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Effect of fasting on the structure and function of the gastrointestinal tract of house sparrows (*Passer domesticus*)

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ABSTRACT

Starvation is a condition that often affects animals in nature. The gastrointestinal tract is the organ system displaying the most rapid and dramatic changes in response to nutrient deprivation. To date, little is known about starvation phases and effects on the organ morphology and digestive function in small passerine birds. In this study, we determined the phases of starvation and examined the effect of final stage of starvation in the organ morphology and, intestinal histology and enzymatic function in the small intestine. Our results show the three phases of the classical model of fasting in a shorter period of time. The mass of heart, pancreas, stomach, small intestine and liver of long-term fasted birds was reduced between 20 and 47%. The mass decrease in small intestine was correlated with reduction in small intestinal histology: perimeter, mucosal thickness, villus height and width. In contrast, the enzyme activity of sucrase–isomaltase and aminopeptidase-N in enterocytes, all expressed per µg of protein, was higher in long-term fasted birds than fed animals. This suggest that, while autophagy of digestive organs is induced by starvation, consistent with phenotypic plasticity, the activity of sucrase–isomaltase and aminopeptidase-N remains high, probably as an anticipatory strategy to optimize digestion at re-feeding time.
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1. Introduction

Starvation is an ecological relevant situation, in which animals do not have enough nutrients to feed on, as a result of some extrinsic limitation on food resources. In nature, starvation occurs as a natural event and can vary widely in frequency and duration, i.e.: rainstorm, snowstorm (McCue, 2010). When animals are exposed to changes in nutrient quality and quantity, they exhibit adaptive biochemical., physiological and molecular responses such as a reduction in body weight and visceral organ mass, atrophy of intestinal mucosa, immune dysfunction and decrease of activity enzyme, among others (Ferraris and Carey, 2000; Chappell et al., 2003; Starck, 2003; Houston et al., 2007).

The response to food deprivation in birds and mammals has been characterized by three consecutive phases defined by progressive metabolic and physiological changes (Wang et al., 2006; McCue, 2010). Phase I occurs immediately after the last food has been absorbed at the small intestine. It is characterized by both the use of liver glycogen stores and a significant body mass reduction in a short time. Phase II is based on the energy economization and oxidation of lipids. Since lipids have high amount of energy, weight loss is slow during this stage. Finally, at phase III, when lipid deposits are depleted, muscle protein catabolism begins combined with a decrease in protein synthesis. As a result of protein degradation, the rate of body mass loss increases along with nitrogenous waste (uric acid) production (Wang et al., 2006). The first objective of this work was to characterize the degradation response to starvation in the house sparrows by biochemical parameters in plasma and body mass changes. We expected to see the same three phases of the classical model of fasting but in a shorter period of time, since small animals are more susceptible to the complete absence of food than those of a larger size. The increased energetic demand to keep homeostasis of the body during starvation is reflected in the down regulation of tissue and organ mass (Bauchinger et al., 2005). In vertebrates, the digestive system is the most affected and displays dramatic changes (Ferraris and Carey, 2000; Starck, 2003; McCue, 2010). Consequently, morphological studies during fasting have showed changes in villus length and thickness, as well as in enterocyte phenotype. Besides, generative components (crypts) of the mucosa are preferentially preserved in comparison to the absorptive part (villi) (Dunel-Erb et al., 2001; Karasov et al., 2004).

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The second objective was to evaluate the effects of long period of food deprivation (phase III of starvation) on organ size and histological parameters in small intestine (perimeter, mucosal layer, villus length and crypt size).

It is known that the structure and function of the gastrointestinal tract in vertebrates is flexible. Previous studies have been performed mainly in mammals, poultry or migratory birds (Yamauchi et al., 1997; Ferraris and Carey, 2000; Fassbinder-Orth and Karasov, 2006; Wang et al., 2006), but there is little information about the mechanisms (i.e. enzyme activity, intestinal absorption, gene expression) involved in the energy administration process in small non-migratory passerine birds during fasting (Karasov and Martínez del Rio, 2007). A reason to study digestive changes in small non-migratory passerine birds in response to a total lack of food is because migrant birds may be under a selective pressure for fasting, (Lindstrom and Alerstam, 1992). In addition, flexibility of the digestive capacity during fasting in non-migrant birds could play a significant role in the economization of energy use (Klaassen et al., 1997). Several studies in mammals had shown a down regulation of enzyme activity during starvation (Holt and Yeh, 1992; Ihara et al., 2000) because the activities of different digestive enzymes and nutrient transporters are regulated positively by their substrate concentration (Sanderson and Naik, 2000; Ferraris, 2001). In contrast, there is little information about this process in birds, in poultry and passerine migratory birds (*Dendroica coronate*) with 54% of food restriction, but not total restriction, the activity of digestive enzymes decreases (Lee et al., 2002; Fassbinder-Orth and Karasov, 2006). On the other hand, in poultry, there is an interesting pattern of plasticity of the intestinal enzyme activities in response to diet, where variations in activity of disaccharidases, but not aminopeptidase-N, in the small intestine have been detected (Biviano et al., 1993; Ciminari, 2011). In contrast, passerine birds modulated the aminopeptidase-N activity in response to diet but disaccharidases activity remained invariable (Caviedes-Vidal et al., 2000; Ciminari, 2011).

The third goal was to investigate the effects of long period of food deprivation (phase III of starvation) on the hydrolytic enzymes (sucrose–isomaltase and aminopeptidase-N) activity in the enterocytes along the intestine. In addition we investigated a parallel change between mRNA of sucrose–isomaltase and activity, to integrate molecular and cellular level.

2. Materials and methods

2.1. Animal care and housing

Adult house sparrows (*Passer domesticus*) were captured with live traps near the Universidad Nacional de San Luis Campus (San Luis, Argentina). The birds were housed in cages (40×25×25 cm) indoors under relatively constant environmental conditions (23±1 °C) on a photoperiod of 14:10 h (light:dark) with food and water *ad libitum* (seeds supplied with vitamins and minerals). Animals were acclimated to laboratory conditions for two weeks prior to use in experiments. Animal care and trial protocols (protocol number N° B69/09) were approved by the committee of Universidad Nacional de San Luis (CICUA).

2.2. Experimental design

2.2.1. Experiment 1: determination of starvation phases

Seven house sparrows were used in experiment 1. Food was removed 2 h after lights turned on (8:00 h), and then blood samples (~40 μL) were collected from the brachial vein with a heparinized capillary tube at the following times: 8:00, 12:00, 20:00, 8:00 (2nd day) and between 15:00 and 18:00 (2nd day, when animals loss around 15 to 20% of body mass). We collected five blood samples (200 μL total, which accounts for <10% of total blood volume; (Stangel, 1986)). The samples were centrifuged 3 min at 10,000 rpm in a hematocrit centrifuge (Eavour model VT-1224) and the plasma was separated and stored in refrigerator at 2–8 °C for biochemical analysis (triglycerides and uric acid).

2.2.2. Experiment 2: effect of long-term fasting on organ mass and enzymatic activity of small intestine

Fourteen house sparrows were randomly assigned to either the feeding group (with food and water *ad libitum*, see Section 2.1) or long-term fasting group (water *ad libitum*). The mean body masses of both groups were similar at the beginning of the experiment (26.31±1.29; 25.66±0.31, $p>0.68$). For long-term fasting group, the body mass of birds were measured after 24 h of fasting every 2–3 h until the end of the experiment (phase III of fasting, when animals lost more than 15% of their body mass, ~31–34 h). After that, the birds were anesthetized using ketamine and xilacyne (Paul-Murphy and Fialkowski, 2001), the abdominal cavity was opened and the entire gastrointestinal tract removed and chilled in ice-cold avian saline buffer (Caviedes-Vidal and Karasov, 1996). In fourteen birds, stomach, heart and liver were removed, cleaned of extraneous tissue and weighed. Pancreas was carefully excised, cleaned of extraneous tissue, weighed and immediately frozen in a −120 °C freezer. The content of the small intestine was removed and the small intestine weighed. A segment of the proximal, medial and distal intestine (proximal = near to pyloric sphincter) were sectioned for enzyme and RNA isolation assays. The width and height of each segment was measured with a caliper, weighted and used for enterocytes isolation for enzymatic activity and mRNA extraction.

Remarkably, the intestinal chyme of birds in phase III fasted was dark brown to black in color, probably due to the presence of carbon, sodium sulfur, chloride, calcium and iron, as reported earlier (Habold et al., 2006).

2.2.3. Experiment 3: intestinal morphometric parameters

In another set of experiments, eight animals were randomly assigned to either the feeding (with food and water *ad libitum*, see Section 2.1) or long-term fasting group (water *ad libitum*). The mean body mass of both groups was not significantly different (25.23±1.033; 24.52±0.815, $p>0.61$). The gastrointestinal tract was dissected out, the small intestine cleaned of extraneous tissue and the lumen washed with ice cold 1% NaCl solution to remove digesta. Two transversal portions of 1 cm each was excised from the proximal, medial and distal intestine and placed in their own individual vials containing fresh Bouin solution (saturated solution of picric acid, with glacial acetic acid and formol 40%).

2.3. Analytical procedures

2.3.1. Triglycerides and uric acid

Plasma samples were used for determination of triglycerides and uric acid using commercial kits from Wiener lab (Wiener Laboratorios SAIC, Rosario, Argentina) following standard protocols.

2.3.2. Enterocytes isolation

Enterocytes were isolated using a modification of classical chemical method adjusted for birds (Mac Donal et al., 2008). Briefly, small intestinal segments were washed with ice-cold Hanks' balanced salt solution supplemented with mannitol (HBSS–mannitol, pH 7.2). Intestinal segments were submerged in 1 mL of ice-cold chelation buffer containing: 30 mM EDTA, 1.94 mM DL-dithiothreitol, 52 mM NaCl, 4.39 mM KCl, 10 mM L-1N-2-hydroxyethylpiperazine-N′-2-ethanosulfonic acid (HEPES), 60 mL/L HCl 1 N (pH 7.1) for 75 min at 4 °C without shaking. After that, chelation buffer was gently discarded; 1 mL of HBSS–mannitol added and cells vigorously shaken for 30 s. Cells were collected and centrifuged at 100 *g*

during 5 min at 4 °C. The pellet was washed three times by successive resuspensions in 1 mL of HBSS–mannitol, cells dispersed by passing them several times through a hypodermic needle and centrifugation at 100 g for 5 min at 4 °C. The final pellet was divided into two aliquots, weighed and stored at −120 °C. One of the aliquots was used to study mRNA expression and the other for enzyme activity assays. Isolated cells obtained using low-temperature has been demonstrated to be suitable for mRNA expression studies (Flint et al., 1991). All solutions were sterilized and prepared using RNAse free water. Isolated cells obtained for enzyme activity assays were homogenized for 30 s by passing them several times through a hypodermic needle in 350 mM mannitol in 1 mM Hepes/KOH buffer (pH 7), using 10 mL per g of tissue.

2.3.3. Enzyme assays

2.3.3.1. Sucrase–isomaltase assay. Activity of the disaccharidase sucrase (EC 3.2.1.48) was determined in the isolated enterocytes sample. We used the colorimetric method developed by Dahlqvist (1984) and modified by Martinez del Río (1990). In brief, aliquots of 40 μL of diluted enterocytes homogenate, were incubated with 40 μL of 56 mM sucrose solutions in 0.1 M maleate/NaOH pH 6.5. After 10 min of incubation at 40 °C the reaction was stopped by adding 1 mL of enzymatic glucose assay (Glicemia Enzimática reagent -Wiener Laboratorios SAIC, Rosario). Sample solutions were allowed to stand for 20 min at room temperature and then the absorbances were measured at 505 nm. Enzyme activity (μmoles of glucose hydrolyzed per min) was determined using a glucose standard curve. The protein concentrations of the enterocytes samples in the three intestinal portions were estimated using the Bradford protocol.

2.3.3.2. Aminopeptidase-N assay. Aminopeptidase-N (E.C. 3.4.11.2) was assayed using L-alanine-p-nitroanilide as a substrate (Maroux et al., 1973). Aliquots of 10 μL of the enterocytes homogenate were added to 1 mL assay solution (2.0 mM L-alanine-p-nitroanilide in 0.2 M phosphate buffer (NaH_2PO_4/Na_2HPO_4, pH 7)). The reaction was incubated for 10 min at 40 °C and then stopped with 3 mL of chilled 2 M acetic acid. Absorbance was measured at 384 nm, and activity was determined using a p-nitroanilide standard curve.

2.3.4. Standardization of enzyme activity and kinetics parameters

Activities are presented as total hydrolytic activity (nmol · min^{-1}), activity per μg of enterocyte protein (μmol · min^{-1} · μg $protein^{-1}$), activity per enterocyte mass (nmol · min^{-1} · mg $enterocytes^{-1}$) and activity per unit of surface area (nmol · min^{-1} · cm^2). On the other hand, most fasting studies show activity per unit whole intestinal mass. We used activity per intestinal cells, because we are interested in activity of enzymes located in the enterocytes. Despite that muscle mass have no changes during starvation (Karasov et al., 2004), we do not know about mass of the underlying tissues (i.e. connective layer), that could be decreased during fasting, so activity per whole tissue seemed to increase. So, we then normalized activity by different parameters to compare with other activities from literature.

Finally the apparent binding constants K_m and V_{max} for the disaccharidase were assayed at pH 6.5 and substrate concentration varying from 2 to 100 mM (Martinez Del Río, 1990; Ciminari et al., 2005).

2.3.5. RNA extraction and RT-PCR assays for sucrase–isomaltose

Total RNA was prepared from isolated enterocytes of the proximal and medial intestinal portions using TRIzol™ according to the manufacturer's protocol (Ambion, Austin, TX, USA). The resulting total RNA was DNase I treated (Invitrogen, Carlsbad, CA, USA) and then quantified (260:280 ratio between 1.9 and 2). RNA quality was confirmed by electrophoresis on a 1% agarose gel stained with GelRed. Single strand cDNA was made from 10 μg total mRNA by reverse transcription (RT) using oligo dT primers, and reverse transcriptase (MMLV), both supplied by Sigma-Aldrich (St Louis, MO, USA) through 1 cycle of RT (42 °C for 1 h followed by 95 °C for 5 min). For each primer pair, concentrations of $MgCl_2$ and Taq DNA polymerase, as well as annealing temperatures were optimized. The polymerase chain reaction (PCR) was achieved with a gradient thermocycler (Mastercycler™ gradient 5331-Eppendorf).

The final PCR reaction mixture used for each primer pair was the same: 1× PCR buffer, 200 μM dNTP mix, 3 mM $MgCl_2$, 0.8 unit Taq polymerase, 1 μM each forward and reverse primer, 2 μL cDNA template brought to a final volume of 35 μL with nuclease free water. The reaction mixture (35 μL) was subjected to the following cycles: 1 cycle of initial denaturation (94 °C for 3 min), 39 cycles of amplification at the following temperatures: melting temperature (94 °C for 45 s); annealing temperature at 57 °C for 1 min (for the β-actin primers), or 60 °C for 30 s (for sucrase–isomaltase primers, Genbank accession number GQ919054 (Gatica Sosa et al., 2009)); elongation temperature (72 °C for 1 min.); with a final elongation (72 °C for 5 min). PCR buffer, MgCl2, dNTP and Taq DNA polymerase were supplied by Sigma; primers were supplied by Invitrogen Argentina S.A. and nuclease free water was from Fermentas, Life Sciences.

PCR products were electrophoresed on agarose gels (2.5%) in a Mini-Sub® Cell GT (Agarose Gel Electrophoresis System, Bio-Rad) for 90 min at 50 mV, visualized by GelRed staining and documented using a gel documentation system. The relative content of mRNA for each gene was expressed as a ratio of band intensity relative to β-actin. No products were observed in negative controls in which reverse transcriptase was replaced by water. For primers design for β-actin of house sparrows we used the sequence of another passerine bird, zebra finch (Genbank accession number AY045726). Staining intensity of each cDNA band was determined using densitometric analysis (ImageJ).

2.3.6. Histological procedure

The proximal, medial and distal intestinal portions were placed in individual vials containing fresh Bouin solution and allowed to fix between 6 and 12 h at room temperature, for posterior processing. The intestinal pieces were dehydrated in increasing ethanol solutions and finally were embedded in paraffin. Sections were mounted on slides, stained with hematoxylin-eosin stains and covered with cover glasses. All histological studies were performed on 5 μm sections, and examined by a light microscope. Pictures were taken whit an Olympus BX40 light microscope and analyzed with the program Image Pro-Plus 5.0.

From each section we measured the circumference of the serosal surface, length and width of villi and the width of the cripts. We took 20 measurements per section, resulting in 60 measurements per individual. We measured only those villi that were cut in their midline, from tip to base, as verified by observations of similarly sized and shaped enterocytes. These data were used to estimate the surface area enlargement factor (SEF) by a simple method (Kisielinski et al., 2002). To avoid inflation of degrees of freedom by repeated measurements within individuals, means and standard deviation were calculated for individual bird. These means were used in statistical analyses.

2.4. Data analysis

Results are given as means ± 1 SE, the number of individuals (n) was 7 for all experiments except where otherwise indicated. Repeated measures analysis of variance (RM-ANOVA), followed by Fisher's LSD (least significant difference) post-hoc test, was used to examine the effect of fasting on measured parameters (biochemical., morphological., histological and enzyme activity) at different intestinal positions. If required, data were first transformed to achieve homogeneity of variances and normality of distribution, using natural

logarithm or square root transformations. Morphological parameters (body mass, intestine, pancreas, liver, stomach and heart mass) were analyzed by *t*-tests for independent samples. Standard least-squares methods were used to estimate linear regression parameters. Kinetic parameters were determined by fitting the kinetic data by non-linear curve fitting (gauss Newton routine, SYSTAT Wilkinson, 1992) to the equation relative activity = $(V_{max} \times \text{concentration})/(K*_m + \text{concentration})$.

Normality of data was checked by Shapiro–Wilk test, and homoscedasticity by Levene's test and homoscedasticity of the covariance tested with the Box M test. The significance level selected to accept a difference for all statistical analyses performed was $\alpha < 0.05$.

3. Results

3.1. Body mass loss and biochemical changes at phases of fasting

In order to explain the changes in body mass (BM) throughout the fasting period and their correspondence with the classical model of three phases, we analyzed total BM loss and rate of BM loss (experiment 1). Fasted animals (n = 7) lost an average of 17% of initial BM (initial BM: 25.66 ± 0.288; final BM: 21.18 ± 0.29, $p < 0.001$). During the first h of fasting (until 4 h, phase I) the rate of BM loss (mg/g∗h) was markedly reduced ($p < 0.002$); from intermediate h (~12 h to 28 h, phase II) the rate of BM loss remained at this lower value ($p > 0.1$) and it was subsequently increased ($p < 0.001$) in the latest 5–7 h of fasting (after ~28 h, phase III) (Fig. 1).

Plasma triglyceride level (g/L) was high in food-deprived animals at the beginning of the experiment (t = 0 h), and this value was decreased throughout all times of fasting ($p < 0.02$) (Fig. 1). Uric acid level (g/L) decreased at the phase I ($p < 0.02$), and then remain constant during phase II, and increased markedly at the end of experiment ($p < 0.01$) (Fig. 1).

3.2. Variation in organ masses

All organs weighted after starvation (experiment 2) showed a decrease in mass compared to control values ($p < 0.01$, means ± SE for several variables are presented for the control group followed by the fasted group). Liver and small intestine masses (g) decreased markedly, around 41% for small intestine (1.08 ± 0.15 to 0.64 ± 0.042, $p < 0.01$); and around 47% for liver (0.92 ± 0.080 to 0.48 ± 0.021, $p < 0.002$), whereas pancreas and stomach showed reductions around 20 to 25% (pancreas: 0.14 ± 0.012; 0.11 ± 0.007, $p < 0.006$; stomach: 0.86 ± 0.078; 0.63 ± 0.034, $p < 0.01$). In the same way, the mass of heart (a nonalimentary tissue) was 24% lower in fasted birds than controls (0.37 ± 0.015; 0.28 ± 0.016; $p < 0.001$), see Fig. 2.

3.3. Intestinal morphometric parameters

Small intestine length (cm) was significant reduced (17.23 ± 0.742; 14.76 ± 0.442, $p < 0.02$) after prolonged fasting compared to control values. The nominal surface area (cm^2) declined (12.28 ± 0.773 to 9.65 ± 0.859; $p < 0.018$) and protein concentration (μg per mg of enterocytes) decreased 35% between control group and fasted group in whole intestine (0.114 ± 0.0149 to 0.073 ± 0.0104, $p < 0.028$). In the same way, mg of enterocytes per mg of whole intestinal tissue decreased after prolonged fasting ($p < 0.048$).

Compared to normally fed animals, phase III fasted birds showed several morphological changes through proximal to distal position (Table 1). Overall, perimeter decreased around ~15% in proximal and medial portions but not in distal position, mucosal width ~30%, villus height ~30% in proximal and medial portions but not in distal position, villus width ~27% and crypts width ~22%. The surface area enlargement factor (SEF) decrease from proximal to distal position and proximal portion show significant difference between fed vs. fasted birds. The enterocytes showed a phenotypic change in height (~25%) and width (~12%).

3.4. Intestinal enzymes

3.4.1. Sucrase–isomaltase activity

The enzymatic activity of sucrase–isomaltase showed a significant decrease from proximal to distal position in both control and fasted groups (RM-ANOVA $p < 0.01$). During starvation, intestinal sucrase–isomaltase total hydrolytic activity, specific activity per μg of protein, mg of enterocytes and per nominal surface area increased by ~100% in proximal and medial sections of small intestine (RM-ANOVA $p < 0,05$ for treatment and position and the interaction was not significant for all groups $p > 0.1$, Fig. 3).

3.4.2. Aminopeptidase-N activity

Enzyme activity also exhibited a significant positional effect along the small intestine for control and fasted group, but increased from proximal to distal position (RM-ANOVA $p < 0.05$). During starvation, intestinal aminopeptidase-N total hydrolytic activity, specific activity per μg of protein increased around 80% in proximal and medial sections and around 200% in distal section (RM-ANOVA $p < 0.05$ for treatment, position and interaction, Fig. 4A,B). Enzyme activity

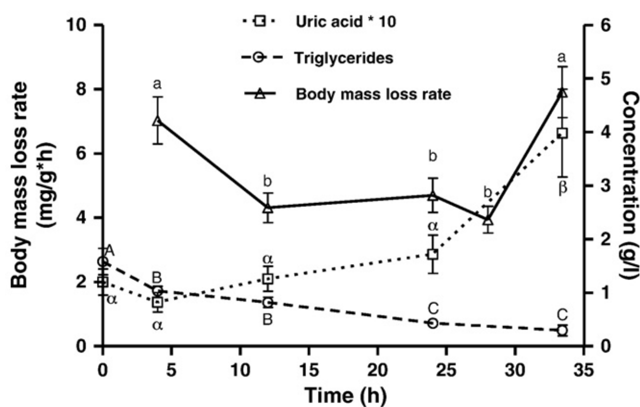

Fig. 1. Variation of biochemical parameters and body mass during fasting. The values represent mean plasma values (±SE) of triglycerides, uric acid and body mass loss rate during fasting (h) of house sparrows (n = 7). Groups marked with different letter are significantly different ($p < 0.05$), for statistical details see Materials and methods.

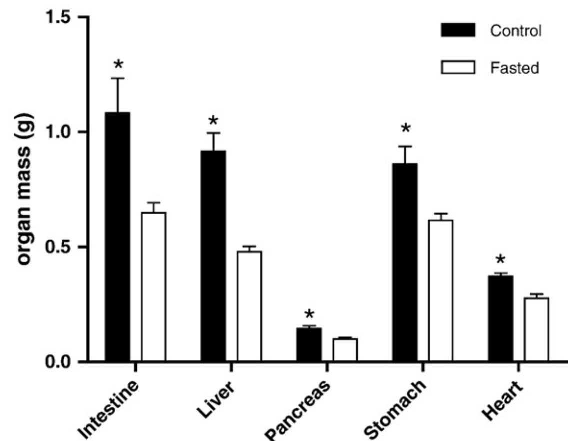

Fig. 2. Organ mass in fasted and control animals. The values are mean (±SE) of organ mass for control group (closed bars) and fasted (open bars) house sparrows (n = 7). Statistical differences are represented by asterisks ($p < 0.01$).

Table 1
Histological measurements of small intestine.

	Control			Fasted		
	Proximal	Medial	Distal	Proximal	Medial	Distal
Perimeter (mm)	9.19 ± 0.31[a]	7.21 ± 0.14[b]	6.31 ± 0.22[c]	7.53 ± 0.37[a*]	6.45 ± 0.21[b*]	5.77 ± 0.12[c]
Mucosal width (mm)	0.87 ± 0.04[a]	0.59 ± 0.03[b]	0.41 ± 0.02[c]	0.62 ± 0.03[a*]	0.40 ± 0.02[b*]	0.28 ± 0.03[c*]
Villus high (μm)	622.45 ± 21.4[a]	363.40 ± 13.2[b]	286.80 ± 17.9[c]	387.80 ± 28.1[a*]	288.40 ± 20.1[b*]	242.35 ± 16.8[b]
Villus width (μm)	88.95 ± 5.7[a]	73.90 ± 6.4[b]	71.40 ± 1.8[b]	61.20 ± 1.7[a*]	54.15 ± 3.1[a*]	55.45 ± 1.8[a*]
Crypts width (μm)	60.85 ± 2.2[a]	55.40 ± 1.2[a]	48.55 ± 1.6[b]	47.90 ± 1.3[a*]	41.40 ± 2.6[b*]	39.15 ± 2.5[b*]
Surface area enlargement factor	10.55 ± 0.31 [a]	7.05 ± 0.20 [b]	6.40 ± 0.37 [b]	8.71 ± 0.69 [a*]	7.70 ± 0.89 [ab]	6.63 ± 0.15 [b]
Enterocytes length (μm)	26.92 ± 1.6[a]	23.45 ± 1.1[b]	22.16 ± 0.7[b]	18.96 ± 0.7[a*]	16.54 ± 0.6[a*]	16.29 ± 0.6[a*]
Enterocytes width (μm)	8.54 ± 0.3[a]	7.96 ± 0.2[ab]	7.77 ± 0.3[b]	7.96 ± 0.2[a]	7.08 ± 0.3[b*]	6.13 ± 0.1[c*]

Data are shown as average ± SE of 4 animals of each treatment (see Materials and methods).
Different superscript characters represent significant differences ($P<0.05$) between intestinal portions within groups (control and fasted group). Asterisks indicate significant differences ($P<0.05$) between groups. For statistical details see Materials and methods.

per mg of enterocytes and per nominal surface area increased significantly by ~60% in distal section of small intestine (Fig. 4C,D), and no interaction between position and treatment was significant ($p>0.08$).

3.4.3. K_m and V_{max} determination of sucrose–isomaltase

There was no significant effect of prolonged fasting on K_m (mM) for sucrase–isomaltase (26.50 ± 3.71 to 29.13 ± 6.37, $p>0.7$) although an increase of 56% was observed on the sucrase–isomaltase V_{max} (nM/min) after fasting (165.36 ± 25.76; 293.13 ± 37.80; $p<0.02$).

3.4.4. Sucrase-isomaltase mRNA changes

Despite the limitations of technique (semi-quantitative RT-PCR), fasting did not alter expression of mRNA sucrase–isomaltase on proximal ($n=4$; feed group: 0.18 ± 0.095 to fasted group: 0.23 ± 0.103; unpaired *t*-test $p>0.53$) and medial portion (0.36 ± 0.180 to 0.31 ± 0.134; unpaired *t*-test $p>0.55$) of small intestine (Fig. 5). The relative content of mRNA for each gene was expressed as a ratio of band intensity relative to β-actin expression as a housekeeping gene.

4. Discussion

According with the first objective of this study the three metabolic phases of fasting were established in house sparrows; this pattern is consistent with the classic profile of the three stages of fasting previously described in larger mammals and birds (Alonso-Alvarez and Ferrer, 2001; Wang et al., 2006). Based on blood sampling times, the time limits (in hours) of the phases of fasting in house sparrows (Fig. 1) were established as follows: phase I is characterized by the use of nutrients from the last meal and the use of glycogen stores, there is a significant reduction in body mass, covering approximately the first 4 h of fasting. Phase II, or economizing phase, is the longest phase (from 4 and up to 24 h.), and is characterized by the use of lipids reserves. Finally, phase III, or critic phase, begins with the use of skeletal muscle proteins, which occurs by a sharp increase in the concentration of uric acid in plasma and a marked decrease in body mass (reaching a loss of 15%–22% of initial body mass). This occurs after 24 h of fasting until 34 h. Several factors, including initial body mass, affect the rate at which starving animals (birds and mammals) lose body mass, making it highly variable. On a comparative level, mammals and birds with similar body masses such as sparrows and mice (~25 g each) have a notable difference regarding the onset of phase III of fasting (3–4 days for the mouse (Wang et al., 2006; Sokolović et al., 2007) and 34 h for sparrow). This disparity between fasting duration in mammals and birds may be due primarily because birds have a high basal metabolic rate, between 30 and 40% more than mammals (McNab, 2009).

4.1. Adaptive changes in organ mass and digestive features

The intestinal tract is a metabolically expensive organ to maintain, the regulation of its physiological functions consumes around 17–25% of oxygen of the whole body (Cant et al., 1996). So, an effective way to minimize energy expenditure during food scarcity is a decrease in organ mass. Our data show that weight loss in response to fasting is more pronounced in the small intestine and liver than in the total body (Fig. 2). Small intestine mass of fasted sparrows was reduced by 41% compared with controls (Fig. 2), similar value than the 45% reduction by the migrating passerine *Sylvia atricapilla* (Karasov et al., 2004). Masses of other important organs involve in nutrient assimilation, such as the liver, also declines to a similar extent, while pancreas and stomach decline around 35% and 30%, respectively. The reduction of organ mass was disproportionately large compared with the decline in body mass and nonalimentary tissues such as heart (around 24%). These values are similar to those obtain in fasted blackcaps by Karasov et al., 2004. Thus, while the reduction of small intestine mass was around 41%, the reduction of length and nominal surface area was not as pronounced (around 15% and 22% respectively). By preliminary histological studies of small intestine, we found that decreased weight could be associated with a diminished extension of mucosal layer and density of villi (in addition to significant changes in villus height and width). The ratio of villous area relative to nominal area, or surface area enlargement factor, decrease in proximal position but remains unchanged in medial and distal positions (Table 1). The value in fed birds is lower than data obtained in bats by the same method (Caviedes-Vidal et al., 2008), but similar than data in pigeons (Lavin et al., 2007). Besides, we found a phenotypic change in enterocytes (smaller size for fasted birds). Accordingly, the intestinal mucosal layer is the most affected by fasting in birds (Ferraris and Carey, 2000; Dunel-Erb et al., 2001; Karasov et al., 2004), but not the thickness of muscle layer (Karasov et al., 2004). Theoretically, the mucosal layer reduction could affect the biochemical digestive capacity, but interestingly we found the sparrows increase its digestive function (Figs. 3 and 4) that we discuss beneath.

4.2. Plasticity in digestive enzymes

In vertebrates, starvation may be associated with an apparent decline in basal metabolism that actually could be ascribed to a gradual cessation of digestive processes (Wang et al., 2006). Previous studies performed in mammals, domestic fowl and migrant birds showed a significant reduction in disacharidases activity, expressed per mg of tissue protein or per mg of tissue, in response to fasting and food restriction, according to the hypothesis of adaptative modulation (Holt and Yeh, 1992; Ihara et al., 2000; Lee et al., 2002; Fassbinder-Orth and Karasov, 2006). Moreover, in fish, maltase has an interesting pattern of expression in which their activity

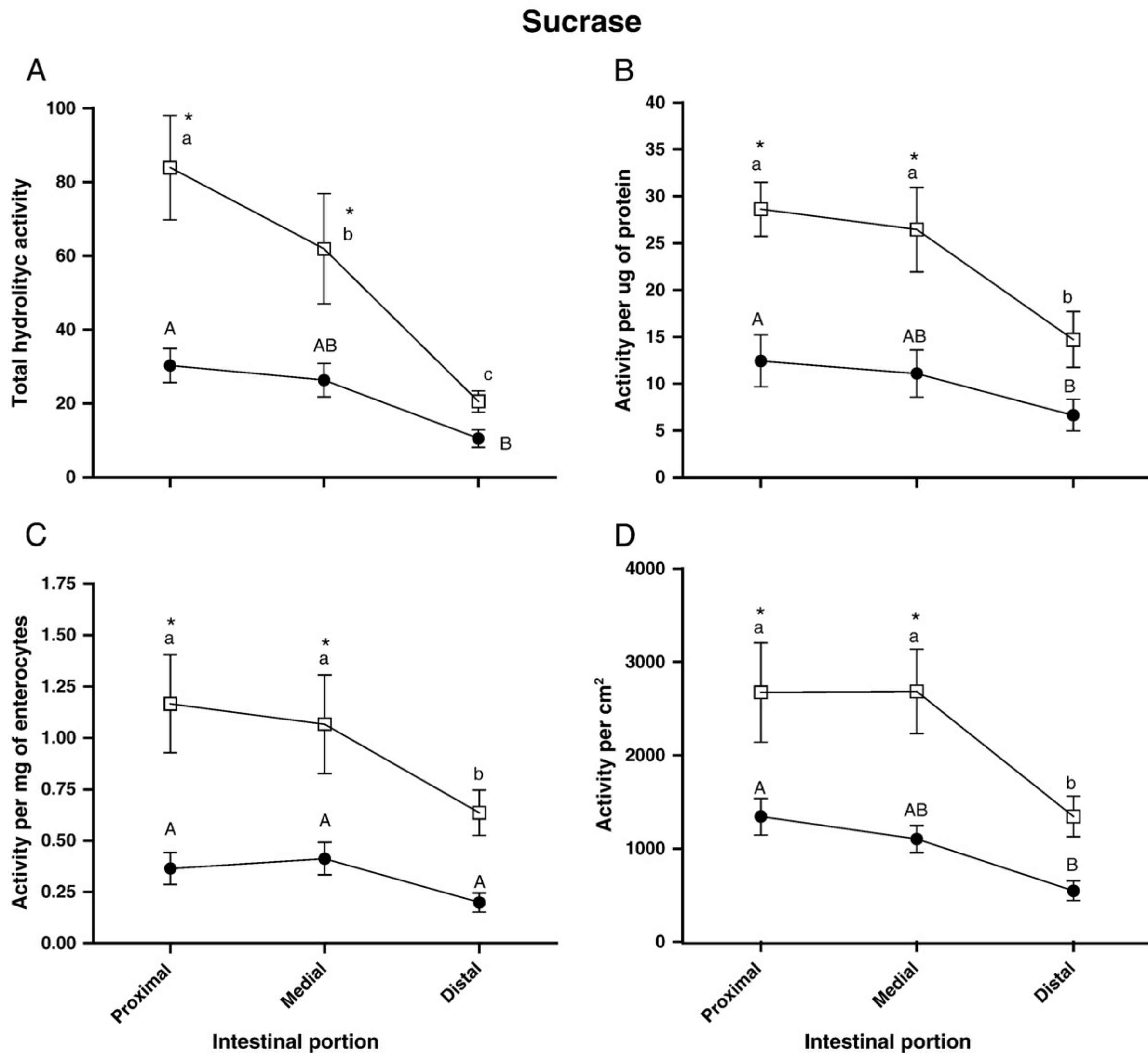

Fig. 3. Effect of fasting on the sucrose activity. Plots of sucrase activity expressed by total hydrolytic activity (panel A), μg of protein (panel B), mg of enterocytes (panel C) and cm^2 of nominal surface area (panel D), in the three portions of small intestine. The values are mean (± SE) of control group (filled circle) and fasted (open square) house sparrows (n = 7). Statistical differences between intestinal portions in the same treatment are represented by letters, and asterisks represent differences between treatments ($p < 0.05$), for statistical details see Materials and methods.

expressed per mg of tissue protein rapidly decrease with the onset of fasting (1–2 days) through day 11, and then have a slightly increase until day 40, while a protease (leucine aminopetidase) increased its activity during all fasting periods (Krogdahl and Bakke-McKellep, 2005). According with these findings, Ihara et al., 2000 found an increasing activity of aminopeptidase-N during fasting in rodents. In sparrows fasted at phase III, the specific activity per μg of enterocytes protein of sucrose–isomaltase and aminopeptidase-N increased by over 100% compared to control animals (Figs. 3 and 4). This significant increase in the disaccharidase specific activity was also observed when we normalized the absolute activity per nominal surface area of the small intestine (Fig. 3). These results seem not to agree with the hypothesis of adaptative modulation (Ferraris and Diamond, 1989), in which expression of digestive enzymes should be modulated in response to the intake of their respective substrates. This pattern is observed in migrant birds which digestive capacities decreased as a result of reduction of digestive organ masses, during long migrations (Lee et al., 2002). But, in non-migratory house sparrows, it is tempting to speculate that the biological significance of the increased activity of sucrase–isomaltase and aminopeptidase-N could be considered a short-term anticipatory strategy. Thus, the increase of enzyme activities would allow the organism to be highly efficient in the hydrolysis of nutrients at the time of the re-feeding to optimize its digestion. This pattern is interesting because there is evidence of significant increase of disaccharidases activity in insulin-deficient mammals (Tandon et al., 1975; Adachi et al., 2003; Liu et al., 2011), indicating that sucrase activity could be inhibited by insulin. On the other hand, in mammals and chickens it is known that insulin levels decrease in blood throughout fasting (Simon et al., 2011), suggesting that the activity of disaccharidases is probably the result of a lack of inhibition by insulin. However, there is no evidence of insulin effect on aminopeptidase-N activity.

Studies of sucrase–isomaltase regulation in mammals showed a coordinated variation in mRNA and protein content, suggesting transcriptional regulation of this enzyme (Yasutake et al., 1995; Boudreau et al., 2001). Through indirect quantification of the functional properties of proteins (kinetic parameters), we found that there were no significant differences between the K_m values of control animals and fasted animals although we observed a significant increase in the V_{max}. One interpretation of these results is that the enzyme affinity does not change during starvation, but there is an

Fig. 4. Effect of fasting on the N-aminopeptadase activity. Plots of N-aminopeptidase activity expressed by total hydrolytic activity (panel A), μg of protein (panel B), mg of enterocytes (panel C) and cm^2 of nominal surface area (panel D), in the three portions of small intestine. The values are mean (±SE) of control group (filled circle) and fasted (open square) house sparrows (n = 7). Statistical differences between intestinal portions in the same treatment are represented by letters, and asterisks represent differences between treatments ($p<0.05$), for statistical details see Materials and methods.

increase in the activity in response to fasting as a result of increased expression of the enzyme or its stabilization (Debnam and Levin, 1975). It is known that the phase III of fasting leads not only to a reduction in body protein content but also to a reduction in protein synthesis (Bertile et al., 2003), however our observation of increased activity and amount of sucrase–isomaltase enzyme suggests the existence of a differential regulation of protein synthesis (while some proteins are being repressed at this stage other are preserved or increased) probably as a response to the optimization of energy expenditure.

Several studies have documented that sucrase–isomaltase mRNA directly correlates to the variations in enzyme activity or protein abundance (Yasutake et al., 1995; Ihara et al., 2000). When we analyzed the mRNA abundance of sucrase–isomaltase in the sparrows, despite the limitations of technique (semi-quantitative RT-PCR), we did not detect an increase accompanying the increase in enzyme activity and amount of the enzyme after the fast (Fig. 5). These results would indicate that exist a regulation to post-transcriptional level for sucrase–isomaltase, i.e. translational regulation, mRNA stabilization process (Guhaniyogi and Brewer, 2001) or protein phosphorylation (Keller et al., 1995). This process will allow the sparrows to increase the amount of protein synthesized from the same RNA

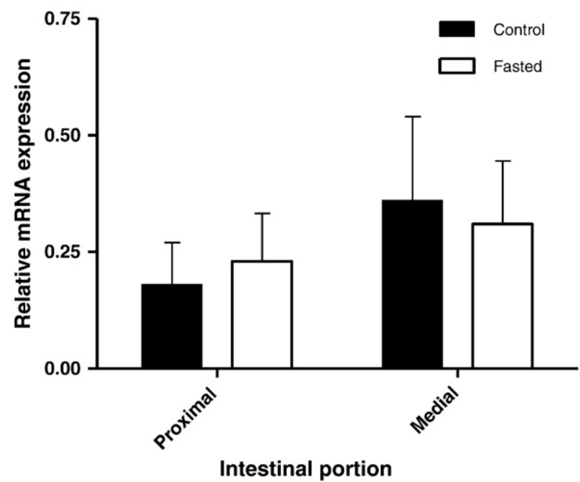

Fig. 5. The relative mRNA expression of sucrose. The relative expression of mRNA of sucrose gene normalized by β-actin gene, in control (closed bars) and fasted (open bars) animals for proximal and medial portions of small intestine. The values represent mean values (±SE) of n = 4 for each group. There was no statistical difference between groups ($p>0.53$).

molecule. Further analysis is clearly needed to investigate in detail the molecular mechanism involved.

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