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Rapid dietary shift in *Podarcis siculus* resulted in localized changes in gut function

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Abstract

Natural dietary shifts offer the opportunity to address the nutritional physiological characters required to thrive on a particular diet. Here, we studied the nutritional physiology of *Podarcis siculus*, populations of which on Pod Mrčaru, Croatia, have become omnivorous and morphologically distinct (including the development of valves in the hindgut) from their insectivorous source population on Pod Kopište. We compared gut structure and function between the two island populations of this lizard species and contrasted them with an insectivorous mainland outgroup population in Zagreb. Based on the Adaptive Modulation Hypothesis, we predicted changes in gut size and structure, digestive enzyme activities, microbial fermentation products (SCFAs), and plant material digestibility concomitant with this dietary change. The Pod Mrčaru population had heavier guts than the mainland population, but no other differences in gut structure. Most of the enzymatic differences we detected were between the island populations and the outgroup population. The Pod Mrčaru lizards had higher amylase and trehalase activities in their hindguts compared to the Pod Kopište population, the Pod Kopište lizards had greater SCFA concentrations in their hindguts than the omnivorous Pod Mrčaru population. Interestingly, the differences between the Pod Mrčaru and Pod Kopište populations are primarily localized to the hindgut and are likely influenced by microbial communities and a higher food intake by the Pod Mrčaru lizards. Although subtle, the changes in hindgut digestive physiology impact digestibility of plant material in adult lizards— Pod Mrčaru lizards had higher digestibility of herbivorous and omnivorous diets fed over several weeks in the laboratory than did their source population.

Keywords

digestion, enzyme, gut, intestine, lizard, omnivory, physiology

Introduction

Based on Optimal Foraging Theory (Pyke et al. 1977) and Chemical Reactor Theory (Penry and Jumars 1987), the Adaptive Modulation Hypothesis (AMH; Karasov and Diamond 1983; Karasov 1992; Karasov and Martínez del Río 2007) uses economic principles, arguing that the gut is expensive to maintain, and thus, there should be a match between gut function (digestive enzyme activities, nutrient transport rates) and the food ingested by an animal. Thus, to maximize net nutrient gain, a diet shift should lead to changes in gut physiology to match the new diet, on short or long timescales. For example, increased digestive substrate concentration (e.g., starch) requires increases in matched enzyme activities (e.g., amylase activity) to achieve high digestibility of the nutrient (Karasov and Martínez del Río 2007). Indeed, correlations between carbohydrase activities in the gut and carbohydrate intake in the natural diet has been observed in fishes, birds, and mammals, even when analyzed in a phylogenetic context (Schondube et al. 2001; Horn et al. 2006; Perry et al. 2007; German et al. 2010; Kohl et al. 2011; German et al. 2015). Some herbivorous fishes that consume starch-rich diets cannot down regulate their digestive enzyme activities towards carbohydrates (German et al. 2004) or intestinal glucose transport rates (Buddington et al. 1987) when fed low-starch foods in the laboratory, suggesting that expression of the requisite enzymes and transporters can be fixed in some animals. The same cannot be said for proteolytic enzymes, where evolutionary correlations of dietary protein and gut proteolytic activity are weak or non-existent (e.g., Schondube et al. 2001; German et al. 2010; Kohl et al. 2011); although there are examples of short term flexibility

in proteolytic enzyme activities in many species (reviewed in Caviedes-Vidal et al. 2000 and Leigh et al. 2018a).

In terms of gut size, increased food intake (e.g., due to high-fiber content of the diet) speeds up digesta transit rate (Dadd 1960; Pritchard and Robbins 1990; Diamond 1991; Castle and Wunder 1995), and more rapid digesta transit rates requires an increase in gut size and/or some way to slow down digesta transit to maintain digestibility (Sibly 1981; Karasov and Hume 1997; Karasov and Martínez del Río 2007; Karasov and Douglas 2013). Hence, herbivores generally have larger guts than carnivores (Wagner et al. 2009; Stevens and Hume 2004; Dearing 1993) to accommodate more voluminous meals (Pough 1973; Wilson and Lee 1987). Hindgut valves (like the ones observed in a Lacertid lizard: see below) can both increase overall gut surface area and can slow the passage of digesta (Iverson 1982; Stevens and Hume 2004; Bignell 1984; Bell et al. 2005; Godon et al. 2016). Digesta high in fiber (i.e. plant material) is particularly viscous, characterized by low Reynolds Numbers (Lentle and Jansen 2008). Because of these flow characteristics, digesta high in fiber leads to decreased mixing in a smooth-bore tube (Love et al. 2013). By acting as “baffles,” hindgut valves can increase mixing of viscous digesta in what would otherwise be a plug-flow environment with unidirectional flow (Lentle and Jansen 2008), thus helping to maintain nutrient digestibility by increasing the residence time of digesta in that gut region (Stevens and Hume 2004; Karasov and Martínez del Río 2007). We, therefore, predict that animals eating plants would have features of gut structure (e.g., larger gut, hindgut valves) and function (e.g., elevated carbohydrase activities, in congruence with AMH) that facilitate digestion of plant material (Table 1; Karasov and Martínez del Río 2007).

Beyond endogenous digestive processes like digestive enzyme synthesis and secretion, many herbivores and omnivores rely on microbial symbioses, usually in the hindgut, to digest

more fibrous portions of plants (e.g., cellulose) and can derive some portion of their energy intake from microbial fermentation (McBee and McBee 1982; Bjorndal 1997; Stevens and Hume 1998). These microbial fermentations produce short chain fatty acids (SCFA) that can be assimilated across the gut wall by the host (Bergman 1990; Foley et al. 1992). Thus, animals consuming more plant material may have increased reliance on microbial fermentation (in this study, indirectly measured via the products of fermentation, SCFA) in their hindguts to assimilate their plant meals (Stevens and Hume 1998).

One of the most interesting examples of a rapid dietary shift in a vertebrate animal is in the Italian Wall Lizard, *Podarcis siculus*. In 1971, five male-female pairs of *P. siculus* were moved from the island of Pod Kopište (0.09 km²), Croatia, to the nearby island of Pod Mrčaru (0.03 km²) as part of a biological invasion study (Fig. 1; Nevo et al. 1972). Returning to the Croatian islets in 2004-2006 (<30 *P. siculus* generations later), Herrel et al. (2008) found the new population on Pod Mrčaru had morphologically and behaviorally diverged from their source population on Pod Kopište. Although the Pod Kopište lizards were insectivorous, consuming 4-7% plant material (by mass), plants made up 34-61% of the Pod Mrčaru population's intake. Plant material was more available on Pod Mrčaru, but arthropod abundance and diversity was similar between islands (A. Herrel, *unpublished data*). The Pod Mrčaru lizards were larger, had different head shapes, and larger bite forces. Additionally, both adult and neonate lizards from Pod Mrčaru had developed valves in their hindguts, a feature not found in the Pod Kopište population. Hindgut valves in lizards are generally associated with highly derived herbivory (Iverson 1982; Bjorndal 1997; Stevens and Hume 2004). Morphological and dietary changes have been documented between the Pod Mrčaru lizards and their source population on Pod

Kopište (Herrel et al. 2008); however it is unknown if the appearance of hindgut valves is concomitant with other shifts in gut morphology or function.

In this study, we examined gut structure (gut length, histological surface area) and function (digestive enzyme activities, organic matter digestibility) in the context of diet (proportion plant material, mass of stomach contents) in *P. siculus* from the two Croatian islets, as well as from Zagreb on the mainland (Fig. 1). Omnivory and herbivory in lizards is rare, with herbivory encompassing <1-4 % of extant lizards (Espinoza et al. 2004; Cooper and Vitt 2001; King 1996). Yet, strict herbivory has originated independently >30 times (Espinoza et al. 2004) adding to the compelling reasons to investigate the mechanisms behind such shifts to plant-rich diets. We tested the assumption that there have been unique shifts in gut structure and function in the Pod Mrčaru lizards allowing them to digest plant material better than lizards from Pod Kopište or the mainland (Zagreb). If pancreatic or brush border enzymes aimed at digesting components of plant material are higher in the Pod Mrčaru lizards than their insectivorous counterparts on Pod Kopište (Fig. 2; Table 1), this would support the AMH, and we can conclude that the lizards have increased relevant enzyme activities (e.g., via increased expression of digestive enzyme genes; German et al. 2016). If digestive enzyme activities aimed at digesting plant material, particularly the fibrous portions, are elevated in the lizards' hindguts, these enzymes would likely be microbially derived (Potts and Hewitt 1973; Nakashima et al. 2002; Mo et al. 2004; Skea et al. 2005; German et al. 2015; Jhaveri et al. 2015; Leigh et al. 2018b; Fig. 2; Table 1). Elevated SCFA concentrations would also support a role of symbionts (Table 1). All of these possibilities are not mutually exclusive, as lizard tissue changes and microbial community shifts may both contribute to the Pod Mrčaru omnivores' ability to subsist on a diet rich in plants.

Materials and Methods

Diet analysis

From August 29-September 2, 2013, we flushed the stomachs of *Podarcis siculus* from the islets of Pod Mrčaru ($n=36$) and Pod Kopište ($n=31$, reduced to $n=30$ due to an empty stomach), Croatia, following Herrel et al. (2006, 2008). Stomach contents were stored in 70% ethanol. Contents from Zagreb lizards ($n=7$, reduced to $n=4$ due to three empty stomachs) were obtained from frozen-stored stomachs from previously dissected animals. We divided stomach contents into plant matter, arthropods, and “other,” weighing each category to the nearest 0.1-mg. We treated stomach contents as a proxy for ingested diet and determined the total mass and the relative proportion of plant and arthropod prey.

Animal collection, dissection, measurements of gut size, and tissue preservation

From August 26-29, 2013, we collected 13 male *P. siculus* from each islet, Pod Kopište and Pod Mrčaru. We captured all lizards in the morning after they became active. Lizards were kept individually in cloth bags and were euthanized and dissected upon returning to the laboratory (within four hours). As an outgroup (Podnar et al. 2005), we collected 13 *P. siculus* from an urban population in Zagreb from September 15-October 4, 2013.

Lizards were weighed to the nearest 0.1-g and euthanized via intramuscular injections of sodium pentobarbital (~ 0.1 mg/g-tissue). We measured snout-vent length (SVL) and dissected the lizards on sterilized, chilled dissecting trays ($\sim 4^{\circ}\text{C}$). We removed and measured the length of the gut from beginning of the esophagus to the end of the hindgut (hereafter: gut length).

We divided the gut into stomach (including esophagus), proximal intestine (PI), mid intestine (MI), and hindgut (H). The hindgut was easily identifiable (see Fig. 2) and the proximal and mid intestine portions were separated by dividing the remaining intestine in half. In seven individuals from each population, we removed the gut contents from the proximal, mid, and hindgut sections (e.g., Proximal Intestine Gut Contents, PIGC; Hindgut Contents, HC) and flushed out the intestinal tissue with chilled 25 mM tris-HCl, pH 7.5. We used pH indicator paper (Macherey-Nagel, Düren, Germany: pH 1-14, 5.5-9.0, and 8.0-10.0) on dissected Zagreb lizards to measure pH of the contents of each gut region as follows: stomach pH 3.4 ± 2.0 , proximal intestine pH 8.17 ± 0.6 , mid intestine pH 8.96 ± 0.7 , and hindgut pH 8.75 ± 0.5 .

Gut tissues and contents from each gut region and pancreases were frozen separately in 1.5 mL vials in liquid nitrogen for storage and transport. Vials were transported on dry ice to the University of California, Irvine, where they were stored at -80°C until used. We weighed frozen gut sections and gut contents (excluding stomachs) to the nearest 0.001 g. For three lizards from each population, we preserved the PI, MI, and H in McDowell Trump's fixative (4% formaldehyde, 1% glutaraldehyde, McDowell and Trump 1976) for subsequent histological analyses. The remaining three lizards from each population were used for microbiome analyses in a different study (Lemieux-Labonté et al., *Unpubl data*).

Estimation of intestinal surface area using histology

Gut sections preserved in Trump's Solution were further sectioned into 3-10 mm sections with a razor blade and rinsed in phosphate buffer pH 7.5 (PBS) for 3 x 20 min., and overnight in PBS at 4°C under constant shaking. The PBS rinsed tissues were flushed with running deionized water

for 2 x 20 min., and then were subjected to serial ethanol dilutions of 30%, 50%, and 75%. We selected the proximal portions of the PI, MI, and H from Pod Mrčaru and Pod Kopište lizards, and portions starting at the halfway point of the hindgut (H+) from all three populations. Tissue portions were placed in tissue cassettes wrapped in ethanol-soaked cheesecloth, sealed in plastic bags, and were sent to Mass Histology Services (Worcester, MA, USA) for embedding in paraffin wax. We stained 7- μ m sectioned samples with hematoxylin and eosin and imaged them with a Zeiss Axioplan 2 epifluorescence microscope and Zeiss and Cannon cameras. Tiled images were assembled using the Photomerge function of Adobe Photoshop CS3. We analyzed 1-25 sections of each sample by measuring the perimeters of mucosa and serosa. We then calculated the epithelial surface magnification (ESM) as the ratio of mucosal to serosal perimeters (Hall and Bellwood 1995; German 2009) to observe how much the mucosal folds increase the inner surface area of the intestine relative to a smooth bore tube.

Homogenate preparation

We homogenized frozen tissues following German and Bittong (2009). We diluted the tissues in the following chilled buffers: pancreases (P) diluted 50-300 volumes and gut contents (PIGC, MIGC, HC pellet) diluted 5-300 volumes in 25 mM tris-HCl buffer, pH 8.6 and, intestinal wall tissues (PI, MI, or H) diluted 10-50 volumes in 350 mM mannitol in 1 mM Tris-HCl, pH 8.6. We chose buffers at pH 8.6 because it was the average pH we measured in the intestinal contents of the *P. siculus* from Zagreb. For all tissues, we used a Polytron homogenizer (Binkmann Instruments, Westbury, NY) with a 12mm generator set to 1100-3000 rpm for 3 x 30 s, with 30 s between pulses to homogenize tissues. Tissue homogenates were centrifuged at 9400 x g for 2 min. To ensure the rupture of the microbial cells and the release of all enzymes within gut

content samples, these samples were sonicated (CL-18 Sonicator, Fisher Scientific, Waltham, Massachusetts, USA) at 5 W output for 3 x 30 s, with 30 s intervals between pulses, followed by homogenization, as described for the gut tissues. The gut content samples were centrifuged at 12000 x g for 10 min. All supernatants were stored in 100-200µl aliquots at -80° C until just before use in digestive enzyme assays. For the HC, we thawed the sample enough to transfer the contents to a spin column (Corning Costar Spin-X Centrifuge cellulose acetate tube filters, 0.22 µm pores) and centrifuged at 14000 x g at 4°C to gather H fluid. The filtered fluid was frozen at -80°C for use in SCFA measurements. The remaining HC pellet was then prepared for enzyme assays in the same manner as the other gut contents (German and Bittong 2009).

Biochemical Assays of Digestive Enzyme Activity

We conducted digestive enzyme assays following protocols outlined in German and Bittong (2009) and German et al. (2015). We ran all assays at 25°C, the mean temperature from May–September (confirmed by iButtons, Maxim Integrated, San Jose, CA, USA; Supplemental Fig. S1) on the islands and within the preferred temperature range of *P. siculus* populations outside of their native geographic range (Liwanag et al. 2018). We measured enzyme activities in duplicate or triplicate and read absorption or fluorescence in flat-bottomed 96-well microplates using a BioTek Synergy H1 Hybrid spectrophotometer/ fluorometer equipped with a monochromator (BioTek, Winooski, VT, USA). Our primary buffer was 25mM Tris-HCl, pH 8.6 (referred to henceforth as “buffer,” any deviations are noted), measured at room temperature (22°C). Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). We optimized each assay for duration and homogenate volume. Each enzyme activity was measured in each gut region (PI, MI, H, PIGC, MIGC, HC) for each lizard. Pancreatic tissue was only used for measuring

pancreatic enzyme activity (i.e.: α -amylase, trypsin, and lipase). We simultaneously conducted control experiments using homogenate or substrate blanks in buffer to check for endogenous substrate and/or product in the substrate solutions. For all kinetic assays, we determined the slope of the longest linear section of absorbance vs. time and used the standard curve of the product to calculate enzymatic activity U per gram wet mass of tissue.

Carbohydrate degrading enzymes— Following German and Bittong (2009) and German *et al.* (2015), we measured α -amylase activity using 1% potato starch dissolved in buffer containing 1 mM CaCl_2 . Maltase and trehalase activities were measured using 112 mM maltose or trehalose, respectively, in buffer. We incubated each of these assays as end-point reactions. Post termination, we determined glucose concentration by measuring absorbance at 650 nm (α -amylase) and 550 nm (maltase and trehalase). The α -amylase, maltase, and trehalase activities were determined from glucose standard curves and expressed in U (μmol glucose liberated per minute) per gram of tissue.

We measured β -glucosidase, β -galactosidase, and N-acetyl- β -D-glucosaminidase (NAG) at following German et al. (2011) and German et al. (2015) using 200 μM solutions of 4-methylumbelliferyl- β -D-glucoside, methylumbelliferyl- β -D-galactopyranoside, and 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide, respectively. Because of the sensitivity of the fluorometric assays, these concentrations far exceed the K_m for these enzymes in soils and animals (German et al. 2011). These assays were run as kinetic fluorometric assays read at 365 nm excitation and 450 nm emission for 30 min to detect a 4-methylumbelliferone – MUB product as U (nmol MUB released min^{-1}) per gram of tissue. β -glucosidase and NAG were chosen since β -glucosides or β -glucoaminides would be common in the digestion of plant fiber

or insect exoskeletons, respectively. β -galactosidase was chosen since is known to be present in lizard genomes (on chromosome 1 in the *A. carolinensis* genome; ensembl.org) and is produced in lizard guts (Kohl et al. 2016a). Thus, it was used as a comparison with β -glucosidase to better understand the activity patterns of this latter enzyme and whether it is endogenously or exogenously (i.e., microbially) produced.

Assays of protein and lipid degrading enzymes— Modified from German and Bittong (2009) and German et al. (2015), we measured trypsin, aminopeptidase, and lipase activities as kinetic assays. To measure trypsin activity, we used 2mM N α -benzoyl-L-arginine-p-nitroanilide hydrochloride (BAPNA) substrate dissolved in 100 mM Tris-HCl buffer. For aminopeptidase activity, we used 2.04 mM L-alanine-p-nitroanilide in buffer. These protease assays were read at 410 nm absorbance for 30 min to detect a p-nitroaniline product as U (μ mol p-nitroaniline released min⁻¹) per gram of tissue. For trypsin activities measured in pancreatic tissue homogenates, we pre-incubated the homogenates with 15 μ l enterokinase (4 U mL⁻¹ in 40 mM succinate buffer, pH 5.6)/ 100 μ l homogenate for 15 min to change trypsinogen from its zymogen form to active trypsin enzyme, then proceeded with the assay as with the other tissues.

We activated lipase in the homogenates via a 15 min pre-incubation in 5.2 mM sodium cholate at 25°C, using 2-methoxyethanol as a solvent. We commenced the assay by adding 0.55 mM p-nitrophenyl myristate substrate (in ethanol) and measured absorbance at 405 nm for 60 min to detect the p-nitrophenol product as U (nmol p-nitrophenol released min⁻¹) per gram of tissue.

In addition to the regional enzyme activities (U x g⁻¹), we calculated the total gut enzyme activities as the sum of mass-specific activity for each region multiplied by the tissue mass to

yield total U (μmol product released min^{-1}). We did not include pancreatic samples in total gut enzyme activities as this region does not interact directly with nutrients.

Fermentation Analyses

To determine symbiotic microbial fermentation, we measured the relative concentrations of short chain fatty acids (SCFAs) in the HC fluid (following methodology in Pryor and Bjorndal 2005; German and Bittong 2009; and German et al. 2015) from Pod Kopište ($n=3$), Pod Mrčaru ($n=4$), and Zagreb lizards ($n=1$). (Despite attempting to collect HC fluid from seven Zagreb lizards, we were only able to collect $\geq 1 \mu\text{L}$ from one lizard from this population.) We hand injected $2 \mu\text{L}$ of thawed HC fluid into a 2-m long stainless steel column (3.2 mm ID) packed with 10% SP-1000 and 1% H_3PO_4 on 100/120 Chromosorb W AW (Supelco, Inc., Bellefonte, PA, USA) attached to a Shimadzu GC-mini-2 gas chromatograph with flame ionization detector (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). We quantified SCFA concentrations via a Hewlett-Packard HP3392A (Hewlett-Packard Co., Palo Alto, CA, USA) integrator attached to the gas chromatograph. We calibrated the system with an external standard of 100 mg L^{-1} each of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate. The SCFA concentrations are expressed as mM of gut fluid.

Digestibility

From September 1-5, 2013, we collected male *P. siculus* from both Pod Kopište and Pod Mrčaru ($n=15$ from each island), transported them in individual cloth bags to the University of Zagreb, where they were housed in individual plastic terraria $30 \times 19 \times 14$ or 20 cm with rock substratum, a hide box, and a water dish. For one week, the lizards were allowed to acclimate to

lab conditions, during which they were offered live cockroaches (*Blatta sp.*) and finely chopped brussels sprouts (*Brassica oleracea*) daily. Lizards had *ad lib* access to water throughout the trials. The lab was kept at 25-31°C on a 10:14 h light/dark schedule. Five lizards from each population were assigned to one of three diets: insectivore, omnivore, or herbivore. On the first day, each lizard was fed $1.56 \pm 0.07\%$ of its body mass of its assigned diet (or $\sim 0.33 \text{ kJ/g}$), and thereafter $0.77 \pm 0.01\%$ of its body mass of its assigned diet daily ($\sim 0.16 \text{ kJ/g}$) for a duration of 11-32 days. Initial SVLs and masses are presented in Supplemental Table S1.

Diets— Triplicate samples of empty gelatin capsules (size 4) and of each diet were combusted in an IKA c2000 calorimeter. The insectivore diet (24.7 kJ/g) was made of cockroaches, the omnivore diet (21.4 kJ/g) was a 50:50 by dry mass mixture of the insectivore and herbivore diets, and the herbivore diet (18.1 kJ/g) was 30% by mass dried plant material collected from Pod Mrčaru, including leaves, flowers, and seeds, and 70% commercial birdseed (primarily millet, flax, hemp seed, and barley). All diets were dried for >2 days at 50°C, ground to $\leq 1 \text{ mm}$ particle size, and supplemented with Herptivite multivitamins and calcium with vitamin D3 (Rep-Cal, Los Gatos, CA) per manufacturer instructions. Diets were weighed out in approximately isocaloric ratios and packed into gelatin capsules. The gelatin capsules add about 14 kJ/g of protein to each diet, equally. We recognize that grinding the food to small particle sizes may take away natural particle size differences in the diets of the Pod Kopište and Pod Mrčaru lizards. However, in seeking some control over dietary intake, we chose to grind the food into a common size, and thus, measure digestibility based on more uniform food. This removed potential physical and chemical processing of differentially sized food particles in the lizards, but allowed us to focus on pure chemical digestibility in the laboratory. One previous investigation of whole

meal worm digestibility in these same lizard populations found similar results to ours (Vervust et al. 2010), suggesting that our grinding of the food didn't mask any performance differences among the lizard populations.

Feeding— Each lizard was weighed, and gently force fed the gelatin capsule of a known mass of experimental diet using a plunger from a syringe to push the pill into their esophagus. After the pill was in the esophagus or swallowed, we administered water (of equal mass to the experimental diet) into the lizard's mouth via pipette. We adjusted the mass of diet fed at each feeding to maintain lizard body mass $\pm 10\%$. We prepared three sizes of meals (+10mg, baseline, -10mg) for each diet. If a lizard's body mass increased by $\geq 10\%$ compared to its initial measured body mass (or if that individual vomited its previous meal), we fed it the smaller meal size at the next feeding. Individuals that lost $\geq 10\%$ of their initial body mass were given the larger sized meal at their subsequent feeding. We collected all feces and urates daily and measured the SVL of each lizard weekly.

Digestibility Analyses— Compiled feces (without urates) from each individual lizard were dried at 50°C for >1 week and weighed to the nearest 0.001 g.

We estimated the organic matter of each diet, of gelatin capsules, and of the dried feces by combusting a portion of the sample (Bjorndal 1989). Samples were dried at 105°C in a drying oven for at least 3 hours to remove all moisture, weighed, then combusted in a Lindburg/ Blue M combusting oven (Ashville, NC, USA) at 550°C for 3 hours. The combusted remains were considered non-organic ash and we subtracted that mass from the initial mass to determine the

proportion of organic material in the original sample. We calculated apparent organic matter digestibility as

$$\frac{(\text{mass food and gelatin capsules ingested} - \text{ash}) - (\text{feces mass} - \text{ash})}{\text{mass food and gelatin capsules ingested} - \text{ash}}.$$

We recognize that this is apparent organic matter digestibility since we didn't account for endogenous organic matter (e.g., sloughed intestinal cells) losses in feces (McConnachie and Alexander 2004; German 2011).

Statistical Analyses

We performed all statistical analyses in R (versions 3.3.2-3.6.0). All data were screened for equal variances using a Bartlett's test and normality of residuals using a Shapiro-Wilk's test. If the data were not naturally parametric, we employed transformations. For propionate concentration comparisons, we used Wilcoxin Signed Rank tests. We used Tukey's HSD test with a family error rate of $P=0.10$ for digestibility analyses and $P=0.05$ for all other analyses to identify pairwise differences following any ANOVAs that indicated significant differences. We compared all data among (or between) populations. We compared gut length (including stomach and esophagus) among populations with an ANCOVA, using SVL as a covariate. We summed the masses of the intestinal tissues for each individual lizard to get total intestinal mass and compared both regional and total intestinal masses among populations with ANCOVA, using body mass as a covariate. We divided gut content mass for each region by total gut content mass to determine the proportion of digesta retention in each gut region.

Additionally, we compared gut content mass and β -glucosidase activity among gut regions within populations. All data were normalized to mass or were proportions, except body mass itself, SVL, and total enzyme activities. We found no effect of covariance between total

enzyme activity and any measured parameter (lizard mass, SVL, intestinal mass, gut length, and gut content mass), thus report only the results of ANOVAs.

To analyze digestibility, we compared data from lizards on the same experimental diets across populations using ANCOVAs. We found instances of covariance between organic matter digestibility and lizard mass, and between digestibility and total intake. No other measured parameter (SVL, duration in days individual was in digestibility trial) covaried with digestibility.

Results

Podarcis siculus from Pod Kopište (SVL: 64.39 ± 2.40 mm; average \pm standard deviation) were significantly shorter in body length ($F_{2,35}=11.83$, $P<0.001$) than the populations from Pod Mrčaru (SVL: 68.73 ± 2.50 mm) and Zagreb (SVL: 68.43 ± 5.80 mm), which were not different from each other. The Zagreb lizards (body mass: 8.9 ± 1.5 g) were significantly more massive ($F_{2,35}=13.75$, $P<0.001$) than either island population lizards, and Pod Mrčaru lizards (body mass: 7.6 ± 0.7 g) were significantly heavier than Pod Kopište lizards (body mass: 6.6 ± 0.9 g; Supplemental Table S2).

Diet & Gut Size

The newly omnivorous *P. siculus* population on Pod Mrčaru ate larger, more plant rich meals, yet showed none of the increases in gut size expected from such a shift. Lizard stomach content mass varied significantly among the populations ($F_{2,68}=16.88$, $P<0.001$), with Pod Mrčaru lizards having more than double the stomach content masses of the other populations, implying that they consumed more food, at least on an instantaneous scale, than the Pod Kopište and Zagreb

populations. Pod Mrčaru *P. siculus* (64% of intake) consumed significantly more plant material ($F_{2,63}=15.35$, $P<0.001$) in comparison to 24% in Pod Kopište lizards and 2% in Zagreb lizards (Table 2).

Relative intestinal masses were heavier in lizards from Pod Kopište (3.6% of total body mass) and Pod Mrčaru (3.3%) than in lizards from Zagreb (2.4%; ANCOVA Population: $F_{2,18}=9.53$, $P<0.007$; body mass: $F_{1,15}=6.94$, $P<0.030$, model run without interaction term as it was non-significant; Fig. 3). We found no significant differences in gut length ($F_{2,36}=2.55$, $P=0.092$) among the three populations. Regional intestinal masses (i.e.: PI, MI, H) did not differ by population nor covary with lizard body mass. (See Supplemental Table S2 for values.) Mass of total gut contents did not vary significantly (ANCOVA with body mass as covariate, $F_{2,18}=2.10$, $P=0.163$) among the three populations. Gut contents were evenly distributed throughout the PI, MI, and H. None of the populations retained more contents in a particular gut region than did the other two populations, excepting the higher mass of digesta in the Pod Mrčaru lizards' stomachs, mentioned above. Additionally, we observed nematodes in the HC of multiple lizards from each of the three populations.

The epithelial surface magnifications (ESM) were not different in Pod Kopište versus Pod Mrčaru lizards in any gut regions (Fig. 4). As such, the following reported values are pooled across the two populations. The mucosa of the proximal intestine was 6.26 ± 0.33 x serosa ($t=0.82$, $P=0.44$). This ratio decreased distally along the gut: the mid intestine, 3.63 ± 0.33 x serosa ($t=-0.14$, $P=0.89$), a proximal part of the hindgut, 2.44 ± 0.32 x serosa ($t=0.64$, $P=0.54$), and more distal portions of the hindgut (H+), 1.95 ± 0.24 x serosa ($t=1.86$, $P=0.11$). Figure 4 also shows representative sections. We did not identify any qualitative differences between the cross

sections of either population, including not identifying any valves captured in the histological sections.

Digestive Enzyme Activities

Carbohydrases—We measured the majority of population differences in carbohydrate activities in H or HC tissues. The newly omnivorous Pod Mrčaru lizards showed greater amylase activity, but no other increases in plant-related enzyme activities compared to the Pod Kopište source population.

The mass-specific α -amylase activity in the hindgut contents was almost 6-fold higher in Pod Mrčaru lizards compared to that measured in the Pod Kopište population ($t=-0.27$, $P=0.038$, Fig. 5a, Supplemental Table S3). Amylase activity was undetectable in all but one HC sample from the Zagreb population. We found no other differences in amylase activity. In most MI and H samples, amylase activity was undetectable or replicates were too variable for confident analyses. No other gut region nor pancreas showed differences in amylase activity among the three populations (Pancreas: $F_{2,23}=2.97$, $P=0.072$; PI: $F_{2,10}=3.41$, $P=0.074$; PIGC: $F_{2,14}=0.49$, $P=0.626$; MIGC: $F_{2,13}=0.63$, $P=0.549$), nor did we find any significant differences in the total gut α -amylase activity among populations ($F_{2,10}=2.05$, $P=0.180$).

The total β -glucosidase activity was >2.5-fold higher in the Pod Mrčaru and Pod Kopište lizards compared to the Zagreb lizards ($F_{2,17}=13.38$, $P=0.003$; Fig. 6a). Mass specific β -glucosidase activity was nearly double in the PI of Pod Mrčaru and Pod Kopište lizards compared to the Zagreb lizards ($F_{2,15}=14.33$, $P<0.001$; Supplemental Table S3). We found no other differences in regional β -glucosidase activities among the populations (Fig. 5b). Within individuals, β -glucosidase activity was significantly higher in the PI and HC regions in

comparison to some regions (e.g. MI, PIGC, etc; Fig. 5b; Pod Kopište: $F_{5,29}=8.02$, $P<0.001$; Pod Mrčaru: $F_{5,28}=11.7$, $P<0.001$; Zagreb: $F_{5,25}=3.433$, $P=0.017$). This pattern appeared in all three populations but was most pronounced in the two island populations. β -galactosidase activity patterns throughout the gut were distinct from those of β -glucosidase activity (e.g. with β -galactosidase activity spiking in the PIGC and MI gut regions whereas we found the opposite pattern with β -glucosidase activity). See Supplemental Table S3 for β -galactosidase activity measures and Supplemental Fig. S2 for a comparison of the regional activities of these two enzymes.

Neither regional mass specific nor total maltase activities ($F_{2,17}=2.28$, $P=0.133$) were different among the three populations (Fig. 5c). In the PI of the Pod Kopište lizards, the N-acetyl- β -D-glucosaminidase (NAG) activity was 2x greater than in the PI of the Pod Mrčaru and Zagreb populations ($F_{2,15}=5.87$, $P=0.013$; Supplemental Table S3). There were no other regional differences among the populations (Fig. 5d), including total NAG activity. In the Pod Mrčaru and Zagreb populations, the greatest NAG activity was found in the HC compared to any other region ($F_{5,50}=23.60$, $P<0.001$; Pod Mrčaru and Zagreb populations pooled due to low sample sizes for PIGC and H regions).

In both the H and HC, the Pod Kopište population had lower regional trehalase activity than one of the other two populations (Fig. 5e). Still, there were no population differences in trehalase activity in more proximal regions, nor in total activity of the enzyme (Supplemental Table S3). The Pod Mrčaru population's H trehalase activity (0.058 ± 0.020 $\mu\text{mol glucose liberated min}^{-1} \text{g}^{-1}$) was higher than the H activity in the Pod Kopište population (0.0045 ± 0.003 $\mu\text{mol glucose liberated min}^{-1} \text{g}^{-1}$; $F_{2,9}=5.50$, $P=0.024$). The Zagreb population's HC activity

(0.254 ± 0.134 $\mu\text{mol glucose liberated min}^{-1} \text{ g}^{-1}$) was higher than Pod Kopište HC activity (0.025 ± 0.011 $\mu\text{mol glucose liberated min}^{-1} \text{ g}^{-1}$; $F_{2,12}=4.28$, $P=0.041$).

Proteases—Protease activity differed by population, but not along the diet lines we predicted.

In the HC, the mass specific trypsin activity was >5-fold higher in the Pod Mrčaru population than in the Pod Kopište population ($F_{2,11}=5.33$, $P=0.024$; Fig. 5f, Supplemental Table S3). The trypsin activity in the HC of the Zagreb population (Supplemental Table S3) was not different from the other two populations. No other gut region showed differences in activity among the three populations (Fig. 5f), nor did we find difference in the total trypsin activity among populations. The mass specific trypsin activity in the pancreas was >2.5x higher in Pod Kopište and Pod Mrčaru lizards (0.454 ± 0.050 $\mu\text{mol p-nitroaniline released min}^{-1} \text{ g}^{-1}$) than in the pancreases of Zagreb lizards (0.190 ± 0.040 $\mu\text{mol p-nitroaniline released min}^{-1} \text{ g}^{-1}$; P : $F_{2,23}=5.14$, $P=0.014$, Fig. 7).

The total aminopeptidase activity throughout the gut (Fig. 6b) was nearly 2.5-fold higher in the Zagreb population than in the Pod Kopište population ($F_{2,17}=7.23$, $P=0.005$; Supplemental Table S3), although neither differed from the Pod Mrčaru population. Compared to the island lizards, the Zagreb population had a >32-fold higher mass specific aminopeptidase activity in PIGC ($F_{2,12}=14.58$, $P<0.001$), as well as higher (although not quite as pronounced) activities in MI ($F_{2,17}=15.14$, $P<0.001$) and MIGC ($F_{2,12}=12.33$, $P<0.001$) tissues (Fig. 5g).

Lipase—There was no difference in lipase activity across the three lizard populations (Supplemental Fig. S3 and Table S3; total lipase $F_{2,18}=0.879$, $P=0.432$).

Microbial Fermentation

Microbial fermentation was considerably lower in the newly omnivorous Pod Mrčaru lizards. The Pod Kopište *P. siculus* had concentrations of 61.90 ± 4.90 mM of total SCFA concentrations in their HC, >3x higher than the 19.20 ± 8.60 mM in Pod Mrčaru lizards ($t=6.42$, $P=0.001$, Table 3). This phenomenon was primarily due to nearly four-fold higher total acetate ($t=9.06$, $P<0.001$) and total isobutyrate ($t=3.80$, $P<0.001$) in the Pod Kopište population compared to Pod Mrčaru lizards. However, even with these two SCFAs omitted, non-significant increases in propionate, butyrate, and valerate (but not isovalerate) contributed to significantly increased total SCFA concentrations ($t=4.00$, $P=0.010$) in the hindguts of the Pod Kopište lizards. When considered as a proportion of the total SCFAs within individuals, only the acetate concentration was higher in the Pod Kopište lizards (Table 3; $t=2.17$, $P=0.049$). Although total SCFAs were lower in the Pod Mrčaru population, isobutyrate ($t=-2.74$, $P=0.041$) and isovalerate ($t=-4.76$, $P=0.005$) were higher proportionally.

The Zagreb individual we measured had very low concentrations of all SCFAs, only 0.60 mM total. Overall, the proportions of each SCFA (e.g. acetate, isobutyrate, etc.) measured in the Zagreb lizard were intermediate to the SCFA proportions found in the island lizards, excepting the nearly absent valerate concentration.

Organic Matter Digestibility

The newly omnivorous Pod Mrčaru lizards were better at digesting plants than were the lizards of the Pod Kopište source population. At 61% OM digestibility, the Pod Mrčaru lizards had 1.1x higher organic matter digestibility (by mass) than the Pod Kopište lizards (55% OM digestibility) on the herbivore diet (Fig. 8, Supplemental Table S4; ANCOVA: population $F_{1,6}=6.17$, $P=0.048$,

body mass $F_{1,6} < 0.001$, $P = 0.983$). On an omnivore diet, Pod Mrčaru lizards had an almost 4% higher organic matter digestibility than their Pod Kopište counterparts (ANCOVA: population $F_{1,7} = 5.875$, $P = 0.0458$; body mass $F_{1,7} = 3.065$, $P = 0.1235$). The two populations did not differ in digestibility of an all insect diet (ANCOVA: population $F_{1,7} = 0.670$, $P = 0.440$, body mass $F_{1,7} = 0.619$, $P = 0.457$). For all three diets, analyses using other covariates are available in Supplemental materials. Final and percent change in body masses and SVL are available in Supplemental Table S1.

Discussion

Subtle changes in performance

The *P. siculus* of Pod Mrčaru eat more plants than their Pod Kopište counterparts after ~35 years of divergence (Herrel et al. 2008) and their digestibility performance has shifted. Overall, the newly omnivorous Pod Mrčaru population was slightly better able to digest plant material in the laboratory than the Pod Kopište lizards, and this difference is supported by subtle changes in gut function. Our expectations of gut-wide shifts in form and function based on the framework of the AMH and other theoretical considerations (Table 1) were not supported, and thus, we largely accept the null hypothesis of little differences in gut function. Indeed, the potential mechanisms (i.e., slight differences in hindgut amylase and trypsin activity; Fig. 5) underlying the increase in digestibility of plant material are subtle and localized within the hindgut. These results are more consistent with Pod Mrčaru lizards—and perhaps even Pod Kopište *P. siculus*—as facultative omnivores (Herrel et al. 2004), agreeing with the greater plant intake by the Pod Kopište *P.*

siculus observed in this study (24% of gut content mass) than in the past (4-7%; Herrel et al. 2008).

As reviewed across vertebrate taxa by Leigh and colleagues (2018a), shifts to high protein diets often lead to higher protease activities, yet show mixtures of increases and decreases in carbohydrases, but no changes in lipases. Shifts to lower protein diets, such as to a plant-rich diet (e.g. like in the Pod Mrčaru lizards) yielded more erratic results in digestive enzyme activities with both increases and decreases in proteases, carbohydrases, and lipases. In the *P. siculus* system, we found both increases and decreases in carbohydrases, and an increase in a protease with the Pod Mrčaru lizards' shift to a plant-rich diet. Yet, all of the studies reviewed by Leigh *et al.* (2018a) considered animals in feeding experiments on shorter time scales (weeks to months), not wild individuals following a natural dietary shift over decades.

As the majority of endogenous nutrient digestion and absorption in non-ruminant vertebrates occurs in the proximal portion of the intestine (Vonk and Western 1984; Karasov and Martínez del Río 2007; Le et al. 2019), differences in the structure and function of the hindgut point to differences in the function of microbial symbionts in this gut region (McBee and McBee 1982; Bergman 1990; Bjorndal 1997). According to the plug flow reactor model of digestion (Penry and Jumars 1986, 1987; Karasov and Hume 1997; Stevens and Hume 2004), nutrients are digested and absorbed down their gradient as they flow through the gut. Thus, a shift in gut function in the proximal region will promote downstream changes, from more potential digestion to more opportunities for nutrient absorption. For example, an increase in trypsin activity in the proximal intestine could lead to greater digestion of proteins into dipeptides that can serve as a substrate for aminopeptidase in the mid intestine and hindgut. There is subsequently more gut remaining over which the dipeptides can be absorbed. Changes in the lizard hindgut, therefore,

may represent a small portion of overall nutrient acquisition. For example, the hindgut of two iguanids (*Dipsosaurus dorsalis* and *Sauromalus ater*) showed <5% of the transport capacity of D-Glucose and L-Proline (a non-essential amino acid) in comparison to the proximal intestine in these species (Karasov et al. 1985). The mammalian large intestine is also known as a site for ion, water, SCFA, and amino acid absorption, particularly essential amino acids (e.g., lysine) (with SCFA and essential amino acids coming from microbial sources; Stevens and Hume 1998; van der Wielen et al. 2017; Moran et al. 2019). Hence, the L-Proline absorption measured in Karasov et al. (1985) may not be representative of all amino acid absorption in the lizard hindgut, as there are separate transporters for charged amino acids (Karasov and Martinez del Rio 2007; van der Wielen et al. 2017), like lysine, which also appears to be an essential amino acid for reptiles (Herbert and Coulson 1976).

Even though the Pod Mrčaru lizards digested plant material organic matter ~10% better than the Pod Kopište lizards, the functional differences we were able to detect amongst *P. siculus* populations were localized to the hindgut. Based on these differences, we may infer that microbial communities, generally localized to the hindgut, play a role in these differential abilities (Karasov and Douglas 2013; Moran et al. 2019). Interestingly, modest shifts in the hindgut microbial communities of the Pod Mrčaru and Pod Kopište lizards included more of the Archaeal, *Methanobrevibacter*, in the Pod Mrčaru lizards (Lemieux-Labonté et al., *Unpubl data*), and these taxa can affect the digestive function, especially of glycans and protein (Samuel and Gordon 2006; Mathur et al., 2013). A study of the microbiome of the Croatian lizards is nearing publication (Lemieux-Labonté et al., *Unpubl data*).

Between the new and source populations of lizards, Pod Mrčaru and Pod Kopište, the only functional difference we measured outside of the hindgut was higher NAG activity in the

proximal intestines of Pod Kopište lizards. Higher NAG activity could contribute to the digestion of chitin from arthropods carapaces, and the cell walls of fungi and nematodes (Skoczylas 1978; Vonk and Western 1984). Although we expected patterns of NAG activity to conform to that of brush border digestive enzymes (German et al. 2015), NAG activity was the highest in the HC, consistent with microbial synthesis, not endogenous synthesis in lizard tissue (Jhaveri et al. 2015). Moreover, *P. siculus* appears to have considerably lower endogenous NAG activity compared to other lizards (Table 4; Jeuniaux 1961, 1963; Marsh et al. 2001).

As we had predicted that the Pod Mrčaru lizards would rely on fermentative pathways to digest their plant-material rich diet, the higher SCFA concentrations in Pod Kopište lizards were opposite of what we expected. Higher SCFA concentrations are an indication of more microbial fermentation (Bjorndal 1997; Pryor and Bjorndal 2005). Indeed, high acetate, propionate, isobutyrate, and butyrate are all associated with fermentation of plant material. Surprisingly, the acetate and isobutyrate concentrations and ratios of total SCFAs were higher in the Pod Kopište lizards. In a study of *Uromastix aegyptius* (Foley et al. 1992), a strict herbivore, acetate, propionate, and butyrate concentrations were similar to our measurements in the Pod Kopište *P. siculus*, suggesting that the insectivorous lizards of this study can employ fermentative digestion more than expected. In the *U. aegyptius* study, Foley and colleagues noted that >98% of the SCFAs they measured in the hindgut were then absorbed prior to defecation. Similarly, we assume our measurements are indicative of SCFA production in the hindgut and not lower SCFA absorption rates in Pod Kopište lizards, but this assumption should be tested.

A potential explanation for lower levels of fermentation in the Pod Mrčaru lizards lies with intake and transit time (Stevens and Hume 1998; Macfarlane and Macfarlane 2003). Lower quality food leads to higher intake, and higher intake leads to faster gut transit rates (Dadd 1960;

Pritchard and Robbins 1990; Diamond 1991; Horn and Messer 1992; Fris and Horn 1993; Castle and Wunder 1995; Karasov and Martinez del Rio 2007). One of the keys of microbial fermentation is that microbes need time to digest insoluble fiber, eventually producing SCFAs (Bergman 1991; Stevens and Hume 1998; Macfarlane and Macfarlane 2003). Animals that are reliant on fermentation to make a living on plant material have ways (e.g., hindgut chambers in iguanas, rumens of cattle) to slow gut transit rates to allow microbes the time they need (Stevens and Hume 1998, 2004). With 2.2x greater stomach content masses than Pod Kopište lizards, Pod Mrčaru lizards likely have higher intake of their lower quality food. Thus, the lower SCFA concentrations in Pod Mrčaru lizards could reflect gut transit rates that are too fast for adequate fermentation in the hindgut. The hindgut valves themselves may have arisen to slow transit of material in the hindgut, but the extent to which the valves act as baffles should be examined. Furthermore, hindgut microbes can play roles other than fermentation in terms of aiding the host in the digestive process (e.g., amino acid and vitamin synthesis; Moran et al. 2019). In fact, the slight differences in organic matter digestibility among the Pod Mrčaru and Pod Kopište lizards argues against significant fiber digestion in the Pod Mrčaru lizards, but fiber digestibility should be measured in these lizards. It should be noted that we attempted to measure cellobiohydrolase activities in *P. siculus*, but detected no activity anywhere in their guts.

Although hindgut valves are present in Pod Mrčaru hindguts (Herrel et al. 2008; Vervust et al. 2010; Wehrle 2018), we did not observe differences in epithelial magnification by population. For whatever reason, the Pod Mrčaru lizards we examined for hindgut valves histologically in summer 2013 ($n=3$) did not have clear epithelial folds (valves) in their hindguts. This stands in contrast to all other Pod Mrčaru lizards ($n=28$, spanning four seasons over three additional years) we examined, which had clearly identifiable folds in the proximal to mid

portion of their hindgut, dividing the gut in the transverse plane (Supplemental Fig. S4; Wehrle 2018). The Pod Kopište lizards ($n=29$) lacked these valves entirely. The same methods were used across years, so there were no methodological differences. These data, combined with those from Herrel et al. (2008) and Vervust et al. (2010) convince us that, although usually present, the hindgut valves in *P. siculus* may be flexible in their morphology or even presence, and they are not fixed features in the Pod Mrčaru lizards. The general pattern of surface area decreasing along the gut (i.e.: $PI > MI > H$, Fig. 4) was consistent with our expectations (Skoczylas 1978; Stevens and Hume 2004) considering we did not observe the valves in our sections from summer 2013 (even the H+ sections).

Whereas shifts in gut structure and function in wild lizards may be the result of plasticity due to dietary differences, the Pod Mrčaru lizards' higher digestibility of a plant diet (including the omnivore diet) in the laboratory support their ability to make a living on a plant-rich diet. It is possible that a lifetime of acclimation to their respective wild diets has influenced digestive performance, and this may be mediated through the microbiome of the hindgut (i.e., not necessarily an evolved difference in the lizards themselves; Garland and Adolph 1991).

Lizard Digestive Physiology

Digestive physiology in reptiles has been less studied than in other taxa such as mammals and fishes (Karasov et al. 1985; Stevens and Hume 2004; Kohl et al. 2016b), particularly in wild populations. We included the Zagreb population of *P. siculus* to give context to the magnitude of differences between the Pod Mrčaru and Pod Kopište populations, and identify which structural and physiological characteristics are unlikely to change (e.g., gut length) even between distantly related populations; the Zagreb population is not as closely related to the island populations as

the island populations are to each other (Podnar et al. 2005; Herrel et al. 2008). We predicted that the Pod Kopište and Zagreb populations, which both mostly consume invertebrates, would show the most similarity in gut form and function. However, we found that the closely related island populations show greater similarities in gut form and function than do the insectivorous populations. Sagonas and colleagues (2015) found more similarities in the gut physiology and morphology of lizards with more similar habitats (i.e. insular vs. mainland) than populations that had more similar diets. Indeed, island effects on physiology, morphology, and ecology often outweigh other factors in lizards, including effects on diet (Van Damme 1999; Cooper and Vitt 2002; Spiller et al. 2010) and digestion (Pafilis et al. 2007; Vidal and Sabat 2010; Sagonas et al. 2015). It may also be that the shared evolutionary history of the Pod Mrčaru and Pod Kopište lizards leads to their similarity in gut structure and function (Garland and Adolph 1994; Karasov and Martinez del Rio 2007).

The more massive guts of the Pod Mrčaru and Pod Kopište lizards compared to the outgroup Zagreb population suggest that the new and source population lizards allocate more tissue resources to digestion. Exactly why this is the case remains unknown. The Zagreb lizards live in an urban area with copious plant cover and potential food sources, whereas the Pod Kopište and Pod Mrčaru lizards live on small, densely populated islets that are likely challenging, from space and resource perspectives (Herrel et al. 2008). Aminopeptidase was the only digestive enzyme that showed higher activity in the Zagreb population in comparison to the Pod Kopište and Pod Mrčaru lizards. The elevated aminopeptidase activity, which was within the range measured in other lizards (Table 4) may compensate for the Zagreb population's lower gut tissue mass, leading to similar nutrient acquisition outcomes for protein among the populations.

656 Interestingly, the elevated aminopeptidase activities of the PIGC in the Zagreb lizards suggests
657 inherent aminopeptidase activity in their ingested prey (mostly ants).

658 Still, as more than two-thirds of the enzymatic differences we identified were between
659 island and outgroup, and not between the two recently diverged island populations, it appears
660 that endogenous enzyme activities are not as flexible amongst the island populations, despite the
661 dietary differences among them. Feeding behavior and microbial symbionts may dampen
662 selection on the digestive system, as changes in behavior can offset evolutionary changes in
663 physiology and morphology (Sibley 1981; Huey et al. 2003; Clements and Raubenheimer 2006).
664 By increasing food intake and through shifts in microbiome function (Lemieux-Labonté et al.,
665 *Unpubl data*), Pod Mrčaru lizards may not need to dramatically shift their gut function to digest
666 plant material more efficiently.

667 Amylase, trypsin, and lipase activities were highest in the pancreas (Figure 7) and
668 decreased distally along the gut, patterns consistent with pancreatic enzymes (Fig. 2; Stevens and
669 Hume 2004; Clements and Raubenheimer 2006; German et al. 2015). We predicted brush border
670 enzymes would peak in the mid intestine, consistent with dimer (substrate) concentrations being
671 highest in the mid intestine following polymer degradation in the proximal intestine. This pattern
672 is seen in other vertebrates (Fig. 2; Vonk and Western 1984; Stevens and Hume 2004; German et
673 al. 2015), yet in the lizards of this study, maltase and trehalase were more active proximally in
674 the gut. Trehalose—the substrate for trehalase—is, of course, a disaccharide as it is ingested, and
675 maltose—the substrate for maltase—may also be more available proximally from rapid starch
676 digestion (Sibley 1981; German and Bittong 2009; Karasov and Douglas 2013; German et al.
677 2015).

Perhaps most unexpected is the enzyme activity pattern of β -glucosidase (Fig. 5b). No non-avian reptile has been recorded to produce endogenous β -glucosidases in their digestive tracts (Stevens and Hume 2004; Karasov and Douglas 2013), and thus must rely on microbial symbionts to produce this enzyme for digesting breakdown products of cellulose (e.g., cellobiose). As predicted, *P. siculus* has a spike of β -glucosidase activity in the HC, consistent with microbial synthesis (Fig. 2; German et al. 2015; Leigh et al. 2018b). However, the β -glucosidase activity is just as high in the PI as in the HC. Of interest, the high activity in the PI, and as a result, higher total β -glucosidase activity, is most starkly found in the island populations, with present, but much diminished activity observed in the mainland lizards. A β -glucosidase is present in the *Anolis carolinensis* genome (on chromosome 5; ensembl.org), and one is expressed in the intestinal tissues of the herbivorous fish *Cebidichthys violaceus* (Heras et al. 2020), but it remains unknown if this enzyme is expressed in the digestive system of *P. siculus*, and liver, as in mammals (de Graaf et al. 2001; Hayashi et al. 2007). Overall, these patterns suggest that *P. siculus* may produce β -glucosidase endogenously in the gut or acquire it from the enteric microbiome in the PI in addition to in the HC. Kohl and colleagues (2016a) propose that β -galactosidase, known to be endogenously produced in reptiles (on chromosome 1 in the *A. carolinensis* genome; ensembl.org), is active against β -glucosidase substrates. Still, the β -galactosidase activity patterns we measured varied from the β -glucosidase activity patterns throughout the gut (Supporting information Figure S2). Thus, there is some evidence that *P. siculus* may endogenously produce β -glucosidase or house β -glucosidase producing microbes in their PI. We acknowledge that β -glucosidase may have functions outside of digestion in the intestine (e.g. in lysosomes), but this increased activity in the PI opposed to other intestinal

tissues is suggestive of digestive function. The mechanisms and function of this finding warrant further investigation to tease apart these possibilities.

Conclusions, Potential Limitations, and Future Directions

The *P. siculus* system offers a rare opportunity to observe a natural diet shift in wild populations, and the subsequent digestive responses. Few studies investigate animals' digestive physiology on a natural diet, within their ecosystem. In this newly omnivorous population of lizards, the only detectable changes in gut form and function—including valves (presence or absence), enzyme activity, and microbial fermentation—start from the hindgut. Although we know the Pod Mrčaru lizards have changed their body morphology over ecological time (Herrel et al. 2008), any shifts in their digestive physiology appears to be more constrained on this timescale, or mitigated by differences in behavior and ecology. We currently do not know whether the observed changes represent flexibility (Piersma and Drent 2003), or evolved differences with underlying genetic changes (Garland and Adolph 1991). Some dietary shifts may not be as limited by physiology as they are by these animals' ecology. Future investigations in this system should focus on microbiome function. Moreover, as the stomach can play a major role in the digestive process, and can account for greater than 50% of gut transit time (Karasov and Martínez del Río 2007), more work on gastric function is warranted, and is the subject of our own work on this system.

We do recognize that this study can be mistaken as a “two species comparison”, which comes with caveats and cautions (Garland and Adolph 1994), especially when comparing the island populations with the mainland population in Zagreb. However, most of our work is focused on comparing the two island populations, which according to their 16s rRNA sequences, have not diverged much genetically over 30+ years of separation (Herrel et al. 2008). The

similarity among the two island populations may, thus, simply represent that they are still very much the same taxon phylogenetically, and thus, may not be treated as separate entities (Karasov and Martínez del Río 2007). The similarity of the Pod Mrčaru and Pod Kopište lizards in comparison to the Zagreb population, should, therefore, not be surprising given the short period of time of the island populations' separation (Nevo 1972; Herrel et al. 2008). Similar results among populations of insular vs mainland populations of lizards have been observed in other species (Pafilis et al. 2007; Sagonas et al. 2015). Nevertheless, the greater plant organic matter digestibility displayed by the Pod Mrčaru lizards shows that they have indeed achieved mechanisms for digesting plant material, and our work suggests such an ability derives from the hindgut.

Author Contributions

BAW, DPG, AH, and ZT designed the experiment. BAW, AH, and DPG performed the fieldwork. BAW, B-Q N-P, SM, RKD, RA, and DPG conducted the lab work. BAW analyzed the data. BAW and DPG led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Data Accessibility

Data are available at <http://german.bio.uci.edu/Supplements.html>.

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Figure Captions

Figure 1: *Podarcis siculus* island collection sites showing Pod Kopište (source population) and Pod Mrčaru (newly omnivorous population). The box in the bottom map shows the area of the inset. Zagreb (mainland population) not pictured.

Figure 2: Potential patterns of digestive enzyme activities across intestinal regions and representative examples of lizard guts from each population (with stomachs). Pancreatic digestive enzymes are secreted into the proximal intestine and are expected to decrease along the gut. Brush border enzymes are produced at the brush border of the epithelial cells and the intestinal lumen, generally peaking in activity in the mid intestine. High enzyme activities in the proximal or mid intestines and/or their contents would be due to the lizards themselves increasing those enzyme activities. Microbial enzymes produced by the microbiome tend to peak in the hindgut where symbiotic microbes are housed. Increased enzyme activities in the hindgut contents are likely produced by microbial symbionts. Modified from German *et al.* 2015. For enzymatic analyses, we removed the esophagus and stomach from the intestines at the pyloric sphincter. The hindgut was easily identified as its diameter is enlarged compared to the proximal and mid intestine. We defined the proximal and mid intestines as half of each portion of the remaining tissue length.

Figure 3: (a) Regional and (b) total intestinal mass (without contents) in Pod Kopište (source), Pod Mrčaru (new omnivore), and Zagreb (mainland) populations. Gut regions are proximal intestine (PI), mid intestine (MI) and hindgut (H) and presented as a percentage of body mass. Values are mean \pm standard deviation. $n=7$ in all except $n=6$ in Pod Mrčaru PI. Comparisons of populations were done via ANCOVA with body mass as a covariate. No particular region showed differences in mass (a), but the Zagreb population had lower total intestinal masses than the Pod Mrčaru population (b) that were also significantly affected by body mass. Pod Kopište intestinal masses were not different from either population.

Figure 4: Epithelial Surface Magnification (ESM), the ratio of inner perimeter length of mucosa to inner perimeter length of serosa in proximal intestine (PI), mid intestine (MI), proximal half of hindgut (H), distal half of hindgut (H+). Values are mean \pm standard deviation, $n=3$. Comparisons of populations were done via equal variance t-test. We found no differences by population. Cross section images are representative stained histological sections from each gut region of Pod Kopište (source) and Pod Mrčaru (new omnivore) populations. Not to scale: each perpendicular black scale bar represents 500 μ m for the two images it lies between.

Figure 5: (a) amylase activity in μmol glucose liberated $\text{g}^{-1} \text{min}^{-1}$, “X” denotes undetectable activity. (b) β -glucosidase and (c) N-acetyl- β -D-glucosaminidase (NAG) activities in nmol MUB liberated $\text{g}^{-1} \text{min}^{-1}$, (d) trehalase activity in μmol glucose liberated $\text{g}^{-1} \text{min}^{-1}$, (e) trypsin in nmol and (f) aminopeptidase activities μmol of p-nitroaniline liberated $\text{g}^{-1} \text{min}^{-1}$ throughout the gut in Pod Kopište (source), Pod Mrčaru (omnivore), and Zagreb (mainland) populations. Values are mean \pm standard deviation, Pod Kopište $n=4-7$, Pod Mrčaru $n=3-7$, Zagreb $n=3-6$. In (b) populations by tissue and tissues within populations were compared via separate ANOVAs, shared letters above icons denote no differences. In (a, c-f) comparisons of populations were done via ANOVA where lines of a different elevation for a gut region indicate significant differences for that population and overlapping lines indicate no differences.

Figure 6: Total β -glucosidase activity (a) in nmol MUB liberated min^{-1} and total aminopeptidase activity (b) in μmol p-nitroaniline liberated min^{-1} . Values are mean \pm standard deviation, $n=7$, Zagreb $n=6$. Populations compared via ANOVAs, different letters above icons denote significant differences.

Figure 7: Trypsin activity in the pancreas in nmol p-nitroaniline liberated $\text{g}^{-1} \text{min}^{-1}$ in Pod Kopište (source), Pod Mrčaru (new omnivore), and Zagreb (mainland) populations. Values are mean \pm standard deviation, $n=10$, Zagreb $n=6$. Populations compared via ANOVA, different letters above icons denote significant differences.

Figure 8: Organic matter digestibility on experimental diets in lizards from in Pod Kopište (blue, source) and Pod Mrčaru (green, new omnivore) presented as box and whisker plots representing each quartile ($n=5$). Populations compared via ANOVA separately for each diet. † denotes significant differences at $P<0.05$ level.

Table 1. Predictions of relative diet, gut morphology, enzyme activities, fermentation products, and digestibility in Pod Mrčaru (new omnivore), Pod Kopište (source), and Zagreb (mainland) populations and whether these hypotheses were supported by the results of this study. *Note that gut length refers to the combined length of the esophagus, stomach, and intestines

Characteristics	Pod Mrčaru	Pod Kopište	Zagreb	Supported?
Mass of stomach contents	highest	low	low	yes
Diet: % plant matter	highest	low	low	yes
Gut Length*	long	short	short	no
Intestinal mass	heaviest	light	light	no
ESM	largest	least	least	no
Enzyme activities (substrate)				
pancreatic				
α -amylase (starch ^a)	moderate	low	low	in HC only
Trypsin (protein)	moderate/high	high	high	no
Lipase (fats)	moderate	moderate	moderate	yes
intestinal				
N-acetyl- β -D-glucosaminidase	low	moderate	moderate	no
Trehalase (arthropod sugars)	low	moderate	moderate	no
Maltase (disaccharides ^a)	moderate	low	low	no
Aminopeptidase (dipeptides)	moderate/high	high	high	no
microbial				
β -glucosidase (β -glucosides ^b)	high	low	low	no
SCFAs				
acetate	high	low	low	no
propionate	high	low	low	no
butyrate	moderate	low	low	no
isobutyrate	moderate	low	low	no
valerate	low	moderate	moderate	no
isovalerate	low	moderate	moderate	no
Digestibility			Not measured	
plants	moderate	low		yes
plants + insects	high	moderate		yes
insects	high	high		yes

^aFrom plants, seeds, glycogen sources; ^bFrom plant cell wall sources. HC=hindgut contents.

Table 2. Average stomach contents (\pm SD) by mass of lizards from Pod Kopište, Pod Mrčaru, and Zagreb. We found empty stomachs in additional lizards (not included in the counts) from Pod Kopište ($n_{empty}=1$) and Zagreb ($n_{empty}=3$), but not Pod Mrčaru. Plant material is broken down into the percentages of each type, adding up to 100% of total plant material. “Other” consisted of rocks and feces.

	Pod Kopište	Pod Mrčaru	Zagreb
Mass of stomach contents	94.50 \pm 68.0 mg <i>n=30</i>	207.41 \pm 16.1 mg <i>n=36</i>	98.79 \pm 123.2 mg <i>n=4</i>
	<i>(does not include individuals with empty stomachs)</i>		
Plant material %	24.49 \pm 33.1	64.24 \pm 30.8	2.37 \pm 8.3
<i>leaves</i>	6.25 \pm 25.0	7.65 \pm 12.0	0
<i>seeds</i>	81.25 \pm 40.3	91.18 \pm 13.3	0
<i>wood</i>	12.50 \pm 34.2	1.18 \pm 3.0	0
<i>fruit</i>	0	0	100
Animal material %	75.36 \pm 33.0	35.58 \pm 30.9	70.48 \pm 44.2
Other %	0.15 \pm 0.8	0.19 \pm 0.8	25.37 \pm 22.8

Table 3. Total short-chain fatty acid (SCFA) concentrations and ratios of acetate: propionate: butyrate: isobutyrate: valerate: isovalerate to total SCFAs in hindguts of Pod Kopište (source; $n=4$), Pod Mrčaru (omnivore; $n=3$), and Zagreb (mainland outgroup, $n=1$) populations. Values are mean \pm standard deviation. Compared between populations using equal variance t-tests, * denotes significant differences between populations.

Population	Total SCFA	Ratio
Pod Kopište	61.86 \pm 3.95 mM *	65*: 19: 8: 5*: 1: 1*
Pod Mrčaru	19.22 \pm 8.63 mM *	56*: 16: 16: 7*: 2: 4*
Zagreb	0.60 mM	65 : 16: 11: 6 : 0: 3

Table 4. Comparison of digestive enzyme activities ranges between the *Podarcis siculus* of the current study and previous work on lizard digestive physiology. Values have been converted so that units are directly comparable, however differing methodology may confound these comparisons. Bolded species and values are similar to those we measured in *P. siculus*. ^aN-acetyl-β-D-glucosaminidase

Enzyme	<i>Podarcis siculus</i> : This Study	Previous Studies	
Amylase (pancreas)	80-137 $\mu\text{mol min}^{-1}\text{g}^{-1}\text{ tissue}$	<i>Tupinambis meriange</i> Parry et al. 2009	5.82-12.7x10 ⁵ $\mu\text{mol min}^{-1}\text{g}^{-1}\text{ protein}$
Aminopeptidase	Total: 0.22-0.81 $\mu\text{mol min}^{-1}$ Total/g tissue: 0.96-3.65 $\mu\text{mol min}^{-1}\text{g}^{-1}$	<i>Liolaemus pictus</i> Vidal and Sabat 2010	0.11-0.21 $\mu\text{mol min}^{-1}$
		<i>Lophognathis temporalis</i> Iglesias et al. 2009	10.22-25.35 $\mu\text{mol min}^{-1}$
		<i>Liolaemus nigriviridis</i> Naya et al. 2009	2-4 $\mu\text{mol min}^{-1}\text{g}^{-1}$
		<i>Liolaemus ruibali</i> Kohl et al. 2016a	2.13±0.3 $\mu\text{mol min}^{-1}\text{g}^{-1}$
Trehalase	Total: 0.004-0.28 $\mu\text{mol min}^{-1}$ Total/ g tissue: 0.023-1.38 $\mu\text{mol min}^{-1}\text{g}^{-1}$	<i>Heloderma suspectum</i> Christel et al. 2007	9-21 $\mu\text{mol min}^{-1}\text{g}^{-1}$
		<i>Liolaemus pictus</i> Vidal and Sabat 2010	5.9-9.4 $\mu\text{mol min}^{-1}$
Maltase	Total: 0.06-0.18 $\mu\text{mol min}^{-1}$ Total/ g tissue: 0.273-0.782 $\mu\text{mol min}^{-1}\text{g}^{-1}$ PI/MI: 0.458-0.872 $\mu\text{mol min}^{-1}\text{g}^{-1}$	<i>Liolaemus nigriviridis</i> Naya et al. 2009	~2-6 $\mu\text{mol min}^{-1}\text{g}^{-1}$
		<i>Liolaemus pictus</i> Vidal and Sabat 2010	5.92-11.31 $\mu\text{mol min}^{-1}$
		<i>Lophognathis temporalis</i> Iglesias et al. 2009	2.39-3.89 $\mu\text{mol min}^{-1}$
		<i>Liolaemus ruibali</i> Kohl et al. 2016a	34.55±2.68 $\mu\text{mol min}^{-1}\text{g}^{-1}$
NAG ^a	Total: 2.16-4.311 nmol min^{-1} Total/g tissue: 9.6-17.9 $\text{nmol min}^{-1}\text{g}^{-1}$	<i>Liolaemus nigriviridis</i> Naya et al. 2009	20-60 $\mu\text{mol min}^{-1}\text{g}^{-1}$
		<i>Lacerta viridis</i> Jeuniaux 1961	1.2x10 ⁷ nmol min^{-1}
		<i>Uromastix acanthinurus</i> Jeuniaux 1963	no detectible activity
		<i>Anolis carolinensis</i> Jeuniaux 1963	no detectible activity
		<i>Chamaeleo chamaelon</i> Jeuniaux 1963	2.7x10 ⁶ nmol min^{-1}
		<i>Sceloporus undulatus</i> Marsh et al. 2001	1.4x10 ⁶ $\text{nmol min}^{-1}\text{g}^{-1}$

Figure 1

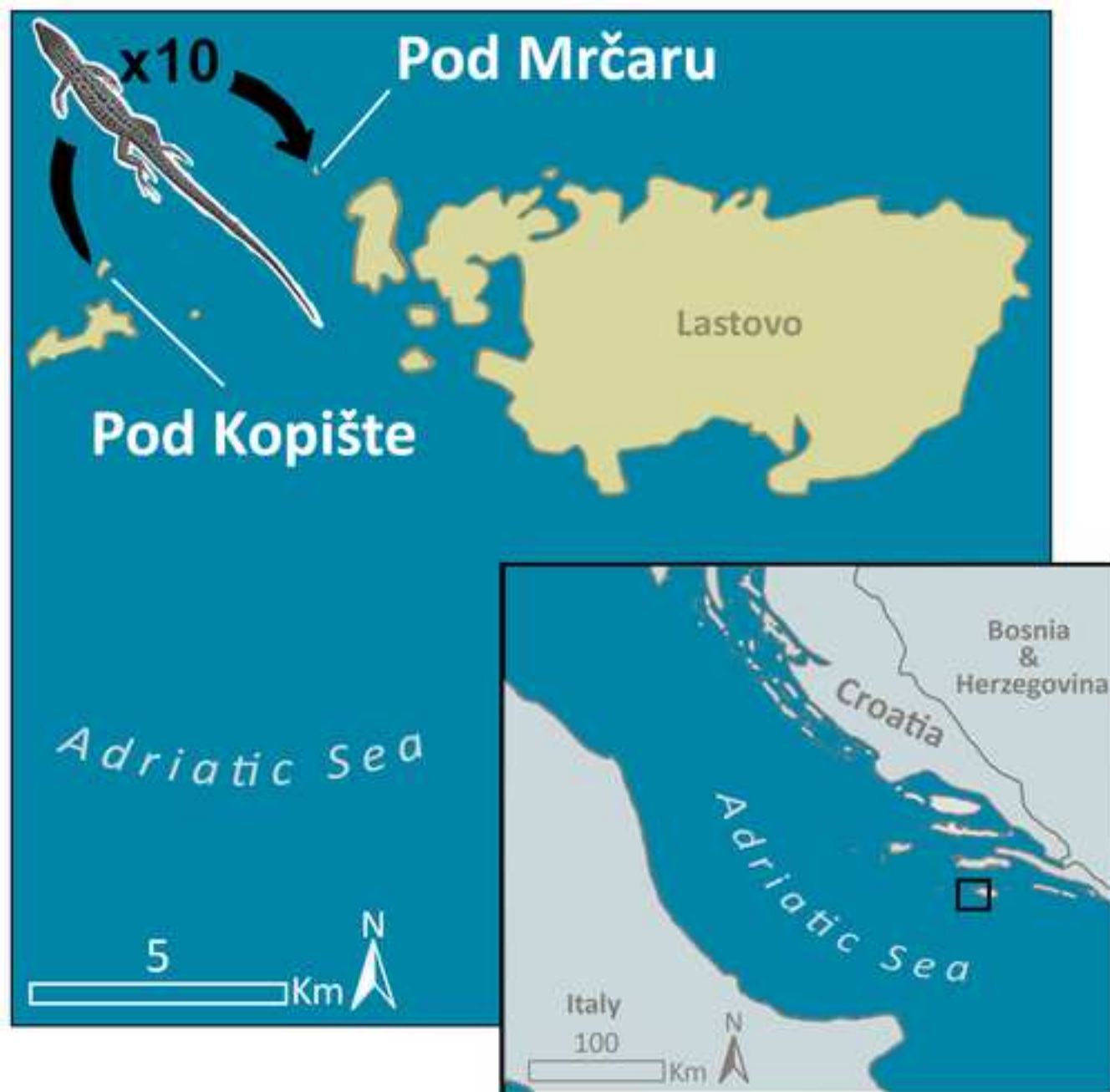


Figure 1 grayscale



Figure 2

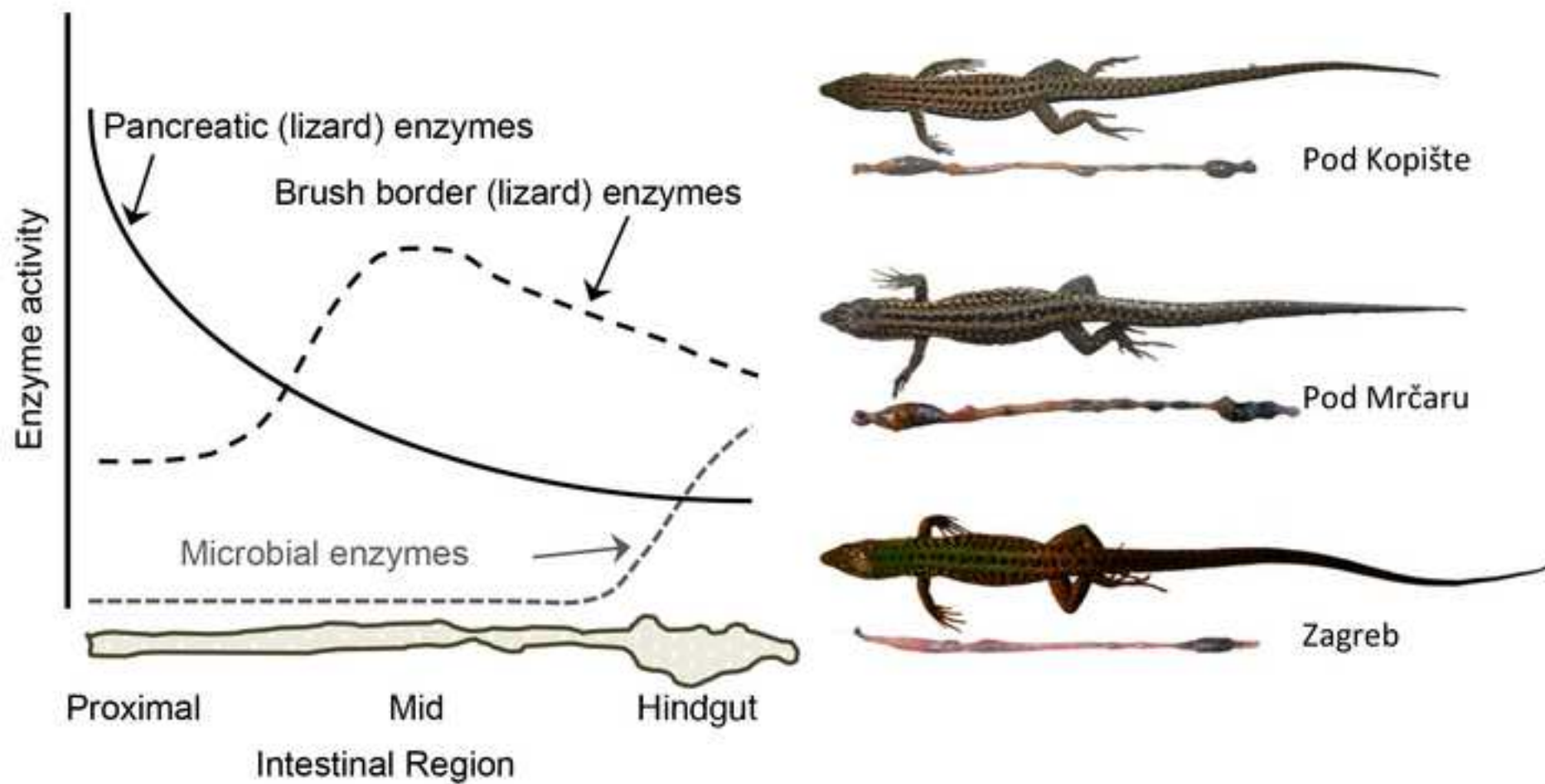


Figure 2 grayscale

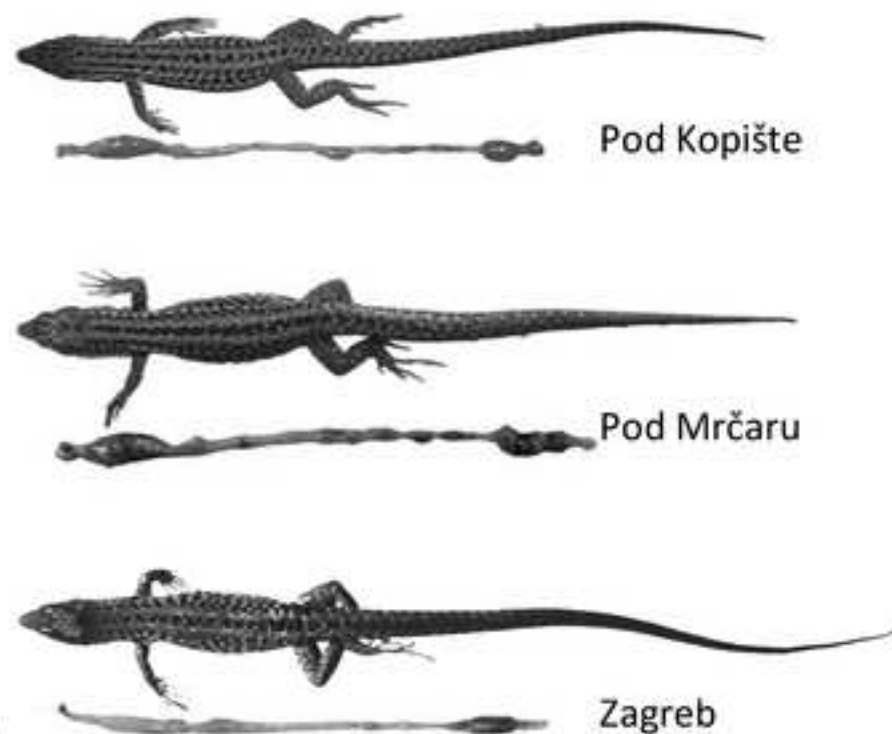
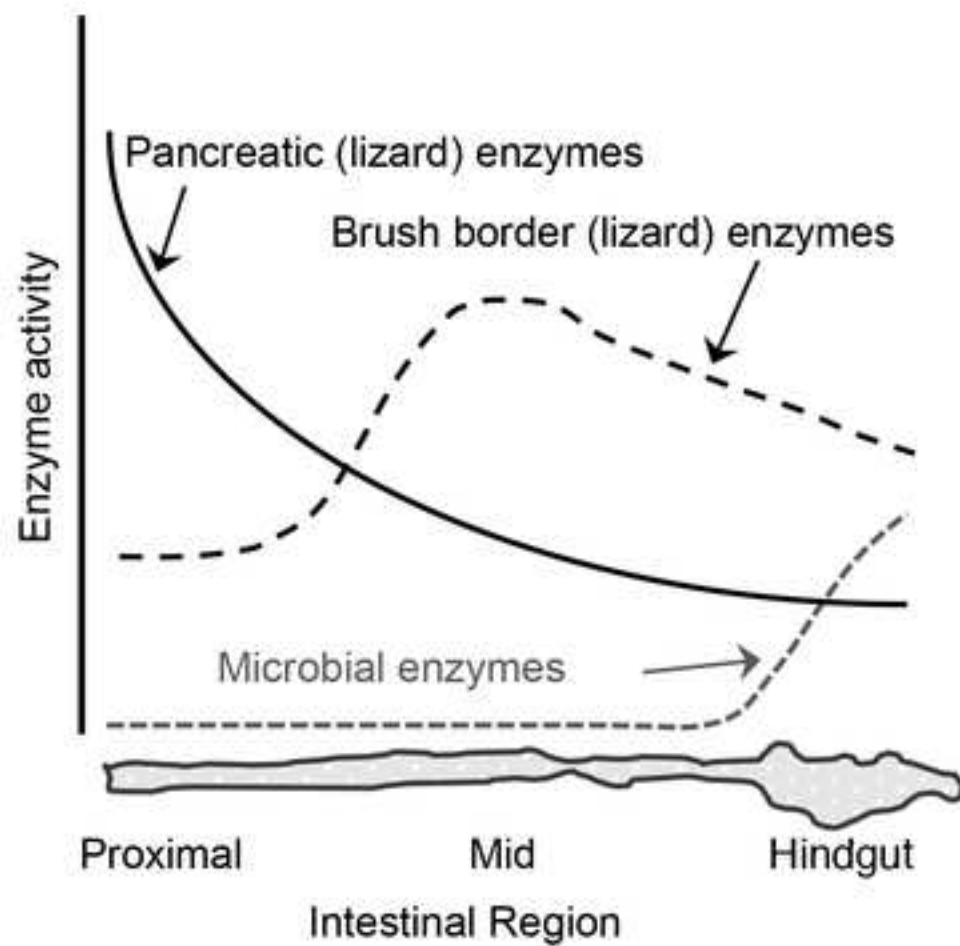


Figure 3

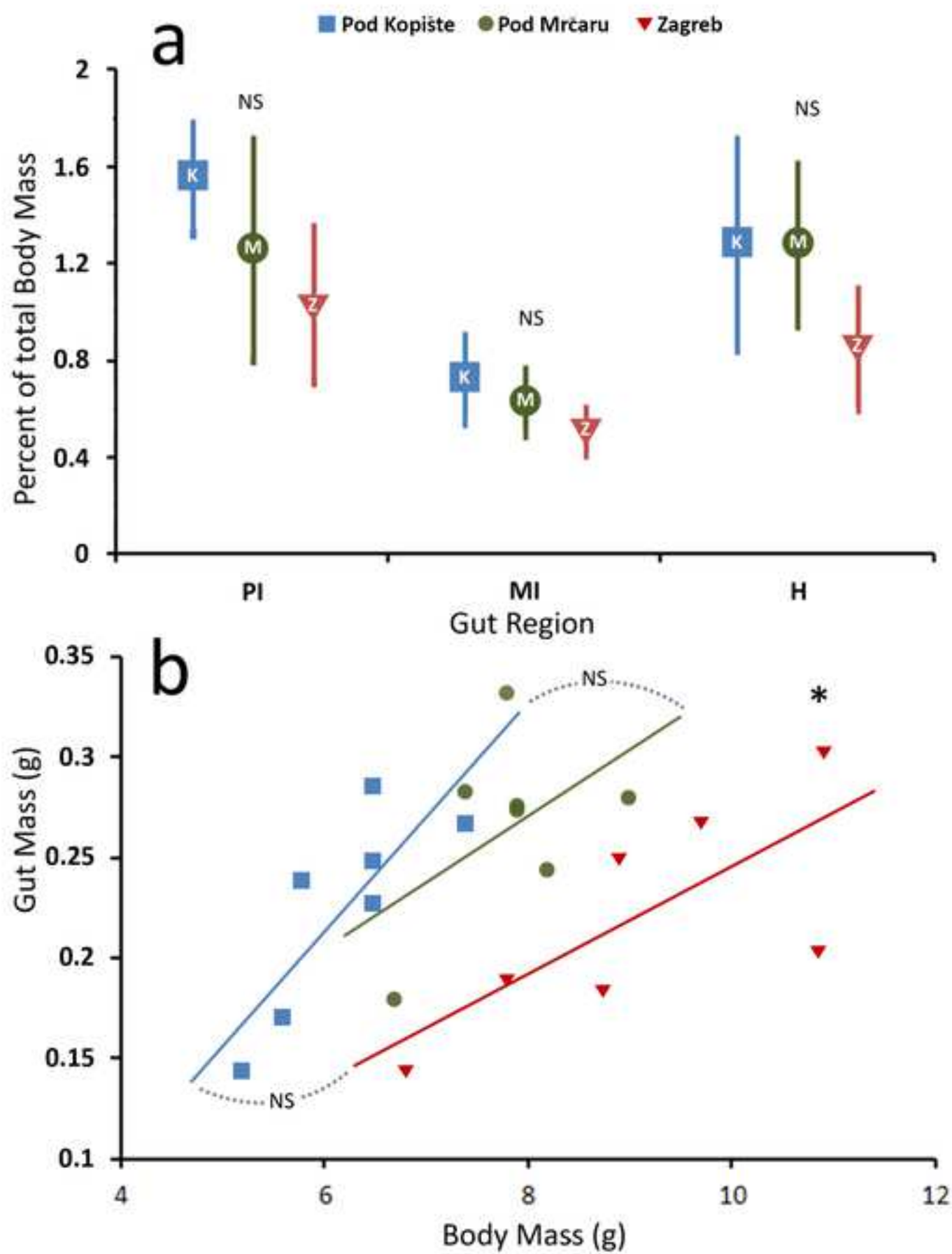


Figure 3 grayscale

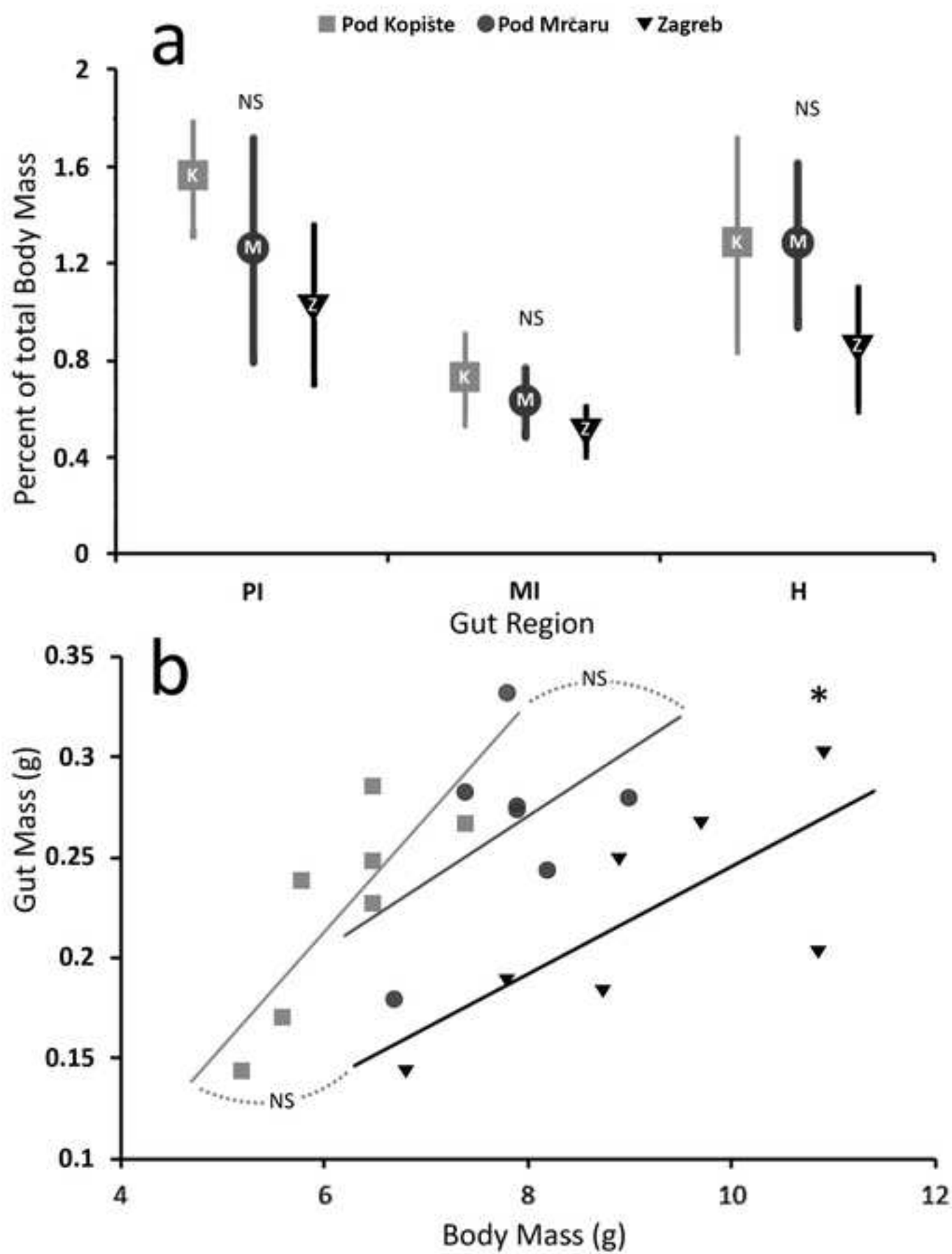


Figure 4

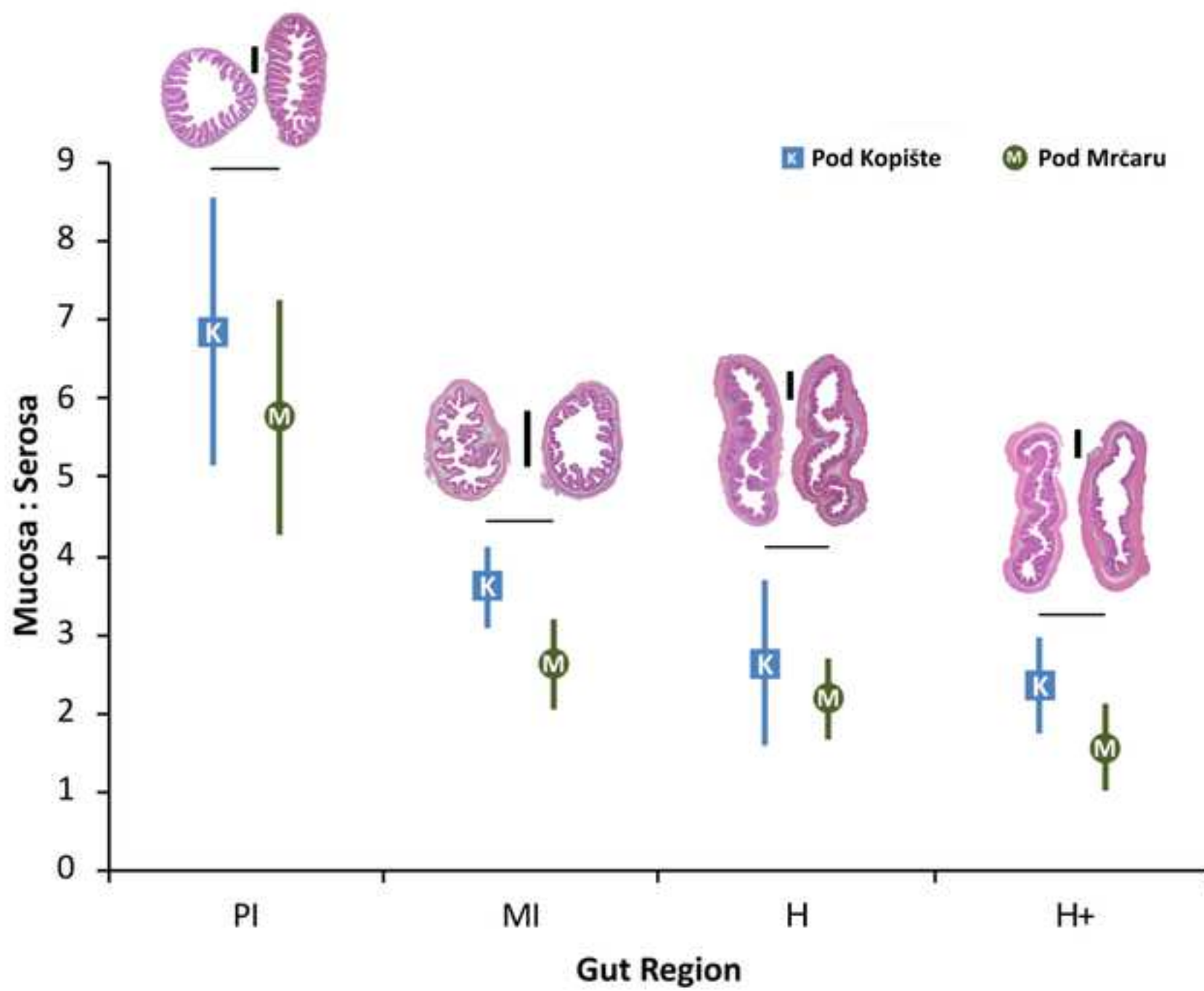


Figure 4 histology color/grayscale graph

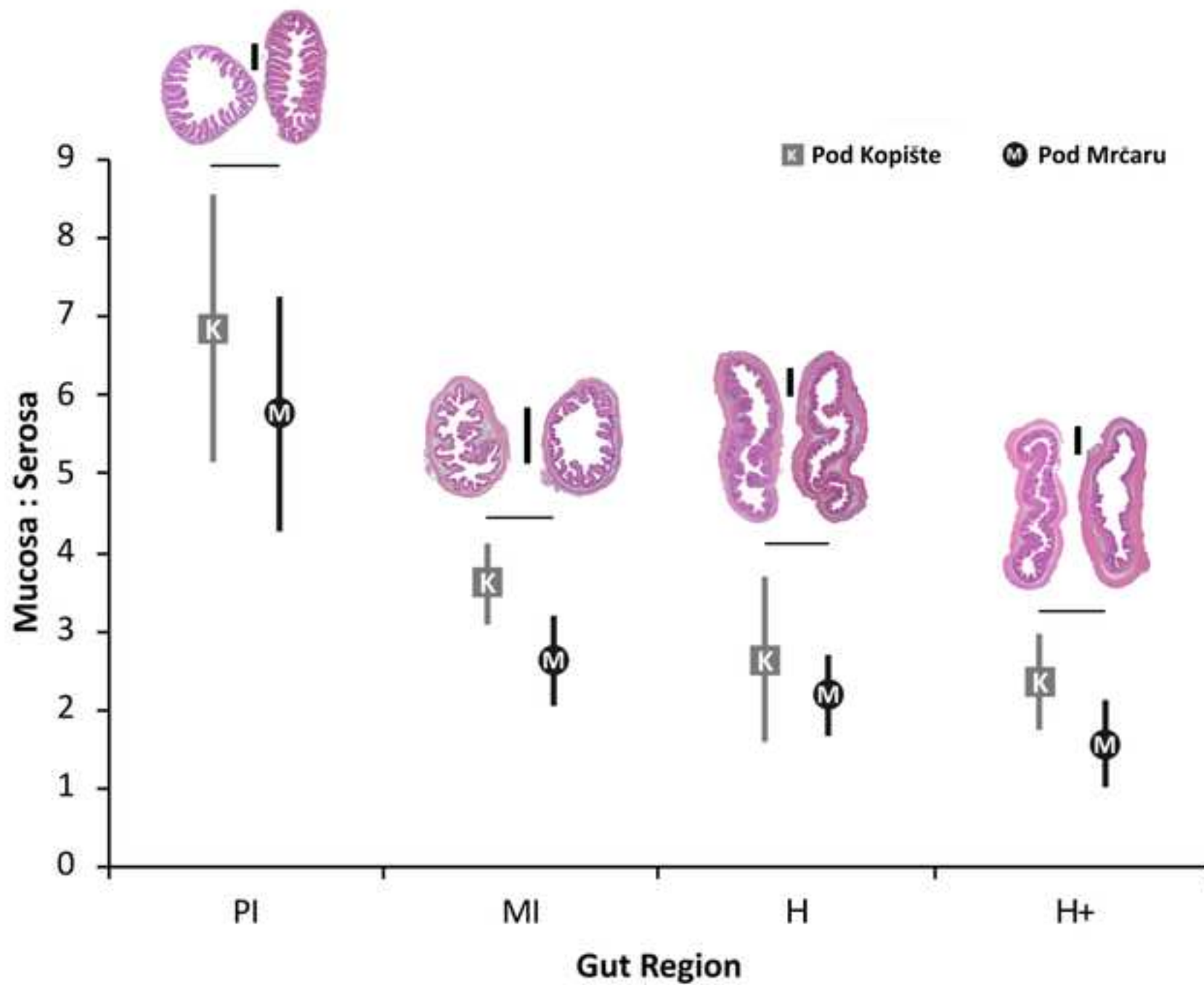


Figure 5

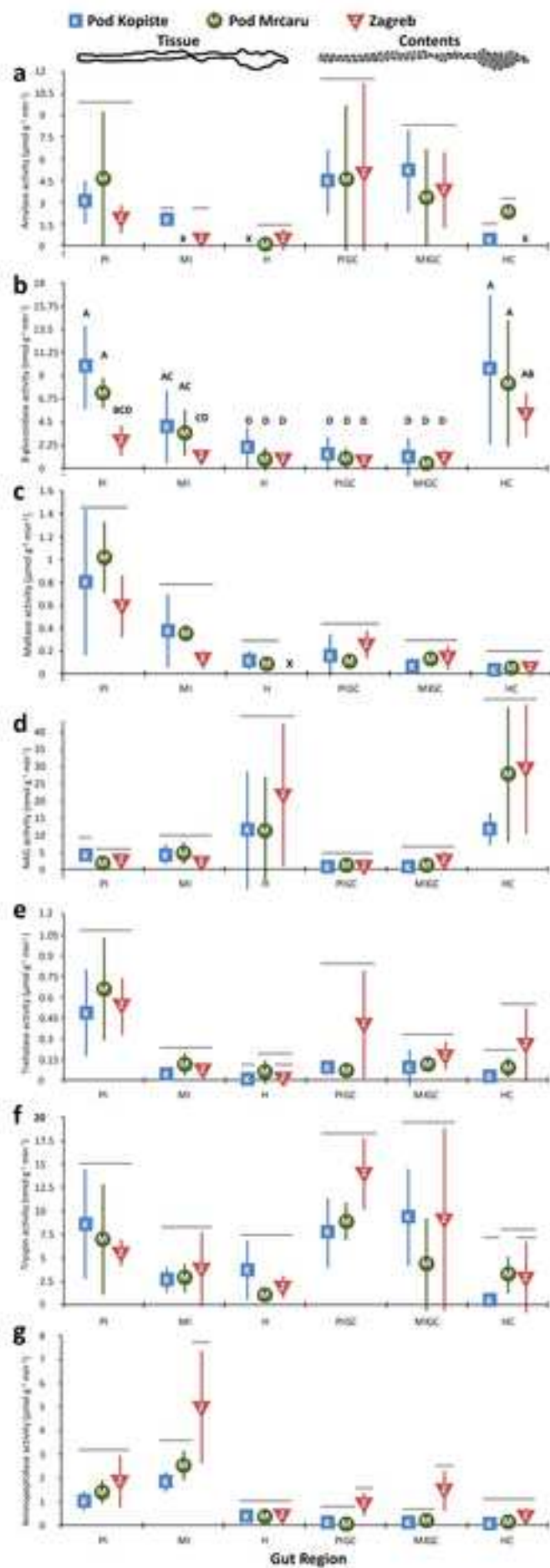


Figure 5 grayscale

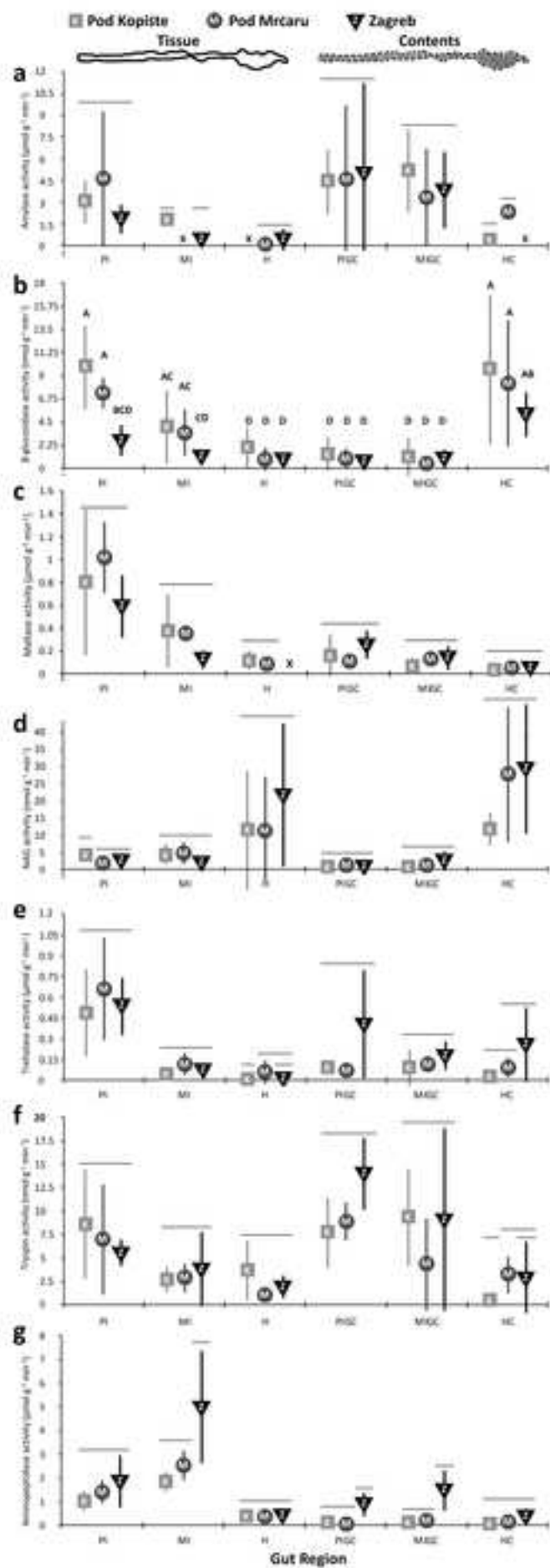


Figure 6

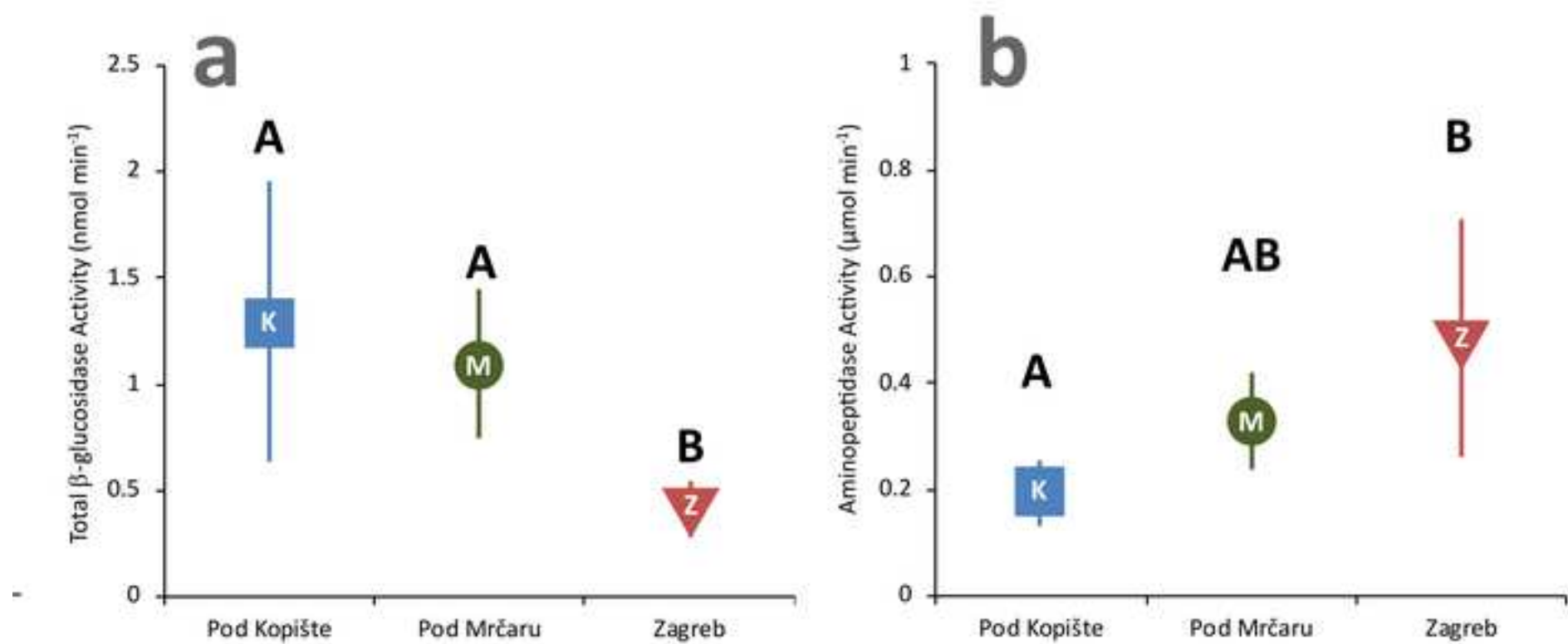


Figure 6 grayscale

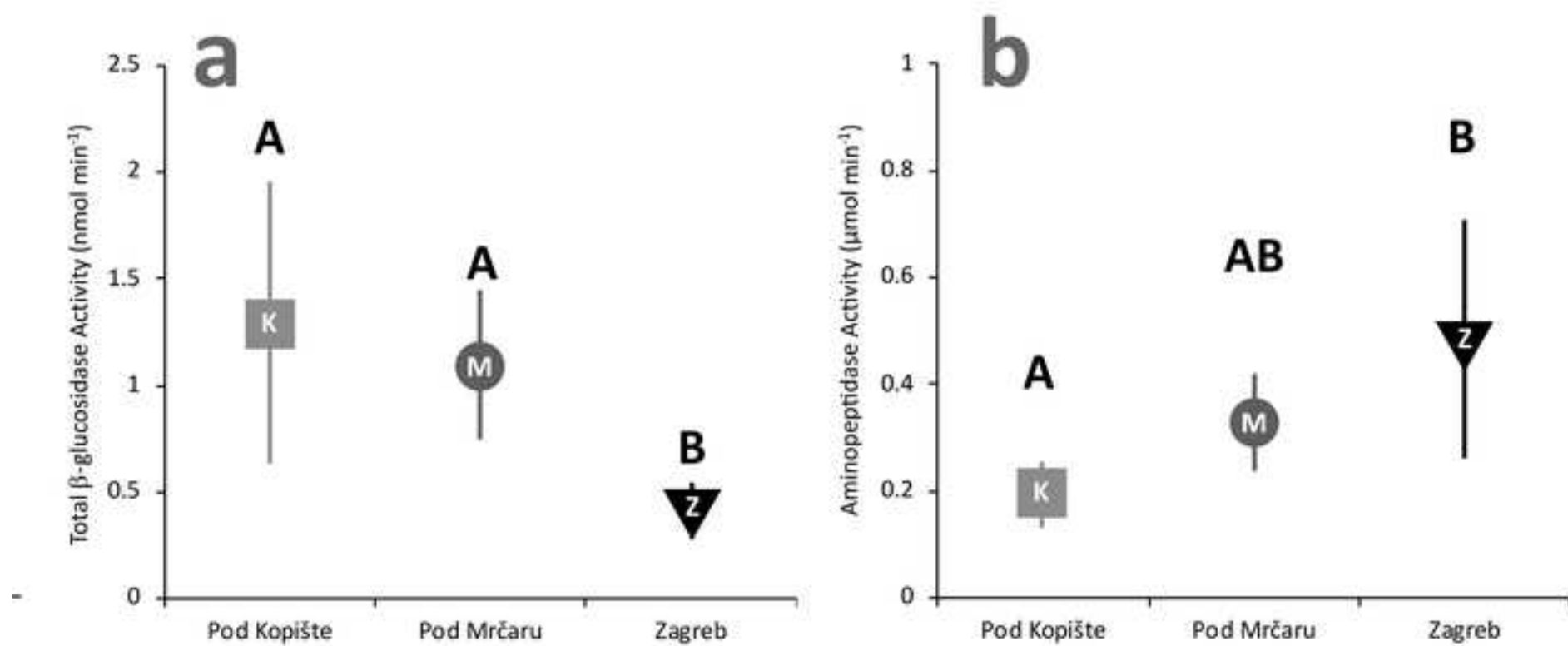


Figure 7

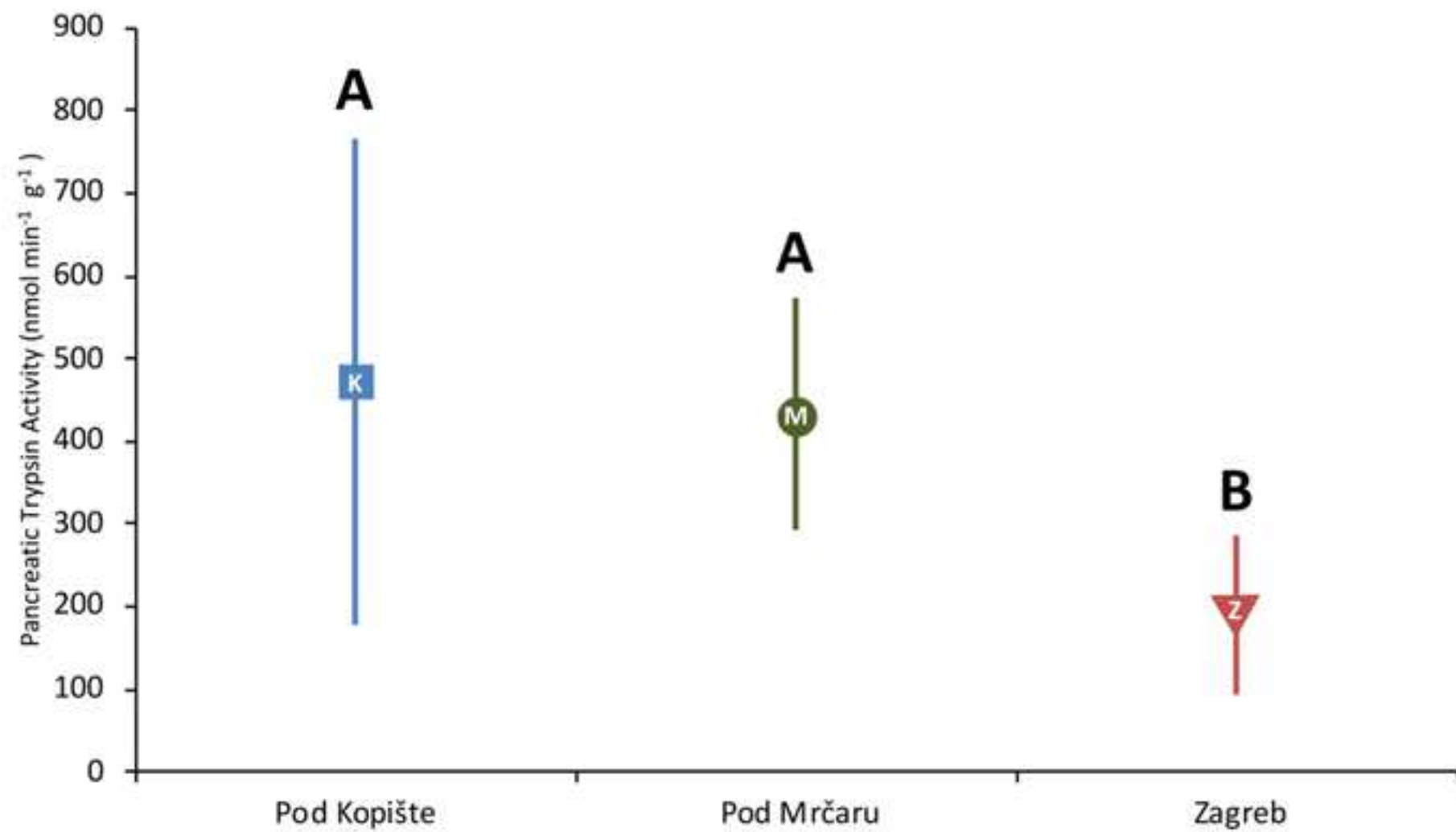


Figure 7 grayscale

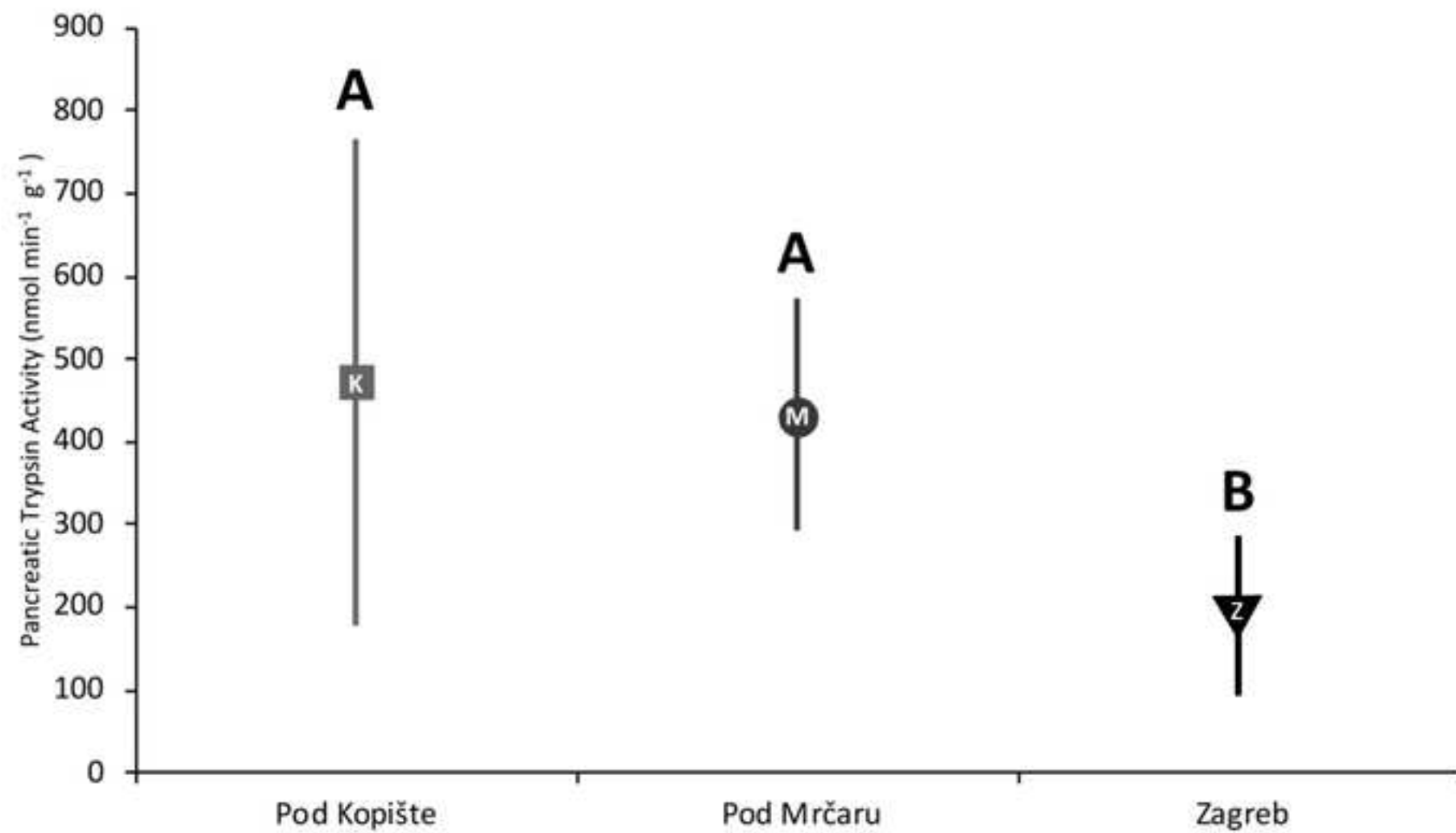


Figure 8

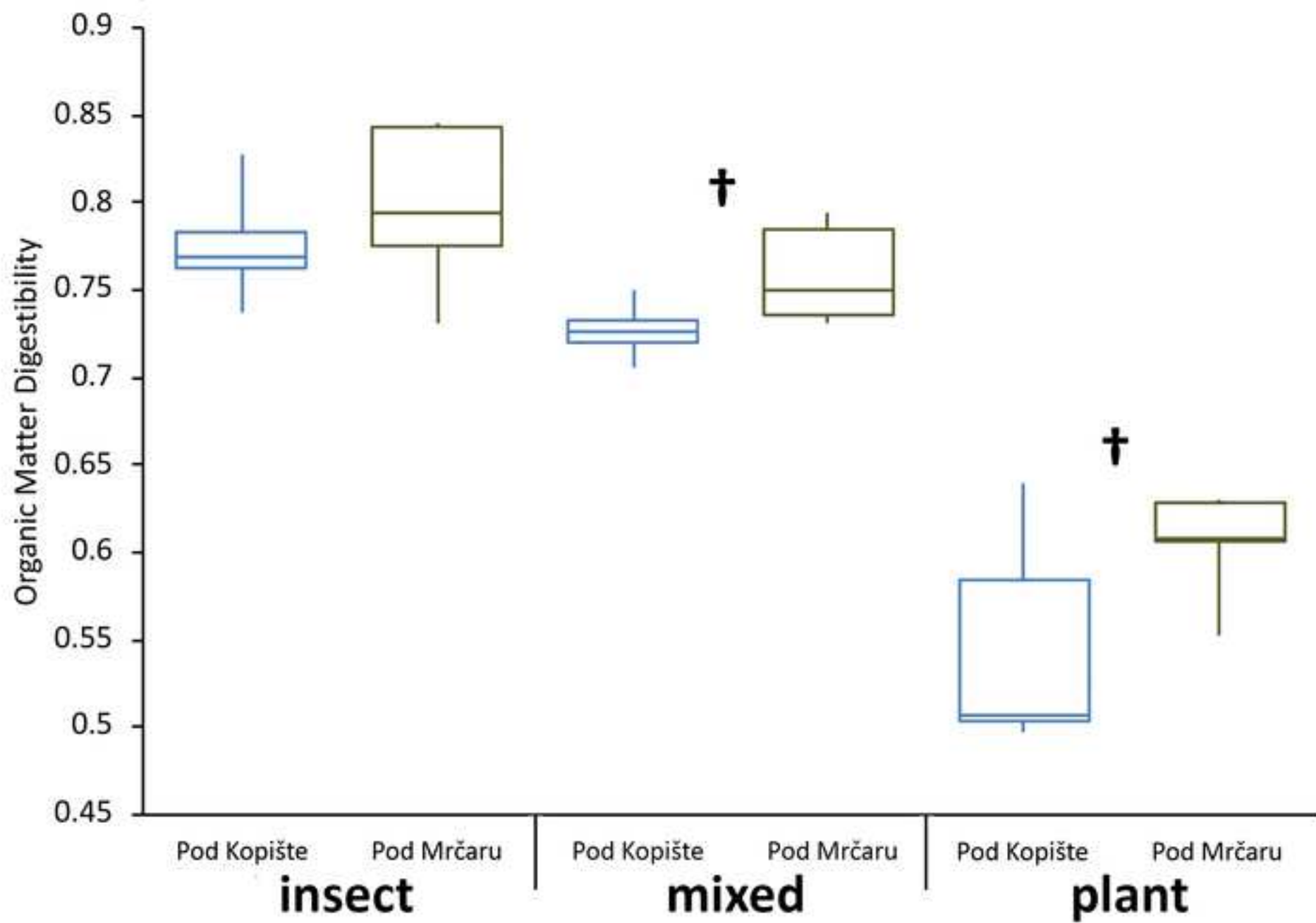
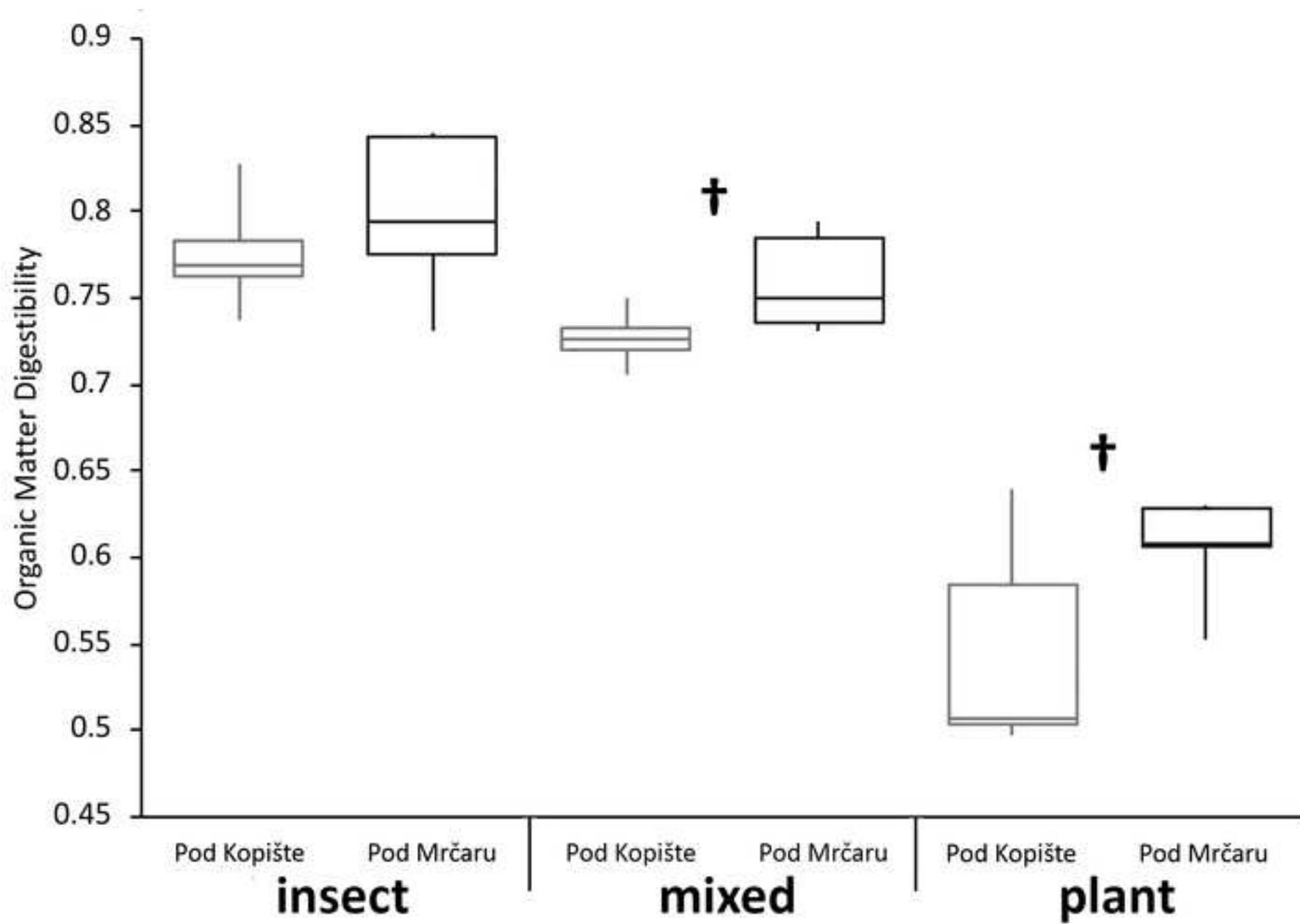


Figure 8 grayscale



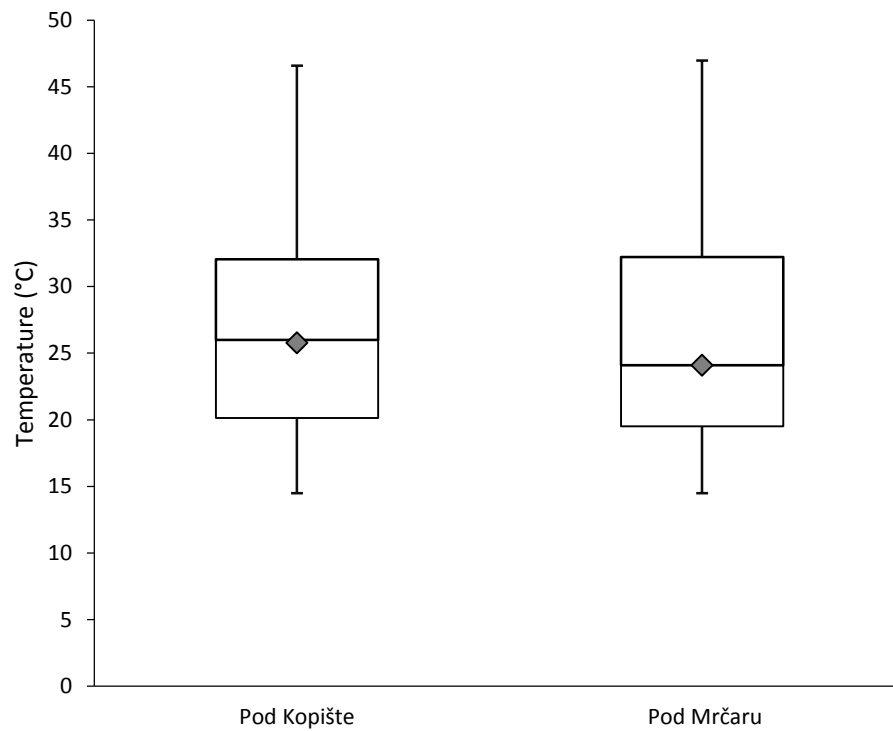
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Figure S1. Box-plot of iButton temperature data collected on islands from May–September. Temperatures were collected from three loggers/ island every 1.5-2 hours, with both full sun and shade represented. Quartile data are represented by boxes and whiskers, with means denoted by ♦. Temperatures are not different by island.

Table S1. (a) Average SVL (\pm SD) and (b) average body mass (\pm SD) of lizards in digestibility trial from Pod Kopište and Pod Mrčaru. Initial measured from first measurement during the feeding trial, final from measurement at sacrifice. Percent change is calculated with respect to initial measurement. Pooled includes all lizards from a population regardless of diet treatment.

a	Pooled SVL			Change in SVL by diet treatment		
	Initial	Final	% Change	insectivore	omnivore	herbivore
Pod Kopište	61.32 \pm 2.8 mm	62.72 \pm 3.3 mm	2.31 \pm 3.8%	5.07 \pm 4.5%	1.22 \pm 3.7%	0.65 \pm 1.6%
Pod Mrčaru	64.42 \pm 3.7 mm	67.6 \pm 2.9 mm	5.16 \pm 5.6%	9.37 \pm 6.2%	1.69 \pm 3.3%	4.42 \pm 4.8%

b	Pooled mass			Change in mass by diet treatment		
	Initial	Final	% Change	insectivore	omnivore	herbivore
Pod Kopište	6.23 \pm 0.6 g	6.19 \pm 0.6 g	-0.45 \pm 6.5%	-0.77 \pm 5.9%	1.75 \pm 8.9%	-2.34 \pm 5.0%
Pod Mrčaru	8.06 \pm 5.8 g	8.04 \pm 0.5 g	0.02 \pm 7.1%	-2.65 \pm 5.1%	5.01 \pm 5.4%	-2.31 \pm 8.5%

Table S2. Values (mean, with 95% confidence interval below) for SVL, body mass, gut length, and gut mass Values that share a superscript letter for a particular measurement are not significantly different.

	<u>Pod Kopište</u>	<u>Pod Mrčaru</u>	<u>Zagreb</u>
SVL	64.39 mm ^A (63.08-65.70)	68.73 mm ^B (67.36-70.11)	68.43 mm ^B (65.30-71.56)
Body mass	6.6 g ^A (6.3-7.0)	7.6 g ^{AB} (7.3-7.9)	8.6 g ^B (8.3-8.8)
Gut length	115.47 mm ^A (108.29-122.66)	116.15 mm ^A (110.65-121.64)	126.02 mm ^A (117.93-134.11)
Gut mass	0.225 g ^A (0.189-0.277)	0.266 g ^A (0.233-0.312)	0.221 g ^A (0.182-0.277)

Table S3. Average enzyme activities \pm standard deviation. ^a N-acetyl- β -D-glucosaminidase

Enzyme, units	Region	Pod Kopište	Pod Mrčaru	Zagreb
Amylase, $\mu\text{mol glucose liberated min}^{-1} \text{ g}^{-1}$	HC	0.400 \pm 0.30	2.387 \pm 1.99	undetectable
maltase, $\mu\text{mol glucose liberated min}^{-1}$	total	0.0435 \pm 0.027	0.0694 \pm 0.023	0.0701 \pm 0.029
trehalase, $\mu\text{mol glucose liberated min}^{-1}$	total	0.0548 \pm 0.041	0.0900 \pm 0.075	0.0569 \pm 0.020
β -glucosidase, $\text{nmol MUB released min}^{-1} \text{ g}^{-1}$	PI	9.834 \pm 4.08	7.362 \pm 1.50	2.706 \pm 1.49
β -galactosidase, $\text{nmol MUB released min}^{-1} \text{ g}^{-1}$	PI	22.461 \pm 8.51	14.647 \pm 6.82	17.332 \pm 8.57
NAG ^a , $\text{nmol MUB released min}^{-1} \text{ g}^{-1}$	PI	4.022 \pm 1.43	1.845 \pm 0.70	2.076 \pm 1.50
Trypsin, $\text{nmol p-nitroaniline released min}^{-1} \text{ g}^{-1}$	HC	0.545 \pm 0.23	3.150 \pm 2.01	2.852 \pm 3.85
aminopeptidase, $\mu\text{mol p-nitroaniline released min}^{-1}$	total	0.196 \pm 0.05	0.326 \pm 0.09	0.485 \pm 0.09
Lipase, $\text{nmol p-nitrophenol released min}^{-1}$	total	0.411 \pm 0.09	0.524 \pm 0.30	0.599 \pm 0.29

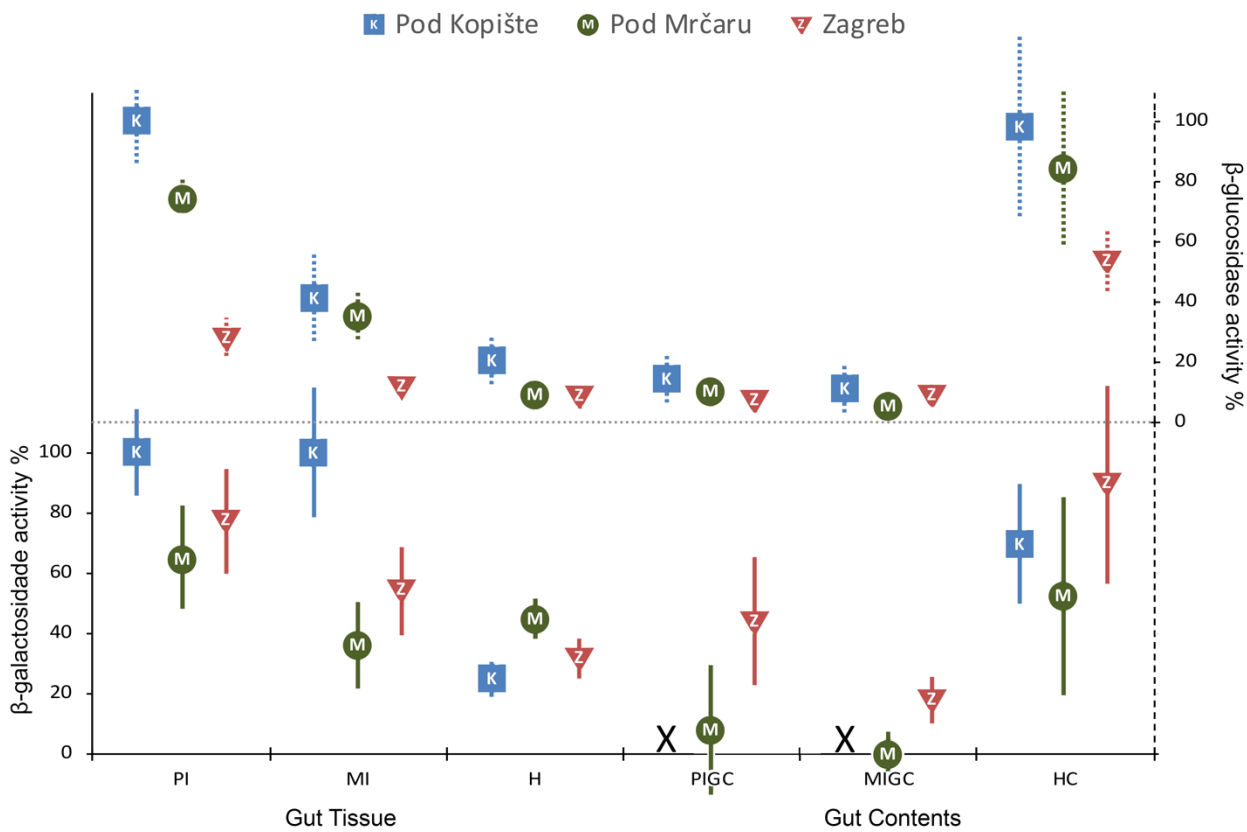


Figure S2. β-galactosidase (top graph, left axis, solid lines) and β-glucosidase (bottom graph, right axis, dashed lines) activities in each gut region as a percentage of the highest activity (i.e.: Pod Kopište PI activity is 100% for both enzymes as it has the highest activity of any gut region and lizard population). Values are averages ± SEM.

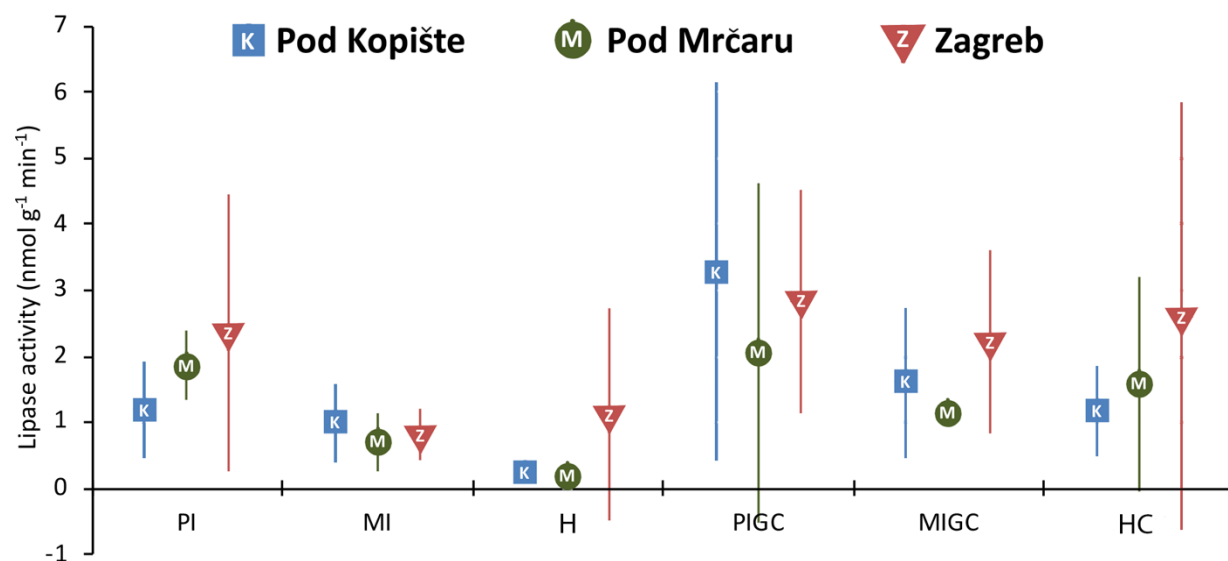


Figure S3. Lipase activity in nmol p-nitrophenol liberated min⁻¹ for each gut region in Pod Kopište (source), Pod Mrčaru (new omnivore), and Zagreb (mainland) populations. Gut regions are proximal intestine (PI), mid intestine (MI), hindgut (H), proximal intestinal gut contents (PIGC), mid intestinal gut contents (MIGC), and hindgut contents (HC). Values are mean \pm standard deviation. Pod Kopište $n=4-7$, Pod Mrčaru $n=4-6$ (MIGC region $n=2$), Zagreb $n=5-7$. There are no differences among populations by region.

Table S4. Average percent organic matter digestibility (\pm standard deviation) on experimental diets of plant material, 50:50 plant material: insects, or insects in lizards from Pod Kopište and Pod Mrčaru ($n=5$). † denotes significantly different at a $P=0.05$ level.

	Pod Kopište		Pod Mrčaru
<i>plant</i>	54.6 \pm 6.3	†	60.5 \pm 3.2
<i>mixed</i>	72.0 \pm 1.6	†	75.9 \pm 2.9
<i>insect</i>	77.6 \pm 3.3		79.8 \pm 4.8

Note: Plant diet comparison: ANCOVA: population $F_{1,6}=6.17$, $P=0.048$, body mass $F_{1,6}<0.001$, $P=0.983$). Omnivore diet comparison: ANCOVA: population $F_{1,7}=5.875$, $P=0.0458$; body mass $F_{1,7}=3.065$, $P=0.1235$.

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Herbivore diet

- ANOVA $F_{1,8}=3.48$, $P=0.099$
- ANCOVA: SVL $F_{1,7}=0.717$, $P=0.425$, population $F_{1,7}=3.357$, $P=0.110$
- ANCOVA: duration $F_{1,7}=2.793$, $P=0.1386$, population $F_{1,7}=4.261$, $P=0.0779$
- ANCOVA: intake $F_{1,6}=3.176$, $P=0.1250$, population $F_{1,6}=5.700$, $P=0.0542$, population*intake $F_{1,6}=3.926$, $P=0.0949$

Omnivore diet

- ANOVA $F_{1,8}=4.67$, $P=0.0627$
- ANCOVA: SVL $F_{1,7}=3.346$, $P=0.1101$, population $F_{1,7}=6.040$, $P=0.0436$
- ANCOVA: duration $F_{1,7}=0.4342$, $P=0.5292$, population $F_{1,7}=4.342$, $P=0.0757$
- ANCOVA: intake $F_{1,7}=1.271$, $P=0.297$, population $F_{1,7}=4.829$, $P=0.064$

Insectivore diet

- ANOVA $F_{1,8}=0.703$, $P=0.426$
- ANCOVA: SVL $F_{1,7}=2.22$, $P=0.180$, population $F_{1,7}=0.811$, $P=0.398$
- ANCOVA: duration $F_{1,7}=1.37$, $P=0.281$, population $F_{1,7}=0.735$, $P=0.281$
- ANCOVA: intake $F_{1,7}=2.57$, $P=0.153$, population $F_{1,7}=0.841$, $P=0.390$

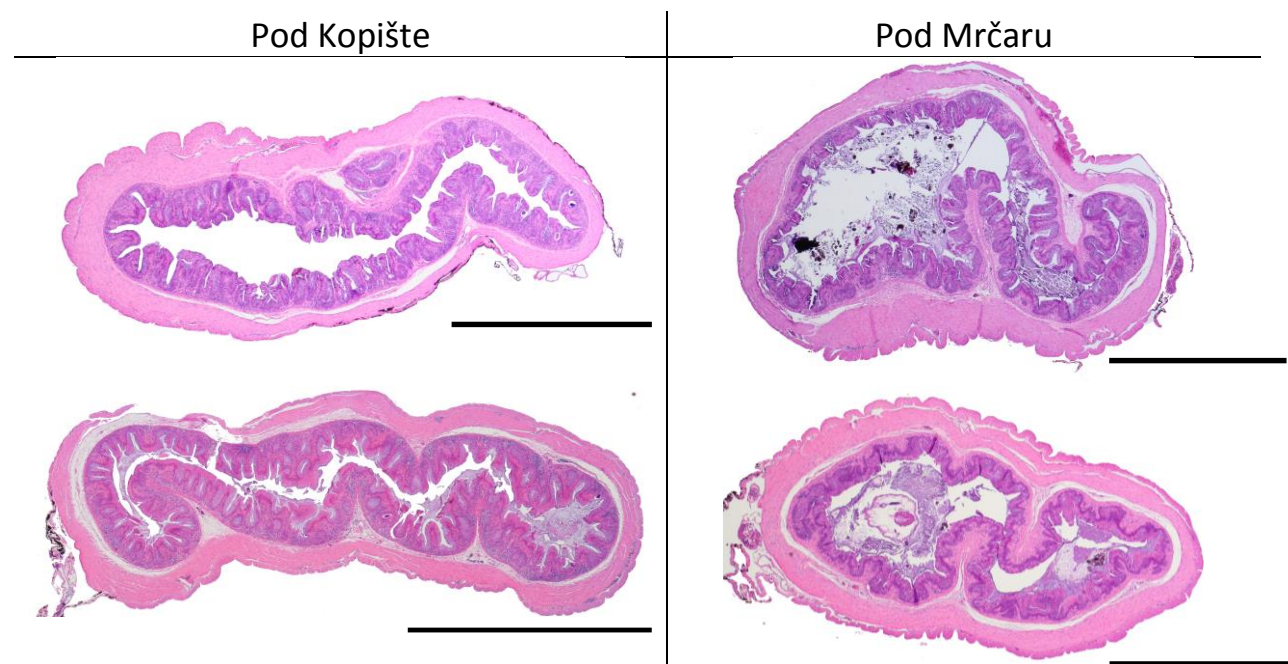


Figure S4. Cross section images are representative stained histological sections from the hindgut of Pod Kopište (source) and Pod Mrčaru (new omnivore) populations (not to scale). Each section from a different individual collected in spring 2014 (Wehrle 2018). Scale bars to the lower right of each image are 100 μm for that image.

GENERAL COMMENTS

We are supportive of this paper overall, yet we request that you be more upfront regarding the limitations or caveats of the study. I suggest adding these in a final paragraph titled something like "Limitations of the Present Study and Future Directions."

We changed the final section of the manuscript to “*Conclusions, Potential Limitations, and Future Directions*”, and we added in the information below.

Into a Future Directions could also go mention of things like "We are analyzing gastric enzymes (including pepsin and chitinase) for a different study and did not include them here."

We added the following sentence to the final section of the manuscript: “Moreover, as the stomach can play a major role in the digestive process, and can account for greater than 50% of gut transit time (Karasov and Martínez del Río 2007), more work on gastric function is warranted, and is the subject of our own work on this system.”

1. Unless I totally missed something, this is not a common-garden experiment, so we cannot know that the differences are evolved, genetically based. Please say this more clearly and cite this paper:
Garland, Jr., T., and S. C. Adolph. 1991. Physiological differentiation of vertebrate populations. *Annual Review of Ecology and Systematics* 22:193–228.

We added the following sentences to the final section of the discussion: “Although we know the Pod Mrčaru lizards have changed their body morphology over ecological time (Herrel et al. 2008), any shifts in their digestive physiology appears to be more constrained on this timescale, or mitigated by differences in behavior and ecology. We currently do not know whether these changes represent flexibility (Piersma and Drent 2003), or evolved differences with underlying genetic changes (Garland and Adolph 1991). Some dietary shifts may not be as limited by physiology as they are by these animals’ ecology.”

I don't see a claim that the population differences reflect evolved differences, but that is the impression one gets in reading over the paper. We need to make sure that readers don't think you have demonstrated something that you have not demonstrated. You do use "rapid evolution" as a keyword (which is a little sneaky, but probably OK to help attract readers). Please consider changing wording that leaves readers with an incorrect impression of your findings.

We removed “rapid evolution” from the key words because we agree that we don’t want to give this impression. We have been careful to say that we don’t know whether any differences (which we argue are subtle) are evolved or flexibility. We do note the different digestibilities measured in the laboratory, but don’t argue that this is an evolved ability.

2. The study is very close to a 2-species comparison, so please explain for the reader why the problems indicated in this paper below are not of major concern in the present study:
Garland, Jr., T., and S. C. Adolph. 1994. Why not to do two-species comparative studies: limitations on inferring adaptation. *Physiological Zoology* 67:797–828. Please keep in mind that PBZ's readers include younger scholars seeking direction from published studies.

The final paragraph of the manuscript now reads: “We do recognize that this study can be mistaken as a “two species comparison”, which comes with caveats and cautions (Garland and Adolph 1994), especially when comparing the island populations with the mainland

population in Zagreb. However, most of our work is focused on comparing the two island populations, which according to their 16s rRNA sequences, have not diverged much genetically over 30+ years of separation (Herrel et al. 2008). The similarity among the two island populations may, thus, simply represent that they are still very much the same taxon phylogenetically, and thus, may not be treated as separate entities. The similarity of the Pod Mrčaru and Pod Kopište lizards in comparison to the Zagreb population, should, therefore, not be surprising given the short period of time of the island populations' separation (Nevo 1971; Herrel et al. 2008). Similar results among populations of insular vs mainland populations of lizards have been observed in other species (Pafilis et al. 2007; Sagonas et al. 2015)."

I do see the paragraph on lines 601-605, but this is somewhat facile. Also, what data are being referred to in the phrase "in the laboratory?" Is that from the present study or something else?

We did include in this study an experiment in the laboratory in which we fed male lizards from the two island populations carnivore, omnivore, and herbivore diets. We collected feces and measured digestibility.

FIGURES

Regarding the color figures: We will provide free color figures in print for the histology photos. For the graphs, please provide grayscale images. If you would like the graphs to be color online: when you submit your revised paper, retain the color images in your manuscript as well as uploading the grayscale versions. We will ask our publisher to use the grayscale versions for the print edition and the color versions for the electronic edition.

We have provided color and gray scale versions of the figures, but kept the histological images in Fig.4 in color (while making the rest of the figure grayscale).

You might consider changing the line style of Figure S2 to match anything similar in the main text.

This graph has been modified to look like the other enzyme figures.

SPECIFIC COMMENTS

Line 29: Is this supposed to say "The Pod Mrčaru populationS?"

No. It is one population on Pod Mrčaru.

Line 33-34: "than the plant-eating Pod Mrčaru population" This sounds like the two populations on Pod Mrčaru differ in diet, but that difference was not made clear earlier in the Abstract. I may be confused by the sampling scheme, which is clear in Table 1 but confusing here in the Abstract.

We understand. We changed this to read “omnivorous” since that is how we described the Pod Mrčaru lizards earlier in the abstract.

Line 38: Say how long they had been acclimated "in the laboratory" and indicate if this was just adults or over ontogeny.

We rewrote this sentence as follows: “Although subtle, the changes in hindgut digestive physiology impact digestibility of plant material in adult lizards— Pod Mrčaru lizards had higher digestibility of herbivorous and omnivorous diets fed over several weeks in the laboratory than did their source population.”

Lines 45-51: The idea that organs are expensive and so likely to be downregulated via phenotypic plasticity is not new and indeed is discussed in MANY areas of physiology/morphology. This argument is, of course, similar to the evolutionary arguments about use and disuse, e.g., regarding blind cave fish, the human appendix, etc., etc., etc. These arguments probably go back to Aristotle and are received wisdom in both physiology and evolutionary biology. They are common sense. A couple of sentences should be added to the start to say this. Then transition into the gut via "For example, ..." You might also add that the gut is known to change rapidly as compared with some other organs and organ systems, and give some comparative data showing this.

I cannot find Krasov (1992) in the literature cited, so I could not check it, but is this really the first place someone made such arguments for the digestive system? Hard to believe. Anyway, how is the AMH anything more than common sense?

Line 46: AMH. Was this stated with respect to phenotypic plasticity, evolutionary adaptation, or both?

The AMH deals with both, short-term flexibility, and long-term adaptive patterns. The history of this hypothesis is more than “just so” stories, emerging from the laboratory of Jared Diamond in the 80’s and 90’s. The reason we cite it here is because it is specific to the gut, and to the function of digestive enzymes and nutrient transporters in particular. A direct quote from Karasov and Diamond (1983): “Dietary substances that yield calories but are not nutritionally essential will specifically stimulate their own absorption. The prime example is likely to be the effect of dietary carbohydrate on glucose transport. The mechanism will be substrate-dependent induction of carriers, expressed kinetically in increased maximal transport rate (V_{max}).” This paper from 1983 led to the formal coining of the AMH and the highly-cited review (Karasov 1992). The AMH has spurred dozens of studies in nutrient transporters and digestive enzymes, and whether their rates of activity are stimulated by their substrates. The AMH is well-supported for carbohydrates in particular, from the physiological (i.e., digestibility) to the genetic level (discussed in Heras et al. 2020). So, although there are other “non-use” arguments for why certain traits may change, the AMH is more functional because it uses Chemical Reactor Theory (CRT) to make some first order approximations and predictions about specific aspects of gut function. For our intent here, these are the best citations to use as a starting point for the specific predictions we make about enzyme activities in the Croatian lizards. We have adjusted the opening paragraph so that it is clear that this can be viewed on short or long time scales, and provide examples of both (long time scales for carbohydrates, more short time scale for proteins). The fact that we largely reject the AMH in the island populations is of interest to a general audience, and thus, we focus on this fact to open the discussion.

Although we have included the CRT proportions (that are currently in the supplements) in earlier versions of this manuscript, we are hesitant to bring them in here because Bill Karasov (previous reviewer of this manuscript) and Steve Secor (current

editor of this manuscript) didn't like our use of the CRT proportions in this context. So, our compromise in earlier versions was to move the CRT discussion into the supplements. Thus, from our perspective, being asked to reintegrate the CRT proportions in the introduction goes against the recommendations of two prominent digestive physiologists, and the author of the AMH itself. Karasov and Secor were fine with us using the AMH to make predictions, but leaning on the CRT proportions seemed to make them uncomfortable. Hence, we are hesitant to reintegrate the CRT proportions into the introduction at this stage. We would prefer to just eliminate the supplemental introduction section altogether and refer readers to the citations.

We added Karasov (1992) as well as Karasov and Diamond (1983) to the literature cited.

I agree with Reviewer 1 that the introductory material currently in Supporting Information makes little sense there and instead needs to go into the Introduction.

Please see our response above. We prefer to eliminate the supplemental introduction altogether and lean more on citations provided in the introduction to refer readers to the theoretical underpinnings of the AMH itself.

Reviewer 3 previously commented about particle size. What you say in response is fine, but please add some of that into the manuscript, either in Methods or maybe in the Final Caveats section.

We added the following to lines 309-317 in the methods, "We recognize that grinding the food to small particle sizes may take away natural particle size differences in the diets of the Pod Kopište and Pod Mrčaru lizards. However, in seeking some control over dietary intake, we chose to grind the food into a common size, and thus, measure digestibility based on more uniform food. This removed potential physical and chemical processing of differentially sized food particles in the lizards, but allowed us to focus on pure chemical digestibility in the laboratory. One previous investigation of whole meal worm digestibility in these same lizard populations found similar results to ours (Vervust et al. 2010), suggesting that our grinding of the food didn't mask any performance differences among the lizard populations."

Lines 94 -95 and elsewhere. Change "While" to "Whereas" or "Although." "While" means literally later in time.

These have all been changed or removed.

Line 103: Change punctuation to be "; however,"

Change made.

Line 639-640: Change to "may dampen selection on ..." Selection does not have units of pressure.

Pressures removed.

Line 640-641: Change to "as changes in behavior can offset evolutionary changes in physiology and morphology"

Change made.

Line 963-964: Decapitalize title and check other references.

Decapitalized and references checked.

Table 2: Unclear if these empty stomach individuals are an additional number of animals versus the sample sizes listed inside the table. Clarify so that someone could calculate % empty.

To the Table 2 caption, we added that the empty stomachs were not included in the counts, but we provided the number of empty stomachs. Thus, if someone wants to redo the calculations with the empty stomachs, they have that information now.

FYI, this is relevant:

Supporting Materials for the Introduction. As noted above, please move all of this into the text and make it flow. In the middle of the third paragraph here, add something like "either in terms of phenotypic plasticity (ontogenetic acclimatization) and/or evolved differences" to help make it clear that both things may be going on. This will also foreshadow the Caveats section where you make clear that you don't know which of these explains the population differences you have found.

We believe we have clarified this throughout the manuscript. Please see our response about the supplemental material above.

Reviewer 1 Comments

I have carefully read through the manuscript and the response to reviewers, and the authors have done an excellent job in revising the manuscript and responding to my comments and criticisms. I look forward to seeing this work in its final and published form.

Thank you.