampled the gastrointestinal tract, blood, and hair (whiskers) from achieved samples of fishers (n = 6) and Pacific martens (*Martes caurina*: n = 6) from the Sierra Nevada Mountain Range in California (Pacific Northwest). We sampled approximately 13 cm of small intestine and large intestine for each individual in the Pacific Northwest. Due to the unrecorded location of the small intestinal piece in the overall gastrointestinal tract for the Pacific Northwest samples, we treated this section as the representative for the whole small intestine.

Trehalase protein identification

Protein identification was conducted in proximal small intestine samples generally following Brun et al. (2020a) and briefly described herein (detailed protocols are in the Supplementary

Materials). To test for endogenous trehalase activity in the proximal small intestine, we collected enterocytes and isolated brush border membranes from martens and fishers from Minnesota. The small intestine was divided longitudinally, with one half used for enterocyte collection and the other for trehalase activity assays. Enterocytes were scraped from the intestinal lining, rinsed in phosphate-buffered saline, and stored at -80°C. For trehalase activity, tissue was homogenized in mannitol buffer using a tissue homogenizer with pulse cycles to prevent overheating. Enterocytes were further homogenized in buffer and subjected to differential centrifugation to isolate brush border membranes using a sucrose gradient (McConnell et al. 2011). The membranes were washed and suspended in mannitol buffer before storage at -80°C. Protein concentration in the samples was determined using a Bradford assay, and trehalase activity was measured via enzymatic assays in triplicate. Samples with high protein concentrations and detectable trehalase activity were selected for further analysis.

Trehalase activity was confirmed using non-denaturing gel electrophoresis (Brun et al. 2020a). Proteins were solubilized with native sample buffer, loaded onto a 12% Tris-Acetate SDS-PAGE gel, and run at 100V for two hours. The gel was incubated with trehalose solution to visualize activity as orange-brown staining, which was excised for in-gel digestion and peptide extraction. Peptides were analyzed using nanoLC-MS/MS on a hybrid linear ion trap-Orbitrap mass spectrometer. Chromatography was performed with a capillary emitter column, employing a gradual acetonitrile gradient over 80 minutes. Peptides were fragmented using CID-MS/MS, with monoisotopic precursor selection and charge state screening enabled. Data were acquired in the Orbitrap with high resolution, and the results were used to identify proteins associated with trehalase activity (Brun et al. 2020a).

Raw MS/MS data were converted to the mgf file format using MSConvert (ProteoWizard). The resulting mgf files were searched against the UniProt database for Mustelidae amino acid sequences (178,659 protein entries) and a list of common laboratory contaminants (172 entries) using the Mascot search engine (version 2.7.0, Matrix Science). Variable modifications included methionine oxidation and deamidation of asparagine and glutamine, with carbamidomethylation of cysteine as a fixed modification. Peptide mass tolerance was set at 10 ppm, and fragment mass tolerance at 0.6 Da. Protein annotations, statistical validation, and spectral-based quantification were conducted using Scaffold software (version 4.11.0, Proteome Software Inc.). Peptide identifications were accepted at >80.0% probability (Scaffold Local FDR), while protein identifications required >95.0% probability and at least two peptides (Protein Prophet algorithm). Proteins sharing significant peptide evidence were grouped into clusters based on parsimony principles.

We used the American mink (*Neogale vison*) amino acid sequence derived from its genome RefSeq (NCBI: ASM_NN_V1) for trehalase identification. To support the use of this sequence for martens and fishers, we searched and downloaded all available trehalase amino acid sequences belonging to Mustelidae in the NCBI database (Sayers et al. 2022) and used rats (*Rattus norvegicus*) as an outgroup. We then aligned the amino acid sequences using MAFFT (Katoh et al. 2002) from Jalview software version 2.11.3.3 (Waterhouse et al. 2009) with the default setup. The alignment analysis revealed high conservation, quality, and consensus of all these trehalase sequences, allowing us to infer with high probability that trehalase in fishers and martens can be identified from the protein RefSeq chosen. Additionally, we analyzed the jpg-gel images using ImageJ software (Schneider et al. 2012) to detect the color produced by the trehalase-specific reaction. Our workflow included splitting color channels and selecting the blue

channel for analysis. Using the area selection tool, we drew parallel rectangles along each sample lane axis and produced a profile plot of the pixel intensities within the selected area.

Diet estimation

We quantified the proportional diets via the analysis of stable isotopes for each species and study area. Analysis of stable isotopes was conducted using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Results are expressed in parts per mil (‰) relative to the international standards of Peedee Belemnite ($\delta^{13}C$) and atmospheric nitrogen ($\delta^{15}N$) calibrated with internal laboratory standards. From animals sampled in the Great Lakes, we analyzed hair from 11 martens and 8 fishers. These hair samples represent the assimilated diets from late summer into fall (July to October; (Pauli et al. 2009)). From animals sampled in the Pacific Northwest, we analyzed hair from 6 martens and 6 fishers. The Pacific Northwest samples were collected from various seasons and, therefore, represent different three-month time periods of the year. We estimated the proportional diet using $\delta^{13}C$ and $\delta^{15}N$ stable isotopes with a Bayesian isotopic mixing model in the R (R Core Team 2024) package *MixSIAR* (Stock et al. 2018). We applied a tissue-specific trophic discrimination factors estimated using the R (R Core Team 2024) package *SIDER* ($\delta^{13}C$ = 2.3; $\delta^{15}N$ = 3.7; (Healy et al. 2018). We used published isotopic values of diet sources for the individuals in California (Smith et al. 2022) and Minnesota (Kirby et al. 2017, Smith et al. 2023) for martens and fishers (Figure S2). Using location specific mixing spaces, we estimated proportional dietary inputs using a 'generalist prior' and by running 3 chains of 300,000 iterations, removed the first 200,000 iterations as burn-in, and then thinned posterior samples to every 100th sample (Stock et al. 2018). We assessed model convergence with trace plots and

Gelman-Rubin statistics ($R\ hat \leq 1.01$; (Gelman and Rubin 1992)). To make comparisons between locations and species, we *a posteriori* combined sources (i.e., prey) of the Bayesian mixing model using *MixSIAR* (Stock et al. 2018) into the consistent dietary groups across locations. We compared three functional dietary groups shared between locations that included plants, fungi and insects, and meat (vertebrate prey).

Digestive hydrolase activities of intestinal tissues

We divided the thawed small intestine of martens and fishers from the Great Lakes into three sections of identical length: proximal (first third), medial (second third) and distal (remaining third). We cut a small piece (~1cm) from the middle of each section, four in total per individual; proximal small intestine., medial small intestine, distal small intestine, and large intestine. Similarly, we cut a small piece (~1cm) from the middle of the small intestine and large intestine (two in total per individual) for individuals from the Pacific Northwest. We then rinsed the pieces of intestine with phosphate buffered saline and blotted dry. We weighed each piece of intestine and added 10mL of mannitol homogenization buffer (hereafter, mannitol buffer; 1:11 w/v, 300mM mannitol in 1mM Hepes-KOH, pH 6.5) per gram of intestinal tissue. We then homogenized the pieces on ice three times for 30 seconds on, 30 seconds off and placed in a -80°C freezer for storage (Brzęk et al. 2013).

We performed enzymatic assays on these samples to determine levels of trehalase activity, maltase activity, sucrase activity, and APN activity, as previously described (Schondube et al. 2001). We first diluted the homogenized tissue with mannitol buffer dependent on the assay type: trehalase (1:50), maltase (1:250), and sucrase (1:10). We did not dilute homogenized tissue for the APN assays. We followed similar procedures for trehalase, maltase, and sucrase assays, after

the tissue had been diluted by assay type. We added 30µl of 56mM sugar (trehalose, maltose, or sucrose) in 0.1M maleate-NaOH buffer (pH 6.5) to 30µl of diluted tissue homogenate at 37°C for 20 minutes. After 20 minutes, we added 400µl of stop-develop reagent (Millipore Sigma: G3550-1Cap and D2679-1VL), vortexed, and incubated at 37°C for 30 minutes. Finally, we added 400µl of 12N H_2SO_4 and we read the absorbance at 540nm using the BioTek Epoc microplate reader. For the APN assay, we added 2.5µl of homogenized tissue to 250µl of 2 mML-alanine-p-nitroanilide (diluted in 0.1M Na_2HPO_4 buffer, pH 6.5), vortexed, and incubated at 37°C for 20 minutes. After 20 minutes, we added 750µl of 4N acetic acid to stop the reaction and we read the absorbance at 380nm using the BioTek Epoc microplate reader.

We compared each digestive enzyme activity between species (fisher vs. marten) in relation to intestinal section. We fitted a generalized linear mixed model (glmer, Package MASS) with gamma error structure with log link function predicting the hydrolase activity by species, intestinal section, and the interaction between the two with individual as a random factor. Separate modeling were performed for the Great Lakes populations (proximal, medial, and distal small intestine and colon) and the Pacific Northwest populations (small intestine and colon) because of the uncertainty of the specific small intestinal section that latter sample was from. These analyses would highlight general trends in the species-level difference. Post-hoc Tukey tests were performed using Package lsmeans.

We further compared fishers and martens in the relative investment into carbohydrase relative to APN (index of deviation from carnivory). These analyses were performed by regressing intestinal carbohydrase activity on APN by species, and also intended to control for the confounding effect of sample quality on the assessment of the investment to carbohydrase (under the assumption that digestive hydrolases would degrade at similar rates post mortem). For

each carbohydrase (trehalase, maltase, and sucrase) we used all values from small intestine and fitted a gamma glmer with identity link with species and APN as the core predictors and their interactions as an additional exploratory predictor with individual as a random psudoreplicated factor. In the initial full model, we also included population, intestinal section, and their two-way interactions with APN as potential confounders. Models were reduced sequentially toward a minimal model that only contained species and APN if $P > 0.05$. Type III Wald χ^2 tests are reported for terms that are significant.

Finally, we tested whether species-level differences in hydrolase activities were due to variations in individual foraging strategy or purely species-specific. We estimated proportions of individual diets (vertebrates, fungi and arthropods, and plants) a posteriorly using the same Baysian mixing model with $n = 8$ samples for fishers and martens in Minnesota and $n = 6$ samples for fishers and martens in California. We then used these proportions to derive a dietary specialization index (0 = ultrageneralist and 1 = ultraspecialist) for each individual (Newsome et al. 2012). We then calculated a similar specialization index for the four digestive hydrolase activities. We averaged the hydrolase activities of the proximal, medial, and distal small intestine for the Great Lakes samples in order to match the measurements of the Pacific Northwest samples. The specialization index for individuals was compared between fishers and martens within a region (population) and between each species in different regions using R Package SIBER (Jackson et al. 2011). Because the digestive enzyme specialization index was non-normal in its error structure (R Package MVN, Shapiro-Wilk test) we added 1 to all values and ln-transformed them to meet SIBER's normality assumption. We constructed 95% confidence interval (CI) ellipses around the bivariate means to test for these differences (function plotGroupEllipses). We also constructed standard ellipse areas corrected for small sample sizes

(SEAc) to represent the core area encompassing 40% of data that is robust to sample size (function plotSiberObject). The sizes of SEA (a metric of variation in overall strategy) were compared between fishers and martens across regions by deriving Bayesian estimates of the standard ellipse areas (SEAb) and their 95% CI using function siberMVN with package rjags and function siberEllipses. Specifications for parms to run rjags were: n.iter = 2000, n.burnin = 1000, n.thin = 10, and n.chains = 2. Specifications to define the priors for rjags were: R =1 * diag(2), k = -2, and tau.mu = 1.0E-3). Sizes of the overlap in SEAc between species within a region, if present, were derived by function maxLikOverlap.

Results

Trehalase protein identification

We detected brown stained bands on the zymography gel for $n = 3$ fishers and $n = 1$ marten when the brush border membrane of the small intestine was incubated with trehalose (Figure S1). Mass spectrometry results from excised gel band for identified the presences of trehalase protein both fishers and martens. We detected three peptide sequences for fishers within the trehalase amino acid sequence: AGAESGWDFSSR (all three fishers), RLGNDIQATKY (two fishers), and TNFEVSQK (one fisher) as well as peptide sequences for the marten within the trehalase amino acid sequence: AGAESGWDFSSR (same as all three fishers) and AQEVAFQLAQNWIR Figure 1).

Diets of fishers and martens

Fishers and martens from the Great Lakes region as well as martens from the Pacific Northwest were largely carnivorous, while fishers in the Pacific Northwest consumed non-trivial amounts

of fungi and insects (Figure 2). In the Great Lakes 73% (95% CI: 35–99%) of fisher diet and 63% (95% CI: 20–98%) of marten diet was vertebrates; neither species consumed large amounts of insects, fungi, or plants. Overall, vertebrate consumption for martens in the Pacific Northwest was similar to those in the Great Lakes, with vertebrate consumption averaging 76% (95% CI: 49–98%). However, fishers in the Pacific Northwest consumed less vertebrates (47%; 95% CI: 12–86%) and higher quantities of combined insects and fungi (43%; 95% CI: 23–72%; Figure 2) compared to sympatric martens.

Digestive hydrolase activities across the intestine

We found that trehalase activity was higher in fishers in the Great Lakes region compared to martens throughout the small intestine and colon as expected and decreased from proximal to distal small intestine and colon in both species (Table 1, Figure 3A). Contrary to our expectations, the Pacific Northwest fishers and martens did not show species difference in trehalase activity. The intestinal section effect (small intestine > colon) was significant and so was the interaction between species and intestinal section, but species difference was nonsignificant in post hoc Tukey tests on the small intestine and colon (Table 1, Figure 3A). Maltase activity was similarly higher in fishers than in martens in the Great Lakes region across the intestinal sections, and the main effect of intestinal section was also significant but not their interaction (*post hoc* Tukey, proximal = medial > distal > colon; Table 1, Figure 3B). Similarly to trehalase activities, we did not find species difference in maltase activity in fishers vs. martens in the Pacific Northwest; only small intestine vs. colon was different (Table 1, Figure 3B). Sucrase activity did not differ between fishers and martens in the Great Lakes region or the Pacific Northwest, and the only differences were among intestinal sections (*post hoc* Tukey,

medial > proximal > distal > colon for the Great Lakes region and small intestine > colon in the Pacific Northwest; Table 1, Figure 3C). As expected, APN activity did not differ between fishers and martens in either the Great Lakes region or the Pacific Northwest samples, with the only difference being intestinal position (*post hoc* Tukey, proximal = medial = distal > colon in the Great Lakes region, small intestine > colon in the Pacific Northwest; Table 1 and Figure 3D).

Relative investment in disaccharidases

As expected, we found that the slope of trehalase against APN was significantly larger in the fisher than in the martens (interaction between species and APN; $\chi^2 = 11.89$, df = 1, $P = 0.0006$; Figure 4A). Additionally, we also found that the interaction between intestinal section and APN ($\chi^2 = 10.04$, df = 3, $P = 0.02$, omitted in Figure 4A) and the main effect of APN ($\chi^2 = 15.22$, df = 1, $P < 0.0001$) are also significant. The effect of population (Great Lakes or Pacific Northwest) was nonsignificant and thus removed from this final model. We also found that maltase activity in fishers are higher relative to APN in fishers than in martens (main effect of species; $\chi^2 = 6.24$, df = 1, $P = 0.01$, Figure 4B). Additionally, we found that the main effect of APN was also significant ($\chi^2 = 20.06$, df = 1, $P < 0.0001$). No other terms were significant in the final model. We did not find slope differences between sucrase relative to APN activities between fishers and martens (species and APN interaction term removed) nor did we find the main effect of species in the regression model ($\chi^2 = 0.80$, df = 1, $P = 0.37$). Additional significant terms were the interaction between intestinal section and APN ($\chi^2 = 12.30$, df = 3, $P = 0.006$; omitted in Figure 4C) and the main effect of APN ($\chi^2 = 11.32$, df = 1, $P = 0.0008$).

Diet and digestive specialization indices

As expected, the overall diet and digestive strategies as expressed as specialization indices in fishers differed by region as exhibited by the lack of overlap in the 95% CI ellipses of fishers between regions; but unexpectedly, martens also exhibited similar regional differences (Figure 5). Specialization indices for fishers from the Pacific Northwest tended toward generalist in the diet axis as expected, but unexpectedly toward specialist in the digestive hydrolase axis compared to the Great Lakes fishers (Figure 5). Martens from the Pacific Northwest also exhibited an unexpected shift toward specialist in the digestive hydrolase axis, even though in the diet axis they were similar as expected (Figure 5). Comparing fishers and martens within regions, in the Pacific Northwest we found clear separation of diet and digestive strategies (as was predicted given their niche partitioning), and even in the Great Lakes region the overlap was small (4.2% of fisher SEAc and 5.4% of marten SEAc; Figure 5). The size of SEAb were similar across comparison groups: Great Lakes Fishers: 0.016 (95% CI: 0.006–0.028); Great Lakes martens: 0.012 (95% CI: 0.005–0.023); Pacific Northwest Fishers: 0.025 (95% CI: 0.008–0.046); Pacific Northwest martens: 0.014 (95% CI 0.006–0.039).

Discussion

Endogenous production of trehalase in fishers and martens

The present study is the first to confirm the endogenous production of trehalase by fishers and martens (and by any member of subfamily Guloninae within family Mustelidae). Trehalase activity was measured in the whole intestinal samples and the isolated brush border membrane of small intestinal enterocytes, and the proteomics on the band exhibiting trehalase activity identified unique partial amino acid sequence that match the predicted sequences in closely related members in the family Mustelidae. Indeed, two species of mustelids – sea otters (*Enhydra*

lutris; Jiao et al. 2019) and American mink (*Neogale vison*; (Karimi et al. 2022)) – have functional and non-psuedogenized trehalase gene. Interestingly, both of these species have diverged from the typical diet of terrestrial carnivores. American mink consume a wide variety of prey, but are principally aquatic (Jęˌ drzejewska et al. 2001) while sea otters consume crustaceans and fish (Newsome et al. 2015). While both species consume meat, crustaceans and arthropods have trehalose as their principal hemolymph sugar (Thompson 2003, Bao et al. 2021). Similarly, fishers and martens also consume meat, but have retained the ability to consume other items, like fungi and insects, that have trehalase as their primary disaccharide.

Diets of fishers and martens

We found that fishers and martens were predominantly carnivorous in the Great Lakes (Smith et al. 2023) whereas fungi and insects formed an important part (~40%) of fishers, but not martens, in the Pacific Northwest (Smith et al. 2022, Kuntze et al. 2024, Smith et al. 2024). As all fishers and martens consumed a significant proportion of meat, we could expect that APN activity would be universally important for all individuals studied here. Trehalase activity would appear important only in the CA fishers due to their high consumption of fungi and insects. Maltase activity would be necessary as all diets contain starch (including glycogen), but high starch only occurs in fungi and plant matter (Karasov et al. 2011, Kalač 2013). Sucrase activity would be the least important as sucrose is a plant-based disaccharide (Karasov and Martínez del Rio 2007) and plants were rare in the diet of both species. We used these observations and expectations to interpret our results of small intestinal enzymatic activities of fishers and martens.

Species-level difference in digestive hydrolase activities

We hypothesized that evolutionary history and adaptations to different dietary niches would have resulted in different digestive hydrolase profile between fishers and martens, with specific predictions of fishers having higher disaccharidase activities than in martens. As expected, we found no difference in APN activities between species in the Great Lakes or the Pacific Northwest. Consistent with our prediction we found that the Great Lakes fishers had greater activities of trehalase and maltase (but not sucrase) across the small intestine relative to the sympatric martens. However, this pattern was not detected in the Pacific Northwest populations.

A more rigorous analysis of the relative investment into each disaccharidase activity relative to APN (and controlling for the potential confounding effect of sample quality) showed that fishers have higher relative trehalase and maltase activities (but not sucrase) compared to martens regardless of population (Great Lakes or Pacific Northwest). These differences were found despite their diet composition and hence macronutrient composition being similar in the Great Lakes. It is particularly noteworthy that fishers were sampled during the winter months when fungi and insects are rare in this region (Curras et al. 2025). Thus, our results suggest that fishers have constitutively higher trehalase and maltase activities than martens, representing higher spare capacity for fungal, insect, and plant matter digestion regardless of their current diet. However, the proportional difference between fishers and martens appear larger for trehalase than for maltase activity and investment in sucrase activity did not differ between species. These observations suggest that fishers may be more preadapted to consume fungi and insects than being a generalist omnivore.

This observation may reflect the ancestral adaptation and phylogeny of fishers and martens in the subfamily Guloninae, which also includes tyras (*Eira barbara*) and wolverines (*Gulo gulo*; Buskirk 2023; (Law et al. 2018)) While species within this subfamily are generally

similar in size and typically associated with forests, they exhibit a wide range of foraging strategies and diets that appear linked to their evolutionary history. The subclade of fishers and tyras are omnivorous; fishers consume varied diets based on prey availability including insects (Kuntze et al. 2024; Smith et al. 2022; Smith et al. 2023; Smith et al. 2024) and tayras consume a variety of dietary items including fruits (Galef Jr et al. 1976, Soley and Alvarado-Díaz 2011). On the other hand, the subclade of martens and wolverines are hyper-carnivorous; Martens in North America (*Martes americana* and *M. caurina*) are primarily predators of small mammals (Carlson et al. 2014, Manlick et al. 2017, Manlick et al. 2019), although some species, such as the Japanese marten (*Martes melampus*), consume insects for protein (Hisano et al. 2019), and wolverines are almost entirely scavengers of carrion meat (Lofroth et al. 2007, Dalerum et al. 2009, Inman et al. 2012). Therefore, the relatively high activity of trehalase and maltase in fishers may well reflect the adaptation to omnivory in the common ancestor of fishers and tayras.

Population-level differences in individual digestive strategy

We hypothesized that only fishers would exhibit variation in individual foraging strategies leading to their digestive enzyme profiles matching their diet, specifically predicting that fisher trehalase activities would show Pacific Northwest > Great Lakes region. Contrary to our predictions, we found no difference in trehalase relative to APN activities (or disaccharidase relative to APN in general) in either species, nor did we find population-level differences in diet and digestive enzyme specialization index. These findings are surprising given that our isotope analysis revealed that fishers in the Great Lakes region primarily consumed meat and virtually no insects or fungi. Our dietary estimates align with previous reports from the Great Lakes region showing that martens and fishers are strictly carnivorous (Manlick et al. 2017, Smith et al. 2023),

consuming predominantly small mammals like voles, mice and shrews (Carlson et al. 2014). Furthermore, omnivorous animals tend to have the ability to modulate digestive enzyme profiles to match their diet (Karasov and Hume 1997, Karasov and Douglas 2013), and fishers in CA show clear evidence of omnivory. Our findings support supports the interpretation that fishers constitutively express higher trehalase and maltase activities than martens, representing a greater spare capacity to digest food items high in carbohydrates.

However, it is important to note the differences and limitations in our sampling. While all samples were treated the same, fishers in the Pacific Northwest, unlike the Great Lakes region, were sampled throughout the year. Therefore, this should minimize any consequence of shifting seasonal diets in terms of the availability of insects and fungi due to the timing of sample collection. Tissue samples for fishers and martens in the Pacific Northwest were collected between 2006 and 2018 and, while collection date is known, it is unclear how long these individuals were deceased before collection. It is possible that fisher samples had degraded in quality, which resulted in lower estimates of trehalase activity. This is opposed to our collection in the Great Lakes region where martens and fishers were frozen after upon collection, which favored higher quality samples. Even within the Great Lakes sample variation in quality likely existed because we did not have control over the trapped animal until collection (e.g., some individuals may have been found dead in the trap while others may have been euthanized upon capture, and freezing of the whole carcass was performed after transport from the field). While we controlled this variation among sample quality through evaluating disaccharidase activities relative to APN activity, the implicit assumption was that the rate of degradation is consistent between disaccharidases and APN, which may not have been true.

Furthermore, individual diet and digestive strategies of suggests that phenotypic plasticity of the digestive enzyme profiles is possible in both species. Specifically, fisher exhibited regional differences in both their diet and the digestive enzyme specialization indices; martens exhibited regional differences in the digestive enzyme index. Further, these competing species exhibited clear separation in the overall diet and digestive enzyme strategy in the Pacific Northwest as would be expected under dietary niche partitioning. Unexpectedly, however, both species showed a shift toward specialization in the digestive enzyme in the Pacific Northwest compared to the Great Lakes. This difference appears to be driven by the high absolute values of maltase activity across all individuals compared to other enzymes like APN. This would result in the specialization index for individuals with high maltase activity to be enzymatically specialized. It is important to note that our analysis of digestive enzymes is incomplete (e.g., APN activity is not the sole intestinal peptidase activity), and the timing of the digestive enzyme profiles (which may reflect the diet immediately prior to death) to the diets derived from hair samples (which captures the diet during hair growth) likely complicate the comparison of the two (Pauli et al. 2022). However, this metric of specialization does suggest that foraging and digestive strategies that varies between individuals by region and that foraging strategies exist in both fishers and martens.

Thus, it is important to stress that our results do not rule out the possibility of diet-driven modulation of disaccharidases in fishers or population-level differences in enzyme profiles reflecting individual digestive and foraging strategies. Thus far, we lack studies testing the capabilities of omnivorous wild carnivorans to modulate digestive enzyme profiles, but it is conceivable that they do given that it was found in domestic dogs (Axelsson et al. 2013) and that

this ability appears widespread in other taxa (Karasov and Hume 1997, Wang et al. 2019, Oguchi et al. 2022).

Implications for co-occurrence, competition, and conservation

Fishers and martens exhibit significant competitive overlap throughout their distributional range as they consume similar diets and occupy similar habitats and geographic distributions (Pauli et al. 2022). Recent studies suggest that fishers and martens may predominantly consume small-bodied prey (e.g., voles and shrews); however, where the two species co-occur martens may drive fishers to switch diets to larger-bodied prey (e.g., squirrels and porcupines) or fungi and insects through exploitation competition (limitation of prey availability), through the larger fishers are the dominant predator with respect to interference competition (Pauli et al. 2022, Smith et al. 2023, Smith et al. 2024). Our findings on digestive hydrolase profiles of the two species provide mechanistic explanation on the dominant fishers tend to switch diets. While both carnivores have carbohydrases (trehalase, maltase, and sucrase) that enable them to consume fungi, insects, and plant matter, higher constitutive production of trehalase and maltase would confer them the ability to better exploit non-meat dietary items, particularly fungi and insects when preferred prey density is reduced. Thus, the increased spare capacity for non-meat digestion of fishers may buffer them better against the unpredictability of meat availability that may arise from climate or human land use change as compared to martens (Pauli et al. 2022). Our findings suggest that food items often dismissed as incidental consumption or consumed only in times of desperation by carnivores can have energetic and nutritional value, and that omnivory may be underappreciated aspect of carnivoran dietary niche.

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Figures and Tables

Figure 1. Zymography and proteomics from isolated brush border membrane reveal endogenous trehalase activity in fishers (*Pekania pennanti*, *n* = 3) and American martens (*Martes americana*, *n* = 1). Protein sequences in mustelids were used compare sequences found in the gel bands. The species phylogeny (Kumar et al. 2022) and partial view of amino acid sequences of 10 mustelid species and rats (*Rattus norvegicus*) was aligned using MAFFT. This blast identified three peptide sequences for fishers (green boxes) within the trehalase amino acid sequence: AGAESGWDFSSR (all three fishers), RLGNDIQATKY (two fishers), and TNFEVSQK (one fisher). This blast also identified two peptide sequences for martens (orange boxes) witin the trehalase amino acid sequence: AGAESGWDFSSR (same as all three fishers) and AQEVAFQLAQNWIR.

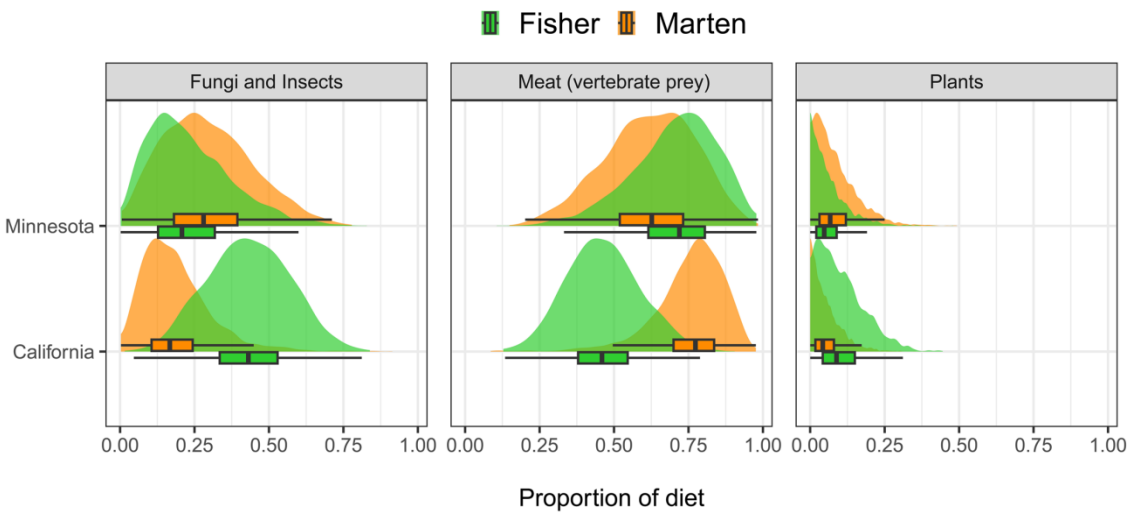

Figure 2. Proportional diet estimates of fishers (*Pekania pennanti*: green) and martens (*Martes* spp.; orange) in the Great Lakes region (Minnesota) and the Pacific Northwest (California) from a Bayesian isotopic mixing model. Diet groups included *a posteriori* combined estimates of fungi and insects, and meat consumption.

Table 1. Type III ANOVA comparing species (fisher vs. marten) effect on intestinal enzyme activity per gram of wet intestine (μmol min^{-1} g^{-1} tissue) in relation to intestinal position. Each comparison group within the Great Lakes region (Minnesota) or the Pacific Northwest (California) was represented by 6–9 individuals. Tests were performed using generalized linear mixed model (glmer) with individual ID as a random effect.

	Trehalase			**Maltase**			**Sucrase**			**APN**		
	χ^2	df	*P*	χ^2	df	*P*	χ^2	df	*P*	χ^2	df	*P*
Minnesota												
Species	8.83	1	0.003	6.45	1	0.01	0.0007	1	0.98	0.02	1	0.894
Intestinal section	110.52	3	<0.0001	72.10	3	<0.0001	91.48	3	<0.0001	25.03	3	<0.0001
Species × intestinal section	6.12	3	0.11	3.86	3	0.27	1.65	3	0.65	2.86	3	0.413
California												
Species	0.11	1	0.73	0.11	1	0.74	0.04	1	0.85	0.76	1	0.38
Intestinal section	93.61	1	<0.0001	53.38	1	<0.0001	32.59	1	<0.0001	50.84	1	<0.0001
Species × intestinal section	7.89	1	0.005	3.23	1	0.07	0.55	1	0.46	3.16	1	0.08

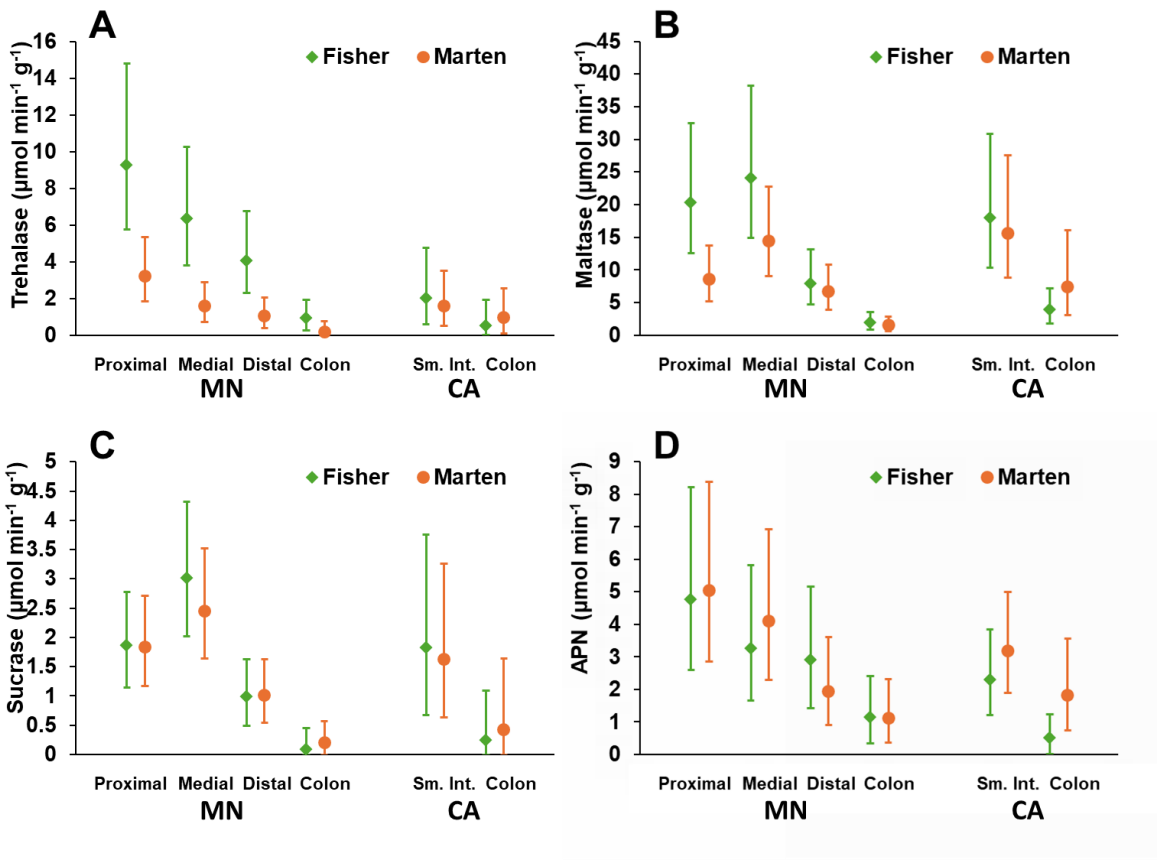

Figure 3. Digestive hydrolase activities of fishers (*Pekania pennanti*, Minnesota $n = 8$ and California $n = 6$) and martens (*Martes americana*, Great Lakes region [MN] $n = 9$; *M. caurina*, Pacific Northwest [CA] $n = 6$) measured at the center of each small intestinal section (proximal, medial, and distal) and colon (mean ± 95% CI) as estimated from generalized linear models. Small intestine samples from California could not be identified to a specific section and thus listed as Sm. Int.

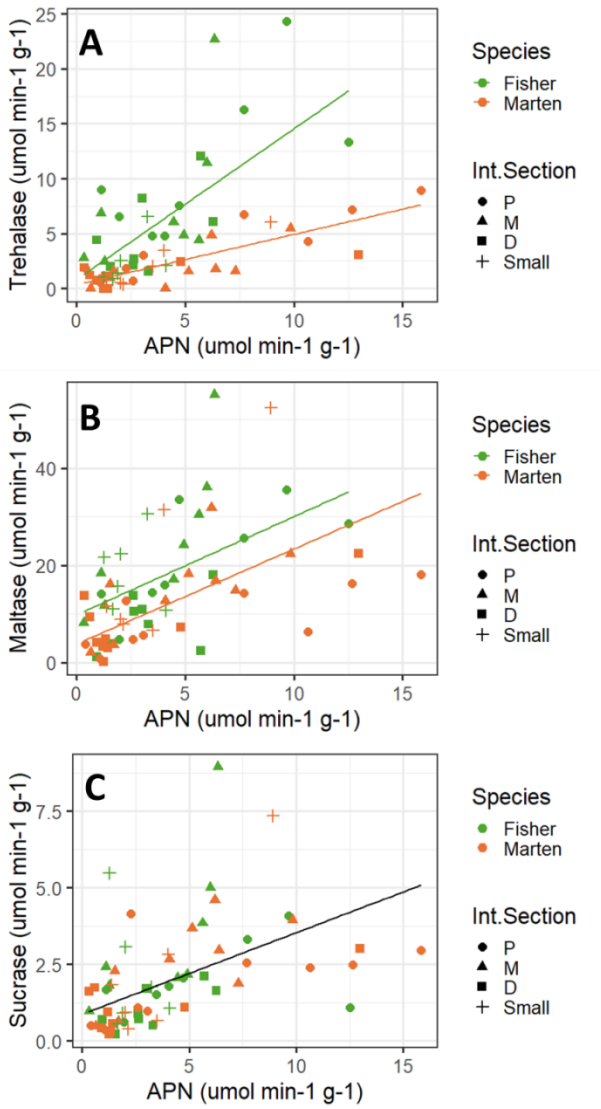

Figure 4. Intestinal disaccharidase activities in relation to intestinal aminopeptidase-N (APN) activities in fishers (*Pekania pennanti*, Great Lakes region $n = 8$ and Pacific Northwest $n = 6$) and martens (*Martes americana*, Great Lakes region $n = 9$; *M. caurina*, Pacific Northwest $n = 6$) measured at the center of each small intestinal section (proximal, medial, and distal). Small intestine samples from the Pacific Northwest could not be identified to a specific section and thus listed as "Small." Species-specific or universal regression lines were derived from generalized linear mixed models with individual ID as a rendom factor.

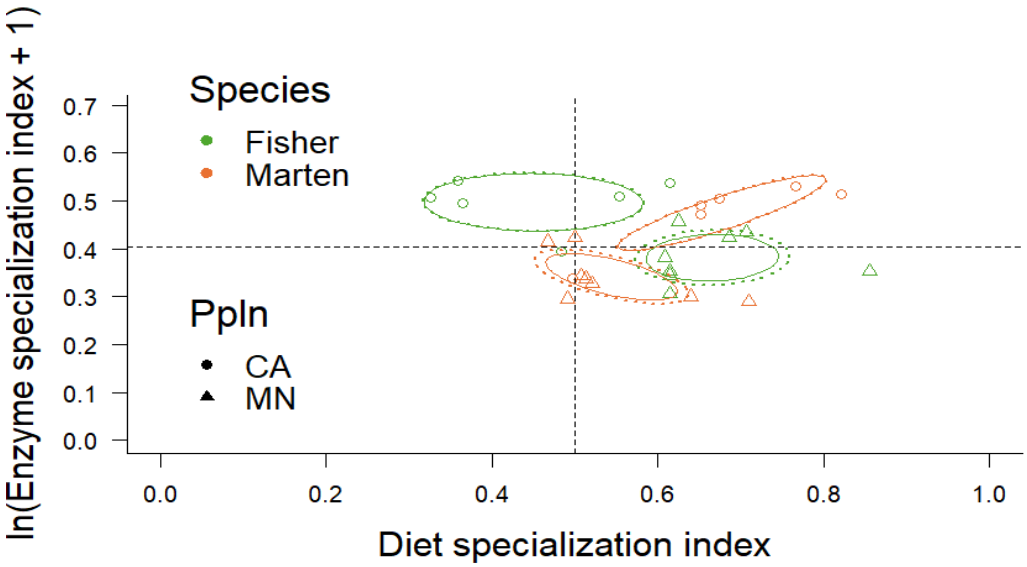

Figure 5. Overview of diet (vertebrates, fungi and arthropods, and plants) and digestive hydrolase (trehalase, maltase, sucrase, and aminopeptidase-N) strategies of fishers (*Pekania pennanti*, Great Lakes region [MN] $n = 8$ and Pacific Northwest [CA] $n = 6$) and martens (*Martes americana*, Great Lakes region [MN] $n = 8$; *M. caurina*, Pacific Northwest [CA] $n = 6$) as indexed as generalist-specialist spectrum. Untrageneralist (unifrom proportion of diet or enzyme activity) would have a value of 0 whereas ultraspecialist (having only a single diet or enzyme activity) would have a value of 1 (ln-transformed to 0.693 in enzyme index). Dotted ellipses represent standard ellipse area corrected for small sample size (SEAc), whereas the solid ellipses represent 95% CI around the bivariate means for statistical comparison.

Supplementary Information

Table S1. Macronutrients and structural biochemicals in dietary items for Gulonids (% of dry matter) (1. Hutchinson 1959; 2. Ewer 1998; 3. Wallis et al. 2012; 4. Oku et al. 1998; 5. Kalač 2012; 6. Donadio and Buskirk 2006; 7. Zielinski et al. 1999; 8. Spencer et al. 2015; 9. Karasov et al. 2011; 10. Axelsson et al. 2013).

Dietary item	Nonstructural carbohydrate		Structural carbohydrate		Other macronutrients	
	Trehalose	Starch[α]	Chitin	Cellulose	Protein	Fat
Meat	nil	~5%[1]	nil	nil	40-75%[1]	5-50%[1]
Invertebrate	up to 33%[2]	~5%[1]	13-30%[3]	nil	40-75%[1]	8-60%[1]
Fungi	10%[4,5]	5-10%[5]	Up to 50%[6]	nil	2-25%[7]	2-10%[8]
Plant	nil	1-80%[9]	nil	9-53%[10]	3-30%[10]	0.1-65%[10]

[α]animal starch is glycogen; plant starch is amylose and amylopectin

Table S2. Diet of subfamily guloninae: fishers (*Pekania pennanti*), tayras (*Eira barbara*), martens (*Martes* spp.), and wolverines (*Gulo gulo*). The subclade of fishers and tayras are more diverse and includes fungi and insects compared to the diets of the subclade of martens and wolverines, which are highly carnivorous.

Species	Meat	Plants	Insects	Fungi	Citation
Fishers (4kg)	13%	19%	20%	48%	Smith et al. 2022; Manlick et al. 2017; Kirby et al. 2018
Tayras (5kg)	Yes	Yes	Yes	Yes	Galef et al. 1976; Soley and Alvarado Diaz 2011
Marten (1kg)	90%	10%	0%	0%	Manlick et al. 2019; Manlick et al. 2017; Carlson et al. 2014
Wolverine (10kg)	100%	Occasional	Occasional	0%	Lofroth et al. 2007; Dalerum et al. 2009; Inman et al. 2012

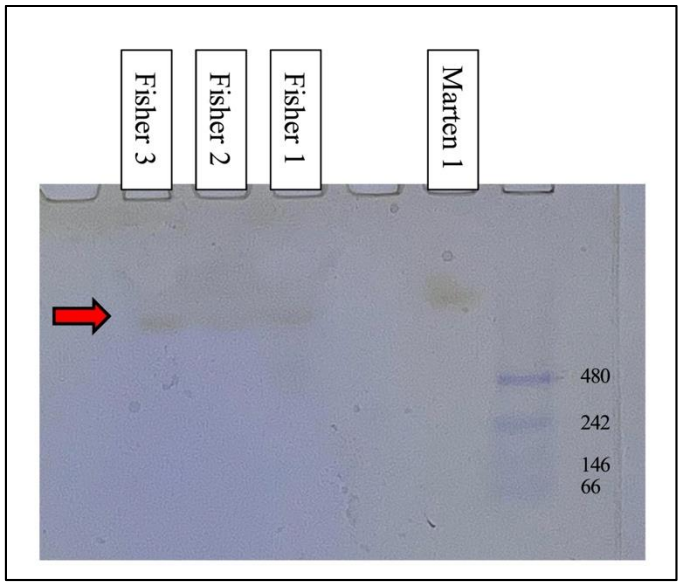

Figure S1. Zymography gel for fishers (*Pekania pennanti*) and martens (*Martes caurina*) in trehalose solution. The red arrow marks the location of the brown stained bands for fishers and martens. These bands indicated the presence of trehalose digestion and were sent to mass spectrometry for further analysis.

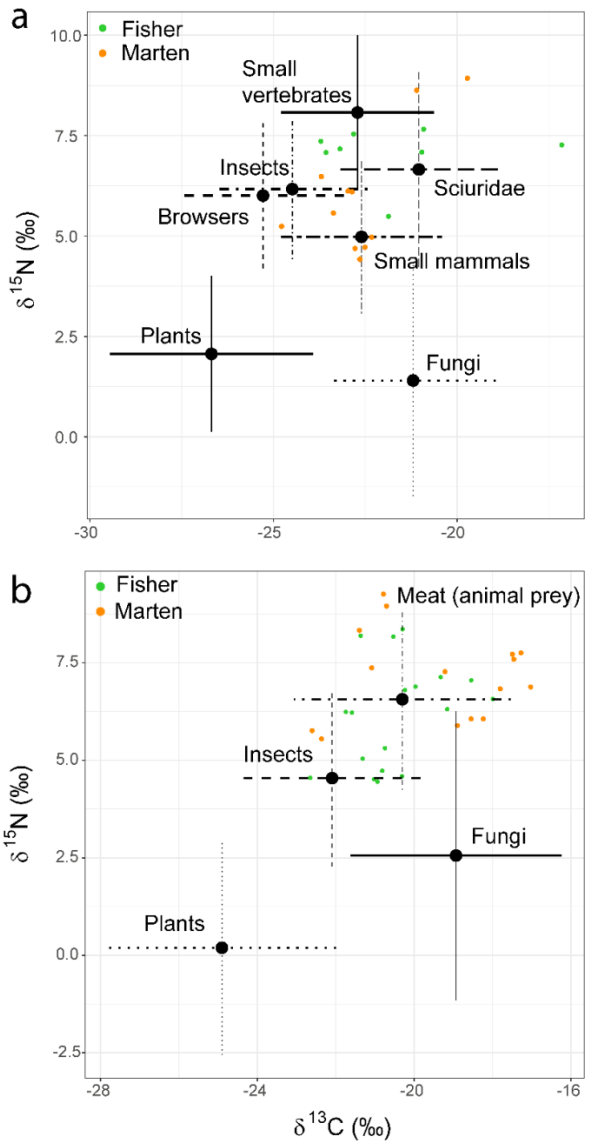

Figure S2. Mean carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope value and standard deviation of dietary source groups including trophically corrected ($\delta^{13}C = 2.3$; $\delta^{15}N = 3.7$) martens (*Martes* spp.) and fishers (*Pekania pennanti*) in Minnesota (a) and California (b). Isotope ratios are reported in delta (δ) notation as parts per mille (‰). Small vertebrates, browsers, Sciuridae, and small mammals were combined *a posteriori* into single prey group (Meat [vertebrate prey]) for Minnesota to compare to estimates in California.

Supplementary Text

Protein identification

We split the remaining proximal small intestine of martens and fishers from Minnesota into two longitudinal pieces. We used one of these longitudinal pieces to collect enterocytes for brush border membrane isolation. After we rinsed the piece with phosphate buffer saline and blotted it dry, we used a glass slide to scrape the enterocytes from the inner lining of the small intestine and kept them at -80°C for storage. We used the second longitudinal piece of proximal small intestine for trehalase assays. Similarly, after rinsing with phosphate buffer saline we blotted it dry, added 10mL of mannitol buffer per gram of tissue and processed it using a omni tissue homogenizer set to maximum speed in pulses for 30 seconds of homogenization followed by 30 seconds of cooling, repeated five times to prevent overheating of the sample. We then followed the same protocol used for measuring trehalase activity in our previous methods.

We rinsed the collected enterocytes by adding phosphate buffer saline and centrifuging at 1000 RCF for five minutes at 4°C. We collected the supernatant and then we added 300µl of rinsed enterocytes through an 18-gauge needle to a new 1.5mL tube. We homogenized the enterocytes on ice with a omni tissue homogenizer, using 15 second on and off pulses. We filled the remainder of the tube with homogenization (McConnell et al. 2011) buffer (10mM imidazole, 4mM EDTA-k, 1mM EGTA-k, 0.02% Na-azide, 1mM DTT, 1mM Pefabloc-SC) and centrifuged for 1000 RCF for 10 minutes at 4°C to pelletize. We discarded the supernatant and washed the pellet with 30ml of solution A (10mM imidazole, 75mM KCl, 1mM EGTA, 5mM, $MgCl_2$, 0.02% Na-azide). We then resuspended the pellet with 300ml of solution.

In a centrifuge tube, we added 1500µl of 60% sucrose solution. We then added 300µl of the homogenized enterocyte mixture into the 60% sucrose solution and mixed until fully

suspended with the sucrose solution. We then added 2200µl of 40% sucrose solution. This solution was centrifuged in weight-balanced centrifuge tubs using an ultracentrifuge at 30,000 RCF for 1 hour and 30 minutes at 4°C. Then, the brush border membrane located in the band between the upper and lower layers of the sucrose gradient were extracted and stored in a new tube. We filled the remainder of the new tube with solution A and centrifuged at 20,000 RCF for 10 minutes at 4°C. We discarded the supernatant, and we washed the pellet with 300µl of solution A twice. We then added 300µl of solution A to the washed pellet, resuspended, and centrifuged at 20,000 RCF for 10 minutes at 4°C for a second time. We discarded the supernatant, and we washed the pellet with 200µl of solution A twice. We suspended the washed pellet (i.e. the collected brush border membrane) in mannitol buffer and kept it at -80°C. We performed a Bradford assay on the final products and used a linearized Bradford assay protocol for protein concentration based on Ernst and Zor (2010) to quantify the concentration of protein in each sample. We also ran trehalase assays (triplicate) on each sample to quantify the amount of trehalase activity occurring in the raw brush border membrane. Once these assays were completed, we selected individuals with high concentrations of protein and measurable amounts of trehalase activity.

We followed the protocol by Brun et al. (2020) to test trehalase activity in a non-denaturing gel. We solubilized samples in a 1:1 ratio of loading buffer (ThermoFisher: Novex™ Tris-Glycine Native Sample Buffer, 6% Triton X 100) with each sample containing at least 20µg of protein. We vortexed the mixture and warmed it in a water bath at 40°C for 10 minutes. Next, we loaded 40µl of the warmed mixture into a 12% Tris-Acetate native gel in running buffer (Tris-glycine, pH 8.2) and run at 100V (60mA) for two hours. We rinsed the gel in distilled water for 15 minutes before being exposing it to a 56mM trehalose solution for 10 hours at 37°C. After

this incubation, we removed the trehalase solution and replaced it with a stop-develop solution (Millipore Sigma: G3660-1Cap and D2679-1VL) for one hour at 37°C. We then removed the solution and took photographs of the gel. Trehalase activity was indicated by a dark orange-brown stain. We cut a rectangle containing the stain out of the gel with a scalpel, and these gel pieces were sent for in-gel digestion followed by mass spectrometric analysis at the Mass Spectrometry Facility at the Biotechnology Center, University of Wisconsin-Madison.

We first excised the gel slices from the native gel based on the activity staining. We then destained the gel pieces for 5 minutes in MeOH/H_20/NH_4HCO_3 [50%:50%:25mM], and then denatured the proteins "in gel" for 10 minutes in SDS/DTT/Tris-HCl solution [2%/1mM/50mM pH7] with subsequent 2 by 5 minutes washes in MeOH/H_20/NH_4HCO_3 [50%:50%:25mM]. We dehydrated the gel pieces for 5 minutes in ACN/H_20/NH_4HCO_3 [50%/50%/25mM] then once more for 30 seconds in 100% ACN. We then dried the gel pieces in a Speed-Vac for 1 minute, then reduced in 25mM DTT [Dithiotreitol in 25mM NH_4HCO_3] for 15 minutes at 56°C, alkylated with 55mM CAA [Chloroacetamide in 25mM NH_4HCO_3] in darkness at room temperature for 15 minutes. We then washed the pieces once in H_20 and dehydrated for 2 minutes in ACN/H_20/NH_4HCO_3 [50%:50%:25mM], then once more for 30 seconds in 100% ACN. We dried the pieces again and rehydrated them with 20µl of trypsin solution with 0.01% ProteaseMAX™ surfactant [10ng/µl *Trypsin* from Promega Corp. in 25mM NH_4HCO_3/0.01% w/v of ProteaseMAX™ from Promega Corp.]. We then left the pieces to sit for 2 minutes at room temperature then we added an additional 30µl of overlay solution [25mM NH_4HCO_3/0.01% w/v of ProteaseMAX™] to keep gel pieces immersed throughout the digestion. We supervised the digestion for 3 hours at 42°C. We transferred the peptides generated from digestion into a new tube and we acidified them with 2.5% TFA [Trifluoroacetic Acid] to 0.3%

final. We then extracted the gel pieces further with ACN:H_2O:TFA [70%:29.25%:0.75%] for 10 minutes while we vortexed and then we combined the solutions and dried completely in a Speed-Vac (~15 minutes). We solubilized the extracted peptides in 30µl of 0.05% TFA. We removed Degraded ProteaseMAX™ via centrifugation [max speed, 10 minutes] and we extracted the peptides solid phase (*Pierce*® C18 tips, 10µl volume from Thermo Scientific) according to manufacturer protocol. We removed the peptides off the C18 SPE column with 5µl of acetonitrile/H_2O/TFA (70%:30%:0.1%) and dried them to completion. We then resolubilized the peptides in 20µl total volume for with 0.1% formic acid and we loaded 2µl on the instrument.

We analyzed the peptides by nanoLC-MS/MS using the Agilent 1100 nanoflow system (Agilent) connected to a hybrid linear ion trap-orbitrap mass spectrometer (LTQ-Orbitrap Elite™, Thermo Fisher Scientific) equipped with an EASY-Spray™ electrospray source (held at constant 35ºC). We accomplished the chromatography of peptides prior to mass spectral analysis using capillary emitter column (PepMap® C18, 3µM, 100Å, 150x0.075mm, Thermo Fisher Scientific) onto which 2µl of extracted peptides were automatically loaded. The NanoHPLC system delivered solvents A: 0.1% (v/v) formic acid, and B: 99.9% (v/v) acetonitrile, 0.1% (v/v) formic acid at 0.50 µL/min to load the peptides (over a 30 minute period) and 0.3µl/min to elute the peptides directly into the nano-electrospray with gradual gradient from 0% (v/v) B to 30% (v/v) B over 80 minutes and concluded with 5 minute fast gradient from 30% (v/v) B to 50% (v/v) B at which time a 4 minute flash-out from 50-95% (v/v) B took place. We ran them for 150 minutes of encompassed column conditioning at 95% B for 1 minute and equilibration at 100% A for 30 minutes. As the peptides eluted from the HPLC-column/electrospray source survey MS scans were acquired in the Orbitrap with a resolution of 120,000 followed by CID-type MS/MS with 2.0 AMU isolation and 10 msec activation time

with 35% normalized collision energy fragmentation of 30 most intense peptides detected in the MS1 scan from 350 to 1800 m/z; the redundancy was limited by dynamic exclusion. Monoisotopic precursor selection and charge state screening were enabled and +1 and undefined charge states were rejected.

We converted the Raw MS/MS data to a mgf file format using MSConvert (ProteoWizard: Open Source Software for Rapid Proteomics Tools Development). We used the resulting mgf files to search against total Unitprot Mustelidae family amino acid sequences (178,659 protein entries, 2024_01_09 download) along with a list of common lab contaminants (172 total entries) using in-house *Mascot* search engine 2.7.0 (Matrix Science) with variable Methionine oxidation, Asparagine, and Glutamine, deamidation plus, fixed cysteine Carbamidomethylation. We set the peptide mass tolerance at 10 ppm and fragment mass at 0.6 Da. We used Scaffold software (version 4.11.0, Proteome Software Inc., Portland, OR) for protein annotations, significance of identification, and spectral based quantification. We accepted peptide identifications if they could be established at greater than 80.0% probability by the Scaffold Local FDR algorithm. We also accepted protein identifications if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Our protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Any of our proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The proteins sharing significant peptide evidence were grouped into clusters.

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