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*, with populations on Pod Mrčaru, Croatia, that 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. On the basis of the adaptive modulation hypothesis, we predicted changes in gut size and structure, digestive enzyme activities, microbial fermentation products (short-chain fatty acids [SCFAs]), and plant material digestibility concomitant with this dietary change. The Pod Mrčaru population had heavier guts than the mainland population, but there were no other differences in gut structure. Most of the enzymatic differences we detected were between the island populations and the out-group population. The Pod Mrčaru lizards had higher amylase and trehalase activities in their hindguts compared with the Pod Kopište population, and 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 the 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.

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Introduction

On the basis of the optimal foraging theory (Pyke et al. 1977) and the chemical reactor theory (Penry and Jumars 1987), the adaptive modulation hypothesis (AMH; Karasov and Diamond 1983; Karasov 1992; Karasov and Martínez del Rio 2007) uses economic principles, arguing that the gut is expensive to maintain and, thus, that 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 have 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, 2015; Kohl et al. 2011). Some herbivorous fishes that consume starch-rich diets cannot downregulate their digestive enzyme activities toward carbohydrates (German et al. 2004) or their 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 nonexistent (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 at al. 2018*a*).

In terms of gut size, increased food intake (e.g., due to high fiber content of the diet) speeds up the digesta transit rate (Dadd 1960; Pritchard and Robbins 1990; Diamond 1991; Castle and Wunder 1995), and more rapid digesta transit rates require 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 Rio 2007; Karasov and Douglas 2013). Hence, herbivores generally have larger guts than carnivores (Dearing 1993; Stevens and Hume 2004; Wagner et al. 2009) to accommodate more voluminous meals (Pough 1973; Wilson and Lee 1974). Hindgut (HG) valves (like the ones observed in a lacertid lizard; see below) can both increase overall gut surface area and slow the passage of digesta (Iverson 1982; Bignell 1984; Stevens and Hume 2004; Bell et al. 2005; Godon et al. 2016). Digesta high in fiber (i.e., plant material) are particularly viscous, characterized by low Reynolds numbers (Lentle and Janssen 2008). Because of these flow characteristics, digesta high in fiber lead to decreased mixing in a smooth bore tube (Love et al. 2013). By acting as baffles, HG valves can increase the mixing of viscous digesta in what would otherwise be a plug-flow environment with unidirectional flow (Lentle and Janssen 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 Rio 2007). We therefore predict that animals eating plants would have features of gut structure (e.g., larger gut, HG valves) and function (e.g., elevated carbohydrase activities, in congruence with AMH) that facilitate digestion of plant material (table 1; Karasov and Martínez del Rio 2007).

Beyond endogenous digestive processes like digestive enzyme synthesis and secretion, many herbivores and omnivores rely on microbial symbioses, usually in the HG, 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 (SCFAs) that can be assimilated across the gut wall by the host (Bergman

Table 1: Predictions of relative diet, gut morphology, enzyme activities, fermentation products, and digestibility in Pod Mrčaru (omnivore), Pod Kopište (source), and Zagreb (mainland) populations and whether these hypotheses were supported by the results of this study

Characteristic	Pod Mrčaru	Pod Kopište	Zagreb	Supported?
Mass of stomach contents	Highest	Low	Low	Yes
Diet: percentage plant matter	Highest	Low	Low	Yes
Gut length[a]	Long	Short	Short	No
Intestinal mass	Heaviest	Light	Light	No
ESM	Largest	Least	Least	No
Enzyme activities (substrate):				
Pancreatic:				
α-amylase (starch)[b]	Moderate	Low	Low	In HGC only
Trypsin (protein)	Moderate/high	High	High	No
Lipase (fats)	Moderate	Moderate	Moderate	Yes
Intestinal:				
N-acetyl-β-D-glucosaminidase (chitins)	Low	Moderate	Moderate	No
Trehalase (arthropod sugars)	Low	Moderate	Moderate	No
Maltase (disaccharides)[b]	Moderate	Low	Low	No
Aminopeptidase (dipeptides)	Moderate/high	High	High	No
Microbial:				
β-glucosidase (β-glucosides)[c]	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:				
Plants	Moderate	Low	...	Yes
Plants + insects	High	Moderate	...	Yes
Insects	High	High	...	Yes

Note. ESM = epithelial surface magnification; HGC = hindgut contents; SCFA = short-chain fatty acid.

[a]Combined length of the esophagus, stomach, and intestines.

[b]From plants, seeds, and glycogen sources.

[c]From plant cell wall sources.

1990; Foley et al. 1992). Thus, animals consuming more plant material may have an increased reliance on microbial fermentation (in this study, indirectly measured via the products of fermentation, SCFAs) in their HGs 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 km^2), Croatia, to the nearby island of Pod Mrčaru (0.03 km^2) 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 that the new population on Pod Mrčaru had morphologically and behaviorally diverged from its 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 and had different head shapes and larger bite forces. Additionally, both adult and neonate lizards from Pod Mrčaru had developed valves in their HGs, a feature not found in the Pod Kopište population. HG 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 whether the appearance of HG 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 [OM] 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 are rare, with herbivory encompassing <1%–4% of extant lizards (King 1996; Cooper and Vitt 2002; Espinoza et al. 2004). 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 that allow them to digest plant material better than lizards from Pod Kopište or from the mainland (Zagreb). If the amount of pancreatic or brush border enzymes aimed at digesting components of plant material is

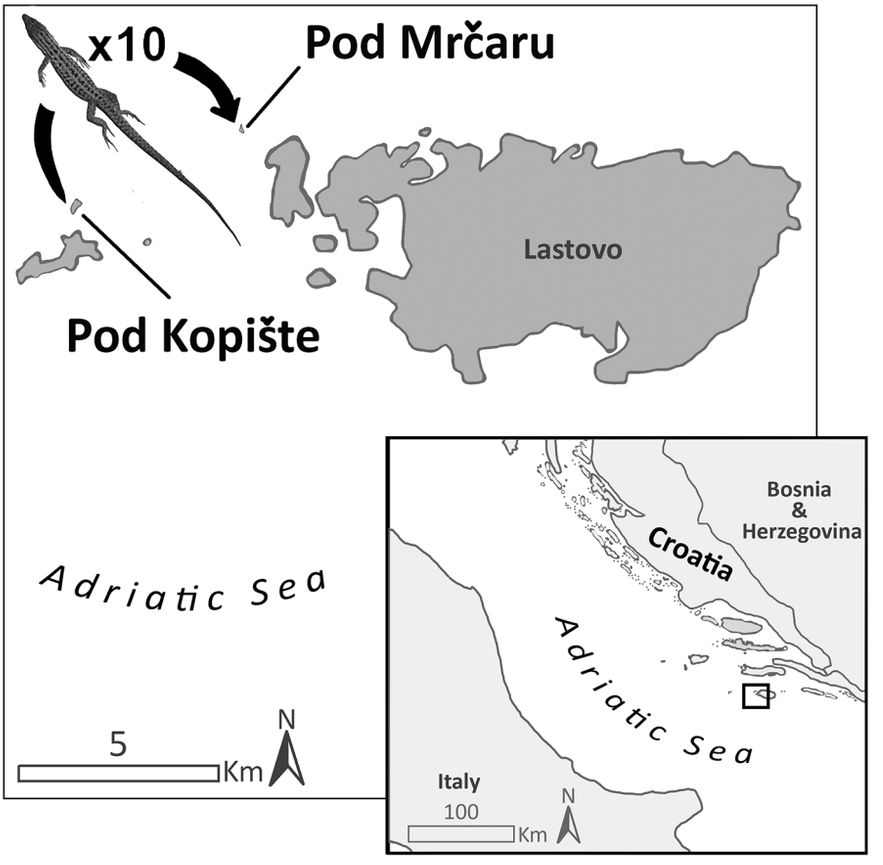

Figure 1. *Podarcis siculus* island collection sites showing Pod Kopište (source population) and Pod Mrčaru (newly omnivorous population). The box in the bottom map shows the location of the area. Zagreb (mainland population) not pictured. A color version of this figure is available online.

higher in the Pod Mrčaru lizards than in 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' HGs, 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. 2018*b*; 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.

Material and Methods

Diet Analysis

From August 29 to 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 $n = 30$ because of 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 $n = 4$ because of 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 the 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 to August 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 4 h). As an out-group (Podnar et al. 2005), 13 *P. siculus* were collected from an urban population in Zagreb from September 15 to 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^{-1}). We measured snout-vent length (SVL) and dissected the lizards on sterilized, chilled dissecting trays (~4°C). We removed and measured the length of the gut from the beginning of the esophagus to the end of the HG (hereafter, "gut length").

We divided the gut into stomach (including esophagus), proximal intestine (PI), midintestine (MI), and HG. The HG was easily

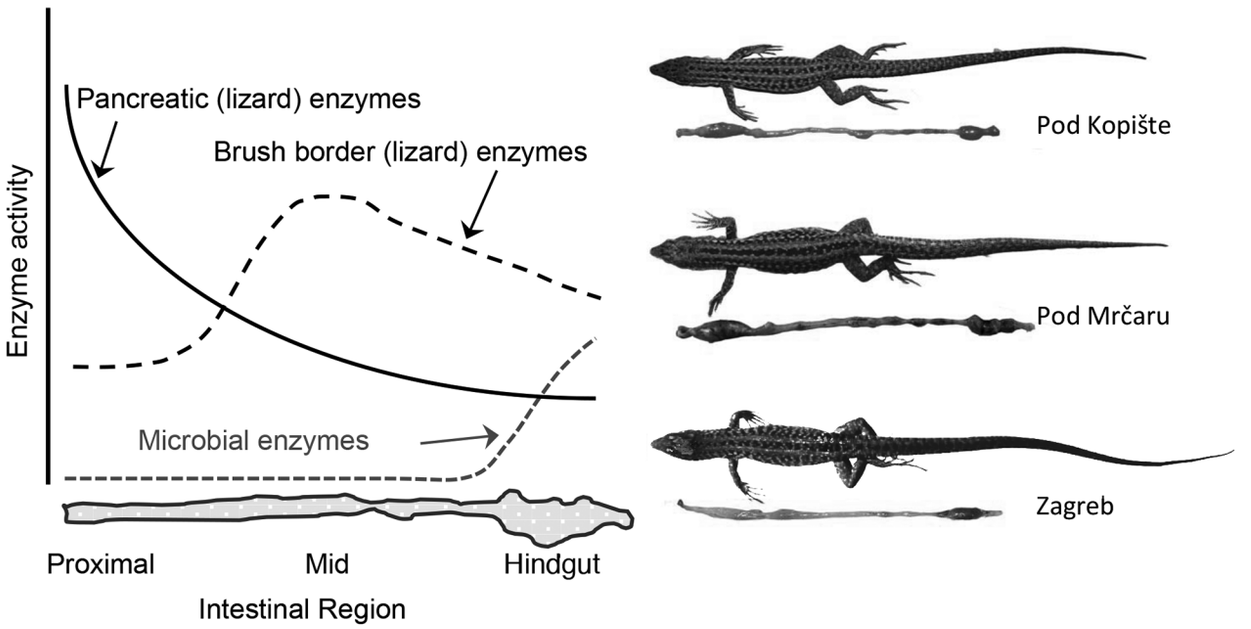

Figure 2. Potential patterns of digestive enzyme activities across intestinal regions and representative examples of lizard guts from each population (with stomachs). Pancreatic digestive enzymes are secreted into the proximal intestine and are expected to decrease along the gut. Brush border enzymes are produced at the brush border of the epithelial cells and the intestinal lumen, generally peaking in activity in the midintestine. High enzyme activities in the proximal intestine or midintestine 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 with the diameters of the proximal intestine and midintestine. We defined the proximal intestine and midintestine as half of each portion of the remaining tissue length. A color version of this figure is available online.

identifiable (see fig. 2), and the PI and MI portions were separated by dividing the remaining intestine in half. In seven individuals from each population, we removed the gut contents from the PI, MI, and HG sections (PI gut contents [PIGC], MI gut contents [MIGC], HG contents [HGC]) 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 the pH of the contents of each gut region as follows: stomach pH 3.4 ± 2.0, PI pH 8.17 ± 0.6, MI pH 8.96 ± 0.7, and HG 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 HG 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 (V. Lemieux-Labonté, C. Vigliotti, S. Dowd, Z. Tadić, B. A. Wehrle, D. P. German, A. Herrel, F. J. Lapointe, P. Lopez, and E. Bapteste, unpublished data).

Estimation of Intestinal Surface Area Using Histology

Gut sections preserved in McDowell Trump's fixative were further sectioned into 3–10-mm sections with a razor blade and rinsed in phosphate buffer (PBS), pH 7.5, for 20 min three times and overnight at 4°C under constant shaking. The tissues rinsed with PBS were flushed with running deionized water for 20 min two times and then subjected to serial ethanol dilutions of 30%, 50%, and 75%. We selected the proximal portions of the PI, MI, and HG from Pod Mrčaru and Pod Kopište lizards and portions starting at the halfway point of the HG (HG+) from all three populations. Tissue portions were placed in tissue cassettes wrapped in ethanol-soaked cheesecloth, sealed in plastic bags, and sent to Mass Histology Service (Worcester, MA) for embedding in paraffin wax. We stained 7-μm-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: 25 mM Tris-HCl buffer, pH 8.6, for 50–300 volumes of pancreases and 5–300 volumes of gut contents (PIGC, MIGC, HGC pellet) and 350 mM mannitol in 1 mM Tris-HCl, pH 8.6, for 10–50 volumes of intestinal wall tissues (PI, MI, or HG). We chose buffers at pH 8.6 because this was the average pH we measured in the intestinal contents of *P. siculus* from Zagreb. To homogenize tissues, we used a Polytron homogenizer (Binkmann Instruments, Westbury, NY) with a 12-mm generator set to 1,100–3,000 rpm for three 30-s pulses, with 30 s between pulses. Tissue homogenates were centrifuged at 9,400 *g* for 2 min. To ensure the rupture of the microbial cells and the release of all enzymes within the gut content samples, these samples were sonicated (CL-18 sonicator, Fisher Scientific, Waltham, MA) at an output of 5 W for 30 s three times, with 30 s intervals between pulses, followed by homogenization, as described for the gut tissues. The gut content samples were centrifuged at 12,000 *g* for 10 min. All supernatants were stored in 100–200-μL aliquots at −80°C until just before use in digestive enzyme assays. For the HGC, 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 14,000 *g* at 4°C to gather HG fluid. The filtered fluid was frozen at −80°C for use in SCFA measurements. The remaining HGC 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 to September (confirmed by iButtons, Maxim Integrated, San Jose, CA; fig. S1; figs. S1–S4 are available online) 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). Our primary buffer was 25 mM Tris-HCl, pH 8.6 (hereafter referred to as "buffer"; any deviations are noted), measured at room temperature (22°C). Reagents were purchased from Sigma-Aldrich (St. Louis, MO). We optimized each assay for duration and homogenate volume. Each enzyme activity was measured in each gut region (PI, MI, HG, PIGC, MIGC, HGC) for each lizard. Pancreatic tissue was used only 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 versus time and used the standard curve of the product to calculate enzymatic activity units per gram wet mass 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. After 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 units (μmol glucose liberated min^{-1}) per gram tissue.

We measured β-glucosidase, β-galactosidase, and *N*-acetyl-β-D-glucosaminidase (NAG) 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 units (nmol MUB released min^{-1}) per gram 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 it is known to be present in lizard genomes (on chromosome 1 in the *Anolis carolinensis* genome; http://ensembl.org) and is produced in lizard guts (Kohl et al. 2016*b*). 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. Using methods modified from German and Bittong (2009) and German et al. (2015), we measured trypsin, aminopeptidase, and lipase activities via kinetic assays. To measure trypsin activity, we used 2 mM *N*α-benzoyl-L-arginine-p-nitroanilide hydrochloride 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 units (μmol p-nitroaniline released min^{-1}) per gram tissue. For trypsin activities measured in pancreatic tissue homogenates, we preincubated the homogenates with 15 μL of enterokinase (4 U mL^{-1} in 40 mM succinate buffer, pH 5.6) per 100 μL of 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 preincubation 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 units (nmol p-nitrophenol released min^{-1}) per gram tissue.

In addition to the regional enzyme activities (units 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 units (μmol product released min^{-1}). We did not include pancreatic samples in total gut enzyme activities, as this region does not directly interact with nutrients.

Fermentation Analyses

To determine symbiotic microbial fermentation, we measured the relative concentrations of SCFAs in the HGC fluid (following methodology in Pryor and Bjorndal 2005; German and Bittong 2009; German et al. 2015) of lizards from Pod Kopište ($n = 3$), Pod Mrčaru ($n = 4$), and Zagreb ($n = 1$). (Despite attempting to collect HGC fluid from seven Zagreb lizards, we were able to collect only ≥ 1 μL from one lizard from this population.) We hand-injected 2 μL of thawed HGC fluid into a 2-m-long stainless steel column (3.2-mm i.d.) packed with 10% SP-1000 and 1% H_3PO_4 on 100/120 Chromosorb W AW (Supelco, Bellefonte, PA) attached to a Shimadzu GC-Mini-2 gas chromatograph with flame ionization detector (Shimadzu Scientific Instruments, Columbia, MD). We quantified SCFA concentrations via an HP3392A integrator (Hewlett Packard, Palo Alto, CA) 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 millimolar gut fluid.

Digestibility

From September 1 to September 5, 2013, we collected male *P. siculus* from both Pod Kopište and Pod Mrčaru ($n = 15$ from each island) and transported them in individual cloth bags to the University of Zagreb, where they were housed in individual plastic terraria (30 cm × 19 cm × 14 cm or 30 cm × 19 cm × 20 cm) with rock substratum, a hide box, and a water dish. For 1 wk, 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 10L : 14D 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% ± 0.07% of its body mass of its assigned diet (or ~0.33 kJ g^{-1}) and thereafter 0.77% ± 0.01% of its body mass of its assigned diet daily (~0.16 kJ g^{-1}) for a duration of 11–32 d. Initial SVLs and masses are presented in table S1 (tables S1–S4 are available online).

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^{-1}) was made of cockroaches, the omnivore diet (21.4 kJ g^{-1}) was a 50 : 50 by dry mass mixture of the insectivore and herbivore diets, and the herbivore diet (18.1 kJ g^{-1}) 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 d at 50°C, ground to ≤1-mm particle size, and supplemented with Herptivite multivitamins and calcium with vitamin D3 (Rep-Cal, Los Gatos, CA) according to manufacturer instructions. Diets were weighed out in approximately isocaloric ratios and packed into gelatin capsules. The gelatin capsules add about 14 kJ g^{-1} of protein to each diet. 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 the digestibility of whole meal worms in these same lizard populations found results similar to ours (Vervust et al. 2010), suggesting that our grinding of the food did not 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 its 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 at each feeding to maintain lizard body mass ± 10%. We prepared three sizes of meals (+10 mg, baseline, −10 mg) for each diet. If a lizard's body mass increased by ≥10% compared with 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 ≥10% of their initial body mass were given the larger meal size 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 wk and weighed to the nearest 0.001 g. We estimated the OM of each diet, gelatin capsules, and 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 h to remove all moisture, weighed, and combusted in a Lindberg/Blue M combusting oven (Ashville, NC) at 550°C for 3 h. The combusted remains were considered nonorganic ash, and we subtracted that mass from the initial mass to determine the proportion of organic material in the original sample. We calculated apparent OM 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 OM digestibility, since we did not account for endogenous OM losses (e.g., sloughed intestinal cells) in feces (McConnachie and Alexander 2004; German 2011).

Statistical Analyses

We preformed all statistical analyses in R (ver. 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 Wilcoxon signed-rank tests. We used Tukey's honestly significant difference 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 the total intestinal mass and compared both regional and total intestinal masses among populations with an 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, we report only the results of the ANOVAs.

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

Results

Podarcis siculus from Pod Kopište (SVL: 64.39 ± 2.40 mm [average ± SD]) 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 lizards from either island population, 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; table S2).

Diet and 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 lizards. Pod Mrčaru *P. siculus* (64% of intake) consumed significantly more plant material ($F_{2,63} = 15.35$, $P < 0.001$) compared with Pod Kopište lizards (24%) and Zagreb lizards (2%; 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 nonsignificant; fig. 3). We

Table 2: Average stomach contents (±SD) by mass of lizards from Pod Kopište, Pod Mrčaru, and Zagreb

	Pod Kopište ($n = 30$)	Pod Mrčaru ($n = 36$)	Zagreb ($n = 4$)
Mass of stomach contents (mg)	94.50 ± 68.0	207.41 ± 16.1	98.79 ± 123.2
Plant material (%)	24.49 ± 33.1	64.24 ± 30.8	2.37 ± 8.3
Leaves (%)	6.25 ± 25.0	7.65 ± 12.0	0
Seeds (%)	81.25 ± 40.3	91.18 ± 13.3	0
Wood (%)	12.50 ± 34.2	1.18 ± 3.0	0
Fruit (%)	0	0	100
Animal material (%)	75.36 ± 33.0	35.58 ± 30.9	70.48 ± 44.2
Other (%)	.15 ± .8	.19 ± .8	25.37 ± 22.8

Note. The number of individuals sampled does not include individuals with empty stomachs. We found empty stomachs in additional lizards from Pod Kopište ($n_{empty} = 1$) and Zagreb ($n_{empty} = 3$) but not from Pod Mrčaru. Plant material is broken down into percentages of each type, adding up to 100% of total plant material. "Other" consisted of rocks and feces.

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, HG) did not differ by population or covary with lizard body mass (for values, see table S2). 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 HG. None of the populations retained more contents in a particular gut region than the other two populations, excepting the higher mass of digesta in the Pod Mrčaru lizards' stomachs, mentioned above. Additionally, we observed nematodes in the HGC of multiple lizards from each of the three populations.

The ESMs 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 PI was 6.26 ± 0.33 times the serosa ($t = 0.82$, $P = 0.44$). This ratio decreased distally along the gut: the MI was 3.63 ± 0.33 times the serosa ($t = -0.14$, $P = 0.89$), a proximal part of the HG was 2.44 ± 0.32 times the serosa ($t = 0.64$, $P = 0.54$), and the HG+ was 1.95 ± 0.24 times the 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 HG or HGC tissues. The newly omnivorous Pod Mrčaru lizards showed greater amylase activity but no other increases in plant-related enzyme activities compared with the Pod Kopište source population.

The mass-specific α-amylase activity in the HGC was almost 6-fold higher in Pod Mrčaru lizards compared with that measured in the Pod Kopište population ($t = -0.27$, $P = 0.038$; fig. 5*a*; table S3). Amylase activity was undetectable in all but one HGC sample from the Zagreb population. We found no other differences in amylase activity. In most MI and HG samples, amylase activity was undetectable, or replicates were too variable for confident analyses. No other gut region or 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: $F_{2, 13} = 0.63$, $P = 0.549$), and we did not 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 with the Zagreb lizards ($F_{2, 17} = 13.38$, $P = 0.003$; fig. 6*a*). Mass-specific β-glucosidase activity was nearly double in the PI of Pod Mrčaru and Pod Kopište lizards compared with the Zagreb lizards ($F_{2, 15} = 14.33$, $P < 0.001$; table S3). We found no other differences in regional β-glucosidase activities among the populations (fig. 5*b*). Within individuals, β-glucosidase activity was significantly higher in the PI and HGC regions in comparison with some regions (e.g., MI, PIGC; fig. 5*b*; 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 table S3 for β-galactosidase activity measures and figure 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. 5*c*). In the PI of the Pod Kopište lizards, the NAG activity was two times greater than in the PI of the Pod Mrčaru and Zagreb populations ($F_{2, 15} = 5.87$, $P = 0.013$; table S3). There were no other regional differences, including differences in total NAG activity, among the populations (fig. 5*d*). In the Pod Mrčaru and Zagreb populations, the greatest NAG activity was found in the HGC compared with any other region ($F_{5, 50} = 23.60$, $P < 0.001$; Pod Mrčaru and Zagreb populations pooled as a result of low sample sizes for PIGC and HG regions).

In both the HG and the HGC, the Pod Kopište population had lower regional trehalase activity than one of the other two populations (fig. 5*e*). Still, there were no population differences in trehalase activity in more proximal regions or in total activity of

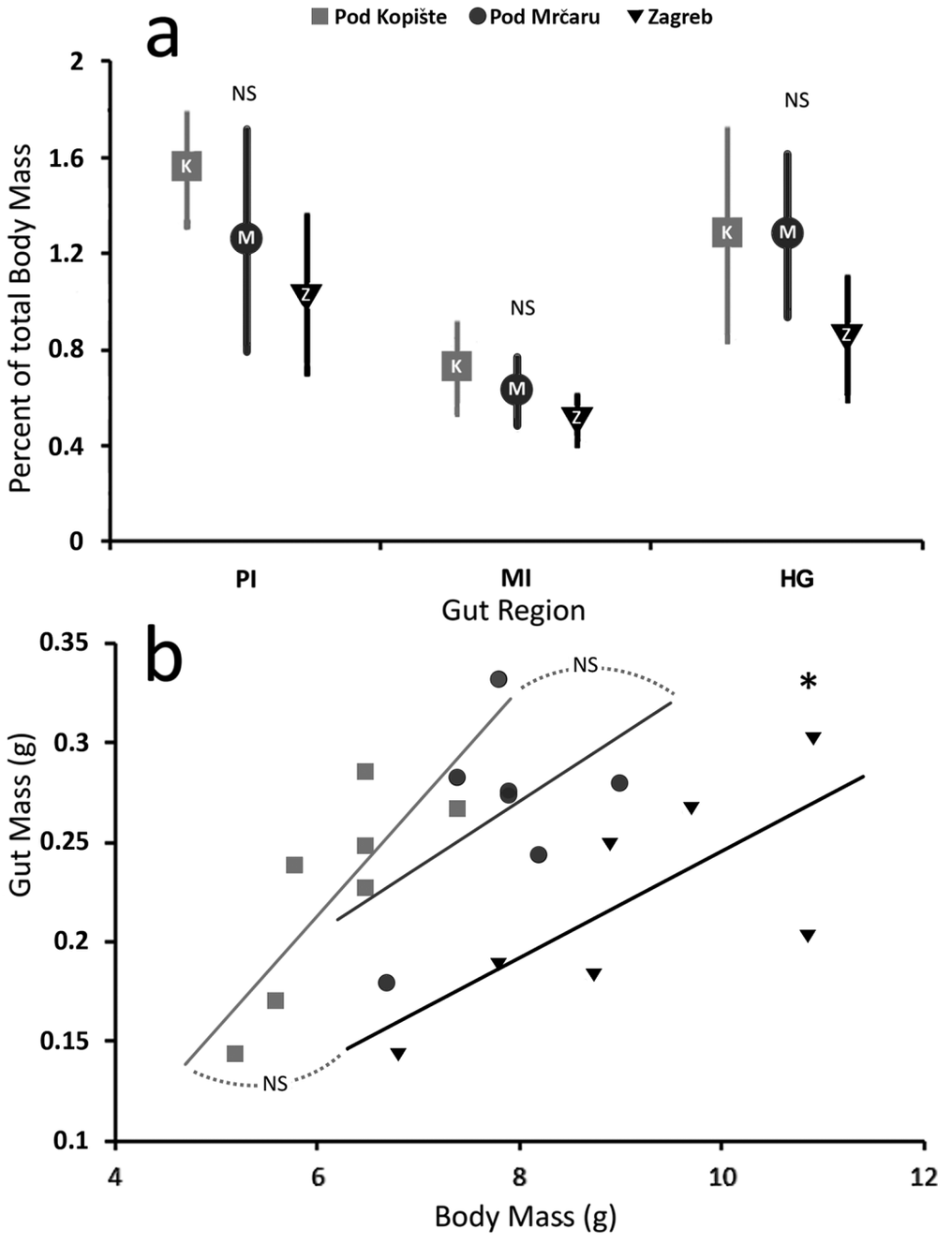

Figure 3. Regional (*a*) and total (*b*) intestinal mass (without contents) in Pod Kopište (source), Pod Mrčaru (omnivore), and Zagreb (mainland) populations. Gut regions are proximal intestine (PI), midintestine (MI), and hindgut (HG) and are presented as a percentage of body mass. Values are mean ± SD. For all, $n = 7$ except $n = 6$ for 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 lizards had lower total intestinal masses than the Pod Mrčaru lizards (*b*) that were also significantly affected by body mass. The asterisk in *b* indicates difference in the ratio of gut mass to body mass between Pod Mrčaru and Zagreb populations. Pod Kopište intestinal masses were not different from either population. A color version of this figure is available online.

the enzyme (table S3). The Pod Mrčaru population's HG trehalase activity (0.058 ± 0.020 μmol glucose liberated min^{-1} g^{-1}) was higher than the HG activity in the Pod Kopište population (0.0045 ± 0.003 μmol glucose liberated min^{-1} g^{-1}; $F_{2,9} = 5.50$, $P = 0.024$). The Zagreb population's HGC activity (0.254 ± 0.134 μmol glucose liberated min^{-1} g^{-1}) was higher than the Pod Kopište population's HGC activity (0.025 ± 0.011 μmol glucose liberated min^{-1} g^{-1}; $F_{2,12} = 4.28$, $P = 0.041$).

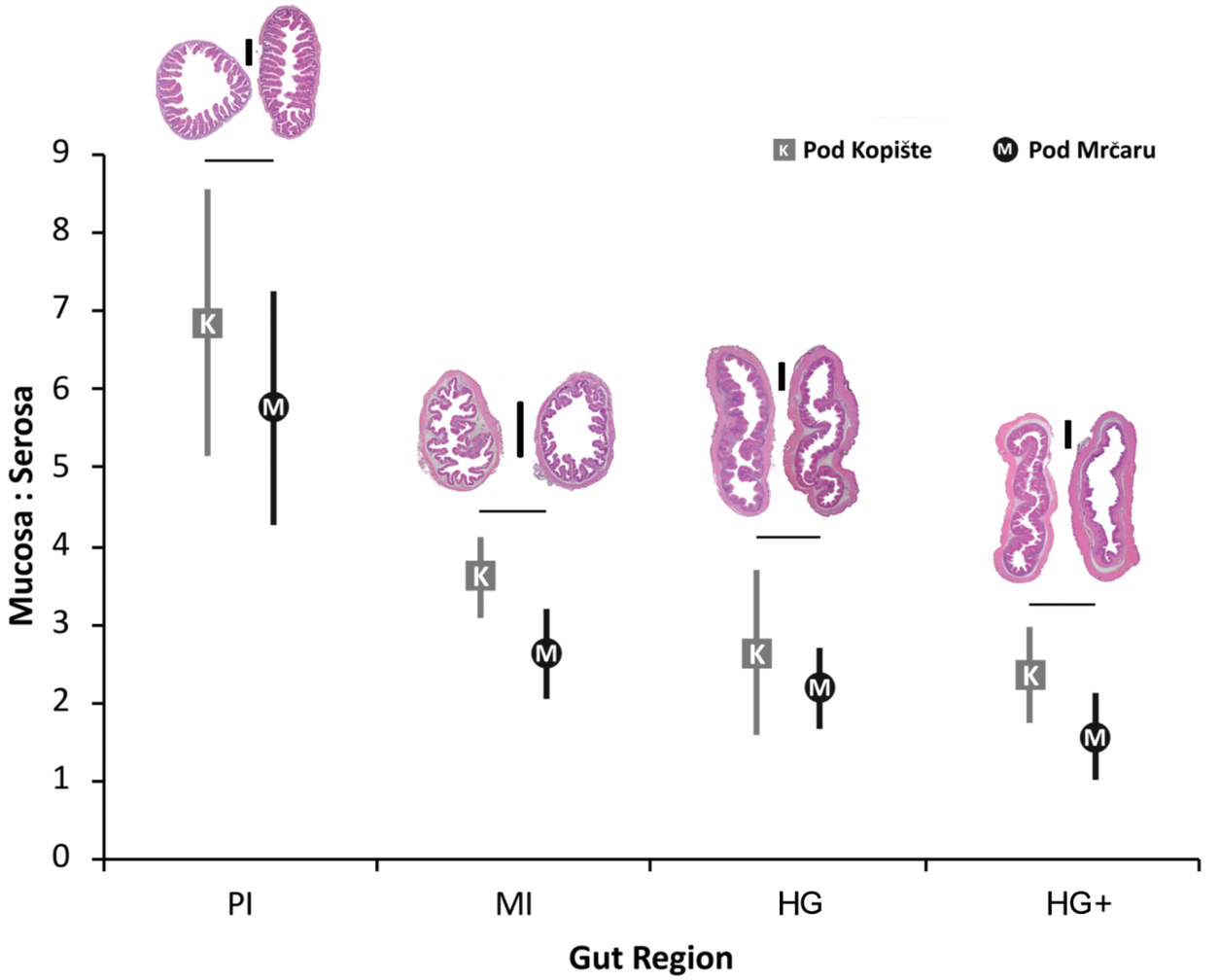

Figure 4. Epithelial surface magnification and ratio of inner perimeter length of mucosa to inner perimeter length of serosa in the proximal intestine (PI), midintestine (MI), proximal half of hindgut (HG), and distal half of hindgut (HG+). Values are mean ± SD; $n = 3$. Comparisons of populations were done an via equal-variance *t*-test. We found no differences by population. Cross-sectional images are representative stained histological sections from each gut region of Pod Kopište (source) and Pod Mrčaru (omnivore) populations. Not to scale: each scale bar represents 500 μm for the two images it lies between.

Proteases. Protease activity differed by population but not along the diet lines that we predicted. In the HGC, 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. 5*f*; table S3). The trypsin activity in the HGC of the Zagreb population (table S3) was not different from the other two populations. No other gut region showed differences in activity among the three populations (fig. 5*f*), and we did not find a difference in the total trypsin activity among populations. The mass-specific trypsin activity in the pancreas was >2.5 times higher in the Pod Kopište and Pod Mrčaru lizards (0.454 ± 0.050 μmol p-nitroaniline released min^{-1} g^{-1}) than in the Zagreb lizards (0.190 ± 0.040 μmol p-nitroaniline released min^{-1} g^{-1}; pancreas: $F_{2,23} = 5.14$, $P = 0.014$; fig. 7).

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

Lipase. There was no difference in lipase activity across the three lizard populations (fig. S3; 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 HGC, >3 times higher than the 19.20 ± 8.60 mM in Pod Mrčaru lizards ($t = 6.42$, $P = 0.001$; table 3). This

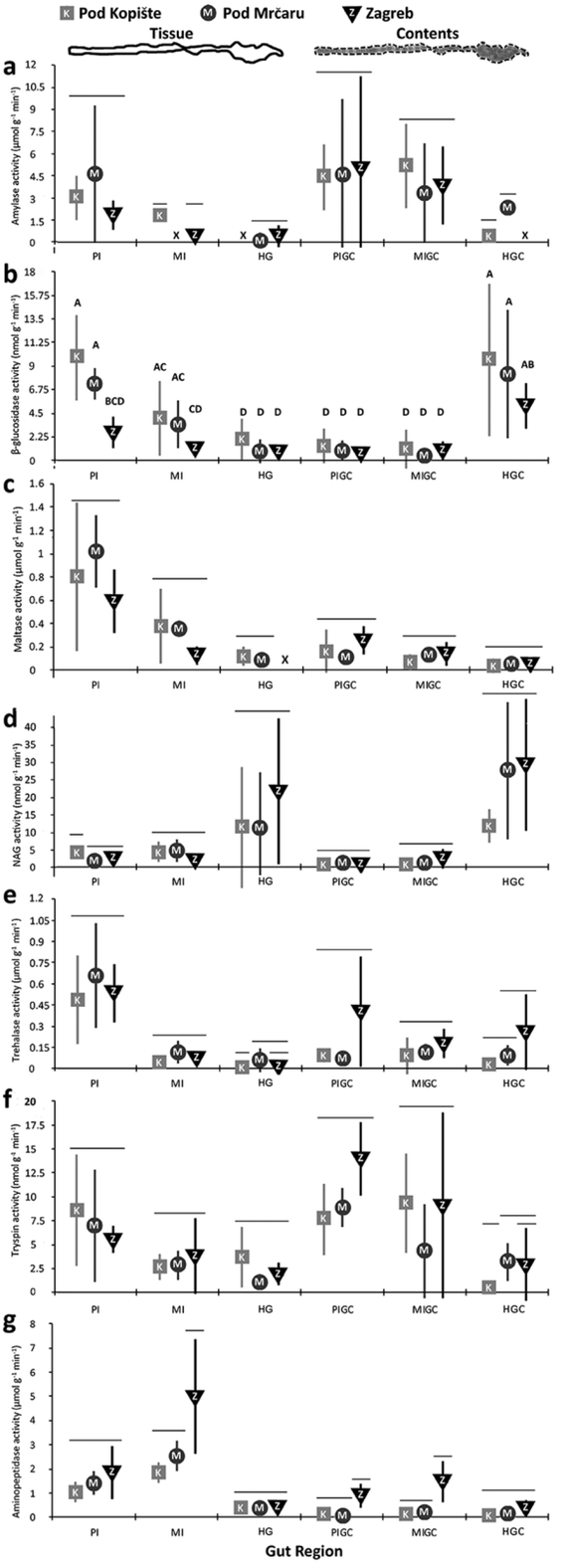

Figure 5. Digestive enzyme activities throughout the gut in Pod Kopište (source; $n = 4$–7), Pod Mrčaru (omnivore; $n = 3$–7), and Zagreb (mainland; $n = 3$–6) populations. Values are mean ± SD. *a*, Amylase activity (μmol glucose liberated g^{-1} min^{-1}; X denotes undetectable activity). *b*, β-glucosidase activity (nmol 4-methylumbelliferone liberated g^{-1} min^{-1}). *c*, Maltase activity (μmol glucose liberated g^{-1} min^{-1}). *d*, *N*-acetyl-β-D-glucosaminidase (NAG) activity (nmol 4-methylumbelliferone liberated g^{-1} min^{-1}). *e*, Trehalase activity (μmol glucose liberated g^{-1} min^{-1}). *f*, Trypsin activity (nmol p-nitroaniline liberated g^{-1} min^{-1}). *g*, Aminopeptidase activity (μmol p-nitroaniline liberated g^{-1} min^{-1}). In *a* and *c*–*g*, comparisons of populations were done via ANOVA; horizontal lines at different levels for a gut region indicate significant differences for that population, and horizontal lines at the same level indicate no differences. In *b*, populations by tissue and tissues within populations were compared via separate ANOVAs; shared capital letters above icons denote no differences. PI = proximal intestine; MI = midintestine; HG = hindgut; PIGC = proximal intestine gut contents; MIGC = midintestine gut contents; HGC = hindgut contents. A color version of this figure is available online.

phenomenon was primarily due to nearly 4-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 with Pod Mrčaru lizards. However, even with these two SCFAs omitted, nonsignificant increases in propionate, butyrate, and valerate (but not isovalerate) contributed to significantly increased total SCFA concentrations ($t = 4.00$, $P = 0.010$) in the HGs 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) 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 1.1 times higher OM digestibility (by mass) than the Pod Kopište lizards (55% OM digestibility) on the herbivore diet (fig. 8; 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 OM 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 thesupplemental material (see ANCOVAs; available online). Final and percent change in body masses and SVLs are available in table S1.

Discussion

Subtle Changes in Performance

The *Podarcis siculus* of Pod Mrčaru eat more plants than their Pod Kopište counterparts after ~35 yr of divergence (Herrel et al. 2008), and their digestibility performance has shifted. Overall, the

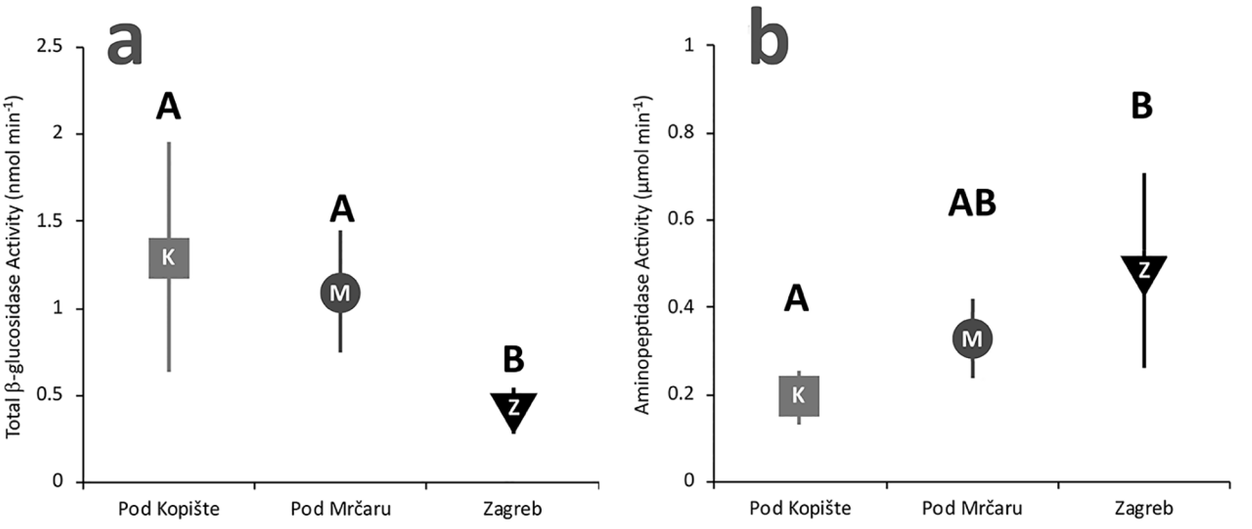

Figure 6. *a*, Total β-glucosidase activity (nmol 4-methylumbelliferone liberated min^{-1}). *b*, Total aminopeptidase activity (μmol p-nitroaniline liberated min^{-1}). Values are mean $\pm$ SD. For the Pod Kopište (source) and Pod Mrčaru (omnivore) populations, $n = 7$; for the Zagreb (mainland) population, $n = 6$. Populations compared via ANOVAs; different capital letters above icons denote significant differences. A color version of this figure is available online.

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 HG amylase and trypsin activity; fig. 5) underlying the increase in digestibility of plant material are subtle and localized within the

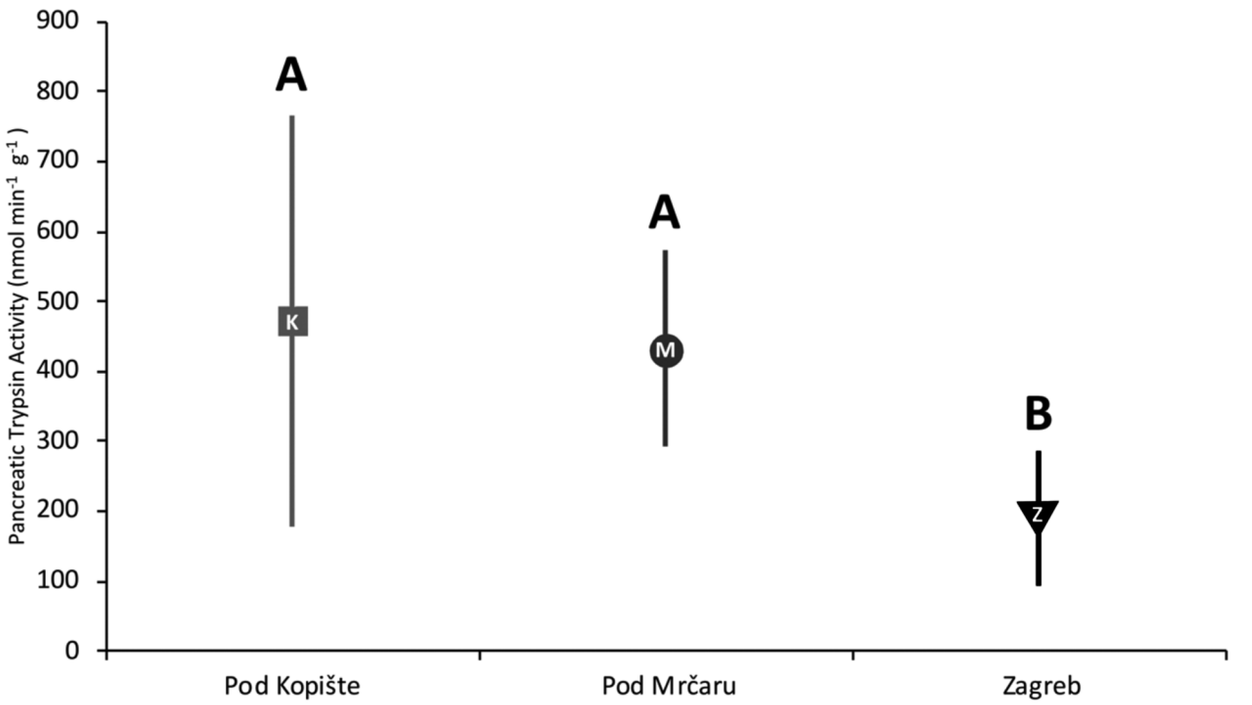

Figure 7. Trypsin activity in the pancreas (nmol p-nitroaniline liberated g^{-1} min^{-1}) in Pod Kopište (source; $n = 10$), Pod Mrčaru (omnivore; $n = 10$), and Zagreb (mainland; $n = 6$) populations. Values are mean $\pm$ SD. Populations compared via ANOVA; different capital letters above icons denote significant differences. A color version of this figure is available online.

Table 3: Total short-chain fatty acid (SCFA) concentrations and ratios of acetate to propionate to butyrate to isobutyrate to valerate to isovalerate with respect to total SCFAs in hindguts of Pod Kopište (source; $n = 4$), Pod Mrčaru (omnivore; $n = 3$), and Zagreb (mainland; $n = 1$) populations

Population	Total SCFA (mM)	Ratio
Pod Kopište	61.86 ± 3.95*	65*:19:8:5*:1:1*
Pod Mrčaru	19.22 ± 8.63*	56*:16:16:7*:2:4*
Zagreb	.60	65:16:11:6:0:3

Note. Values are mean ± SD. Compared between populations using equal-variance *t*-tests.

*Significant differences between populations.

HG. 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 (2018*a*), shifts to high-protein diets often lead to higher protease activities yet show mixtures of increases and decreases in carbohydrases and no changes in lipases. Shifts to lower-protein diets, such as 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. (2018*a*) considered animals in feeding experiments on shorter timescales (weeks to months), not wild individuals following a natural dietary shift over decades.

As the majority of endogenous nutrient digestion and absorption in nonruminant vertebrates occurs in the proximal portion of the intestine (Vonk and Western 1984; Karasov and Martínez del Rio 2007; Le et al. 2019), differences in the structure and function of the HG 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 gradients 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

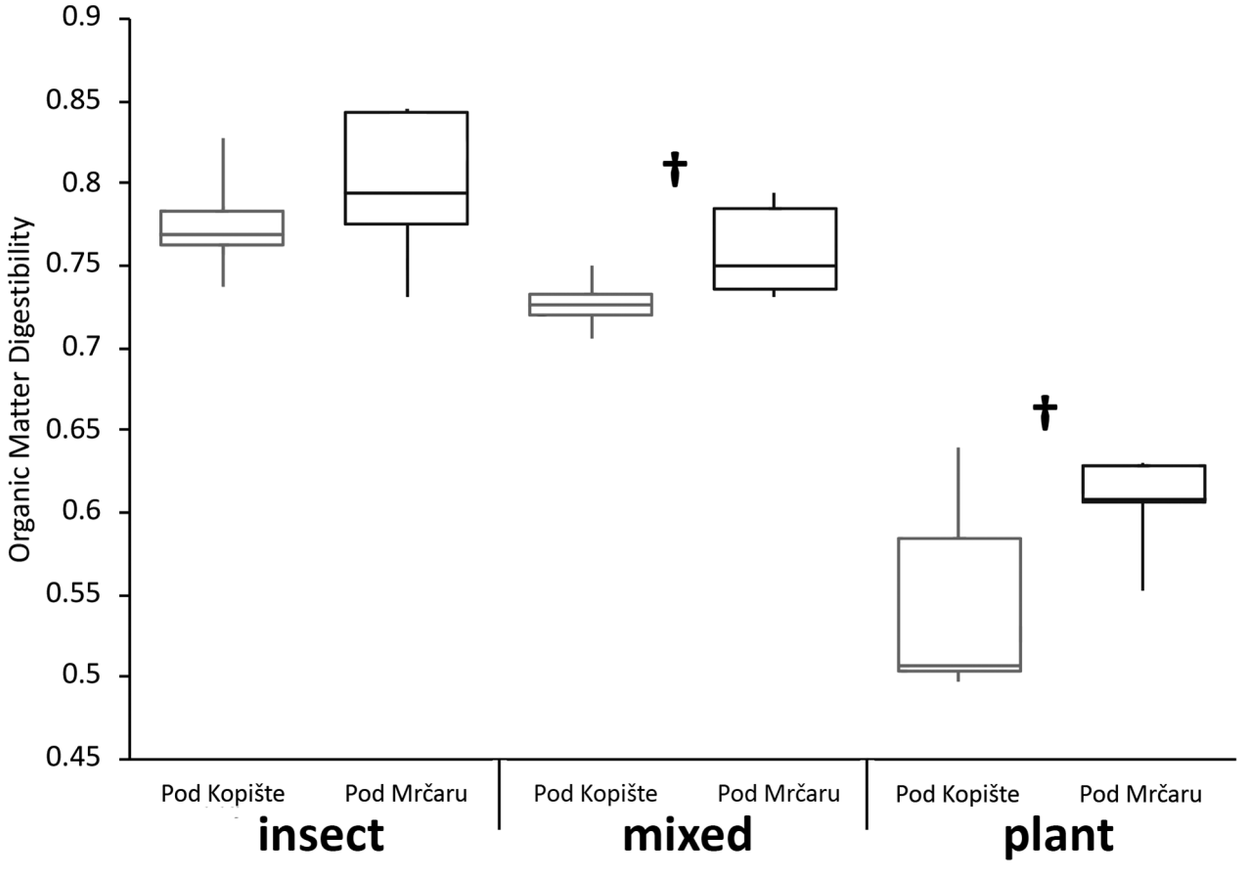

Figure 8. Organic matter digestibility on experimental diets in lizards from Pod Kopište (source) and Pod Mrčaru (omnivore) presented as box and whisker plots representing each quartile ($n = 5$). Populations were compared via ANOVA separately for each diet. A dagger denotes significant differences at $P < 0.05$. A color version of this figure is available online.

absorption. For example, an increase in trypsin activity in the PI could lead to greater digestion of proteins into dipeptides that can serve as a substrate for aminopeptidase in the MI and HG. There is subsequently more gut remaining that can absorb the dipeptides. Changes in the lizard HG, therefore, may represent a small portion of overall nutrient acquisition. For example, the HG of two iguanids (*Dipsosaurus dorsalis* and *Sauromalus ater*) showed <5% of the transport capacity of D-Glucose and L-Proline (a nonessential amino acid) in comparison with the PI in these species (Karasov et al. 1985). The mammalian large intestine is also known as a site for ion, water, SCFA, and amino acid, particularly essential amino acids (e.g., lysine), absorption (with SCFAs 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 HG, as there are separate transporters for charged amino acids (Karasov and Martinez del Rio 2007; van der Wielen et al. 2017), such as 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 OM ~10% better than the Pod Kopište lizards, the functional differences we were able to detect among *P. siculus* populations were localized to the HG. On the basis of these differences, we may infer that microbial communities, generally localized to the HG, play a role in these differential abilities (Karasov and Douglas 2013; Moran et al. 2019). Interestingly, modest shifts in the HG microbial communities of the Pod Mrčaru and Pod Kopište lizards included more of the Archaean *Methanobrevibacter* in the Pod Mrčaru lizards (V. Lemieux-Labonté, C. Vigliotti, S. Dowd, et al., unpublished 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 (V. Lemieux-Labonté, C. Vigliotti, S. Dowd, et al., unpublished data).

Between the new and source populations of lizards, Pod Mrčaru and Pod Kopište, the only functional difference we measured outside of the HG was higher NAG activity in the PIs of Pod Kopište lizards. Higher NAG activity could contribute to the digestion of chitin from arthropod 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 HGC, consistent with microbial synthesis and not endogenous synthesis in lizard tissue (Jhaveri et al. 2015). Moreover, *P. siculus* appears to have considerably lower endogenous NAG activity compared with 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-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 *Uromastyx 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 HG were then absorbed before defecation. Similarly, we assume that our measurements are indicative of SCFA production in the HG and not of 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; Macfarlene and Macfarlene 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 1990; Stevens and Hume 1998; Macfarlene and Macfarlene 2003). Animals that are reliant on fermentation to make a living on plant material have ways (e.g., HG chambers in iguanas, rumens in cattle) to slow gut transit rates to allow microbes the time they need (Stevens and Hume 1998, 2004). With 2.2 times 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 HG. The HG valves themselves may have arisen to slow the transit of material in the HG, but the extent to which the valves act as baffles should be examined. Furthermore, HG microbes can play roles other than in 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 OM 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 we detected no activity anywhere in their guts.

Although HG valves are present in Pod Mrčaru HGs (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 histologically for HG valves in summer 2013 ($n = 3$) did not have clear epithelial folds (valves) in their HGs. This stands in contrast to all of the other Pod Mrčaru lizards ($n = 28$, spanning four seasons over three additional years) we examined, which had clearly identifiable folds in the proximal portion to midportion of their HG, dividing the gut in the transverse plane (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 HG valves in *P. siculus* may be flexible in their morphology or even in their presence, and

Table 4: Comparison of digestive enzyme activity ranges between *Podarcis siculus* of the current study and previous work on lizard digestive physiology

		Previous studies	
Enzyme	*P. siculus* (this study)	Species, source	Activity range
Amylase (pancreas)	80–137 μmol min^{-1} g tissue^{-1}	*Tupinambis meriange*, Parry et al. 2009	5.82–12.7 × 10^{5} μmol min^{-1} g protein^{-1}
Aminopeptidase	Total: .22–.81 μmol min^{-1}	***Liolaemus pictus***, Vidal and Sabat 2010	**.11–.21 μmol min^{-1}**
		Lophognathis temporalis, Iglesias et al. 2009	10.22–25.35 μmol min^{-1}
	Total per gram tissue: .96–3.65 μmol min^{-1} g^{-1}	***Liolaemus nigriviridis***, Naya et al. 2009	**2–4 μmol min^{-1} g^{-1}**
		Liolaemus ruibali, Kohl et al. 2016*b*	**2.13 ± .3 μmol min^{-1} g^{-1}**
		Heloderma suspectum, Christel et al. 2007	9–21 μmol min^{-1} g^{-1}
Trehalase	Total: .004–.28 μmol min^{-1}	*Liolaemus pictus*, Vidal and Sabat 2010	5.9–9.4 μmol min^{-1}
	Total per gram tissue: .023–1.38 μmol min^{-1} g^{-1}	*Liolaemus nigriviridis*, Naya et al. 2009	~2–6 μmol min^{-1} g^{-1}
Maltase	Total: .06–.18 μmol min^{-1}	*Liolaemus pictus*, Vidal and Sabat 2010	5.92–11.31 μmol min^{-1}
		Lophognathis temporalis, Iglesias et al. 2009	2.39–3.89 μmol min^{-1}
	Total per gram tissue: .273–.782 μmol min^{-1} g^{-1}	*Liolaemus ruibali*, Kohl et al. 2016*b*	34.55 ± 2.68 μmol min^{-1} g^{-1}
	PI/MI: .458–.872 μmol min^{-1} g^{-1}	*Liolaemus nigriviridis*, Naya et al. 2009	20–60 μmol min^{-1} g^{-1}
NAG	Total: 2.16–4.311 nmol min^{-1}	*Lacerta viridis*, Jeuniaux 1961	1.2 × 107 nmol min^{-1}
		Uromastyx acanthinurus, Jeuniaux 1963	No detectable activity
		Anolis carolinensis, Jeuniaux 1963	No detectable activity
		Chamaeleo chamaelon, Jeuniaux 1963	2.7 × 106 nmol min^{-1}
	Total per gram tissue: 9.6–17.9 nmol min^{-1} g^{-1}	*Sceloporus undulatus*, Marsh et al. 2001	1.4 × 106 nmol min^{-1} g^{-1}

Note. Values have been converted so that units are directly comparable; however, differing methodology may confound these comparisons. Species and values in bold are similar to those we measured in *P. siculus*. NAG = *N*-acetyl-β-D-glucosaminidase; PI = proximal intestine; MI = midintestine.

they are not fixed features in the Pod Mrčaru lizards. The general pattern of surface area decreasing along the gut (i.e., the PI is greater than the MI, which is greater than the HG; 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 HG+ 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 supports 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 HG (i.e., not necessarily an evolved difference in the lizards themselves; Garland and Adolph 1991).

Lizard Digestive Physiology

Digestive physiology has been less studied in reptiles than in other taxa, such as mammals and fishes (Karasov et al. 1985; Stevens and Hume 2004; Kohl et al. 2016*a*), 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 the Pod

Kopište populations and to 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 consume mostly 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 with the out-group 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 at el. 2008). Aminopeptidase was the only digestive enzyme that showed higher activity in the Zagreb population in comparison with 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. Interestingly, the elevated aminopeptidase activities of the PIGC in the Zagreb lizards suggests inherent aminopeptidase activity in their ingested prey (mostly ants).

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

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

Perhaps most unexpected is the enzyme activity pattern of β-glucosidase (fig. 5*b*). No nonavian reptile has been recorded to produce endogenous β-glucosidases in their digestive tracts (Stevens and Hume 2004; Karasov and Douglas 2013), and thus they 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 HGC, consistent with microbial synthesis (fig. 2; German et al. 2015; Leigh et al. 2018*b*). However, the β-glucosidase activity is just as high in the PI as in the HGC. Of interest, the high activity in the PI and, as a result, higher total β-glucosidase activity are most starkly found in the island populations, with present but much-diminished activity observed in the mainland lizards. A β-glucosidase gene is present in the *Anolis carolinensis* genome (on chromosome 5; https://www.ensembl.org), and one is expressed in the intestinal tissues of the herbivorous fish *Cebidichthys violaceus* (Heras et al. 2020), but in *P. siculus*, it remains unknown whether this enzyme is expressed in the digestive system or in the 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 HGC. Kohl and colleagues (2016*b*) propose that β-galactosidase, known to be endogenously produced in reptiles (on chromosome 1 in the *A. carolinensis* genome; https://www.ensembl.org), is active against β-glucosidase substrates. Still, the β-galactosidase activity patterns that we measured varied from the β-glucosidase activity patterns throughout the gut (fig. S2). Thus, there is some evidence that *P. siculus* may endogenously produce β-glucosidase or house microbes that produce β-glucosidase 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 HG. Although

we know that the Pod Mrčaru lizards have changed their body morphology over ecological time (Herrel et al. 2008), any shifts in their digestive physiology appear 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 of 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 ribosomal RNA sequences, have not diverged much genetically over 30 yr of separation (Herrel et al. 2008). The similarity between the two island populations may simply indicate 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 with 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 versus mainland populations of lizards have been observed in other species (Pafilis et al. 2007; Sagonas et al. 2015). Nevertheless, the greater plant OM digestibility displayed by the Pod Mrčaru lizards shows that they have indeed achieved mechanisms for digesting plant material, and our work suggests that such an ability derives from the HG.

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