

Low plasticity in digestive physiology constrains feeding ecology in diet specialist, zebra finch (*Taeniopygia guttata*)

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SUMMARY

It can be hypothesized that species with a wide or variable food niche are able to adjust their digestive physiology to current food type. In diet specialists, however, the capacity for such presumably costly plasticity is not necessary and flexibility of digestive physiology should be lower. Recently, we found that ontogenetic changes in the activity of digestive enzymes in house sparrow, a species that gradually consumes more carbohydrates during ontogeny, are strongly modified by diet composition. In the present study we examined digestive flexibility of nestling and adult zebra finches, typical diet specialists that consume only seeds after hatching. Both adult and nestling zebra finches could not thrive on a protein-rich and carbohydrate-free diet that supported normal development of young house sparrows. Mass-specific activity of intestinal carbohydrases (maltase and sucrase) was not elevated by higher diet carbohydrate content in both nestling and adult birds. Mass-specific activity of maltase changed less during ontogenetic development in zebra finch than in house sparrow. We conclude that the digestive physiology of zebra finch is adapted to process carbohydrate-rich food after hatching and is much less flexible than in house sparrow. We hypothesize that this difference might reflect the lack of a diet switch during ontogeny or result from high specialization to a narrow diet niche.

Key words: developmental flexibility, digestive physiology, diet composition, digestive enzymes, zebra finch, *Taeniopygia guttata*.

INTRODUCTION

Vertebrates exhibit wide variation in morphological, physiological and biochemical features of their gastrointestinal tract (Stevens and Hume, 1995; Karasov and Hume, 1997; Starck and Wang, 2005; Karasov and Martínez del Río, 2007; Naya et al., 2007; Naya et al., 2008). For a particular species, a high degree of plasticity in its nutritional physiology features can permit high energy flow across a wide range of diets, but a lack of plasticity can constrain it to a more narrow food niche (Afik and Karasov, 1995; Karasov, 1996). Many species change their diet during ontogeny (e.g. Wiens and Johnston, 1977; Durtsche, 2000; Hjelm et al., 2003; Anderson, 2006), or because of seasonal changes in the availability of different types of food. Therefore, such species can be expected to be able to adjust their nutritional physiology to the current food type. However, as capacity for such plasticity is presumably costly (DeWitt et al., 1998; Pigliucci, 2005), natural selection should lead to its loss when the expected variability in food type is low. These assumptions are the evolutionary background for the adaptive modulation hypothesis, which predicts that diet specialists should exhibit a lower level of diet-induced plasticity of the gastrointestinal tract than food generalists (Karasov, 1992; Karasov and Hume, 1997).

These considerations guided our study of the nutritional and digestive plasticity of zebra finch (*Taeniopygia guttata* Vieillot) nestlings and adults. These natives of Australia are diet specialists, frequently relying in any single location mostly on seeds of only one genus of grass, and taking animal food (insects) only occasionally (Zann, 1996). Strikingly, zebra finches seem to have

narrower diet niche than other estrild finches living in the same environment [see Schöpfer cited in Zann (Schöpfer, 1989; Zann, 1996)]. In contrast to most seed-eating altricial species (Wiens and Johnston, 1977), zebra finch adults feed their nestlings seeds throughout development (Zann, 1996) rather than initially feeding them insects followed by a transition to seeds.

In a recent study (Brzęk et al., 2009) we showed that in young house sparrows (*Passer domesticus*), whose diet gradually changes during growth from mostly insects to mostly seeds (Anderson, 2006), ontogenetic increases in the activity of intestinal carbohydrases are partly induced by high dietary carbohydrate and are partly programmed (diet independent). Furthermore, house sparrow nestlings grew normally throughout development on either high protein/carbohydrate-free or lower protein/carbohydrate-containing diets. We think that house sparrows represent a convenient comparator species to highlight possible differences of the specialist zebra finch, although two-species comparisons cannot be seen as conclusive in testing evolutionary adaptive hypotheses (Garland and Adolph, 1994).

We tested three predictions related to the adaptive modulation hypothesis. First, we expected the nutritional physiology of the specialist zebra finch to be relatively constrained and therefore nestlings and adults would fail to thrive properly on a high protein/low carbohydrate diet (in contrast to house sparrows; prediction 1). Second, activity of intestinal carbohydrases should not be induced by high dietary carbohydrate in both nestling and adult zebra finch (in contrast to nestling house sparrows; prediction 2). Finally, we hypothesized that there is no programmed increase

in the activity of intestinal carbohydrases during ontogenetic development in nestling zebra finches (in contrast to nestling house sparrows; prediction 3).

We tested our predictions in several experiments using both hand-fed zebra finch nestlings and freely feeding adults in our captive colony. In experiment I, we tested the range of diet composition that can be accepted by nestling and adult zebra finches. We subsequently selected two diets with the most varying composition that could still support development of nestlings and tested their effect on digestive physiology and development in nestling (experiment II) and adult (experiment III) zebra finches.

MATERIALS AND METHODS

Our study involved several experiments that differed only in diet composition. Therefore, we have divided the description of our methods into three sections: general procedures applied in experiments with adult and nestling birds; description of all experiments and the diet composition used in each of them; and physiological and biochemical assays used in our experiments.

All experimental procedures were accepted by the University of Wisconsin, Madison ethics committee (permit no. RARC A-01269-4-10-06).

General experimental procedures

Non-experimental birds and their maintenance

Zebra finches used in the present study were obtained from a breeding colony maintained by the Department of Forest and Wildlife Ecology, University of Wisconsin, Madison. Birds were maintained at 24–25°C, 20–40% humidity, and 16 h:8 h L:D photoperiod. Birds were fed *ad libitum* with commercial seed mix and Mazuri Small Bird Breeder (www.mazuri.com) food supplement, mixed in a 50:50 ratio. Hard-boiled egg food with vitamin and mineral supplement and vegetables were offered 3 times per week. Birds had unlimited access to water, grit, and cuttlefish bone (source of calcium). Breeding pairs were kept in separate cages with nest boxes and nest material added.

General procedures used in experiments with adult birds

Adult birds used in experiments were raised under the same conditions as non-experimental birds. Birds were grouped in cages, with birds of the same sex and diet in one cage. For 2 days, birds were fed a 50:50 mass ratio mix of standard food (seed mix and Mazuri Small Bird Breeder supplement) and synthetic diet, and afterwards on pure synthetic diet. All birds were weighed every day to check their condition.

General procedures used in experiments with nestlings

All procedures used in experiments with zebra finch nestlings were similar to those used in a previous experiment with house sparrows, and are described in detail elsewhere (Brzęk et al., 2009). The protocol was modified because of the smaller body size and longer nestling period of zebra finches. Briefly, nest boxes of breeding pairs were checked daily to ascertain the exact date of hatching. Hatchlings were marked individually with an indelible marker. The day when hatchlings were found was counted as day 0. Nestlings selected for the experimental treatments were removed from their nests between 10:30 and 12:30 h on day 4. Zebra finches show wide hatching asynchrony; therefore, the obvious runts were excluded from our trials. To control for nest effect, nestlings from the same clutch were randomly assigned to different diets. When nestlings from the second brood of the same pair were collected, they were assigned to trials of different length (see below) from individuals

of the first brood. Collected nestlings were kept individually in tissue-lined plastic containers and housed in an environmental chamber under constant conditions of 15 h:9 h L:D photoperiod, 35°C and 40–45% relative humidity using a water bath system.

Nestlings were fed every hour by gavage using 0.5 and 1 ml syringes a total of 15 times per day, beginning at 06:30 h. Syringe mass (± 0.01 g) was recorded prior to and following feeding to calculate the exact meal mass. The age-specific feeding schedule was 0.15, 0.21, 0.27, 0.33, 0.37, 0.45, 0.51, 0.57, 0.63, 0.69 and 0.75 ml of food per hour for nestlings of ages 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14–15 days, respectively. This feeding pattern was validated in pilot trials and supported body mass growth similar to that observed in nestlings fed in the colony by parents (Fig. 1A). Measured wet mass intake was corrected to dry mass based on daily aliquots of both diets that were weighed, dried and reweighed. Age-

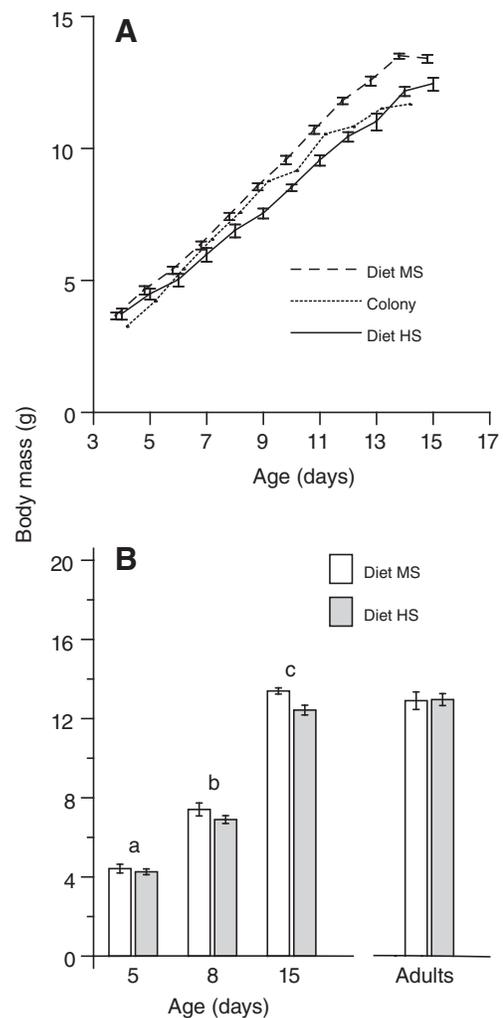


Fig. 1. (A) Repeated measures for body mass against age of zebra finch nestlings. There was a significant interaction between diet and age for body mass reflecting a difference between diet groups that increased with age (see Results). The growth pattern of nestlings in the colony is shown for comparison with that of the hand-fed nestlings on the two synthetic diets (MS, medium starch; HS, high starch). (B) Body mass for nestlings of different ages and for adult zebra finches at necropsy. Means \pm s.e.m. are shown. On this and subsequent graphs, nestling and adult birds are shown on the same graph for easier comparison, although they were analysed separately (see Materials and methods). Different lowercase letters indicate significant differences between nestlings of different ages.

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

	Starch free (SF)	Low starch (LS)	Medium starch (MS)	High starch (HS)	Medium starch and high oil (MSO)
Corn starch	0	12.7	25.4	46.23	25.4
Casein (protein)	59.63	52.93	46.23	25.4	34.23
Corn oil	20	14	8	8	20
Alphacel non-nutritive bulk	4.9	4.9	4.9	4.9	4.9
Silica sand	5	5	5	5	5
Amino acids, vitamins, mineral salt, etc.*	10.47	10.47	10.47	10.47	10.47

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

specific dry matter intake rates never differed between diets by more than 5%. Body mass (± 0.01 g) was recorded daily prior to the first feeding at 06:30 h, again after the 13:30 h feed, and after the last feed at 20:30 h.

Experiments

Composition of applied diets

Synthetic diets used in our experiments were based on a formula developed by E.C.-V. and applied successfully in several previous studies (e.g. Lepczyk et al., 1998; Caviades-Vidal and Karasov, 2001; Brzęk et al., 2009). All diets were composed of protein (casein), corn oil and corn starch (with exception of carbohydrate-free diet SF) in varying proportions, and a constant mix of free amino acids, vitamins and microelements. Details of the composition of all diets are presented in Table 1. We emphasize that diets SF and MS had the same composition as diets '0starch' and '+starch' used in the study by Brzęk and colleagues with nestling house sparrows (Brzęk et al., 2009); they were intended to mimic, respectively, insects consumed by young house sparrows during the first phase of their life, and the mixture of insects and plant (seed) material consumed by older nestlings. In the present study, adult zebra finches were offered dry food (with water provided separately), whereas food fed to nestlings was mixed with water in a 1:3 mass ratio.

Experiment I – capacity of adult and nestling zebra finches to process diets with a different starch content

Young zebra finch nestlings were initially tested on four diets: starch free (SF; no starch; 7 nestlings), low starch (LS; 12.7% starch in dry mass; 10 nestlings), medium starch (MS; 25.4% starch; 8 nestlings) and high starch (HS; 46.23% starch; 7 nestlings). In an analogous experiment with adult zebra finches we used two diets: starch free (SF), and medium starch and high oil (MSO; 25.4% starch; sample size was 9 males per diet). Diet MSO had the same starch content as diet MS given to nestlings and the same oil content as diet SF, to avoid potential confounding effects of different oil content on the activity of digestive enzymes.

Experiment II – developmental plasticity of zebra finch nestlings fed diets containing different starch levels

In this experiment we applied diets MS and HS, which were found in experiment I to support normal development of nestlings (see Results). Performance of nestlings was tested at three time points during their development, selected on the basis of earlier pilot studies of the ontogeny of the digestive anatomy of the zebra finch, and analogous to similar time points used in the study of house sparrows (Brzęk et al., 2009) (see also Caviades-Vidal and Karasov, 2001). They were: day 5 (the phase of rapid development of the gastrointestinal tract); day 8 (the day of the peak in relative intestine mass) and day 15 (3 days before normal fledging, when

adult body mass is already reached). Thus, in experiment II we created six experimental groups: nestlings fed with MS or HS diet, and analysed at day 5, 8 or 15. The total number of nestlings in each group was, for MS diet: 7, 8 and 10; and for HS diet: 10, 9 and 9. Wet food intake differed significantly between nestlings analysed at different ages ($P > 0.0001$ for all comparisons) but not between diets ($P = 0.12$).

Experiment III – effect of different diet starch levels on the digestive physiology of adult zebra finches

We applied the same diets as for nestlings in experiment II. The sample size was 9 adults for MS diet (5 males and 4 females) and 11 adults for HS diet (5 males and 6 females). Birds were raised on pure diets for 15–21 days.

Physiological endpoints – physiological assays applied in our experiments

Organ sizes

Birds were killed with CO₂ and dissected to remove the intestines, stomach, liver, pancreas and pectoral muscles. Pectoral muscles were not dissected from 5 day old nestlings because of their small size. Wet masses of internal organs were recorded (± 0.1 mg). Intestines were flushed with ice-cold avian Ringer solution, weighed, cut into three sections, corresponding to proximal, middle and distal regions of the intestines, and immediately preserved in liquid nitrogen. Similarly, the stomach was emptied, and all organs were cleaned of external fat and tissues, rinsed with ice-cold avian Ringer solution and weighed.

Intestinal enzyme assays

Assays of intestinal digestive enzymes were carried out in the same way as in our earlier study and are described in detail elsewhere (Brzęk et al., 2009). The differences were: (1) intestinal samples analysed in the previous study formed only part of each intestinal section (proximal, medial and distal), and in the present experiment they represented the whole intestine length; (2) we added analysis of the activity of sucrase, in addition to maltase and aminopeptidase-N. The sucrase assay was identical to the maltase assay described previously (Brzęk et al., 2009), with the exception that a solution of sucrose was used as the substrate instead of maltose; (3) we used Omni TH homogenizer (Omni International, Marietta, GA, USA); (4) all volumes in the aminopeptidase-N assay were 4 times lower because of the smaller size of the analysed samples. For 5 day old nestlings, we analysed only 4 birds from each diet, while the remaining individuals were used in other assays. Our results are expressed as micromoles per minute per gram wet tissue (mass-specific activity). We calculated the summed hydrolysis activity of the entire small intestine, which is an index of the total hydrolysis capacity, by multiplying mass-specific activity in proximal, medial and distal intestinal regions by their respective masses, and summed

over the three regions. Finally, we calculated the ratio of mass-specific or summed activity of maltase to aminopeptidase-N (hereafter M/A). We emphasize that the last parameter compares relative investment in the two enzymes and is not affected by potential changes in intestinal morphology or villus area, which can plague comparison between birds of different ages.

Data analysis

Nestling zebra finches in experiment II and adults in experiment III were fed on the same diets. However, nestlings were switched to synthetic diets just 4 days after hatching, whereas the same diets were offered to adult birds long after their development was complete. For this reason, the effect of diet composition on studied traits is not fully comparable between the two experiments and we analysed them in separate sets of ANOVA/ANCOVA tests. However, we present data for nestling and adult birds on the same graphs so that values for each trait in birds of different ages can be easily compared. Sex sometimes had a significant effect on studied traits in adult birds; however, it never affected our main conclusions about diet effect, and was not included in final analyses.

We tested for effects of age and diet on body mass in all nestlings held until age 15 days in experiment II using repeated measures analysis of variance (ANOVA). The size of internal organs in nestlings was analysed with two-way ANOVA or ANCOVA [factors: diet (MS and HS) and age at dissection (5, 8 and 15 days), initial body mass as covariate]. We did not use body mass at dissection as a covariate in these analyses because its range did not overlap for nestlings of different ages, which violates an assumption of ANCOVA. Rather, we re-analysed our data with ANCOVA separately for each age, with diet as a main effect and with body mass at dissection as a covariate. We found some significant differences in these comparisons but because they were always in the same direction as in ANOVA for all ages pooled together we do not show them for the sake of brevity. For the analysis of organ size in adult birds we applied similar two-way ANOVA or ANCOVA [factors=diet (MS and HS) and sex, final body mass as covariate].

Repeated measures analysis of variance was used to examine the effect of diet, intestinal position (proximal, medial, distal) and age (for nestlings) on mass-specific enzyme activities. Data for enzymatic activity summed over the entire length of the intestine were analysed by means of ANOVA similar to our procedure for organ size. All variables describing enzymatic activity were log-transformed prior to analysis. Our enzyme assays were carried out in two batches spread over weeks, and we found a significant difference between them for the activity of disaccharidases but not aminopeptidase-N. We blocked for this effect in the analysis presented in Tables 4 and 5 (therefore tests for aminopeptidase-N have more degrees of freedom than for other enzymes and for M/A ratio).

In all tests, the significance level was set at $P < 0.05$, and $0.05 < P < 0.1$ was taken to indicate a trend. Results are given as means ± 1 s.e.m. We emphasize that the activity of different intestinal disaccharidases cannot be treated as independent traits; for example, intestinal maltase activity is the sum of sucrase-isomaltase (i.e. sucrase) and sucrase-independent maltases [α -glucoamylases (Semenza and Auricchio, 1989)]. We therefore decided not to adjust our significance level for multiple comparisons but rather to rely mostly on M/A ratio, which offers a single estimate of relative investment in carbohydrate- and protein-digestion intestinal enzymes. All tests were carried out using SYSTAT (Wilkinson, 1992) and SAS software (SAS Institute, Cary, NC, USA).

RESULTS

Experiment I – survival of zebra finches on starch-containing and starch-free diets

All 7 nestlings raised on diet SF (starch-free) died before day 15, most of them after just 2 days. For other diets, 6 out of 10 nestlings survived on diet LS (12.7% starch in dry mass), 8 out of 8 on diet MS (25.4% starch in dry mass), and 6 out of 7 on diet HS (46.23% starch in dry mass). Thus, nestlings raised on the SF diet had significantly lower survival than those raised on the other diets (continuity-adjusted chi-square test, $P < 0.05$ for all comparisons). On the other hand, there was no difference in survival among the diets LS, MS and HS (continuity-adjusted chi-square test, $P > 0.1$ for all comparisons).

For adult zebra finches, all 9 males raised on the MSO diet looked normal and showed normal behaviour. However, most birds raised on the SF diet looked obviously weak, and seemed to be less active. One bird had to be excluded because of a very sudden body mass drop, and three more died after 1 week. After that, we decided to stop the experiment and switch all birds back to their normal seed diet; however, two more males that had been consuming the SF diet died soon thereafter. Therefore, only 3 out of 9 males originally fed the SF diet survived this trial in good condition, in contrast to all 9 maintained on diet MSO. This difference between diets was significant (continuity-adjusted chi-square test, $P = 0.012$).

On the basis of these results, all subsequent experiments with nestlings and adults were performed with diets MS and HS (Table 1).

Experiments II and III – effect of diet composition on digestive physiology of nestling and adult zebra finches

Because diet composition usually had the same effect in nestling and adult zebra finches in experiments II and III, we present their results together for the sake of brevity.

Body mass changes

The initial body mass of nestlings in experiment II did not differ among ages and diets ($P > 0.3$ for both effects). Body mass growth of nestlings hand-fed in the laboratory between days 4 and 15 was similar to that observed in nestlings in the colony fed by their parents (Fig. 1A). However, for hand-fed nestlings we found a highly significant interaction between diet and body mass changes ($P < 0.0001$; Fig. 1A), reflecting significantly higher body mass increments in birds fed on the MS diet. When body mass was compared separately for each day by means of a *t*-test, the difference between diets reached the margin of significance on day 8 ($P = 0.05$) and MS-fed birds were heavier for all subsequent days (always $P < 0.005$; see Fig. 1A). A similar pattern was found for body mass measured in the morning, which was not affected by the mass of food in the gastrointestinal tract of nestlings (results not shown). However, the interaction between diet and age was non-significant for the mass of nestlings at necropsy ($P = 0.14$; Fig. 1B).

In the experiment with adult zebra finches, birds assigned to the two diets did not differ in their initial body mass ($P = 0.74$). Diet had no significant effect on final body mass ($P = 0.91$; Fig. 1B) or on the change in body mass during the course of the experiment ($P = 0.93$; initial body mass was significant as covariate).

Organ mass

The mass of most studied internal organs increased with age in nestlings (Fig. 2, Table 2). For intestine mass and length, and pancreas and liver mass all comparisons between ages were

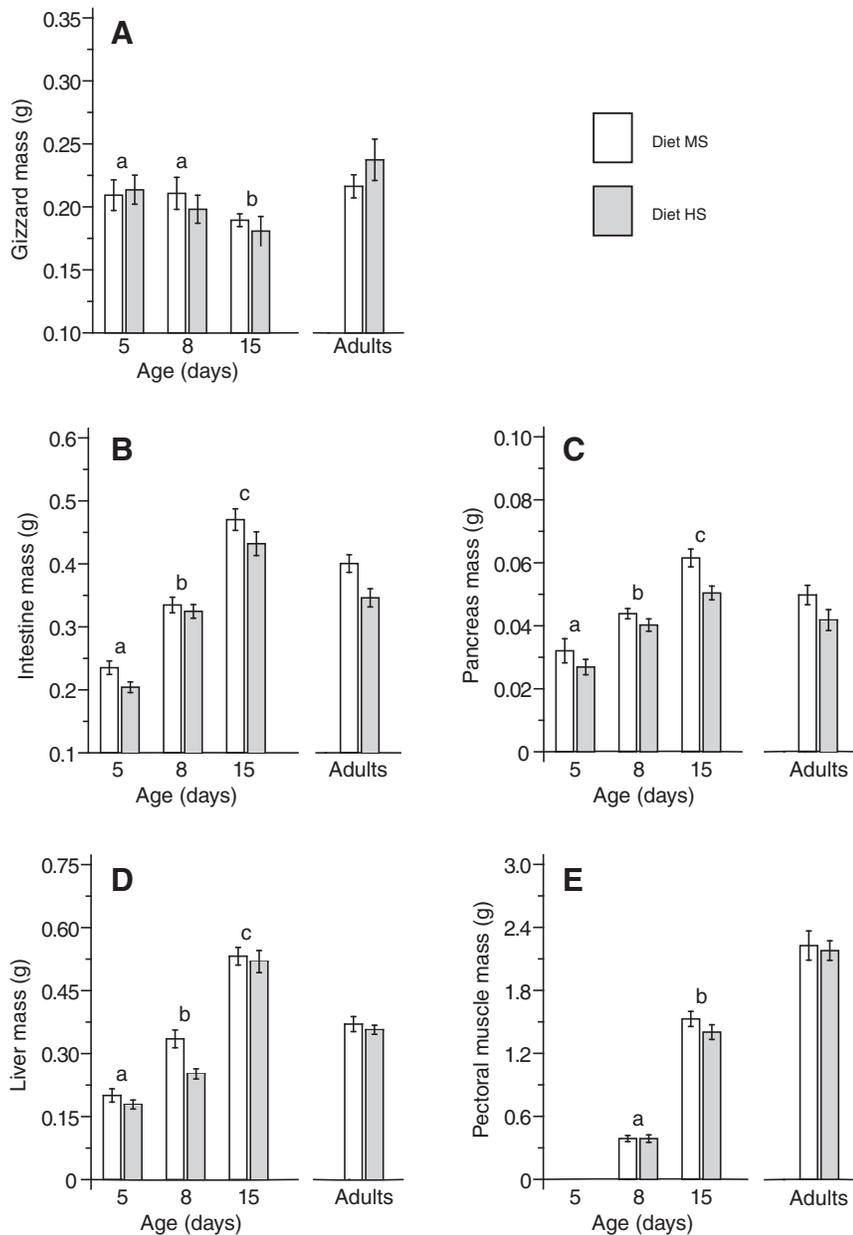


Fig. 2. Size of internal organs in nestling and adult zebra finches as a function of diet and age (for nestlings). For each age, open bars indicate birds raised on diet MS, and filled bars depict birds raised on diet HS, which contained more starch. Different lowercase letters indicate significant differences between nestlings of different ages. See Tables 2 and 3 for significance of diet and age effects.

highly significant ($P < 0.0001$). Only gizzard mass did not follow this pattern (Fig. 2A): it did not differ between days 5 and 8 ($P = 0.23$) but it was significantly smaller in nestlings on day 15 than on day 5 ($P = 0.0018$) and day 8 ($P = 0.041$). Finally, pectoral muscle mass was higher on day 15 than on day 8 (Fig. 2E, Table 2).

The mass of intestines, pancreas and liver was significantly higher in nestlings fed on the MS compared with the HS diet (Fig. 2, Table 2). Likewise, adult birds fed on the MS diet had heavier intestines and longer intestines (almost reaching significance) than individuals fed on the HS diet (Fig. 2, Table 3). When one outlying point was removed, they had also heavier pancreases (Table 3).

Table 2. Summary of results of ANOVA or ANCOVA for the effects of diet and age on size of internal organs in nestlings (experiment II)

	Age			Diet		
	<i>F</i>	d.f.	<i>P</i>	<i>F</i>	d.f.	<i>P</i>
Intestine mass	163.2	2,49	<0.0001	6.39	1,49	0.015
Intestine length	45.37	2,48	<0.0001	2.22	1,48	0.14
Pancreas mass	62.8	2,49	<0.0001	12.21	1,49	0.001
Gizzard mass	4.05	2,49	0.024	0.53	1,49	0.47
Liver mass	184	2,49	<0.0001	6.72	1,49	0.012
Pectoral muscle mass	389	1,33	<0.0001	1.50	1,33	0.23

Interaction between age and diet was always non-significant.

Table 3. Summary of results of ANOVA or ANCOVA for the effects of diet and final body mass on size of internal organs in adult birds (experiment III)

	Diet			Body mass		
	<i>F</i>	d.f.	<i>P</i>	<i>F</i>	d.f.	<i>P</i>
Intestine mass	8.00	1,18	0.011	n.s.	n.s.	n.s.
Intestine mass (without outlying bird)	20.28	1,17	0.0003	n.s.	n.s.	n.s.
Intestine length	4.28	1,17	0.054	12.13	1.17	0.0028
Pancreas mass	3.34	1,18	0.084	n.s.	n.s.	n.s.
Pancreas mass (without outlying bird)	10.15	1,17	0.0054	n.s.	n.s.	n.s.
Gizzard mass	1.24	1,18	0.28	n.s.	n.s.	n.s.
Liver mass	0.93	1,17	0.35	9.06	1,17	0.008
Pectoral muscle mass	0.48	1,17	0.5	16.39	1,17	0.0008

Enzymes

Diet type had no significant effect on the mass-specific activity of all studied enzymes in nestling zebra finches (repeated measures ANOVA; Fig. 3, Table 4). On the other hand, diet MS significantly elevated mass-specific activity of all enzymes in adult zebra finches (Fig. 3, Table 4). However, M/A ratio was not affected by diet composition in both young and adult birds (Fig. 3, Table 4). Nestling age significantly affected all studied parameters (Table 4); for all enzymes, there was a significant increase in mass-specific activity between 5 and 15 days (Fig. 3).

The summed activity of all enzymes increased significantly with age in nestlings and was higher or tended to be higher in birds fed on the MS diet in both nestlings and adults (Fig. 4, Table 5). These differences reflected mainly changes in the size of the intestines, not in mass-specific enzymatic activity (compare Fig. 2B, Fig. 3 and Fig. 4). Not surprisingly, the M/A ratio of summed enzymatic

activity, which is independent of a change in gut size, was not affected by diet (Fig. 4D, Table 5).

DISCUSSION

Prediction 1 – nestlings and adults fail to thrive on high protein/low carbohydrate diets

The results of experiment I showed that both nestling and adult zebra finches could not thrive on diet SF, which had the most extreme composition out of all the applied diets (no carbohydrates and *ca.* 60% of protein in dry mass; Table 1). This contrasts sharply with the nutritional flexibility of the house sparrow, in which both nestlings (Brzęk et al., 2009) and adults (P.B., personal observation) can survive in good condition on diet SF as well as on diets with a lower protein content plus carbohydrate. Moreover, survival of zebra finch nestlings tended to be lower on a diet with 13% starch (LS) than on diets with more starch, although this difference was non-

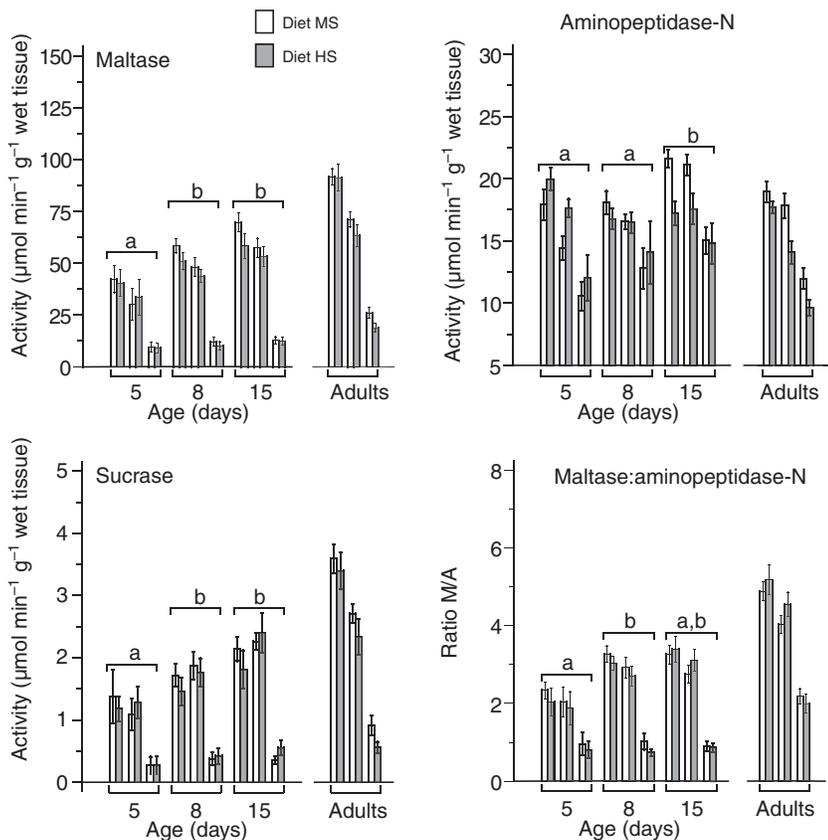


Fig. 3. Mass-specific activity of intestinal enzymes and ratio of maltase to aminopeptidase-N mass-specific activity (M/A) in nestling and adult zebra finches as a function of intestinal position, diet and age (for nestlings). For each age, the three pairs of bars represent, from left to right, the proximal, medial and distal regions of the small intestine. For each pair, the open bars indicate birds raised on diet MS, and the filled bars depict birds raised on diet HS. Different lowercase letters indicate significant differences between nestlings of different ages. See Table 4 for significance of diet and age effects.

Table 4. Summary of results of repeated measure ANOVA for intestinal enzyme mass-specific activities and maltase/aminopeptidase-N ratio (M/A) for nestlings (experiment II) and adults (experiment III)

Effect	d.f.*	Enzyme activity							
		Maltase		Sucrase		Aminopeptidase-N		M/A	
		F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value
Nestlings (experiment II)									
Diet	1,37	1.00	0.32	0.13	0.72	0.03	0.87	1.05	0.31
Age	2,37	4.27	0.021	4.56	0.017	4.57	0.017	3.28	0.049
Diet × age	2,37	0.31	0.74	0.30	0.74	2.97	0.064	1.22	0.31
Position	2,74	264.15	<0.0001	147.73	<0.0001	35.76	<0.0001	309.07	<0.0001
Position × diet	2,74	0.34	0.72	2.32	0.11	0.64	0.53	0.50	0.61
Position × age	4,74	0.71	0.59	1.24	0.30	1.03	0.40	4.12	0.0046
Position × diet × age	4,74	0.29	0.88	0.41	0.80	0.26	0.90	0.18	0.95
Adult birds (experiment III)									
Diet	1,17	8.73	0.0089	15.72	0.001	8.42	0.01	0.00	0.99
Position	2,34	486.69	<0.0001	245.81	<0.0001	100.58	<0.0001	252.43	<0.0001
Position × diet	2,34	5.76	0.007	1.19	0.32	3.03	0.061	3.64	0.037

*Error degrees of freedom for aminopeptidase-N are always larger by 2 for all effects which include position and by 1 for remaining effects.

significant. Although we hypothesized that zebra finches would perform less well (e.g. grow more slowly) on starch-free vs starch-containing diets than did house sparrows, we had no *a priori* reason to expect the difference to be so dramatic when we designed the study.

The transition of adult zebra finches from their normal seed diet to experimental diets was relatively quick (2 days). However, adult house sparrows, which are also granivorous, dealt very well with an even more rapid (1 day) switch from seeds to diet SF (P.B., personal observation). In another Australian seed-eating species, the

cockatiel (*Nymphicus hollandicus*), adult birds were switched gradually over 2 weeks from a diet containing *ca.* 13% crude protein (typical of seeds) to one with 70%, and were able to survive on this new diet for 1 year in good condition (Koutsos et al., 2001). However, any reasonably long transition period would be not feasible in our experiment with rapidly developing altricial nestlings in which we started feeding synthetic diet immediately after nestlings were collected. Therefore, we decided not to apply a long transition period for adult birds to make the results of our different trials with nestling and adult zebra finches more comparable.

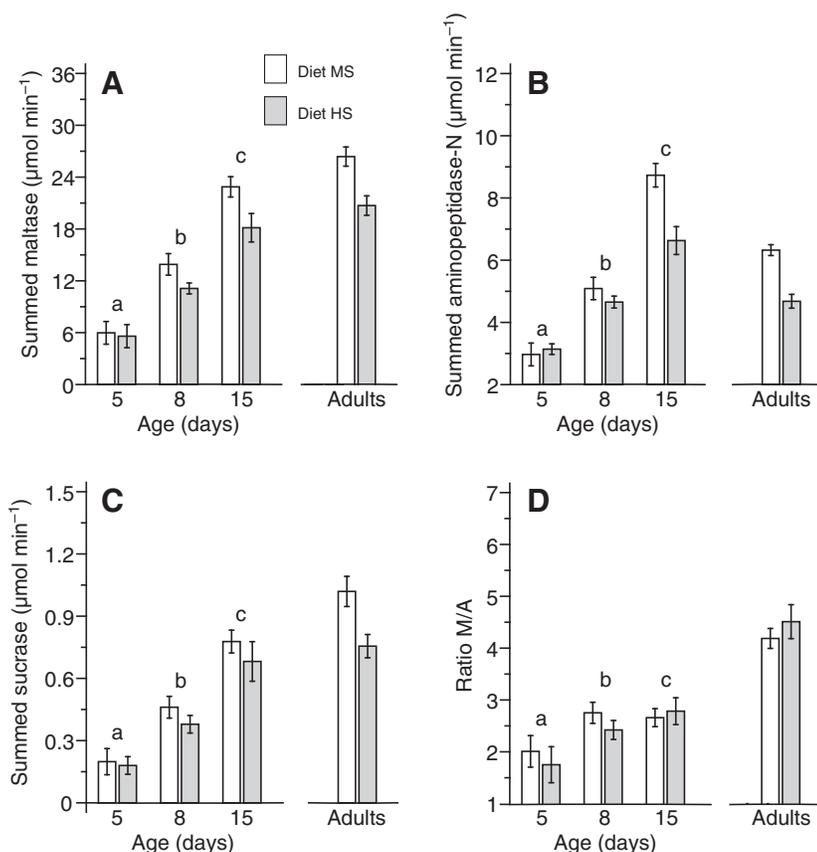


Fig. 4. Summed activity of intestinal enzymes and ratio of summed maltase to aminopeptidase-N activity (M/A) in nestling and adult zebra finches as a function of intestinal position, diet and age (for nestlings). For each age, open bars indicate birds raised on diet MS, and filled bars depict birds raised on diet HS. Different lowercase letters indicate significant differences between nestlings of different ages. See Table 5 for significance of diet and age effects.

Table 5. Summary of results of ANOVA for the effects of diet and age (nestlings; experiment II), and diet (adults; experiment III) on total enzyme activity summed over intestine length

Enzyme	Nestlings						Adults	
	Diet effect		Age effect		Diet vs age		Diet effect	
	F	P	F	P	F	P	F	P
Maltase	6.48	0.015	71.90	<0.0001	0.49	0.62	28.55	<0.0001
Sucrase	2.32	0.14	31.85	<0.0001	0.22	0.81	37.81	<0.0001
Aminopeptidase-N	3.93	0.055	90.53	<0.0001	3.91	0.028	32.32	<0.0001
M/A	1.66	0.21	5.93	0.0059	1.23	0.31	0.40	0.54

d.f. for nestlings: 2,37 for age effect; 1,37 for diet effect; 2,37 for age vs diet interaction; for adults: 1,17 for diet effect; error degrees of freedom for aminopeptidase-N are always larger by 1.

There are at least two possible reasons why zebra finches cannot survive on the SF diet. First, 60% of dietary protein might be too high for a strictly granivorous species and result in problems with denitrification. Indeed, necropsy carried out in one dead adult zebra finch from experiment I revealed possible gout in kidneys. However, both adults and nestlings in experiments II and III were doing well on diet MS, containing 46% protein, which is much higher than is typical of seeds. Following this explanation, then, zebra finches possess a significant spare capacity of amino acid catabolic and nitrogen excretion enzymes for protein metabolism, but their threshold for tolerance of high protein lies between 46% and 60%.

Alternatively, zebra finches might have a low capacity for gluconeogenesis using amino acids as a substrate, and therefore they cannot efficiently use protein as the source of energy (cf. Migliorini et al., 1973). Thus, the limiting factor in diet SF might be not the excess of protein but the lack of carbohydrate. Even on the LS diet with 12.7% starch the zebra finch nestlings survived poorly. For comparison, adult granivorous cockatiels (Koutsous et al., 2001) and pigeons [*Columba livia* (Ciminari et al., 2005)] seemed fine on diets with 15% and 14% starch, respectively. The answer to the question of which factor, high protein level or low carbohydrate content, limits the capacity of zebra finches to thrive on a high protein/starch-free diet will require further experiments with other diet compositions.

Prediction 2 – intestinal carbohydrases are not induced by high dietary carbohydrate in both nestling and adult zebra finches

As predicted, dietary carbohydrate did not significantly increase mass-specific activity of either maltase or sucrase in either nestlings or adults. Indeed, when we found a significant effect of diet on the activity of disaccharidases in adult birds (Tables 4 and 5), its direction was opposite to that predicted by the adaptive modulation hypothesis, as enzymatic activity was higher in birds fed on the MS diet (containing less carbohydrate) than in those fed on the HS diet (Fig. 3). Most significantly, the M/A ratio was always independent of diet type (Tables 4 and 5), and therefore the observed differences in mass-specific activity were presumably caused by a non-specific effect of diet (e.g. changes in composition and fluidity of cell membranes), affecting all digestive enzymes similarly (compare Sabat et al., 1998). Therefore, unlike in young house sparrows (Brzęk et al., 2009), the activity of disaccharidases in young zebra finches was not elevated by a higher dietary starch content. Because young house sparrows face variable and, presumably, unpredictable changes in diet composition (Anderson, 2006), there could be selection for high developmental plasticity of their digestive physiology. On the other hand, we hypothesize there has been little

selection for the evolution and maintenance of such plasticity in zebra finches because they are specialists that face relatively little change in diet composition.

Mass-specific activity of aminopeptidase-N in adults was elevated in birds fed on the higher protein MS diet (Fig. 3, Table 4). Therefore, adult zebra finches seem to follow the pattern found in almost all previous experimental tests of the adaptive modulation theory for intestinal digestive enzymes in adult altricial birds: a varying level of carbohydrates did not modulate the activity of disaccharidases, whereas the activity of aminopeptidase-N was usually elevated by a protein-rich diet (Afik et al., 1995; Martínez del Rio et al., 1995; Sabat et al., 1998; Caviades-Vidal et al., 2000). Thus, even though changes in the activity of aminopeptidase-N seem to follow the adaptive modulation hypothesis, this is not true for disaccharidases. Young house sparrows provide the best example in altricial birds of a carbohydrate-induced increase in intestinal carbohydrases (Brzęk et al., 2009). It is therefore tempting to speculate that the higher flexibility observed in young house sparrows results from the presence of regulatory mechanisms adjusting the activity of digestive enzymes to changing diet type during ontogeny that are missing in zebra finch and adults of other species.

Both nestling and adult zebra finches fed on the MS diet had a higher intestine and pancreas mass, and nestlings on that diet had also larger livers (Fig. 2). These changes probably reflected the necessity to process food with a higher protein content (Brody, 1994). The larger intestine size resulted in significantly higher or almost significantly higher summed activities of all enzymes in zebra finches fed the higher protein MS diet (Fig. 4). Nestlings also grew faster on the MS than on the HS diet (Fig. 1). This is not surprising, as a protein-supplemented diet improves body growth and general development of zebra finch nestlings (Boag, 1987; Birkhead et al., 1999). Diet composition had no significant effect on the body mass of adult zebra finches in experiment III (Fig. 1).

Prediction 3 – no programmed (diet-independent) increase in intestinal carbohydrases during ontogenetic development in nestling zebra finches

The ratio of summed maltase activity to summed aminopeptidase-N activity (M/A) changed significantly during development in nestling zebra finches, but the change reflected only the lower values observed in the youngest nestlings (Fig. 4D, Table 5). The virtue of using this ratio to test prediction 3 is that it uncouples intestinal enzymatic capacity from ontogenetic increases in the size of the intestine, which would increase the capacity for the two enzyme activities similarly. In young zebra finches fed on the MS diet, the M/A ratio increased *ca.* 37% between days 5 and 15 (Fig. 4D). On the other hand, this ratio increased 350% in nestling house sparrows

eating the identical MS diet over the same developmental period (ca. 20–80% of the typical nestling period) (Caviedes-Vidal and Karasov, 2001). Thus, even when the diet is held constant during development, house sparrow nestlings exhibit much larger relative increases in their intestinal carbohydrase activity as expected for a species that naturally shifts from a diet of insects to one of seeds, whereas zebra finches exhibit much lower changes as expected for a specialist that feeds on a seed diet from hatching through to adulthood.

Digestive physiology and feeding ecology of zebra finches

Our study revealed that the digestive physiology of young zebra finches changes little during ontogeny or in response to diet composition manipulation, especially when compared with house sparrow nestlings. Our diet manipulation was strong enough to cause some significant changes in the size of the internal organs (Fig. 2, Tables 2 and 3) but mechanisms controlling the expression of intestinal disaccharidases were not affected by diet (at least not in the direction predicted by the adaptive modulation hypothesis) and were affected relatively little by age. We studied birds that bred in captivity for several generations; however, domestication had little effect on the physiology of zebra finches (Allen and Hume, 2001). Therefore, our results are likely to represent the digestive flexibility typical of wild birds.

Evolution and maintenance of flexibility of the digestive system depend on several factors linked to species' feeding ecology (Starck, 1999; Starck, 2003; Starck and Wang, 2005). There are two mutually related features of the zebra finch feeding ecology that might be responsible for the low plasticity of its digestive physiology: lack of a diet switch during ontogeny and high specialization to a seed diet. Young house sparrows show a gradual switch in their diet type that is absent in zebra finches. Such a change presumably selected for a higher capacity of diet-induced modulation of physiology of the digestive tract than in zebra finches. However, because our comparison is based on only two species of passerines, much broader comparisons are necessary. Moreover, not all results of previous studies have been consistent with the adaptive modulation hypothesis. Comparative study of the effect of diet type on the ontogeny of digestive enzyme activity in prickleback fishes (*Stichaeidae*) unexpectedly found a lower plasticity in species which change their diet during ontogeny compared with that in a whole-life dietary specialist (German et al., 2004).

If the capacity of mounting a flexible digestive response is burdened with costs (DeWitt et al., 1998; Pigiucci, 2005), the low diet-induced flexibility of the digestive tract in zebra finch nestlings can be seen as an adaptation to the best possible utilization of seeds, even at the cost of a lower digestive plasticity. Similarly, the lack of diet-induced changes in the digestive physiology of young *Octodon degus* rodents was interpreted as a result of specialization to herbivory (Sabat and Bozinovic, 2008). In the arid habitat typical of zebra finches, seeds can be quite a reliable food source, at least relative to other types of food (Morton and Davies, 1983). Adult zebra finches show some physiological adaptations to a protein-limited, granivorous diet (Houston et al., 1995; Allen and Hume, 2001). Very narrow diet specialization, including relying only on seeds for feeding nestlings, is possible in granivorous birds when natural seeds have a relatively high protein content (Valera et al., 2005). In zebra finches, such a role can be played by the presence of green seeds following rain, which can provide breeding birds and nestlings with essential amino acids (Allen and Hume, 1997). Our results show that zebra finches have some extra capacity to increase protein intake, as they tolerated the diet with only 25%

starch and 46% protein well, which is presumably very different from the composition of their natural diet. But, their overall low digestive flexibility may limit their capacity for using new food sources. Significantly, zebra finches cannot live on a synthetic starch-free diet, whereas at least three other granivorous passerines possess this capacity [*Zonotrichia capensis* and *Diuca diuca* (Sabat et al., 1998); *Passer domesticus* (Brzęk et al., 2009) (P.B., personal observation)]. However, more similar studies on species with different feeding ecology and phylogenetic position are necessary to make some firm general conclusions.

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