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

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Abstract:	<p>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 <i>Podarcis siculus</i>, 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 plant-eating 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— Pod Mrčaru lizards had higher digestibility of herbivorous and omnivorous diets in the laboratory than did their source population.</p>

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

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19 **Abstract**

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

40

41 **Keywords**

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

43

44 **Introduction**

45

46 Based on Optimal Foraging Theory (Pyke et al. 1977) and Chemical Reactor Theory (Penry and
47 Jumars 1987), the Adaptive Modulation Hypothesis (AMH; Karasov and Diamond 1983;
48 Karasov 1992; Karasov and Martínez del Río 2007) uses economic principles, arguing that the
49 gut is expensive to maintain, and thus, there should be a match between gut function (digestive
50 enzyme activities, nutrient transport rates) and the food ingested by an animal. Thus, to
51 maximize net nutrient gain, a diet shift should lead to changes in gut physiology to match the
52 new diet, on short or long timescales. For example, increased digestive substrate concentration
53 (e.g., starch) requires increases in matched enzyme activities (e.g., amylase activity) to achieve
54 high digestibility of the nutrient (Karasov and Martínez del Río 2007). Indeed, correlations
55 between carbohydrase activities in the gut and carbohydrate intake in the natural diet has been
56 observed in fishes, birds, and mammals, even when analyzed in a phylogenetic context
57 (Schondube et al. 2001; Horn et al. 2006; Perry et al. 2007; German et al. 2010; Kohl et al. 2011;
58 German et al. 2015). Some herbivorous fishes that consume starch-rich diets cannot down
59 regulate their digestive enzyme activities towards carbohydrates (German et al. 2004) or
60 intestinal glucose transport rates (Buddington et al. 1987) when fed low-starch foods in the
61 laboratory, suggesting that expression of the requisite enzymes and transporters can be fixed in
62 some animals. The same cannot be said for proteolytic enzymes, where evolutionary correlations
63 of dietary protein and gut proteolytic activity are weak or non-existent (e.g., Schondube et al.
64 2001; German et al. 2010; Kohl et al. 2011); although there are examples of short term flexibility

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

67 In terms of gut size, increased food intake (e.g., due to high-fiber content of the diet)
68 speeds up digesta transit rate (Dadd 1960; Pritchard and Robbins 1990; Diamond 1991; Castle
69 and Wunder 1995), and more rapid digesta transit rates requires an increase in gut size and/or
70 some way to slow down digesta transit to maintain digestibility (Sibly 1981; Karasov and Hume
71 1997; Karasov and Martínez del Rio 2007; Karasov and Douglas 2013). Hence, herbivores
72 generally have larger guts than carnivores (Wagner et al. 2009; Stevens and Hume 2004; Dearing
73 1993) to accommodate more voluminous meals (Pough 1973; Wilson and Lee 1987). Hindgut
74 valves (like the ones observed in a Lacertid lizard: see below) can both increase overall gut
75 surface area and can slow the passage of digesta (Iverson 1982; Stevens and Hume 2004; Bignell
76 1984; Bell et al. 2005; Godon et al. 2016). Digesta high in fiber (i.e. plant material) is
77 particularly viscous, characterized by low Reynolds Numbers (Lentle and Jansen 2008). Because
78 of these flow characteristics, digesta high in fiber leads to decreased mixing in a smooth-bore
79 tube (Love et al. 2013). By acting as “baffles,” hindgut valves can increase mixing of viscous
80 digesta in what would otherwise be a plug-flow environment with unidirectional flow (Lentle
81 and Jansen 2008), thus helping to maintain nutrient digestibility by increasing the residence time
82 of digesta in that gut region (Stevens and Hume 2004; Karasov and Martínez del Rio 2007). We,
83 therefore, predict that animals eating plants would have features of gut structure (e.g., larger gut,
84 hindgut valves) and function (e.g., elevated carbohydrase activities, in congruence with AMH)
85 that facilitate digestion of plant material (Table 1; Karasov and Martínez del Rio 2007).

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

88 more fibrous portions of plants (e.g., cellulose) and can derive some portion of their energy
89 intake from microbial fermentation (McBee and McBee 1982; Bjorndal 1997; Stevens and Hume
90 1998). These microbial fermentations produce short chain fatty acids (SCFA) that can be
91 assimilated across the gut wall by the host (Bergman 1990; Foley et al. 1992). Thus, animals
92 consuming more plant material may have increased reliance on microbial fermentation (in this
93 study, indirectly measured via the products of fermentation, SCFA) in their hindguts to
94 assimilate their plant meals (Stevens and Hume 1998).

95 One of the most interesting examples of a rapid dietary shift in a vertebrate animal is in
96 the Italian Wall Lizard, *Podarcis siculus*. In 1971, five male-female pairs of *P. siculus* were
97 moved from the island of Pod Kopište (0.09 km²), Croatia, to the nearby island of Pod Mrčaru
98 (0.03 km²) as part of a biological invasion study (Fig. 1; Nevo et al. 1972). Returning to the
99 Croatian islets in 2004-2006 (<30 *P. siculus* generations later), Herrel et al. (2008) found the new
100 population on Pod Mrčaru had morphologically and behaviorally diverged from their source
101 population on Pod Kopište. Although the Pod Kopište lizards were insectivorous, consuming 4-
102 7% plant material (by mass), plants made up 34-61% of the Pod Mrčaru population's intake.
103 Plant material was more available on Pod Mrčaru, but arthropod abundance and diversity was
104 similar between islands (A. Herrel, *unpublished data*). The Pod Mrčaru lizards were larger, had
105 different head shapes, and larger bite forces. Additionally, both adult and neonate lizards from
106 Pod Mrčaru had developed valves in their hindguts, a feature not found in the Pod Kopište
107 population. Hindgut valves in lizards are generally associated with highly derived herbivory
108 (Iverson 1982; Bjorndal 1997; Stevens and Hume 2004). Morphological and dietary changes
109 have been documented between the Pod Mrčaru lizards and their source population on Pod

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

112 In this study, we examined gut structure (gut length, histological surface area) and
113 function (digestive enzyme activities, organic matter digestibility) in the context of diet
114 (proportion plant material, mass of stomach contents) in *P. siculus* from the two Croatian islets,
115 as well as from Zagreb on the mainland (Fig. 1). Omnivory and herbivory in lizards is rare, with
116 herbivory encompassing <1-4 % of extant lizards (Espinoza et al. 2004; Cooper and Vitt 2001;
117 King 1996). Yet, strict herbivory has originated independently >30 times (Espinoza et al. 2004)
118 adding to the compelling reasons to investigate the mechanisms behind such shifts to plant-rich
119 diets. We tested the assumption that there have been unique shifts in gut structure and function in
120 the Pod Mrčaru lizards allowing them to digest plant material better than lizards from Pod
121 Kopište or the mainland (Zagreb). If pancreatic or brush border enzymes aimed at digesting
122 components of plant material are higher in the Pod Mrčaru lizards than their insectivorous
123 counterparts on Pod Kopište (Fig. 2; Table 1), this would support the AMH, and we can
124 conclude that the lizards have increased relevant enzyme activities (e.g., via increased expression
125 of digestive enzyme genes; German et al. 2016). If digestive enzyme activities aimed at digesting
126 plant material, particularly the fibrous portions, are elevated in the lizards' hindguts, these
127 enzymes would likely be microbially derived (Potts and Hewitt 1973; Nakashima et al. 2002; Mo
128 et al. 2004; Skea et al. 2005; German et al. 2015; Jhaveri et al. 2015; Leigh et al. 2018b; Fig. 2;
129 Table 1). Elevated SCFA concentrations would also support a role of symbionts (Table 1). All of
130 these possibilities are not mutually exclusive, as lizard tissue changes and microbial community
131 shifts may both contribute to the Pod Mrčaru omnivores' ability to subsist on a diet rich in plants.
132

133 **Materials and Methods**

134

135 *Diet analysis*

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

144

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

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

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

155 We divided the gut into stomach (including esophagus), proximal intestine (PI), mid intestine
156 (MI), and hindgut (H). The hindgut was easily identifiable (see Fig. 2) and the proximal and mid
157 intestine portions were separated by dividing the remaining intestine in half. In seven individuals
158 from each population, we removed the gut contents from the proximal, mid, and hindgut sections
159 (e.g., Proximal Intestine Gut Contents, PIGC; Hindgut Contents, HC) and flushed out the
160 intestinal tissue with chilled 25 mM tris-HCl, pH 7.5. We used pH indicator paper (Macherey-
161 Nagel, Düren, Germany: pH 1-14, 5.5-9.0, and 8.0-10.0) on dissected Zagreb lizards to measure
162 pH of the contents of each gut region as follows: stomach pH 3.4 ± 2.0 , proximal intestine pH
163 8.17 ± 0.6 , mid intestine pH 8.96 ± 0.7 , and hindgut pH 8.75 ± 0.5 .

164

165 Gut tissues and contents from each gut region and pancreases were frozen separately in
166 1.5 mL vials in liquid nitrogen for storage and transport. Vials were transported on dry ice to the
167 University of California, Irvine, where they were stored at -80°C until used. We weighed frozen
168 gut sections and gut contents (excluding stomachs) to the nearest 0.001 g. For three lizards from
169 each population, we preserved the PI, MI, and H in McDowell Trump's fixative (4%
170 formaldehyde, 1% glutaraldehyde, McDowell and Trump 1976) for subsequent histological
171 analyses. The remaining three lizards from each population were used for microbiome analyses
172 in a different study (Lemieux-Labonté et al., *Unpubl data*).

173

174 *Estimation of intestinal surface area using histology*

175 Gut sections preserved in Trump's Solution were further sectioned into 3-10 mm sections with a
176 razor blade and rinsed in phosphate buffer pH 7.5 (PBS) for 3 x 20 min., and overnight in PBS at
177 4°C under constant shaking. The PBS rinsed tissues were flushed with running deionized water

178 for 2 x 20 min., and then were subjected to serial ethanol dilutions of 30%, 50%, and 75%. We
179 selected the proximal portions of the PI, MI, and H from Pod Mrčaru and Pod Kopište lizards,
180 and portions starting at the halfway point of the hindgut (H+) from all three populations. Tissue
181 portions were placed in tissue cassettes wrapped in ethanol-soaked cheesecloth, sealed in plastic
182 bags, and were sent to Mass Histology Services (Worcester, MA, USA) for embedding in
183 paraffin wax. We stained 7- μ m sectioned samples with hematoxylin and eosin and imaged them
184 with a Zeiss Axioplan 2 epifluorescence microscope and Zeiss and Cannon cameras. Tiled
185 images were assembled using the Photomerge function of Adobe Photoshop CS3. We analyzed
186 1-25 sections of each sample by measuring the perimeters of mucosa and serosa. We then
187 calculated the epithelial surface magnification (ESM) as the ratio of mucosal to serosal
188 perimeters (Hall and Bellwood 1995; German 2009) to observe how much the mucosal folds
189 increase the inner surface area of the intestine relative to a smooth bore tube.

190

191 *Homogenate preparation*

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

201 content samples, these samples were sonicated (CL-18 Sonicator, Fisher Scientific, Waltham,
202 Massachusetts, USA) at 5 W output for 3 x 30 s, with 30 s intervals between pulses, followed by
203 homogenization, as described for the gut tissues. The gut content samples were centrifuged at
204 12000 x g for 10 min. All supernatants were stored in 100-200µl aliquots at -80° C until just
205 before use in digestive enzyme assays. For the HC, we thawed the sample enough to transfer the
206 contents to a spin column (Corning Costar Spin-X Centrifuge cellulose acetate tube filters, 0.22
207 µm pores) and centrifuged at 14000 x g at 4°C to gather H fluid. The filtered fluid was frozen at -
208 80°C for use in SCFA measurements. The remaining HC pellet was then prepared for enzyme
209 assays in the same manner as the other gut contents (German and Bittong 2009).

210

211 *Biochemical Assays of Digestive Enzyme Activity*

212 We conducted digestive enzyme assays following protocols outlined in German and Bittong
213 (2009) and German et al. (2015). We ran all assays at 25°C, the mean temperature from May–
214 September (confirmed by iButtons, Maxim Integrated, San Jose, CA, USA; Supplemental Fig.
215 S1) on the islands and within the preferred temperature range of *P. siculus* populations outside of
216 their native geographic range (Liwanag et al. 2018). We measured enzyme activities in duplicate
217 or triplicate and read absorption or fluorescence in flat-bottomed 96-well microplates using a
218 BioTek Synergy H1 Hybrid spectrophotometer/ fluorometer equipped with a monochromator
219 (BioTek, Winooski, VT, USA). Our primary buffer was 25mM Tris-HCl, pH 8.6 (referred to
220 henceforth as “buffer,” any deviations are noted), measured at room temperature (22°C).

221 Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). We optimized each assay
222 for duration and homogenate volume. Each enzyme activity was measured in each gut region (PI,
223 MI, H, PIGC, MIGC, HC) for each lizard. Pancreatic tissue was only used for measuring

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

229

230 Carbohydrate degrading enzymes— Following German and Bittong (2009) and German *et al.*
231 (2015), we measured α -amylase activity using 1% potato starch dissolved in buffer containing 1
232 mM CaCl_2 . Maltase and trehalase activities were measured using 112 mM maltose or trehalose,
233 respectively, in buffer. We incubated each of these assays as end-point reactions. Post
234 termination, we determined glucose concentration by measuring absorbance at 650 nm (α -
235 amylase) and 550 nm (maltase and trehalase). The α -amylase, maltase, and trehalase activities
236 were determined from glucose standard curves and expressed in U (μmol glucose liberated per
237 minute) per gram of tissue.

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

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

252

253 Assays of protein and lipid degrading enzymes— Modified from German and Bittong (2009) and
254 German et al. (2015), we measured trypsin, aminopeptidase, and lipase activities as kinetic
255 assays. To measure trypsin activity, we used 2mM N α -benzoyl-L-arginine-p-nitroanilide
256 hydrochloride (BAPNA) substrate dissolved in 100 mM Tris-HCl buffer. For aminopeptidase
257 activity, we used 2.04 mM L-alanine-p-nitroanilide in buffer. These protease assays were read at
258 410 nm absorbance for 30 min to detect a p-nitroaniline product as U (μ mol p-nitroaniline
259 released min^{-1}) per gram of tissue. For trypsin activities measured in pancreatic tissue
260 homogenates, we pre-incubated the homogenates with 15 μ l enterokinase (4 U mL^{-1} in 40 mM
261 succinate buffer, pH 5.6)/ 100 μ l homogenate for 15 min to change trypsinogen from its
262 zymogen form to active trypsin enzyme, then proceeded with the assay as with the other tissues.

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

268 In addition to the regional enzyme activities (U \times g^{-1}), we calculated the total gut enzyme
269 activities as the sum of mass-specific activity for each region multiplied by the tissue mass to

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

272

273 *Fermentation Analyses*

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

287

288 *Digestibility*

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

293 lab conditions, during which they were offered live cockroaches (*Blatta sp.*) and finely chopped
294 brussels sprouts (*Brassica oleracea*) daily. Lizards had *ad lib* access to water throughout the
295 trials. The lab was kept at 25-31°C on a 10:14 h light/dark schedule. Five lizards from each
296 population were assigned to one of three diets: insectivore, omnivore, or herbivore. On the first
297 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
298 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-
299 32 days. Initial SVLs and masses are presented in Supplemental Table S1.

300

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

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

319

320 Feeding— Each lizard was weighed, and gently force fed the gelatin capsule of a known mass of
321 experimental diet using a plunger from a syringe to push the pill into their esophagus. After the
322 pill was in the esophagus or swallowed, we administered water (of equal mass to the
323 experimental diet) into the lizard's mouth via pipette. We adjusted the mass of diet fed at each
324 feeding to maintain lizard body mass $\pm 10\%$. We prepared three sizes of meals (+10mg, baseline,
325 -10mg) for each diet. If a lizard's body mass increased by $\geq 10\%$ compared to its initial measured
326 body mass (or if that individual vomited its previous meal), we fed it the smaller meal size at the
327 next feeding. Individuals that lost $\geq 10\%$ of their initial body mass were given the larger sized
328 meal at their subsequent feeding. We collected all feces and urates daily and measured the SVL
329 of each lizard weekly.

330

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

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

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

340
$$\frac{(\text{mass food and gelatin capsules ingested}-\text{ash})-(\text{feces mass}-\text{ash})}{\text{mass food and gelatin capsules ingested}-\text{ash}}.$$

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

344

345 *Statistical Analyses*

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

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

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

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

367

368 **Results**

369

370 *Podarcis siculus* from Pod Kopište (SVL: 64.39±2.40 mm; average ± standard deviation) were
371 significantly shorter in body length ($F_{2,35}=11.83$, $P<0.001$) than the populations from Pod
372 Mrčaru (SVL: 68.73±2.50 mm) and Zagreb (SVL: 68.43±5.80 mm), which were not different
373 from each other. The Zagreb lizards (body mass: 8.9±1.5 g) were significantly more massive
374 ($F_{2,35}=13.75$, $P<0.001$) than either island population lizards, and Pod Mrčaru lizards (body mass:
375 7.6±0.7 g) were significantly heavier than Pod Kopište lizards (body mass: 6.6±0.9 g;
376 Supplemental Table S2).

377

378 *Diet & Gut Size*

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

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

387 Relative intestinal masses were heavier in lizards from Pod Kopište (3.6% of total body
388 mass) and Pod Mrčaru (3.3%) than in lizards from Zagreb (2.4%; ANCOVA Population:
389 $F_{2,18}=9.53$, $P<0.007$; body mass: $F_{1,15}=6.94$, $P<0.030$, model run without interaction term as it
390 was non-significant; Fig. 3). We found no significant differences in gut length ($F_{2,36}=2.55$,
391 $P=0.092$) among the three populations. Regional intestinal masses (i.e.: PI, MI, H) did not differ
392 by population nor covary with lizard body mass. (See Supplemental Table S2 for values.) Mass
393 of total gut contents did not vary significantly (ANCOVA with body mass as covariate, $F_{2,18} =$
394 2.10 , $P=0.163$) among the three populations. Gut contents were evenly distributed throughout the
395 PI, MI, and H. None of the populations retained more contents in a particular gut region than did
396 the other two populations, excepting the higher mass of digesta in the Pod Mrčaru lizards'
397 stomachs, mentioned above. Additionally, we observed nematodes in the HC of multiple lizards
398 from each of the three populations.

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

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

408

409 *Digestive Enzyme Activities*

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

414 The mass-specific α -amylase activity in the hindgut contents was almost 6-fold higher in
415 Pod Mrčaru lizards compared to that measured in the Pod Kopište population ($t=-0.27$, $P=0.038$,
416 Fig. 5a, Supplemental Table S3). Amylase activity was undetectable in all but one HC sample
417 from the Zagreb population. We found no other differences in amylase activity. In most MI and
418 H samples, amylase activity was undetectable or replicates were too variable for confident
419 analyses. No other gut region nor pancreas showed differences in amylase activity among the
420 three populations (Pancreas: $F_{2,23}=2.97$, $P=0.072$; PI: $F_{2,10}=3.41$, $P=0.074$; PIGC: $F_{2,14}=0.49$,
421 $P=0.626$; MIGC: $F_{2,13}=0.63$, $P=0.549$), nor did we find any significant differences in the total gut
422 α -amylase activity among populations ($F_{2,10}=2.05$, $P=0.180$).

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

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

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

445 In both the H and HC, the Pod Kopište population had lower regional trehalase activity
446 than one of the other two populations (Fig. 5e). Still, there were no population differences in
447 trehalase activity in more proximal regions, nor in total activity of the enzyme (Supplemental
448 Table S3). The Pod Mrčaru population's H trehalase activity (0.058 ± 0.020 $\mu\text{mol glucose}$
449 $\text{liberated min}^{-1} \text{g}^{-1}$) was higher than the H activity in the Pod Kopište population (0.0045 ± 0.003
450 $\mu\text{mol glucose liberated min}^{-1} \text{g}^{-1}$; $F_{2,9}=5.50$, $P=0.024$). The Zagreb population's HC activity

451 (0.254±0.134 μmol glucose liberated min⁻¹ g⁻¹) was higher than Pod Kopište HC activity

452 (0.025±0.011 μmol glucose liberated min⁻¹ g⁻¹; $F_{2,12}=4.28$, $P=0.041$).

453

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

455 In the HC, the mass specific trypsin activity was >5-fold higher in the Pod Mrčaru
456 population than in the Pod Kopište population ($F_{2,11}=5.33$, $P=0.024$; Fig. 5f, Supplemental Table
457 S3). The trypsin activity in the HC of the Zagreb population (Supplemental Table S3) was not
458 different from the other two populations. No other gut region showed differences in activity
459 among the three populations (Fig. 5f), nor did we find difference in the total trypsin activity
460 among populations. The mass specific trypsin activity in the pancreas was >2.5x higher in Pod
461 Kopište and Pod Mrčaru lizards (0.454±0.050 μmol p-nitroaniline released min⁻¹ g⁻¹) than in the
462 pancreases of Zagreb lizards (0.190±0.040 μmol p-nitroaniline released min⁻¹ g⁻¹; $P: F_{2,23}=5.14$,
463 $P=0.014$, Fig. 7).

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

470

471 Lipase—There was no difference in lipase activity across the three lizard populations

472 (Supplemental Fig. S3 and Table S3; total lipase $F_{2,18}=0.879$, $P=0.432$).

473

474 *Microbial Fermentation*

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

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

491

492 *Organic Matter Digestibility*

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

497 body mass $F_{1,6} < 0.001$, $P = 0.983$). On an omnivore diet, Pod Mrčaru lizards had an almost 4%
498 higher organic matter digestibility than their Pod Kopište counterparts (ANCOVA: population
499 $F_{1,7} = 5.875$, $P = 0.0458$; body mass $F_{1,7} = 3.065$, $P = 0.1235$). The two populations did not differ in
500 digestibility of an all insect diet (ANCOVA: population $F_{1,7} = 0.670$, $P = 0.440$, body mass
501 $F_{1,7} = 0.619$, $P = 0.457$). For all three diets, analyses using other covariates are available in
502 Supplemental materials. Final and percent change in body masses and SVL are available in
503 Supplemental Table S1.

504

505 **Discussion**

506

507 *Subtle changes in performance*

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

519 *siculus* observed in this study (24% of gut content mass) than in the past (4-7%; Herrel et al.
520 2008).

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

530 As the majority of endogenous nutrient digestion and absorption in non-ruminant
531 vertebrates occurs in the proximal portion of the intestine (Vonk and Western 1984; Karasov and
532 Martínez del Rio 2007; Le et al. 2019), differences in the structure and function of the hindgut
533 point to differences in the function of microbial symbionts in this gut region (McBee and McBee
534 1982; Bergman 1990; Bjorndal 1997). According to the plug flow reactor model of digestion
535 (Penry and Jumars 1986, 1987; Karasov and Hume 1997; Stevens and Hume 2004), nutrients are
536 digested and absorbed down their gradient as they flow through the gut. Thus, a shift in gut
537 function in the proximal region will promote downstream changes, from more potential digestion
538 to more opportunities for nutrient absorption. For example, an increase in trypsin activity in the
539 proximal intestine could lead to greater digestion of proteins into dipeptides that can serve as a
540 substrate for aminopeptidase in the mid intestine and hindgut. There is subsequently more gut
541 remaining over which the dipeptides can be absorbed. Changes in the lizard hindgut, therefore,

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

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

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

565 proximal intestines of Pod Kopište lizards. Higher NAG activity could contribute to the digestion
566 of chitin from arthropods carapaces, and the cell walls of fungi and nematodes (Skoczylas 1978;
567 Vonk and Western 1984). Although we expected patterns of NAG activity to conform to that of
568 brush border digestive enzymes (German et al. 2015), NAG activity was the highest in the HC,
569 consistent with microbial synthesis, not endogenous synthesis in lizard tissue (Jhaveri et al.
570 2015). Moreover, *P. siculus* appears to have considerably lower endogenous NAG activity
571 compared to other lizards (Table 4; Jeuniaux 1961, 1963; Marsh et al. 2001).

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

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

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

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

611 portion of their hindgut, dividing the gut in the transverse plane (Supplemental Fig. S4; Wehrle
612 2018). The Pod Kopište lizards ($n=29$) lacked these valves entirely. The same methods were used
613 across years, so there were no methodological differences. These data, combined with those from
614 Herrel et al. (2008) and Vervust et al. (2010) convince us that, although usually present, the
615 hindgut valves in *P. siculus* may be flexible in their morphology or even presence, and they are
616 not fixed features in the Pod Mrčaru lizards. The general pattern of surface area decreasing along
617 the gut (i.e.: PI>MI>H, Fig. 4) was consistent with our expectations (Skoczylas 1978; Stevens
618 and Hume 2004) considering we did not observe the valves in our sections from summer 2013
619 (even the H+ sections).

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

626

627 *Lizard Digestive Physiology*

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

634 the island populations are to each other (Podnar et al. 2005; Herrel et al. 2008). We predicted that
635 the Pod Kopište and Zagreb populations, which both mostly consume invertebrates, would show
636 the most similarity in gut form and function. However, we found that the closely related island
637 populations show greater similarities in gut form and function than do the insectivorous
638 populations. Sagonas and colleagues (2015) found more similarities in the gut physiology and
639 morphology of lizards with more similar habitats (i.e. insular vs. mainland) than populations that
640 had more similar diets. Indeed, island effects on physiology, morphology, and ecology often
641 outweigh other factors in lizards, including effects on diet (Van Damme 1999; Cooper and Vitt
642 2002; Spiller et al. 2010) and digestion (Pafilis et al. 2007; Vidal and Sabat 2010; Sagonas et al.
643 2015). It may also be that the shared evolutionary history of the Pod Mrčaru and Pod Kopište
644 lizards leads to their similarity in gut structure and function (Garland and Adolph 1994; Karasov
645 and Martinez del Rio 2007).

646 The more massive guts of the Pod Mrčaru and Pod Kopište lizards compared to the
647 outgroup Zagreb population suggest that the new and source population lizards allocate more
648 tissue resources to digestion. Exactly why this is the case remains unknown. The Zagreb lizards
649 live in an urban area with copious plant cover and potential food sources, whereas the Pod
650 Kopište and Pod Mrčaru lizards live on small, densely populated islets that are likely
651 challenging, from space and resource perspectives (Herrel et al. 2008). Aminopeptidase was the
652 only digestive enzyme that showed higher activity in the Zagreb population in comparison to the
653 Pod Kopište and Pod Mrčaru lizards. The elevated aminopeptidase activity, which was within the
654 range measured in other lizards (Table 4) may compensate for the Zagreb population's lower gut
655 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).

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

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

702

703 *Conclusions, Potential Limitations, and Future Directions*

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

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

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

733

734 **Author Contributions**

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

739

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753

754 **Data Accessibility**

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

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757

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1041 **Figure Captions**

1042 Figure 1: *Podarcis siculus* island collection sites showing Pod Kopište (source population) and
 1043 Pod Mrčaru (newly omnivorous population). The box in the bottom map shows the area of the
 1044 inset. Zagreb (mainland population) not pictured.

1045

1046 Figure 2: Potential patterns of digestive enzyme activities across intestinal regions and
 1047 representative examples of lizard guts from each population (with stomachs). Pancreatic
 1048 digestive enzymes are secreted into the proximal intestine and are expected to decrease along the
 1049 gut. Brush border enzymes are produced at the brush border of the epithelial cells and the
 1050 intestinal lumen, generally peaking in activity in the mid intestine. High enzyme activities in the
 1051 proximal or mid intestines and/or their contents would be due to the lizards themselves
 1052 increasing those enzyme activities. Microbial enzymes produced by the microbiome tend to peak
 1053 in the hindgut where symbiotic microbes are housed. Increased enzyme activities in the hindgut
 1054 contents are likely produced by microbial symbionts. Modified from German *et al.* 2015. For
 1055 enzymatic analyses, we removed the esophagus and stomach from the intestines at the pyloric
 1056 sphincter. The hindgut was easily identified as its diameter is enlarged compared to the proximal
 1057 and mid intestine. We defined the proximal and mid intestines as half of each portion of the
 1058 remaining tissue length.

1059

1060 Figure 3: (a) Regional and (b) total intestinal mass (without contents) in Pod Kopište (source),
 1061 Pod Mrčaru (new omnivore), and Zagreb (mainland) populations. Gut regions are proximal
 1062 intestine (PI), mid intestine (MI) and hindgut (H) and presented as a percentage of body mass.
 1063 Values are mean \pm standard deviation. $n=7$ in all except $n=6$ in Pod Mrčaru PI. Comparisons of
 1064 populations were done via ANCOVA with body mass as a covariate. No particular region
 1065 showed differences in mass (a), but the Zagreb population had lower total intestinal masses than
 1066 the Pod Mrčaru population (b) that were also significantly affected by body mass. Pod Kopište
 1067 intestinal masses were not different from either population.

1068

1069 Figure 4: Epithelial Surface Magnification (ESM), the ratio of inner perimeter length of mucosa
 1070 to inner perimeter length of serosa in proximal intestine (PI), mid intestine (MI), proximal half of
 1071 hindgut (H), distal half of hindgut (H+). Values are mean \pm standard deviation, $n=3$.
 1072 Comparisons of populations were done via equal variance t-test. We found no differences by
 1073 population. Cross section images are representative stained histological sections from each gut
 1074 region of Pod Kopište (source) and Pod Mrčaru (new omnivore) populations. Not to scale: each
 1075 perpendicular black scale bar represents 500 μ m for the two images it lies between.

1076
1077 Figure 5: (a) amylase activity in μmol glucose liberated $\text{g}^{-1} \text{min}^{-1}$, “X” denotes undetectable
1078 activity. (b) β -glucosidase and (c) N-acetyl- β -D-glucosaminidase (NAG) activities in nmol
1079 MUB liberated $\text{g}^{-1} \text{min}^{-1}$, (d) trehalase activity in μmol glucose liberated $\text{g}^{-1} \text{min}^{-1}$, (e) trypsin in
1080 nmol and (f) aminopeptidase activities μmol of p-nitroaniline liberated $\text{g}^{-1} \text{min}^{-1}$ throughout the
1081 gut in Pod Kopište (source), Pod Mrčaru (omnivore), and Zagreb (mainland) populations. Values
1082 are mean \pm standard deviation, Pod Kopište $n=4-7$, Pod Mrčaru $n=3-7$, Zagreb $n=3-6$. In (b)
1083 populations by tissue and tissues within populations were compared via separate ANOVAs,
1084 shared letters above icons denote no differences. In (a, c-f) comparisons of populations were
1085 done via ANOVA where lines of a different elevation for a gut region indicate significant
1086 differences for that population and overlapping lines indicate no differences.

1087
1088 Figure 6: Total β -glucosidase activity (a) in nmol MUB liberated min and total aminopeptidase
1089 activity (b) in μmol p-nitroaniline liberated min^{-1} . Values are mean \pm standard deviation, $n=7$,
1090 Zagreb $n=6$. Populations compared via ANOVAs, different letters above icons denote significant
1091 differences.

1092
1093 Figure 7: Trypsin activity in the pancreas in nmol p-nitroaniline liberated $\text{g}^{-1} \text{min}^{-1}$ in Pod
1094 Kopište (source), Pod Mrčaru (new omnivore), and Zagreb (mainland) populations. Values are
1095 mean \pm standard deviation, $n=10$, Zagreb $n=6$. Populations compared via ANOVA, different
1096 letters above icons denote significant differences.

1097
1098 Figure 8: Organic matter digestibility on experimental diets in lizards from in Pod Kopište (blue,
1099 source) and Pod Mrčaru (green, new omnivore) presented as box and whisker plots representing
1100 each quartile ($n=5$). Populations compared via ANOVA separately for each diet. † denotes
1101 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</i> =30	207.41 \pm 16.1 mg <i>n</i> =36	98.79 \pm 123.2 mg <i>n</i> =4
	<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 μmol min ⁻¹ g ⁻¹ tissue	<i>Tupinambis meriange</i> Parry et al. 2009	5.82-12.7x10 ⁵ μmol min ⁻¹ g ⁻¹ <u>protein</u>
Aminopeptidase	Total: 0.22-0.81 μmol min ⁻¹	<i>Liolaemus pictus</i> Vidal and Sabat 2010	0.11-0.21 μmol min⁻¹
		<i>Lophognathis temporalis</i> Iglesias et al. 2009	10.22-25.35 μmol min ⁻¹
	Total/g tissue: 0.96-3.65 μmol min ⁻¹ g ⁻¹	<i>Liolaemus nigriviridis</i> Naya et al. 2009	2-4 μmol min⁻¹ g⁻¹
		<i>Liolaemus ruibali</i> Kohl et al. 2016a	2.13±0.3 μmol min⁻¹ g⁻¹
		<i>Heloderma suspectum</i> Christel et al. 2007	9-21 μmol min ⁻¹ g ⁻¹
Trehalase	Total: 0.004-0.28 μmol min ⁻¹	<i>Liolaemus pictus</i> Vidal and Sabat 2010	5.9-9.4 μmol min ⁻¹
	Total/ g tissue: 0.023-1.38 μmol min ⁻¹ g ⁻¹	<i>Liolaemus nigriviridis</i> Naya et al. 2009	~2-6 μmol min ⁻¹ g ⁻¹
Maltase	Total: 0.06-0.18 μmol min ⁻¹	<i>Liolaemus pictus</i> Vidal and Sabat 2010	5.92-11.31 μmol min ⁻¹
		<i>Lophognathis temporalis</i> Iglesias et al. 2009	2.39-3.89 μmol min ⁻¹
	Total/ g tissue: 0.273-0.782 μmol min ⁻¹ g ⁻¹	<i>Liolaemus ruibali</i> Kohl et al. 2016a	34.55±2.68 μmol min ⁻¹ g ⁻¹
	PI/MI: 0.458-0.872 μmol min ⁻¹ g ⁻¹	<i>Liolaemus nigriviridis</i> Naya et al. 2009	20-60 μmol min ⁻¹ g ⁻¹
NAG ^a	Total: 2.16-4.311 nmol min ⁻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
	Total/g tissue: 9.6-17.9 nmol min ⁻¹ g ⁻¹	<i>Sceloporus undulatus</i> Marsh et al. 2001	1.4x10 ⁶ nmol min ⁻¹ g ⁻¹

Figure 1

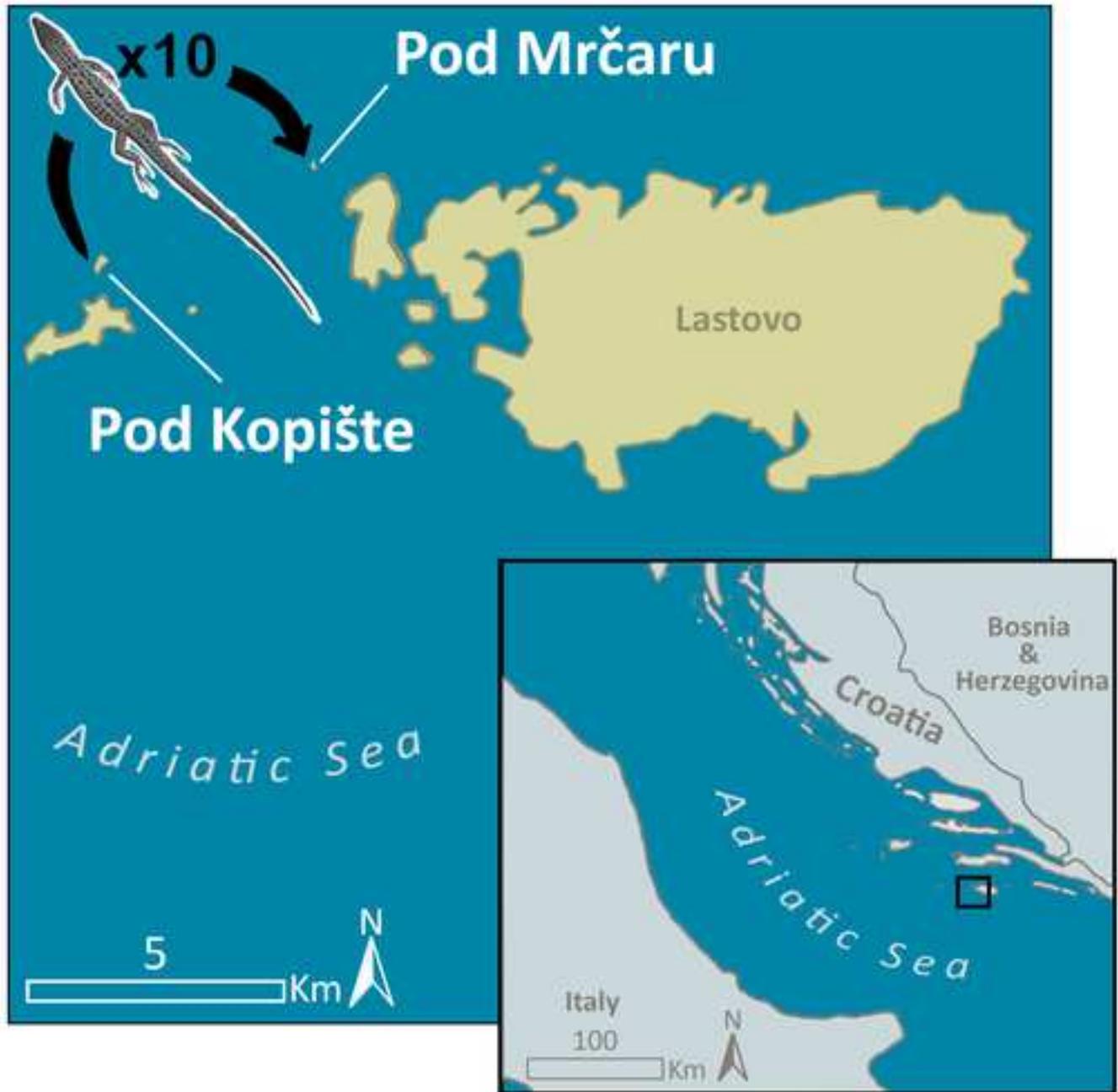


Figure 1 grayscale

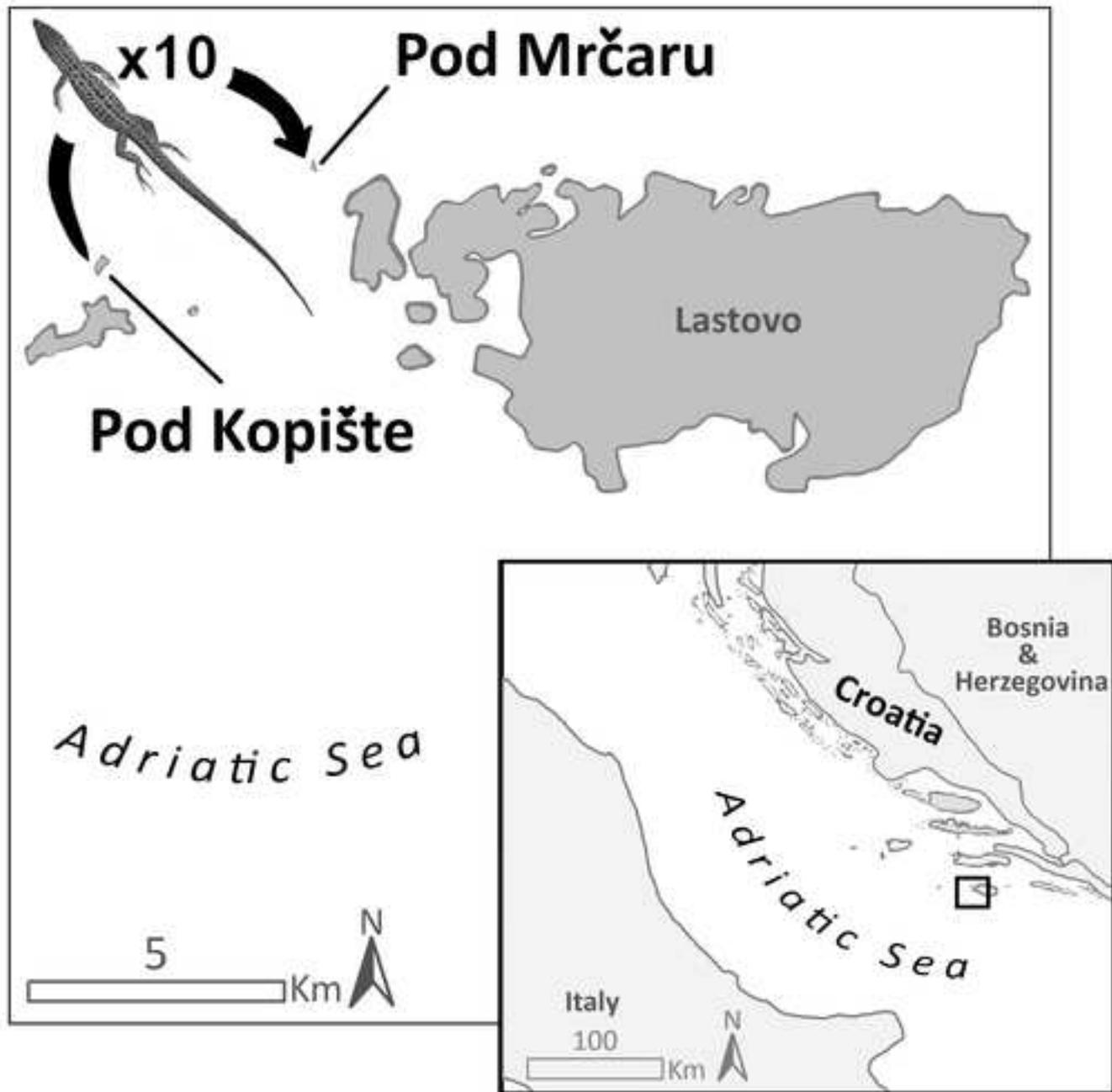


Figure 2

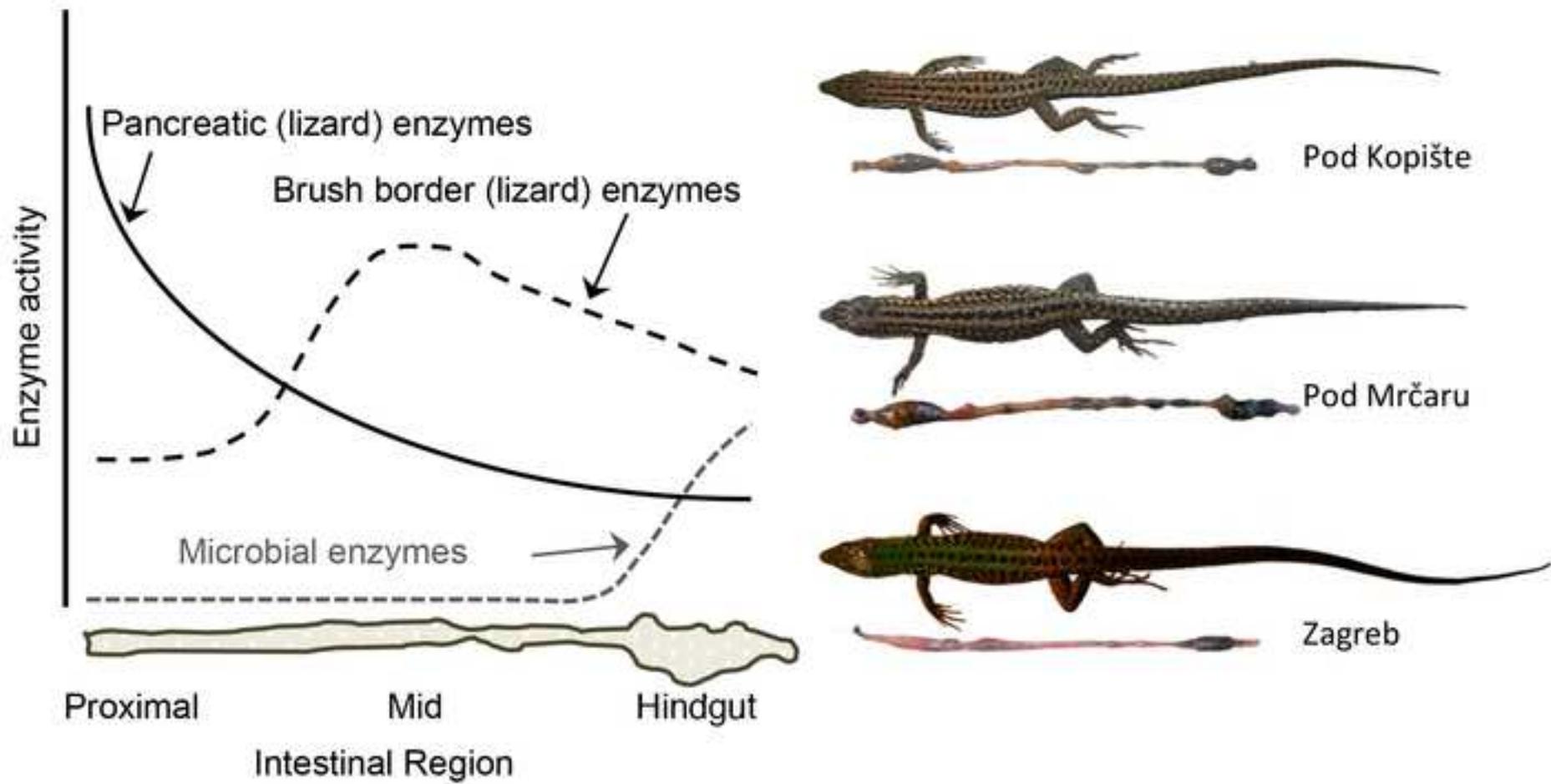


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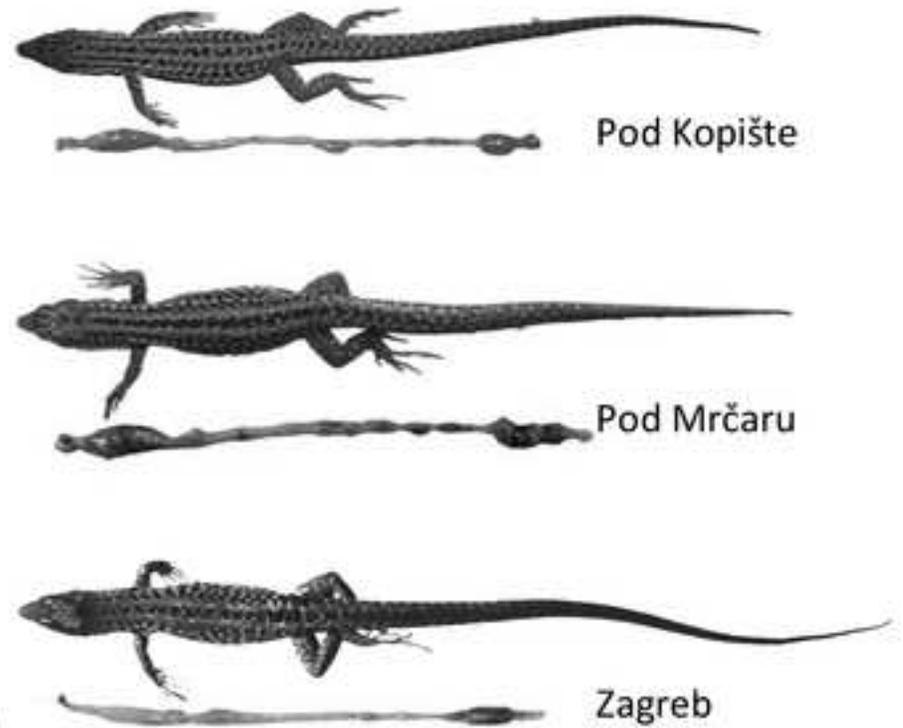
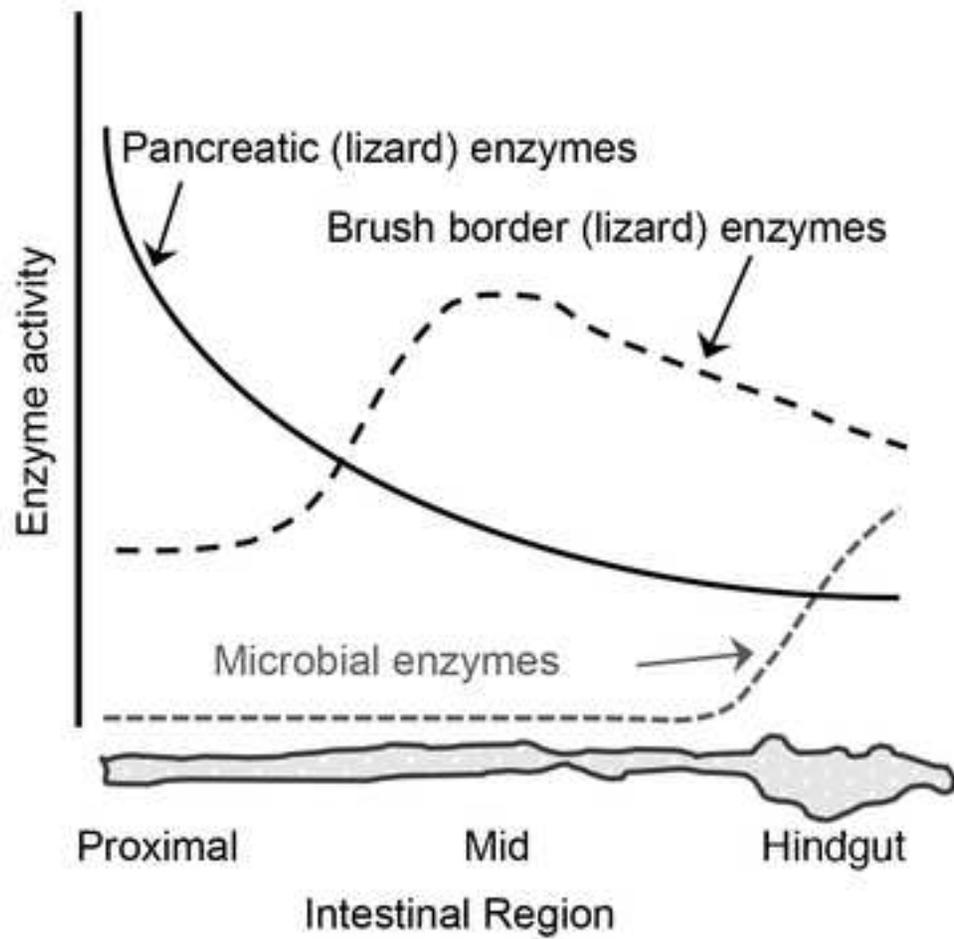


Figure 3

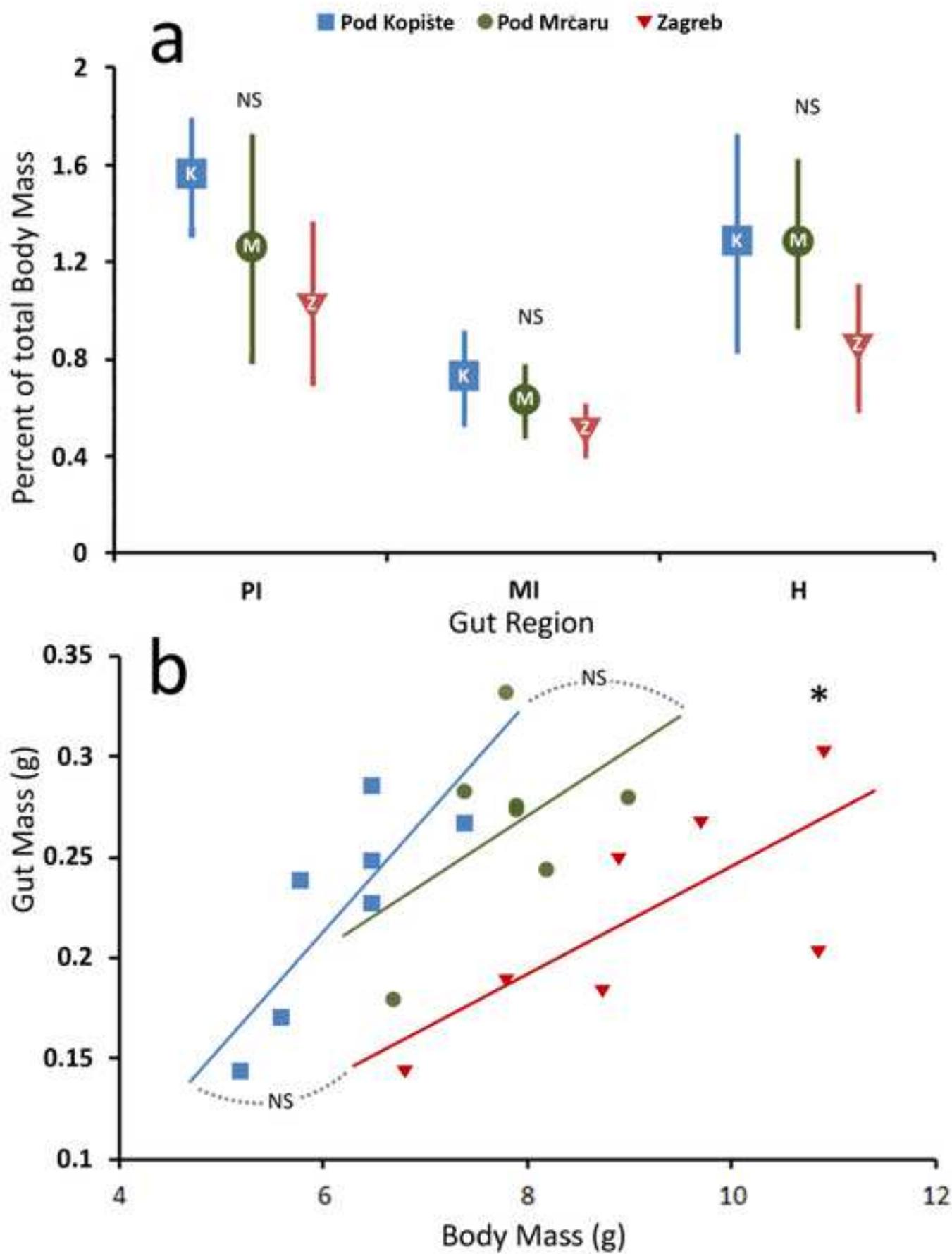


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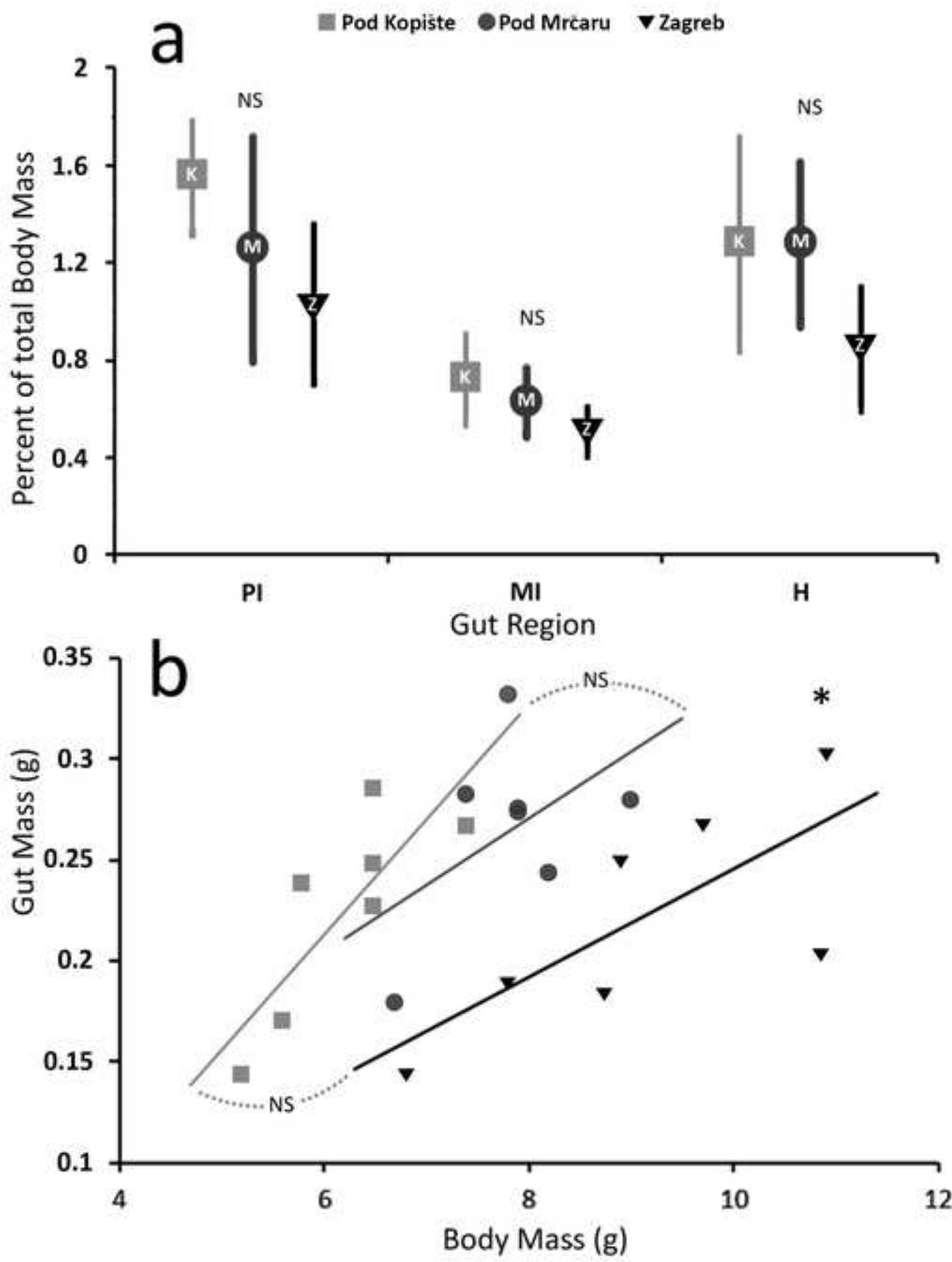


Figure 4

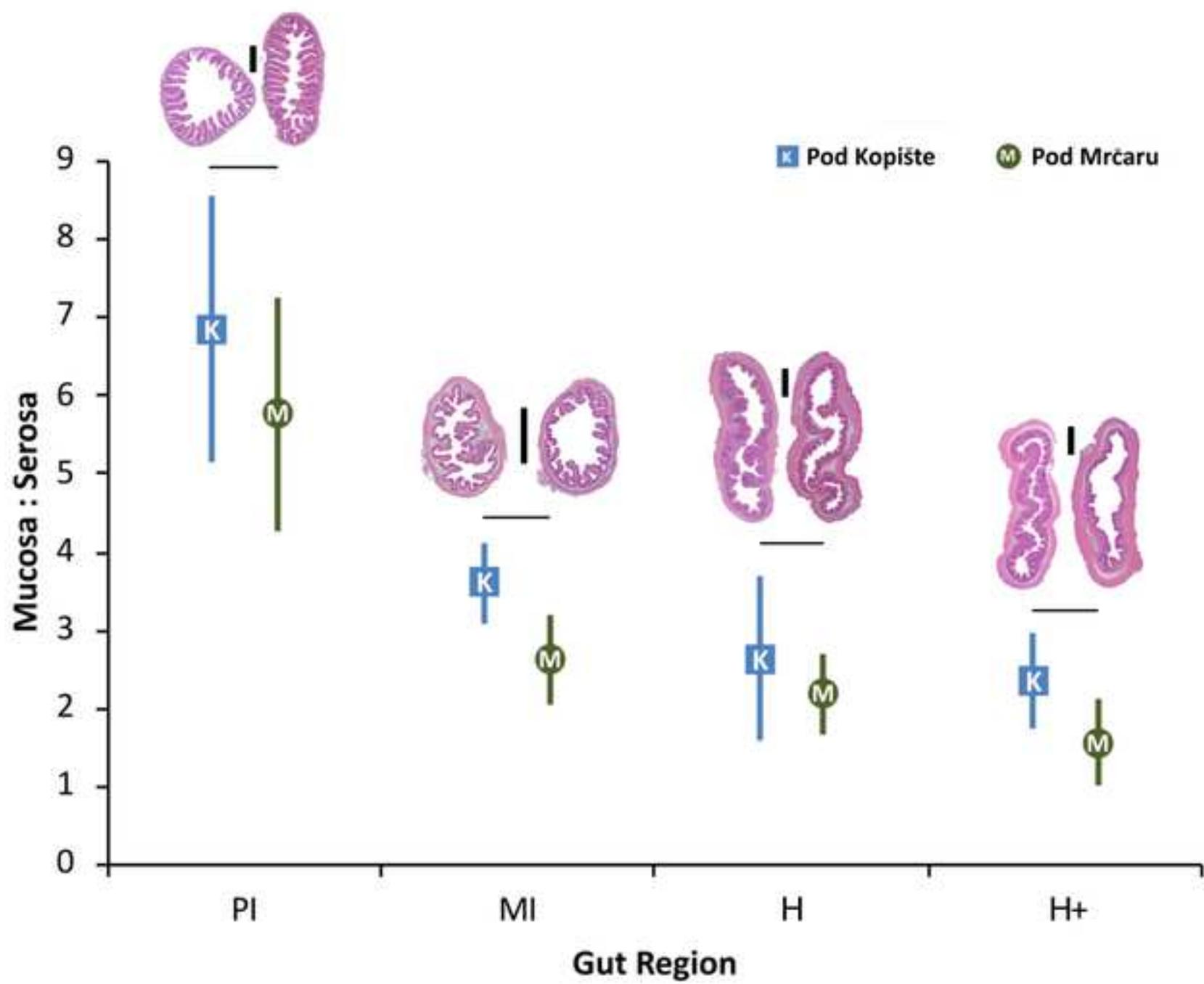


Figure 4 histology color/grayscale graph

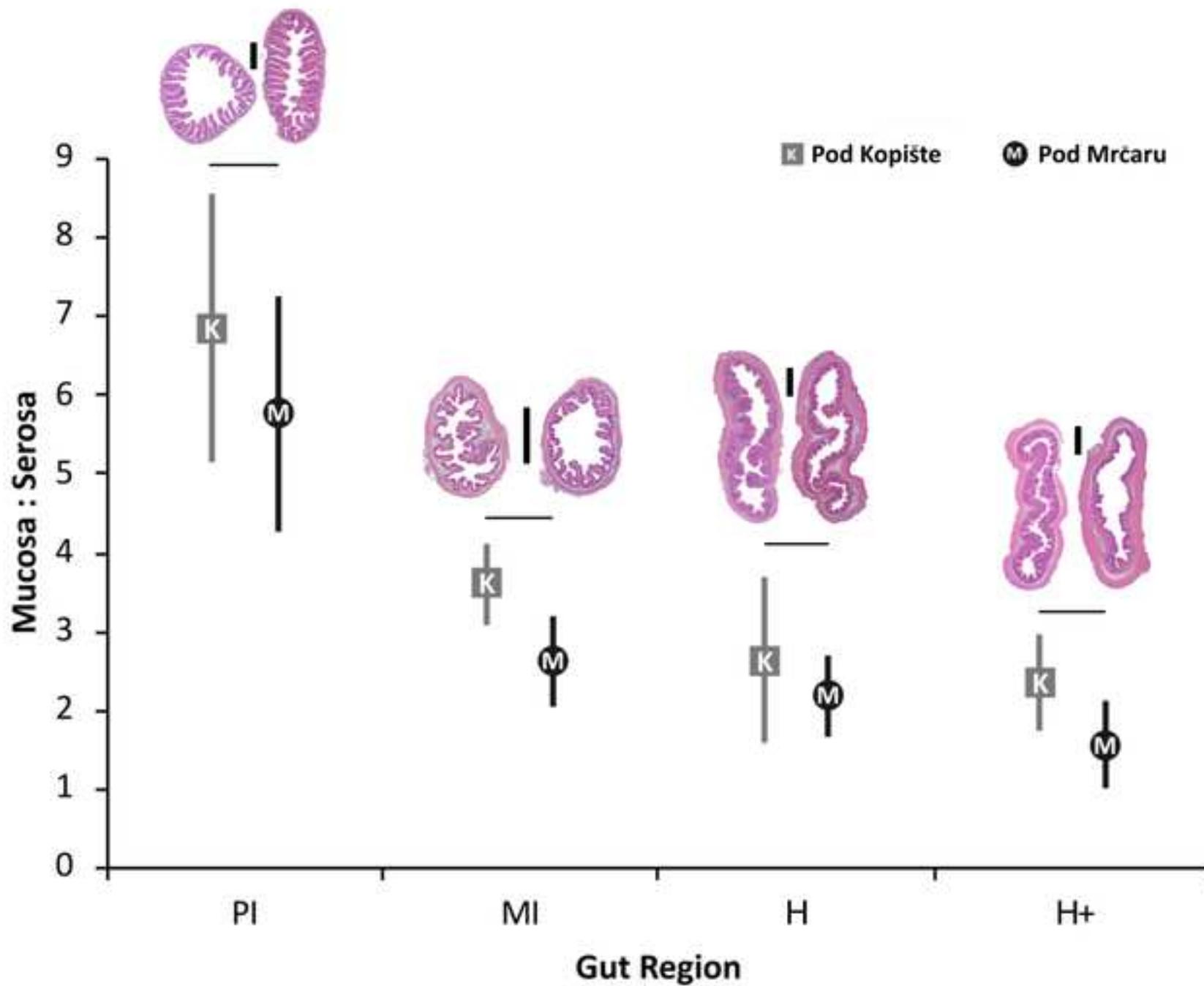


Figure 5

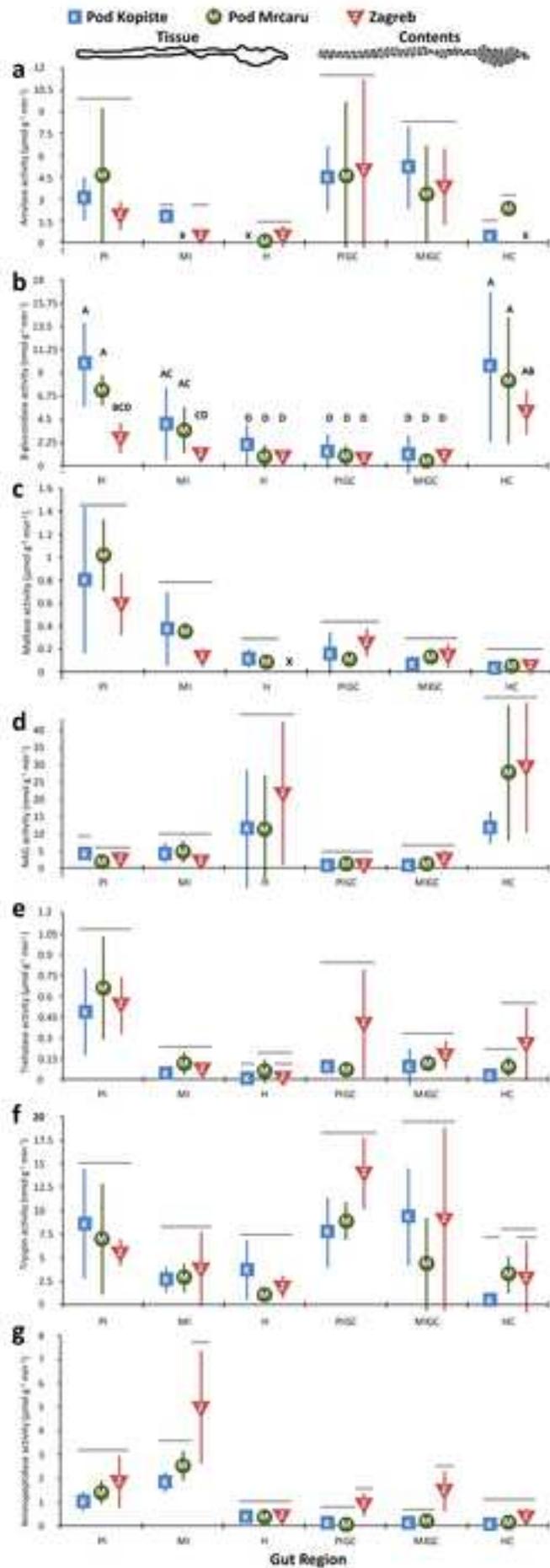


Figure 5 grayscale

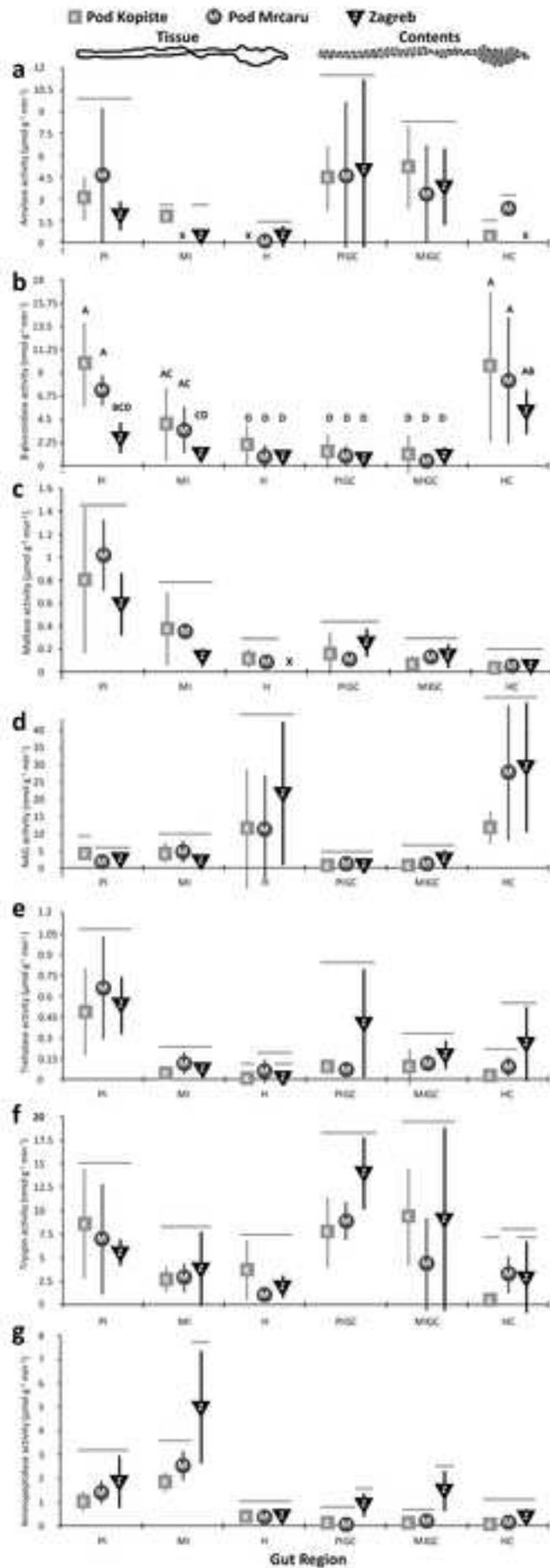


Figure 6

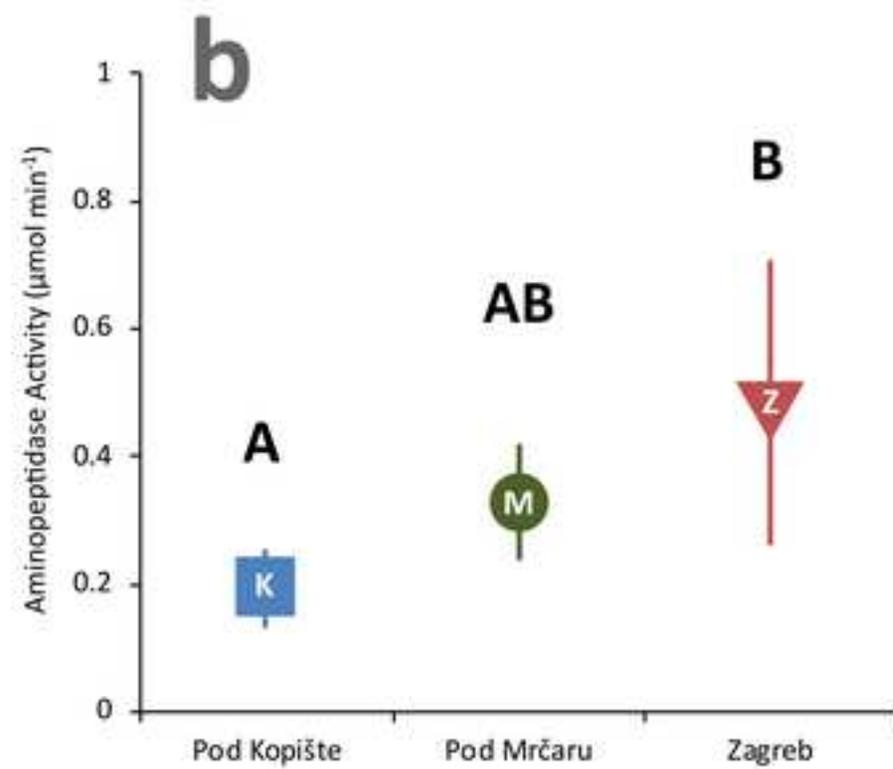
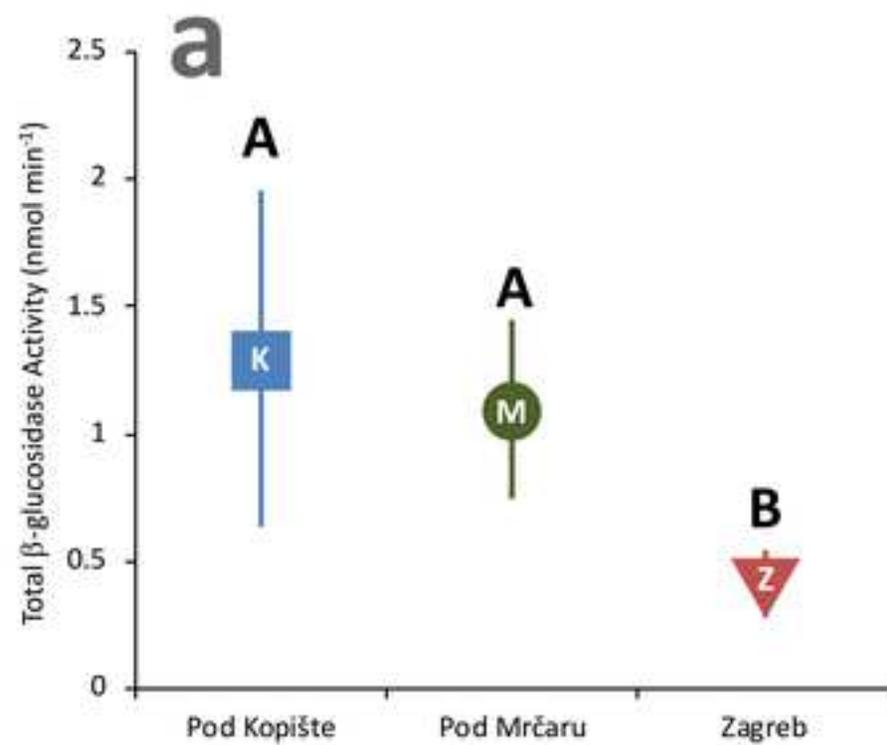


Figure 6 grayscale

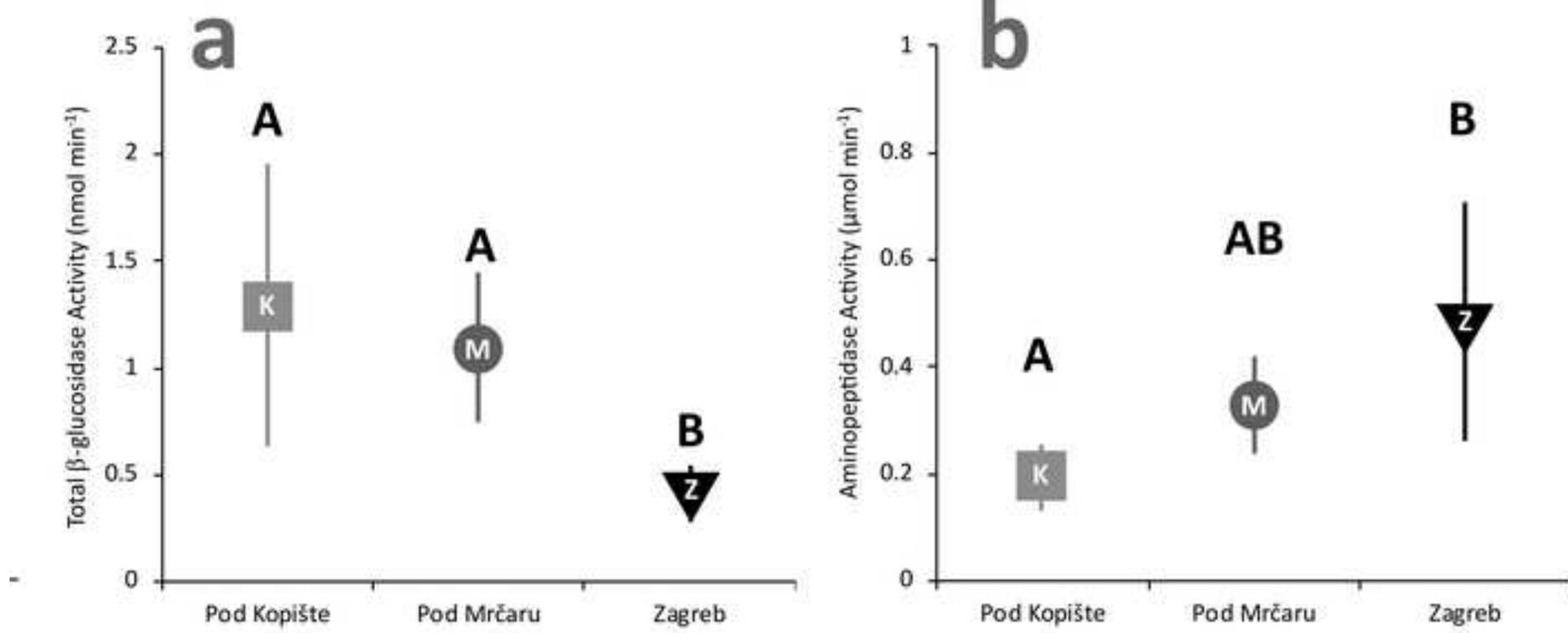


Figure 7

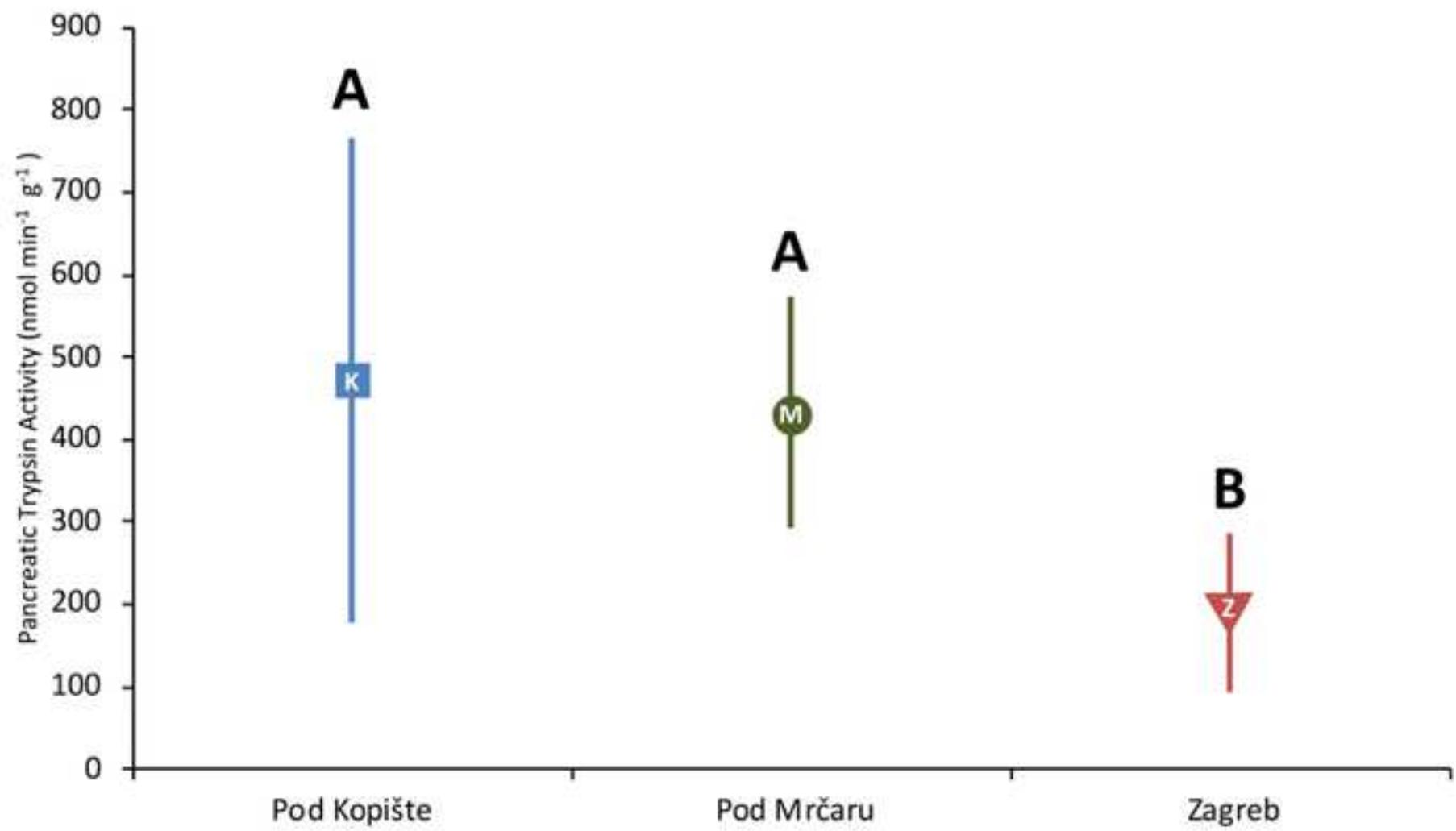


Figure 7 grayscale

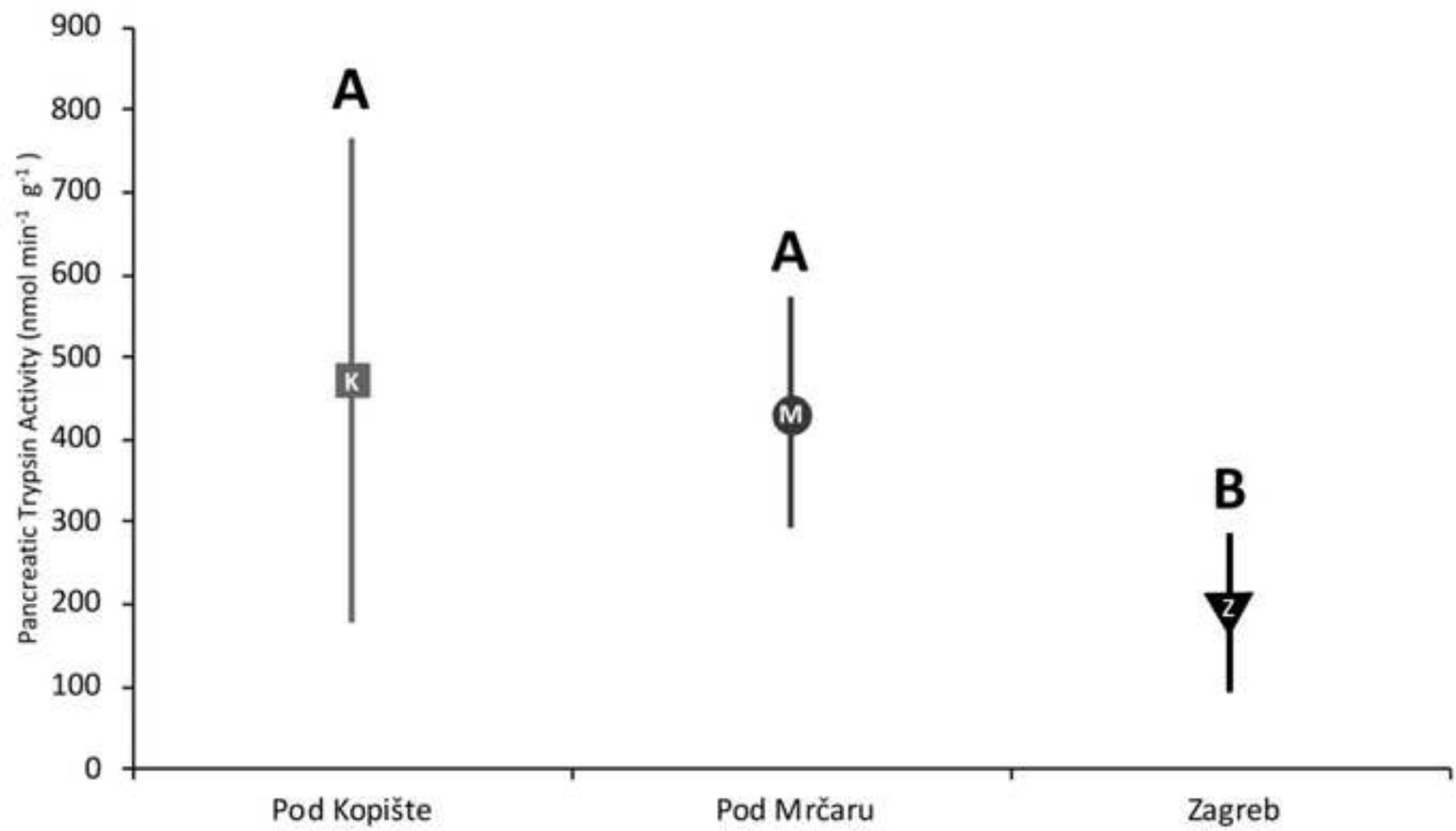


Figure 8

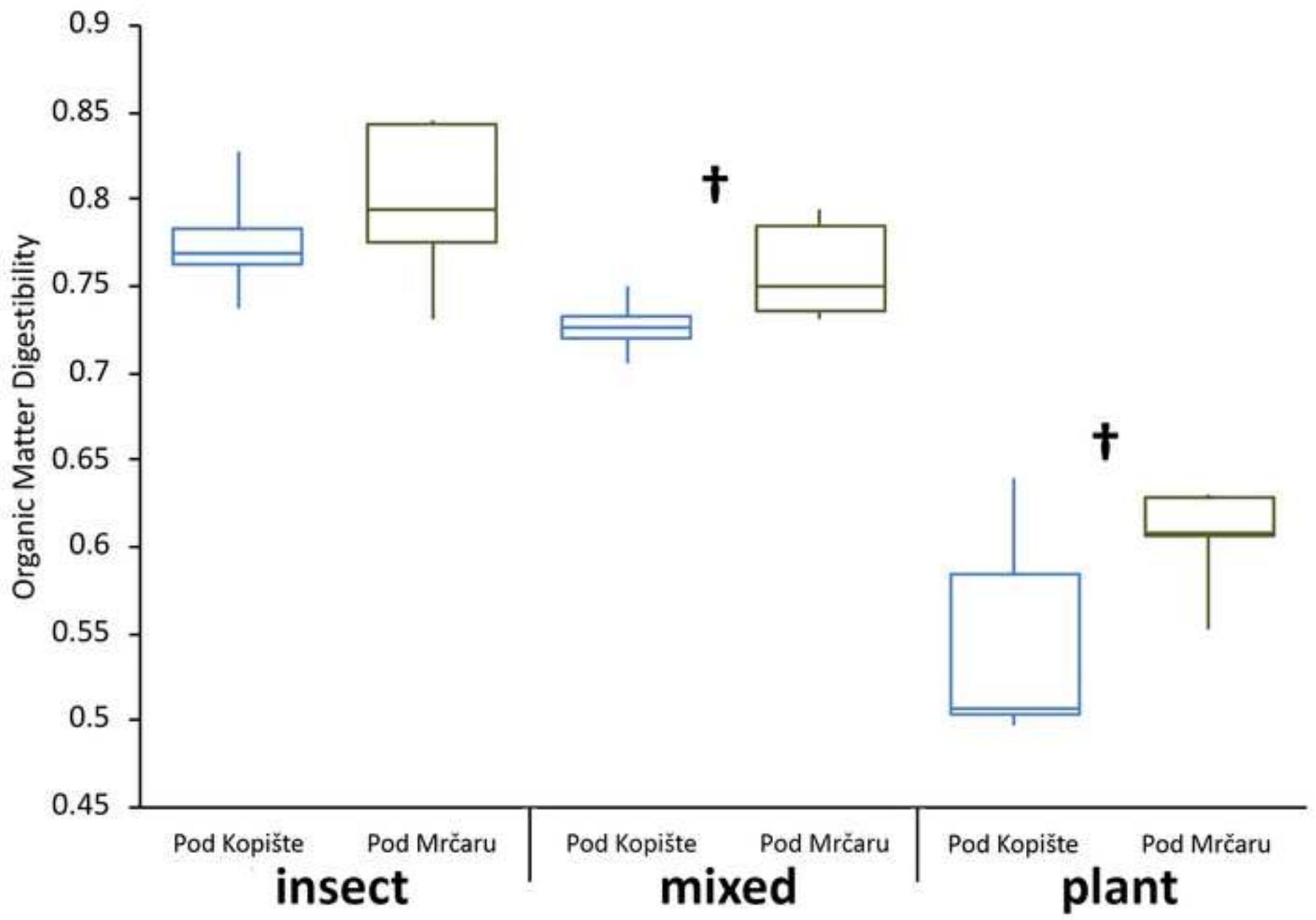
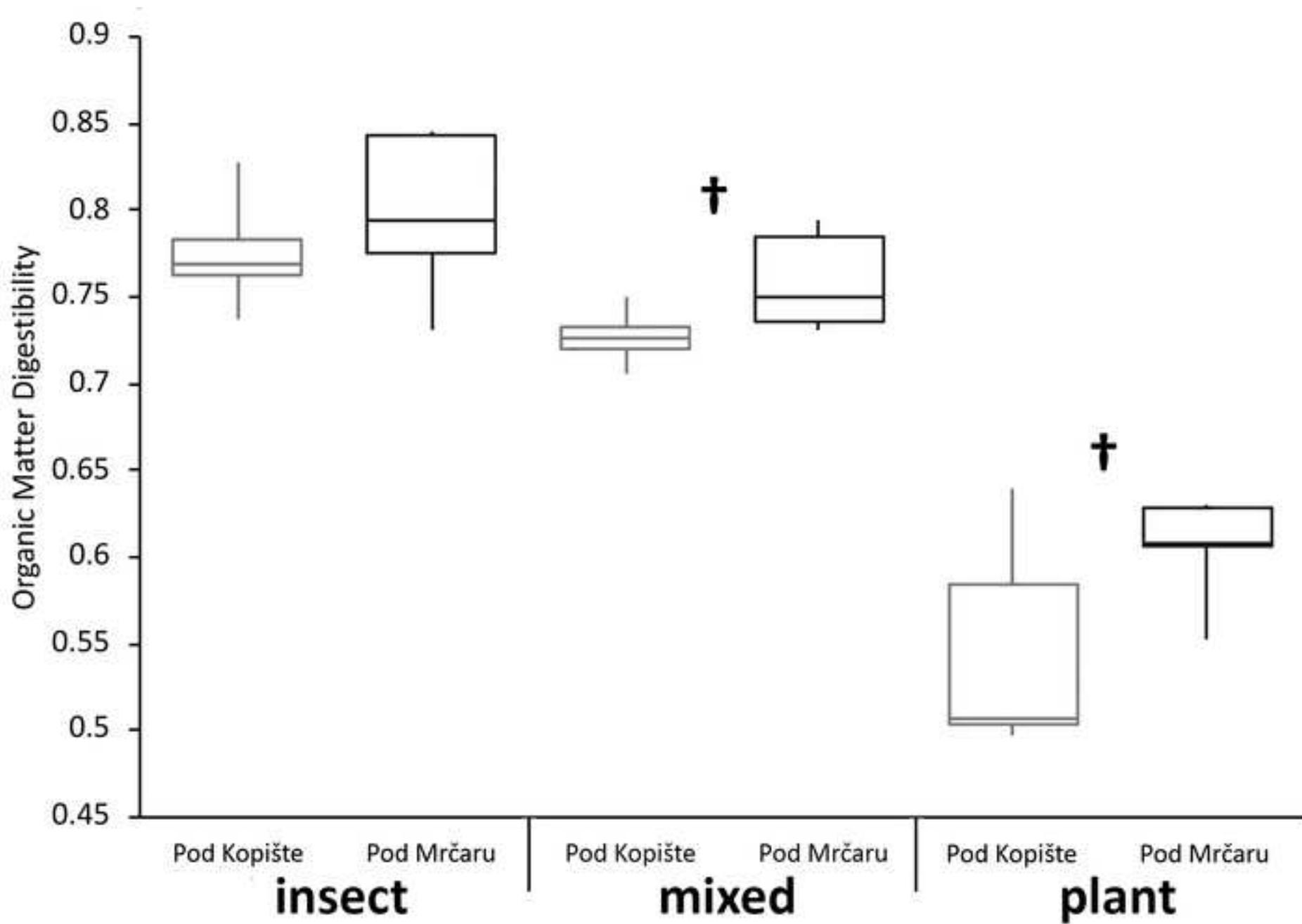


Figure 8 grayscale



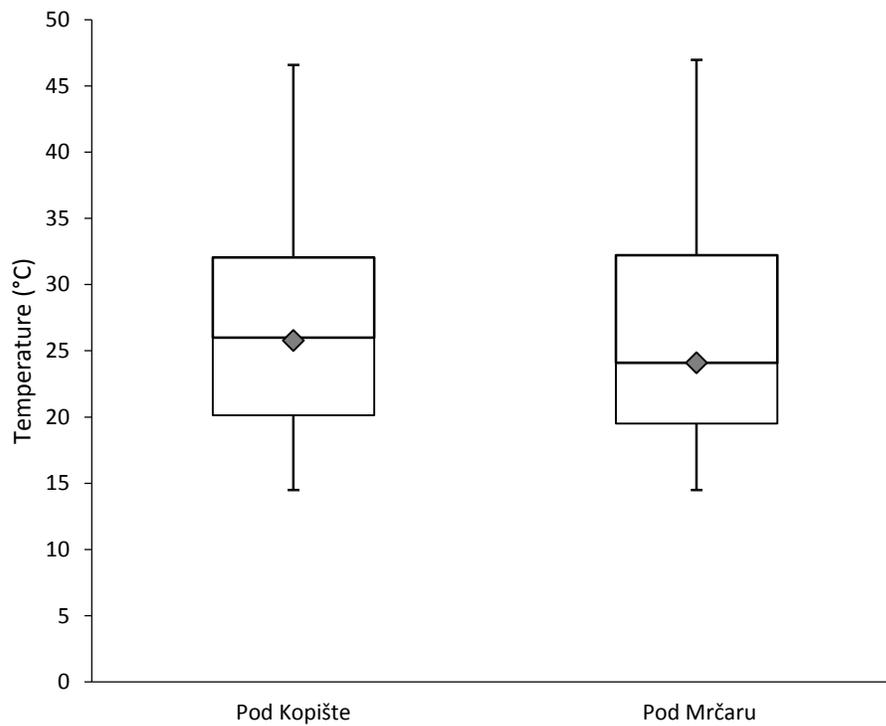
Wehrle *et al.* Supporting Information

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 \blacklozenge . Temperatures are not different by island.

Wehrle *et al.*, Supporting information

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%

Wehrle *et al.* Supporting Information

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

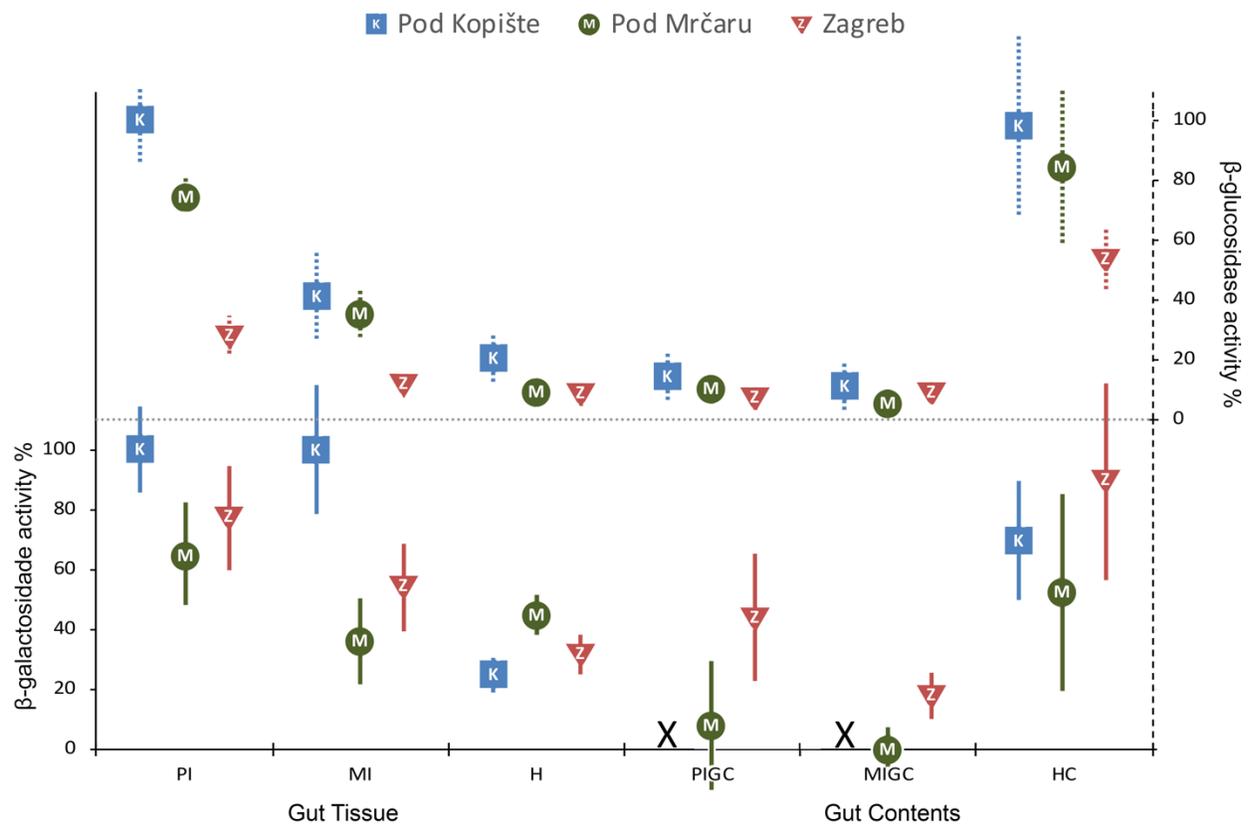
Wehrle *et al.* Supporting Information

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 \pm SEM.

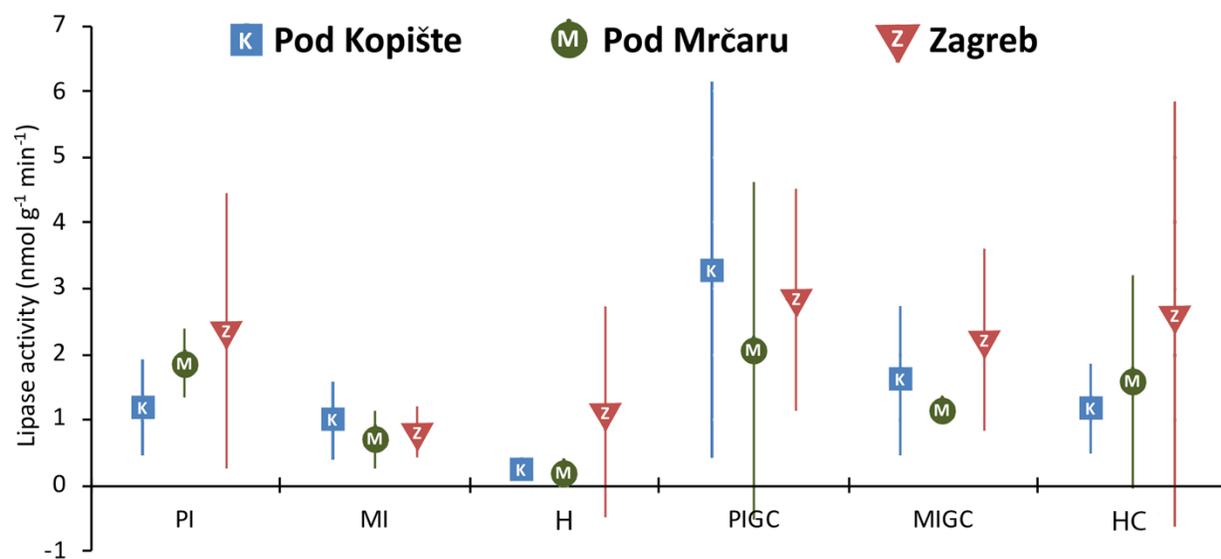
Wehrle *et al.*, Supporting information

Figure S3. Lipase activity in nmol p-nitrophenol liberated min⁻¹ for each gut region in Pod Kopašite (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 Kopašite $n=4-7$, Pod Mrčaru $n=4-6$ (MIGC region $n=2$), Zagreb $n=5-7$. There are no differences among populations by region.

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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 $F_{1,7}=0.841$, $P=0.390$

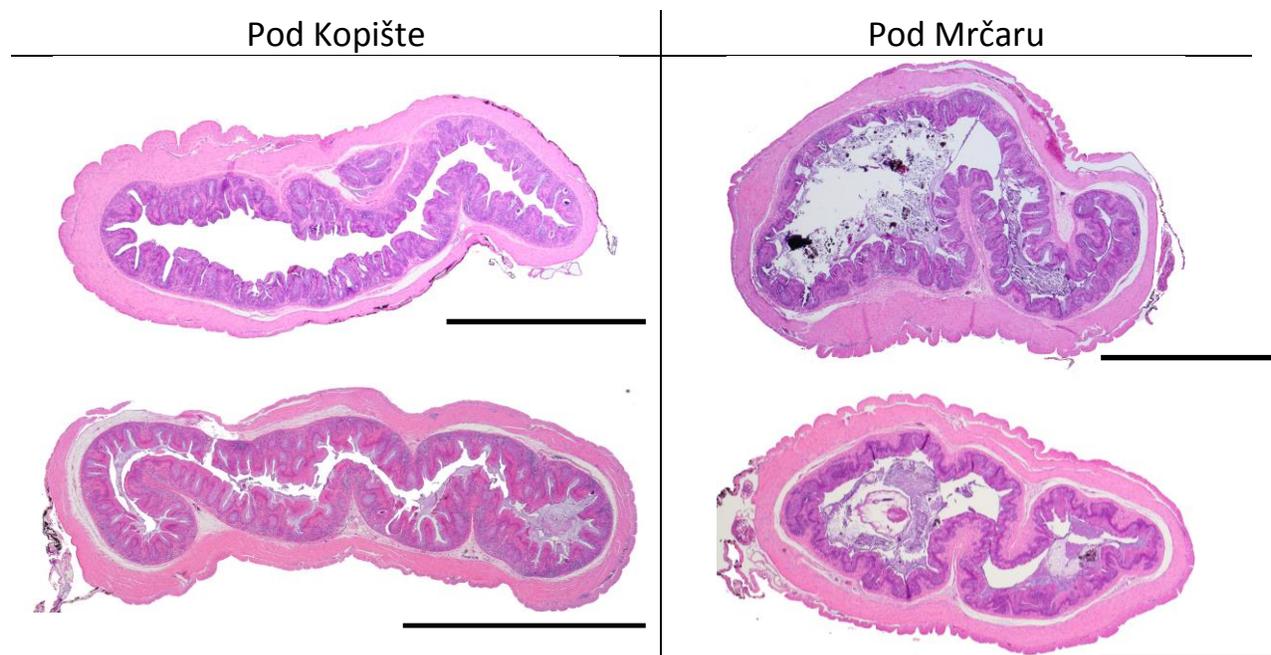


Figure S4. Cross section images are representative stained histological sections from the hindgut of Pod Kopašće (source) and Pod Mrčaru (new omnivore) populations (not to scale). Each section from a different individual collected in spring 2014 (Wehrle 2018). Scale bars to the lower right of each image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