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Who, When, and How Much? The Context Dependency of Rapid Evolution in Response to a Dietary Shift

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Ecology and Evolutionary Biology

by

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2018

DEDICATION

To the trans people who came before and did the work to make life possible
and carve out the niche for others to expand
To the trans people who emerged after and taught that it could be done unapologetically

To all the people putting themselves on the line to make
science and academia safer and more inclusive.

To Drs. Virginia M. Brothers and Joan C. Egrie
for their early science education and persistent encouragement.

To Dr. Thomas J. White for treating me as a colleague from the very beginning.

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UC Irvine is located on the occupied and unceded land of the Tongva and Acjachemen Nations. I recognize that by attending and working on the UC Irvine campus that I am benefitting from the continued denial of these nations' rights to their land.

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Wehrle, B.A. and D. P. German. 2014. Rapid physiological and performance changes in a newly herbivorous lizard. UC Irvine Associated Graduate Students Symposium.

Invited Presentations (continued)

Wehrle, B.A. 2011. Lizard herbivores, digestive microbes, and social aggregations. Capybara Seminar, Smithsonian Tropical Research Institute, Barro Colorado Island, Panamá

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Wehrle, B.A. 2010. Iguanas and microbes. Capybara Seminar, Smithsonian Tropical Research Institute, Barro Colorado Island, Panamá

Contributed Papers

Wehrle, B.A., M. Travern, M. Krajnović, Z. Tadić, A. Herrel, and D.P. German. 2018. Interplay of gut length, diet, and ecology in lacertid lizards. Society for Integrative and Comparative Biology Annual Meeting, San Francisco, CA [oral]

Wehrle, B.A.* 2017. Changes in digestive performance and physiology in a newly herbivorous lizard. Southern California Herpetologists' HerpFest, Riverside, CA [oral]

Wehrle, B.A.; Z. Tadić, M. Krajnović, K. Chernoff, A. Herrel, and D.P. German. 2017. Comparative nutrient digestibility between insectivorous and rapid-evolving herbivorous Italian Wall Lizards. Society for Integrative and Comparative Biology Annual Meeting, New Orleans, LA [oral]

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Wehrle, B.A.; B.Q. Nguyen-Phuc, R.K. Dang, M. Krajnović, Z. Tadić, A. Herrel, and D.P. German. 2016. Seasonal and sex effects on the digestive physiology of a newly herbivorous lizard. Society for Integrative and Comparative Biology Annual Meeting, Portland, OR. [poster]

Wehrle, B.A., M. Krajnović, Z. Tadić, A. Herrel, and D.P. German. 2015. Changes in digestive performance and gut structure and function in a newly herbivorous lizard. Society for Integrative and Comparative Biology Meeting, West Palm Beach, FL. [oral]

Wehrle, B.A., A. Herrel, Z. Tadić, and D.P. German. 2014. Testing the Adaptive Modulation Hypothesis: Physiological changes in a newly herbivorous lizard. Society for Integrative and Comparative Biology Meeting, Austin, TX. [poster]

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Wehrle, B.A. and R.E. Espinoza. 2012. Why do lizards lounge? The role of sociality in exchanging microbial communities among hatchling *Iguana iguana*. World Congress of Herpetology 7, University of British Columbia, Vancouver, BC, Canada. [oral]

Wehrle, B.A. 2012. Why do lizards lounge? The role of sociality in exchanging microbial communities among hatchling Green Iguanas. 2012 Sigma Xi Student Research Symposium, California State University, Northridge. [oral]

Wehrle, B.A. 2012. Gut bugs and lounging lizards: Comparisons of microbial communities in the hindguts of Green Iguanas. 16th Annual Student Research and Creative Works Symposium, California State University, Northridge [oral]

Contributed Papers (continued)

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Wehrle, B.A. 2011. Eat poop and thrive: testing the role of the lizard lounge for transferring digestive microbes. Joint Meeting of Ichthyologists and Herpetologists, Minneapolis, MN. [poster]

Wehrle, B.A. 2011. Why do lizards lounge? The role of sociality in exchanging microbial communities among hatchling *Iguana iguana*. 15th Annual Student Research and Creative Works Symposium, California State University, Northridge [oral]

Wehrle, B.A. 2010. Acquisition of fiber-fermenting microbes in hatchling green iguanas (*Iguana iguana*). 14th Annual Student Research and Creative Works Symposium, California State University, Northridge [oral]

Publications

Wehrle, B.A., and J.A. Guzman. 2012. *Iguana iguana* (Green Iguana). Predation. Herpetological Review 43:134.

As member of the Earth Microbiome Project Consortium

Thompson, L.R, J.G. Sanders, D. McDonald, A. Amir, J. Ladau, K.J. Locey, R.J. Prill, A. Tripathi, S.M. Gibbons, G.Ackermann, J.A. Navas-Molina, S. Janssen, E. Kopylova, Y. Vázquez-Baeza, A. González, J.T. Morton, S. Mirarab, Z. Zech Xu, L. Jiang, M.F. Haroon, J. Kanbar, Q. Zhu, S.-J. Song, T. Kosciolek, N.A. Bokulich, J. Lefler, C.J. Brislawn, G. Humphrey, S.M. Owens, J. Hampton-Marcell, D. Berg-Lyons, V. McKenzie, N. Fierer, J.A. Fuhrman, A. Clauset, R.L. Stevens, A. Shade, K.S. Pollard, K.D. Goodwin, J.K. Jansson, J.A. Gilbert, R. Knight, and The Earth Microbiome Project Consortium. 2017. A communal catalogue reveals Earth's multiscale microbial diversity. Nature 551: 457–463 doi:10.1038/nature24621

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Vigliotti, C., V. Lemieux-Labonté, S. Dowd, Z. Tadić, B. A. Wehrle, D. P. German, A. Herrel, F.-J. Lapointe, P. Lopez, E. Bapteste. Targeted changes in the gut microbiota of natural populations of lizards with different diets.

Wehrle, B. A. and D.P. German. Review: Digestion in reptiles.

Wehrle, B. A., A. D. Luu, M. Krajnović, S. T. Prasertphong, Z. Tadić, A. Herrel, and D.P. German. Nutrient digestibility in a newly omnivorous lizard shows performance differences with feeding frequency and sex.

Wehrle, B. A., G. E. Flores, and R. E. Espinoza. Intergenerational lizard lounges do not explain the variation in the microbiome of Green Iguanas.

ABSTRACT OF THE DISSERTATION

Who, When, and How Much? The Context Dependency of Rapid Evolution in Response to a Dietary Shift

By

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Doctor of Philosophy in Ecology and Evolutionary Biology

University of California, Irvine, 2018

Professor Donovan P. German, Chair

A population of Italian Wall Lizards (*Podarcis sicula*) on a small island in Croatia has become primarily herbivorous and morphologically distinct from its source population on a nearby island in ~30 generations, making it a compelling example of rapid evolution. What changes in digestive physiology and morphology have facilitated this switch to a novel diet? How does this dietary shift affect digestive performance? Do these accommodations for plant eating vary through time or with aspects of the lizards' life history? I compared gut size and histology, eight digestive enzyme activities, and products of microbial fermentation across the two island populations, with selected comparisons to a mainland outgroup of *P. sicula*. The newly omnivorous population had several targeted biochemical differences in their hindguts compared to their source population, but no large-scale changes in physiology or gut morphology to match the scale and direction of divergence in their diets. In fact, island-mainland effects were far more prominent, even between insectivorous populations.

To test digestive performance, in the lab I fed lizards insect, mixed, or plant diets daily (high frequency, island males only) or on alternating days (low frequency, island and mainland males and females). When fed with high frequency, the newly omnivorous population was better

at digesting plant proteins than their source population counterparts. However, when fed at the lower frequency, the males did not differ by population on any diet type. The females from the insectivorous source population, however, digested insect diets less efficiently than the other two populations.

To better elucidate dietary differences between the new and source populations lizards, I used stable isotope analyses to track ^{13}C and ^{15}N enrichment in their livers in concert with measurements of body and gut size over time and in males and females. Isotopic enrichment and gut length vary considerably by both year and season and suggest that the diet these lizards actually digest is not as different between the populations as stomach contents have suggested. Measurements of six digestive enzymes show differing patterns between populations depending on season and sex. Thus, the lizards' responses to this dietary shift are dependent on multiple contexts.

INTRODUCTION

How an animal acquires its nutrients lies at the intersection of its physiology, morphology, behavior, and ecology. Its diet and digestion can be modeled through economic theory (Karasov and Diamond, 1983; Ferraris and Diamond, 1989; Cant *et al.*, 1996), as chemical reactors (Penry and Jumars, 1986, 1986), as driven by nutrient scarcity (Clissold *et al.*, 2010, 2013), or driven by nutrient abundance (Karasov *et al.*, 1986; Levey and Martínez del Rio, 1999). Understanding an animal's methods of nutrient acquisition can have implications for its conservation, animal husbandry and economic impacts, community competition, symbioses and parasitism, ecosystem services, and large-scale nutrient cycling in the environment. Thus, investigations of diet and nutrient processing necessitate and promote integrative understanding of the whole animal and its environment.

The source of an animal's nutrients is subjected to restriction at multiple levels. The animal can only consume material is available in the environment at sufficient frequencies to make that diet profitable (Pyke *at al.*, 1977). It can only ingest food that it is morphologically, physiologically, and behaviorally able to handle (e.g. via sharp teeth, immunity to prey toxins, constriction). Of the ingested food, it can only break down material that has physical and chemical properties compatible with the structure, biomechanics of digesta flow, and enzymes of its gut. The degraded material can only be absorbed via sufficient quantities of the matching transporters in the appropriate areas of the gut. Those nutrients are then subject to further assimilation and metabolism. Through this lens, novel and specialized diets are an especially interesting context to examine the development and interaction of complex traits.

Digestion has been little studied in reptiles compared to other taxa. Lizards, however, provide intriguing examples for study of digestion due to their ectothermy, their broad environmental niches spanning across the globe, and their diverse evolutionary radiations, including dietary specializations for eating ants (Myers *et al.*, 2005), snails (Dalrymple, 1979), large mammals (Goldstein *et al.*, 2013), and plant material (Cooper and Vitt, 2002). Between <1-4% of lizards are primarily herbivorous (Espinoza *et al.*, 2004; Cooper and Vitt, 2002), yet this diet strategy has independently arisen >30 times (Espinoza *et al.*, 2004). Indeed, the transition to herbivory necessitates multiple complex traits to coevolve, nearly simultaneously. The digestive tract is expensive to maintain (Karasov and Diamond, 1983) and acquiring sufficient nutrients is crucial for not only the maintenance of the digestive tract, but survival and fitness. Herbivores generally have longer guts than carnivores with one or more valves in the hindgut to accommodate large, less energetic meals, increase the surface area for nutrient absorption, slow transit of digesta, and increase habitat for microbial endosymbionts (Iverson, 1982, Stevens and Hume, 1998, 2004). Herbivores are generally large bodied to accommodate larger, less energy dense meals, benefitting from metabolic scaling properties (Pough, 1973; Karasov and Martinez del Rio, 2007). They have higher bite forces (Metzger and Herrel, 2005) to more easily shear plant material, and microbial endosymbionts to aid in the breakdown of recalcitrant material (i.e. fiber; McBee and McBee 1982; Foley *et al.*, 1992; Pafilis *et al.*, 2007). Is there a set path for how these changes arise? How does an animal's gut form and function interact with other aspects of its biology with respect to a diet shift?

In this dissertation, I investigate the digestive morphology, function, and performance in a unique system of lacertids (one of the broadest radiations of lizards) in the context of their diet, sex, seasonality, and feeding regime. The Italian Wall Lizard (*Podarcis sicula*) is common

throughout southern Europe and is ancestrally insectivorous. However, in a pair of small islands in the Adriatic Sea of Croatia, there has arisen a prime ecological laboratory in which to observe the origination of a plant-based diet. In 1970, five pairs of *P. sicula* were moved from the island of Pod Kopište, Croatia to the nearby island of Pod Mrčaru as part of a competition experiment (Nevo *et al.*, 1972). Italian Wall lizards had not previously been found on Pod Mrčaru, but after several years, the population was thriving. Thirty-six years later, <30 lizard generations, the new population on Pod Mrčaru had morphologically and behaviorally diverged from the lizards of their source population on Pod Kopište (Herrel *et al.*, 2008). While the Pod Kopište lizards ate mostly insects, the Pod Mrčaru lizards had become omnivorous and developed several of the morphological traits associated with consuming a plant diet. Of particular interest was the presence of valves in the hindgut, a trait thought to be present only in highly derived herbivores. Has gut function also shifted with this diet change? How do these changes affect the functional performance of these lizards? Are strategies the same by sex? By season?

Comparative studies provide opportunities to test the "proximate" mechanisms through which specialization arises, and the consequences of specialization on organismal performance. How efficiently an animal digests and metabolizes its food is an important measure of performance, as this determines the maximum nutrients and energy available for its daily budget, as well as how often and how much it must feed to fuel the rest of its biological processes. Digestibility is affected by biochemical properties (i.e.: food type, enzyme activity), physics (i.e.: particle size, digesta flow), and mechanical constraints (i.e.: gut volume and surface area; Durtsche, 2004; Iverson 1982; Bjorndal *et al.*, 1990). Chemical Reactor Theory positions digestive efficiency as the outcome of the following physiological reactions (Penry and Jumars 1987; Karasov and Hume 1997; Karasov *et al.* 2011):

$$digestive\ efficiency \propto \frac{enzyme\ activity}{[substrate]} \propto \frac{gut\ volume}{digesta\ velocity} \propto time$$

Thus, to increase digestive efficiency of a nutrient, if more of the substrate is ingested, the specific digestive enzyme(s) to degrade that substrate must also increase. The same is true with an increase in the rapidity of digesta transit (or decrease in transit time/ gut passage time) necessitating a longer gut to allow for more contact with absorptive epithelia. With enough time, an animal can improve its digestive efficiency of a given diet. Indeed, these relationships can scale from simple substrates (e.g., dipeptides containing leucine) to whole complex diets (e.g., plants and animal prey) by matching enzyme specificity (e.g., Leucyl-aminopeptidase and total digestive enzyme activity, respectively).

The Adaptive Modulation Hypothesis (AMH; Karasov and Diamond 1983; Ferraris and Diamond 1989; Cant *et al.* 1996) is a modification of Chemical Reactor Theory in the context of Optimal Foraging Theory (Pyke *et al.*, 1977) and symmorphosis (Weibel and Taylor, 1991) as articulated by Diamond (1991). Optimal Foraging Theory postulates that an animal will consume the diet that affords them the highest net energy gain through maximizing food energy (e.g. more nutrient dense foods, larger portions) and minimizing their energy output to obtain that food (e.g. through easier to find foods and thus less energy spent searching, easier to consume foods such as smaller prey with softer shells, etc.). In the context of the AMH, digestive function should function like Optimal Foraging Theory, maximizing energy absorbed from the diet (e.g. producing enough enzymes to degrade the food taken in, maintaining long and complex enough intestines to absorb the energy digested) while minimizing energy spent to digest the food (e.g. producing only the enzymes that are needed, limiting the length and mass of the gut to the minimum necessary to absorb the energy digested). Symmoprohsis was proposed in the context of respiratory performance, hypothesizing that the structure of the lungs and circulatory system

would be exactly matched to respiratory demand. Although this concept was generally found to be unsupported (Dudley and Gans, 1991), in the context of digestion (i.e. the AMH), an increase or decrease in a dietary substrate should lead to a proportional increase or decrease, respectively, in the magnitude of the structure and functional characters of the gut associated with that substrate.

The AMH assumes that the goal of digestion is to maximize nutrient acquisition and minimize energy expenditure. While the Chemical Reactor Theory leads to many of the same hypotheses as the AMH, the AMH is more constrained by matching magnitude of substrate intake with the optimal magnitude of response for gut form and function. Both models lead to expectations of higher activities of enzymes related to plant material degradation (e.g. amylase to digest starch) in the newly omnivorous population, equivalent activities of general use enzymes (e.g. trypsin to digest protein), and lower insect degrading enzymes (e.g. trehalase to digest a sugar found in insect blood). These models also provide frameworks for expectations of seasonal differences. For example, in spring when the new omnivores consume less plant material, Chemical Reactor Theory predicts amylase activity will decrease, the AMH predicts amylase activity will decrease proportionally to the drop in plant matter in the diet. These models also predict shifts in gut size and structure.

As an alternate hypothesis, the Nutrient Balancing Hypothesis (Clissold *et al.*, 2010, 2013) posits that when an essential nutrient is rare in the diet, as the restrictive nature of nutrient acquisition aides, a process of digestion will be disproportionally increased to make up for the scarcity. For example, if carbohydrates are low in the ingested diet, the Nutrient Balancing Hypothesis predicts that amylase activity will be high to ensure that all starch that comes through the gut gets degraded to easily absorbable glucose. While the Nutrient Balancing Hypothesis is

generally most specific to predicitons of enzyme activities, it could be applied to specific nutrient transporters.

Unlike the previously mentioned theory and hypotheses, the Rate vs. Yield model (Sibly, 1981) focuses on nutrient density and intake to predict patterns of nutrient acquisition. This model assumes that nutrient density and intake have a negative relationship (e.g. high nutrient density diet requires low intake of that diet). Within this conceptual framework, a "rate maximizer" is predicted to have a voluminous, nutrient poor diet, a long gut, a high digesta transit rate, and high enzyme activities for degrading easy to digest material, and a lower likelihood of microbial fermentation. This model is not mutually exclusive with the others mentioned as it prioritizes overall intake amount over specific nutrients.

By considering these theoretical frameworks to predict changes in gut form and function and digestive performance in a newly omnivorous lizard and its source population, I am able to integrate multiple complex aspects of these lizards' biology to understand what happens when diet shifts. These studies are among the most comprehensive investigations of the digestive tract in wild-caught lizards to date.

Chapter 1

Rapid evolution and a shift to omnivory in *Podarcis sicula* resulted in localized changes in gut structure and function

Introduction

What an animal eats and digests is often viewed through an evolutionary lens, and differences in gut structure and function accommodating different diets are often considered as adaptive traits gained over evolutionary time scales (Karasov and Martínez del Rio, 2007; German *et al.*, 2004, 2010a). However, both rapid dietary shifts and rapid evolution of complex traits can occur, even in vertebrate animals (Herrel *et al.*, 2008; Lallensack, 2018). For example, in 1971, five male-female pairs of Italian Wall lizards (*Podarcis sicula*) were moved from the island of Pod Kopište (0.09 km^2), Croatia, to the nearby island of Pod Mrčaru (0.03 km^2) as part of a biological invasion study (Fig. 1.1; Nevo *et al.*, 1972). Returning to the Croatian islets in 2004-2006 (<30 *P. sicula* generations later), Herrel and colleagues (2008) found the new population on Pod Mrčaru had morphologically and behaviorally diverged from their source population on Pod Kopište. While the Pod Kopište lizards were insectivorous, consuming 4-7% plant material (by mass), plants made up 34-61% of the Pod Mrčaru population's intake. The Pod Mrčaru lizards were larger, had different head shapes, and larger bite forces. Additionally, both adult and neonate lizards from Pod Mrčaru had developed valves in their hindguts, a feature not found in the Pod Kopište population. Hindgut valves in lizards are generally associated with highly derived herbivory (Iverson, 1982; Bjorndal, 1997; Stevens and Hume, 2004). These valves can slow the passage of digesta to allow more time for chemical processing and increase surface area for nutrient absorption and endosymbiotic microbial attachment. Morphological and dietary

changes have been documented between the Pod Mrčaru lizards and their source population on Pod Kopište (Herrel *et al.*, 2008), however it is unknown if the appearance of hindgut valves is concomitant with other shifts in gut morphology or function. Thus, the island populations of *P. sicula* offer the rare opportunity to examine evolution in action in a vertebrate under natural conditions.

The Chemical Reactor Theory (CRT) of digestion (Penry and Jumars, 1986, 1987) posits that the goal of digestion is to optimize nutrient or energy gain. Thus, the digestive tract may be morphologically and physiologically optimized for the food that is being digested (Karasov and Douglas, 2013). This leads to the following relationships:

$$Digestive\ efficiency \propto \frac{enzyme\ activities}{substrate\ concentration} \propto \frac{gut\ size}{digesta\ transit\ rate}$$

(modified from Sibly, 1981; Karasov and Douglas, 2013).

To maximize net nutrient gain, a diet shift should lead to changes in gut morphology and physiology to match the new diet. For example, per the above proportions, increased digestive substrate (e.g., starch) requires increases in matched enzyme activities (e.g., amylase activity) to maintain the same digestibility of the nutrient. Increased food intake (e.g., due to high-fiber content of the diet) speeds up digesta transit rate, requiring an increase in gut size to balance this proportion. Hindgut valves, like the ones observed in the Pod Mrčaru lizards, can both increase overall gut surface area and can slow the passage of digesta by acting as "baffles," maintaining nutrient digestibility (Karasov and Martínez del Rio, 2007). Since the presence of hindgut valves in the Pod Mrčaru lizards fits the predictions of the CRT, we posit that other shifts in digestive tract form and function have occurred to accommodate their omnivorous diet. Especially because Pod Mrčaru lizards showed approximately twice the organic matter digestibility as Pod Kopište

lizards when they were fed an herbivorous diet in the laboratory ($F_{1,8}$=7.495, P=0.0255; Wehrle, *Chapter 2*).

Herbivores generally have larger guts than carnivores (Wagner *et al.*, 2009; Stevens and Hume, 2004; Dearing, 1993) to accommodate more voluminous meals (Pough, 1973; Wilson and Lee, 1987), increase nutrient absorption (O'Grady *et al.*, 2005; Bjorndal *et al.*, 1990; Bjorndal and Bolten, 1992), and increase microbial habitat (Stevens and Hume, 2004, 1998). With their diet shift, it would follow that Pod Mrčaru lizards should have larger guts to accommodate the larger intake of a plant diet. This may manifest as longer intestines, increased villi, folding, and valves that would increase surface area, or slow digesta transit. These strategies are not mutually exclusive; thus, combinations of increased intestinal length and cross-sectional surface area would lead to exponential increases in overall gut size (Leigh *et al.*, 2018a). We summarize our predictions in Table 1.1.

As reptiles do not masticate their food as extensively as mammals (Fritz *et al.*, 2010), most of their digestion relies on chemical, not physical, breakdown. With a diet richer in plant material, we expected the Pod Mrčaru lizards to have higher biochemical specificity for digesting substrates present in plant material (Table 1.1). In line with the CRT, we predict that carbohydrase activities (e.g., amylase) would be higher in the guts of Pod Mrčaru lizards (German *et al.*, 2010a; Kohl *et al.*, 2011; German *et al.*, 2015) while protease, lipase, and chitinase activities would be higher in the guts of insectivorous Pod Kopište and mainland populations of this lizard species (German *et al.*, 2010a; German *et al.*, 2015; Schondube *et al.*, 2001; Marsh *et al.*, 2001). There should, however, be no differences in enzymes that would be used generally by each population, such as aminopeptidase to cleave dipeptides (Karasov and Martínez del Rio, 2007; German and Bittong, 2009; German *et al.*, 2004). Additionally, the site

of enzyme activity affects absorption, enzyme interactions, and digestive efficiency (Vonk and Western, 1984; Stevens and Hume, 2004; Clements and Raubenheimer, 2006; Tengjaroenkul *et al.*, 2000; German *et al.*, 2015). Based on patterns seen throughout vertebrates, we expect pancreatic, brush border, and microbially-derived enzymes along *P. sicula* guts to present the patterns illustrated in Fig. 1.2 (Clements and Raubenheimer, 2006; German *et al.*, 2015; Stevens and Hume, 2004).

Because vertebrates do not endogenously produce enzymes (e.g., cellulase) to break down plant fiber (e.g., cellulose), many herbivores and omnivores rely on microbial symbioses, usually in the hindgut, to digest these carbohydrates and can derive a large portion of their energy intake from microbial fermentation (Stevens and Hume, 1998; McBee and McBee, 1982; Bjorndal *et al.*, 1997). These microbial fermentations produce short chain fatty acids (SCFA) that can be easily assimilated across the gut wall by the host (Foley *et al.*, 1992; Bergman, 1990). Thus, Pod Mrčaru lizards may be reliant on microbial fermentation, indirectly measured via the products of fermentation, SCFA, in their hindguts.

By differentiating between endogenous and exogenous sources of chemical digestion, we can better understand what kinds of changes in digestive physiology have occurred in such a short time. If pancreatic or brush border enzymes aimed at digesting components of plant material are higher in the Pod Mrčaru lizards than their insectivorous counterparts (Table 1.1), we can conclude that the lizards themselves have developed mechanisms for increasing relevant enzyme activities (e.g., via increased expression of digestive enzyme genes; German *et al.*, 2016). If enzyme activities are increased in the distal intestines and concentrations of SCFAs are higher, it is likely that these digestive responses are due to microbial endosymbionts. These possibilities, too, are not mutually exclusive and lizard tissue evolution and microbial community

shifts may both contribute to the Pod Mrčaru omnivores' ability to subsist on a diet rich in plant material. Overall, our study aims to elucidate what changes— including symbionts, and host physiology and behavior—have occurred over this ecological timescale to accommodate a drastic dietary shift.

Materials and Methods

Diet analysis

From August 29-September 2, 2013, we flushed the stomachs of *Podarcis sicula* from the islets of Pod Mrčaru (N=34) and Pod Kopište (N=30), Croatia, following Herrel *et al*. (2006, 2008). Stomach contents were stored in 70% ethanol. Contents from Zagreb lizards (N=7) were obtained from frozen stomachs of dissected animals. We divided stomach contents into plant matter, arthropods, and "other." We treated stomach contents as a proxy for ingested diet and determined the total mass and the relative proportion of plant and arthropod prey.

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

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

Lizards were weighed to the nearest 0.1-g and euthanized via intramuscular injections of sodium pentobarbital (~0.1mg/g-tissue). We measured snout-vent length (SVL) and dissected the lizards on sterilized, chilled dissecting trays (~4°C). We removed the entire gut from esophagus

to cloaca and measured the whole gut length. We divided the gut into stomach, proximal intestine (PI), mid intestine (MI), and distal intestine (DI). The distal intestine was easily identifiable (see Fig. 1.2) and the proximal and mid intestine portions were separated by dividing the remaining intestine in half. In seven individuals from each population, we removed the gut contents from the proximal, mid, and distal sections (e.g., Proximal Intestine Gut Contents, PIGC) and flushed out the intestinal tissue with chilled 25 mM tris-HCl, pH 7.5. Gut tissues and contents from each gut region and pancreases were frozen separately in 1.5 mL vials in liquid nitrogen for storage and transport. Vials were transported on dry ice to the University of California, Irvine, where they were stored at -80°C until used. We used pH indicator paper (Macherey-Nagel, Düren, Germany: pH 1-14, 5.5-9.0, and 8.0-10.0) on dissected Zagreb lizards to measure intestinal fluid pH for each gut region.

For the remaining six lizards from each population, we preserved the PI, MI, and DI in McDowell Trump's fixative (4% formaldehyde, 1% glutaraldehyde, McDowell and Trump, 1976) for subsequent histological analyses.

We compared gut length (including stomach) among populations with an ANCOVA, using SVL as a covariate.

We weighed frozen gut sections and gut contents (excluding stomachs) to the nearest 0.001 g. We summed the masses of the gut tissues for each individual lizard to get total gut mass and compared both regional and total gut masses among populations with ANCOVA, using body mass as a covariate. Additionally, we divided gut content mass for each region by total gut content mass to determine the proportion of digesta retention in each gut region.

Estimation of intestinal surface area using histology

Gut sections preserved in Trump's Solution were further sectioned into 3-10 mm sections with a razor blade and rinsed in phosphate buffer pH 7.5 (PBS) for 3 x 20 min., and overnight in PBS at 4°C under constant shaking. The PBS rinsed tissues were flushed with running deionized water for 2 x 20 min., and then were subjected to serial ethanol dilutions of 30%, 50%, and 75%. We selected the proximal portions of the PI, MI, and DI from Pod Mrčaru and Pod Kopište lizards, and portions starting at the half way point of the distal intestine (DI+) from all three populations. Tissue portions were placed in tissue cassettes wrapped in ethanol-soaked cheesecloth, sealed in plastic bags, and were sent to Mass Histology Services (Worcester, MA, USA) for embedding in paraffin wax. We stained 7-μm sectioned samples with hematoxylin and eosin and imaged them with a Zeiss Axioplan 2 epifluorescence microscope and Zeiss and Cannon cameras. Tiled images were assembled using the Photomerge function of Adobe Photoshop CS3. We analyzed 1-25 sections of each sample by measuring the perimeters of mucosa and serosa. We then calculated the epithelial surface magnification (ESM) as the ratio of mucosal to serosal perimeters (German, 2009; Hall and Bellwood, 1995) to observe how much the mucosal folds increase the inner surface area of the intestine relative to a smooth bore tube.

Homogenate preparation

We homogenized frozen tissues following German and Bittong (2009). We diluted the tissues in the following chilled buffers: pancreases (P) diluted 50-300 volumes and gut contents (PIGC, MIGC, DIGC pellet) diluted 5-300 volumes in 25 mM tris-HCl buffer, pH 8.6 and, intestinal wall tissues (PI, MI, or DI) diluted 10-50 volumes in 350 mM mannitol in 1 mM Tris-HCl, pH 8.6. We chose buffers at pH 8.6 because it was the average pH we measured in the intestinal fluids of the *P. sicula* from Zagreb. For all tissues, we used a Polytron homogenizer (Binkmann

Instruments, Westbury, NY) with a 12mm generator set to 1100-3000 rpm for 3 x 30 s, with 30 s between pulses to homogenize tissues. Tissue homogenates were centrifuged at 9400 x *g* for 2 min. To ensure the rupture of the microbial cells and the release of all enzymes within gut content samples, these samples were sonicated (CL-18 Sonicator, Fisher Scientific, Waltham, Massachusetts, USA) at 5 W output for 3 x 30 s, with 30 s intervals between pulses, followed by homogenization, as described for the gut tissues. The gut content samples were centrifuged at 12000 x *g* for 10 min. All supernatants were stored in 100-200µl aliquots at -80° C until just before use in digestive enzyme assays. For the DIGC, we thawed the sample at room temperature for transfer to a spin column (Corning Costar Spin-X Centrifuge cellulose acetate tube filters, 0.22 µm pores) and centrifuged at 14000 x *g* at 4°C to gather DI fluid. The filtered fluid was frozen at -80°C for use in SCFA measurements. The remaining DIGC pellet was then prepared for enzyme assays in the same manner as the other gut contents (German and Bittong, 2009).

Biochemical Assays of Digestive Enzyme Activity

We conducted digestive enzyme assays following protocols outlined in German and Bittong (2009) and German et al. (2015). We ran all assays at 25°C, the mean temperature from May–September (confirmed by iButtons, Maxim Integrated, San Jose, CA, USA; Fig. 1.3) on the islands. We measured enzyme activities in duplicate or triplicate and read absorption or fluorescence in flat-bottomed 96-well microplates using a BioTek Synergy H1 Hybrid spectrophotometer/ fluorometer equipped with a monochromator (BioTek, Winooski, VT, USA). Our primary buffer was 25mM Tris-HCl, pH 8.6 (referred to henceforth as "buffer," any deviations are noted), measured at room temperature (22°C). Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). We optimized each assay for duration and homogenate

volume. Each enzyme activity was measured in each gut region (PI, MI, DI, PIGC, MIGC, DIGC) for each lizard. Pancreatic tissue was only used for measuring pancreatic enzyme activity (i.e.: α-amylase, trypsin, and lipase). We simultaneously conducted control experiments using homogenate or substrate blanks in buffer to check for endogenous substrate and/or product in the substrate solutions. For all kinetic assays, we determined the slope of the longest linear section of absorbance vs. time and used the standard curve of the product to calculate enzymatic activity U per gram wet mass of tissue.

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

We measured β-glucosidase, β-galactosidase, and N-acetyl-β-D-glucosaminidase (NAG) following German *et al.* (2011) and German *et al.* (2015) using 200 μM solutions of 4-methylumbelliferyl-β-D-glucoside, methylumbelliferyl-β-D-galactopyranoside, and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, respectively. These assays were run as kinetic fluorometric assays read at 365 nm excitation and 450 nm emission for 30 min to detect a 4-methylumbelliferone – MUB product as U (nmol MUB released min^{-1}) per gram of tissue.

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

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

In addition to the regional enzyme activities (U x g^{-1}), we calculated the total gut enzyme activities as the sum of mass-specific activity for each region multiplied by the tissue mass to yield total U (µmol product released min^{-1}). We did not include pancreatic samples in total gut enzyme activities as this region does not interact directly with nutrients.

Fermentation Analyses

To determine symbiotic microbial fermentation, we measured the relative concentrations of short chain fatty acids (SCFAs) in the DIGC fluid (following methodology in Pryor and Bjorndal,

2005; German and Bittong, 2009; and German *et al.*, 2015) from Pod Kopište (N=3) and Pod Mrčaru (N=4) lizards. We hand injected 2 μL of thawed DIGC fluid into a 2-m long stainless steel column (3.2 mm ID) packed with 10% SP-1000 and 1% H_3PO_4 on 100/120 Chromosorb W AW (Supelco, Inc., Bellefonte, PA, USA) attached to a Shimadzu GC-mini-2 gas chromatograph with flame ionization detector (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). We quantified SCFA concentrations via a Hewlett-Packard HP3392A (Hewlett-Packard Co., Palo Alto, CA, USA) integrator attached to the gas chromatograph. We calibrated the system with an external standard of 100 mg L^{-1} each of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate. The SCFA concentrations are expressed as mM of gut fluid.

Statistical Analyses

We preformed all statistical analyses in R (version 3.3.2). All data were screened for equal variances using a Bartlett's test and normality of residuals using a Shapiro-Wilk's test. If the data were not naturally parametric, we employed transformations. For propionate concentration comparisons, we used Wilcoxin Signed Rank tests. We used Tukey's HSD test with a family error rate of P=0.05 to identify pairwise differences following any ANOVAs that indicated significant differences. We analyzed all data by population. Additionally, we compared gut content mass and β-glucosidase activity among gut regions within populations. All data were normalized to mass or were proportions, except total enzyme activities. We found no effect of covariance between total enzyme activity and any measured parameter (lizard mass, SVL, gut mass, gut length, and gut content mass), thus report only the results of ANOVAs.

Results

Diet

Pod Mrčaru lizards had more massive stomach contents, implying they consumed more food than Pod Kopište and Zagreb populations ($F_{2,68}$=16.88, P<0.001). Plant material consumption was highest in the Pod Mrčaru *P. sicula* (see Table 1.2 for specific data) at 64% the ingested diet by mass compared to 24% in Pod Kopište lizards and 2% in Zagreb lizards ($F_{2,63}$=15.347, P<0.001).

Gut size

Lizards from Pod Kopište (3.6% of total body mass) and Pod Mrčaru (3.3%) had more massive guts than did lizards from Zagreb (2.4%; ANCOVA Population: $F_{2,20}$=9.534, P<0.002; body mass, model run without interaction term as it was non-significant; Fig. 1.4). Moreover, both island populations showed relative regional gut masses of the mid intestines that were lighter than either the proximal or distal intestine, showing that although the distal intestine section may be shorter than the others (Fig. 1.2), the distal intestine is heavier than the mid intestine (P<0.001; Fig. 1.4). We found no other differences in gut morphology or gut content distribution beyond that reported with diet above. We found no differences in gut length ($F_{2,36}$=2.551, P=0.0921) among the three populations.

Regional gut masses (i.e.: PI, MI, DI) and total gut mass (Table 1.3) did not differ by population nor covary with lizard body mass. There were no significant interactions between these factors on regional gut mass.

No population differed in total gut contents with respect to body mass ($F_{2,18}$=2.102, P=0.163). Gut contents were evenly distributed throughout the PI, MI, and DI. None of the populations retained more contents in a particular gut region than did the other two populations, excepting the higher mass of digesta in the Pod Mrčaru lizards' stomachs, mentioned above.

Our comparisons of relative ESM were not different in Pod Kopište versus Pod Mrčaru lizards in any gut regions (Fig. 1.5). The mucosa of the proximal intestine was 6.26±0.33 x serosa (Pod Kopište vs. Pod Mrčaru: t=0.8234, P=0.4418). This ratio decreased distally along the gut: the mid intestine, 3.63±0.33 x serosa (Pod Kopište vs. Pod Mrčaru: t=-0.14131, P=0.8923), a proximal part of the distal intestine, 2.44±0.32 x serosa (Pod Kopište vs. Pod Mrčaru: t=0.64699, P=0.5462), and more distal portions of the distal intestine (DI+), 1.95±0.2422 x serosa (Pod Kopište vs. Pod Mrčaru: t=1.86, P=0.1122). Fig. 1.5 also shows representative sections. We did not identify any qualitative differences between the cross sections of either population.

Digestive Enzyme Activities

Carbohydrases—The mass-specific α-amylase activity in the distal intestinal contents was almost 6-fold higher in Pod Mrčaru lizards compared to that measured in the Pod Kopište population (t=-0.266, P=0.038, Fig. 1.6a, Table 1.4).

Total maltase activity was three times higher in the Zagreb population than in the Pod Kopište lizards ($F_{2,17}$=6.876, P=0.007; Fig. 1.7a). The Pod Mrčaru lizards had a total maltase activity intermediate to, but not different from, that of their source population or the Zagreb outgroup.

Trehalase activity was lowest in the Pod Kopište lizards compared to the other two populations DI and DIGC (Fig. 1.6d).

The β-glucosidase activity was nearly double in the PI of Pod Mrčaru and Pod Kopište lizards compared to the Zagreb lizards ($F_{2,15}$=14.33, P<0.001; Table 1.4). Activity was highest in the PI and DIGC regions for all populations. This pattern was most pronounced in the two island populations. Other than those reported in the PI and DIGC, we found no regional differences among the populations, including total β-glucosidase activity (Fig. 1.6b). β-galactosidase and β-glucosidase showed different regional patterns throughout the gut (Fig. 1.8). See Table 1.4 for values.

In the PI of the Pod Kopište lizards, the N-acetyl-β-D-glucosaminidase (NAG) was 2x greater than in the PI of the Pod Mrčaru and Zagreb populations ($F_{2,15}$=5.867, P=0.013; Table 1.5). In all populations, the greatest NAG activity was found in the DIGC.

Proteases—In the DIGC, the mass specific trypsin activity was >5-fold higher in the Pod Mrčaru population than the Pod Kopište population ($F_{2,11}$=5.334, P=0.024; Fig. 1.6e, Table 1.5). The mass specific trypsin activity in the pancreas was higher in the island lizards than in the Zagreb population ($F_{2,23}$=5.139, P=0.0143, Fig. 1.9). The pancreatic trypsin activity was >2.5x higher in Pod Kopište and Pod Mrčaru lizards (0.4535±0.0504 μmol p-nitroaniline released min^{-1} g^{-1}) than in the pancreases of Zagreb lizards (0.1901±0.0397 μmol p-nitroaniline released min^{-1} g^{-1}).

The aminopeptidase activity was higher in the Zagreb population than in the island lizards in the MI, PIGC, and MIGC. The total aminopeptidase activity throughout the gut (Fig. 1.7b) was nearly 3-fold higher in the Zagreb population than in the Pod Kopište and Pod Mrčaru populations ($F_{2,17}$=18.91, P<0.001; Table 1.4). Compared to the Zagreb population, the island

lizards had a >32-fold higher aminopeptidase activity in PIGC ($F_{2,12}$=14.58, P<0.001). Mass specific aminopeptidase activity in MI ($F_{2,17}$=15.14, P<0.001) and MIGC ($F_{2,12}$=12.33, P<0.001) tissues were considerably higher in Zagreb lizards, but not as exaggerated as in the PIGC (Fig. 1.6f). However, these regional patterns are consistent with typical brush border enzyme activities.

Lipase— We found no differences in lipase among populations.

Microbial Fermentation

The Pod Kopište *P. sicula* had >3x total SCFA concentrations (t=6.422, P=0.001) in their DIGC than Pod Mrčaru lizards (Table 1.5). This was primarily due to nearly four-fold increases in acetate (t=9.058, P<0.001) and isobutyrate (t=3.796, P<0.001) concentrations in the Pod Kopište population. However, even with these two SCFAs omitted, non-significant increases in propionate, butyrate, and valerate (but not isovalerate) contributed to increased total SCFA concentrations (t=3.997, P=0.010) in the hindguts of the Pod Kopište lizards. Proportionally, acetate concentration was higher in the Pod Kopište lizards (Table 1.5; t=2.165, P=0.049). Although total SCFAs were lower in the Pod Mrčaru population, isobutyrate (t=-2.743, P=0.041) and isovalerate (t=-4.761, P=0.005) were higher proportionally.

Discussion

Differences between island populations

While the *P. sicula* of Pod Mrčaru eat more plants than their Pod Kopište counterparts after ~35 years of divergence, their gut morphology and physiology remains similar. Our expectations of

gut-wide shifts in form and function based on the framework of the Chemical Reactor Theory were not supported. These results are more consistent with Pod Mrčaru lizards as facultative omnivores (Herrel *et al.*, 2004). In fact, all but one of the differences (see supplemental material) we observed in gut structure and function were localized to the hindgut or distal intestine. As the majority of endogenous nutrient digestion and absorption in non-ruminant vertebrates occurs in the proximal portion of the intestine (Karasov and Martínez del Rio, 2007; Vonk and Western, 1984), differences in the structure and function of the distal portion of the intestine point to differences in the function of microbial symbionts in this gut region (Bjorndal *et al.*, 1997; McBee and McBee, 1982; Bergman, 1990). According to the plug flow reactor model of digestion (Penry and Jumar, 1986, 1987; Karasov and Hume, 1997; Stevens and Hume, 2004), nutrients are digested and absorbed down their gradient as they flow through the gut. Thus, a shift in gut function in the proximal region will promote downstream changes, from more potential digestion to more opportunities for nutrient absorption. For example, an increase in trypsin activity in the proximal intestine could lead to greater digestion of proteins into dipeptides that can serve as a substrate for aminopeptidase in the mid- and distal intestines. There is subsequently more gut remaining over which the dipeptides can be absorbed. Changes in the distal intestine, however, may represent a small portion of overall nutrient acquisition due to (a) decreased substrate concentrations distally along the gut (German, 2009; German and Bittong, 2009; German *et al.*, 2010b), (b) decreased length along the gut for nutrients to be absorbed, and (c) the possibility that the hindgut is less active for nutrient absorption than more proximal regions (though nutrient transport in the hindguts of lizards remains unstudied). Thus, as most changes observed amongst the *P. sicula* populations are localized to the hindgut, we do not expect large magnitude "shotgun" (i.e., non-specific) differences in digestive performance.

Rather, these changes in digestive physiology take more of a "rifle" approach (i.e., specific), manifesting as small, targeted changes in a gut region having greater flexibility in form and function. We may also infer that microbial communities, generally localized to the hindgut, are a likely driver of this shift. Indeed, the only shifts in the hindgut microbial communities of the Pod Mrčaru and Pod Kopište lizards were among relatively rare taxa (Vigliotti *et al.*, *in prep*), supporting the small changes in digestive biochemistry we observed in this gut region.

The only functional difference we measured outside of the hindgut was higher NAG activity in the proximal intestines of Pod Kopište lizards. Higher NAG activity could contribute to the digestion of chitin from arthropods carapaces, and the cell walls of fungi and nematodes (Skoczylas, 1978; Vonk & Western, 1984). Although we expected patterns of NAG activity to conform to that of brush-border derived digestive enzymes (German *et al.,* 2015), NAG activity was the highest in the DIGC, consistent with microbial synthesis, not endogenous synthesis in lizard tissue. *Podarcis sicula* appears to have considerably lower endogenous NAG activity compared to other lizards (Table 1.6; Jeuniaux, 1961, 1963; Marsh *et al.,* 2001).

The higher SCFA concentrations in Pod Kopište lizards were opposite of what we expected. Higher SCFA concentrations are an indication of more microbial fermentation (Bjorndal, 1997; Pryor and Bjorndal, 2005). Indeed, high acetate, propionate, isobutyrate, and butyrate are all associated with fermentation of plant material. However, the acetate and isobutyrate concentrations and ratios of total SCFAs were higher in the Pod Kopište lizards. In a study of *Uromastyx aegyptius* (Foley *et al.*, 1992), a strict herbivore, acetate, propionate, and butyrate concentrations were similar to our measurements in the Pod Kopište *P. sicula*.

Fermentation is often limited by time rather than substrate. As Pod Mrčaru lizards consume >2.2x more than the Pod Kopište lizards, digesta transit time must be considerably

shorter in Pod Mrčaru lizards. Based on their differences in intake and microbial fermentation, these two populations appear to fit a model of "rate vs. yield" (Sibly, 1981; Clements and Raubenheimer, 2006; German *et al.*, 2015). "Yield maximizers" tend to have measured intake, greater levels of fermentation, and slow digesta transit times (aided by increased structure in the hindgut), absorbing more of the nutrients within their diets and minimizing "wastage" lost in feces. This long retention time offers more opportunity for the distal intestine microbial community to ferment recalcitrant digesta. Consistent with this, only the Pod Kopište lizards had significantly more gut content mass in their distal intestines than the other gut regions ($F_{2,17}$ = 12.96; $P < 0.001$). "Rate maximizers," however, generally have high intake, rapid transit of digesta throughout the gut, and rely primarily on endogenous digestive enzymes to digest the more soluble components of their food. The generally endogenously produced enzymes amylase, trypsin, and trehalase have higher activities in the Pod Mrčaru population, thus consistent with rate maximization. This strategy capitalizes on high intake, leading to shorter retention of digesta in the gut, losing some of the potential nutrients as wastage. The Pod Mrčaru lizards appear to fit this strategy. That trypsin and trehalase are not associated with a plant diet, yet these enzyme activities are higher in the Pod Mrčaru lizards, is additional support for rate maximization, along with elevated proportions of isovalerate in the hindguts of Pod Mrčaru lizards, which suggests that some amino acids are escaping the mid intestine to the distal intestine to be fermented by microbes (Clements *et al.*, 2017). (However, see Leigh *et al.*, 2018a for a summary of conflicting findings on enzyme activities across studies and taxa.)

Although cecal valves are present in Pod Mrčaru hindguts (Herrel *et al.*, 2008; Vervust *et al.*, 2010; Wehrle *pers. obs.*), we did not observe differences in epithelial magnification by population. The general pattern of surface area decreasing along the gut (i.e.: PI>MI>DI,

supporting information) was consistent with our expectations (Stevens and Hume, 2004; Skoczylas, 1978) considering we did not capture the valves in our sections (even the DI+ sections). Rate vs. yield maximizing strategies also predict differences in gut structure. Rate maximizers should have long digestive tracts to accommodate nutrient uptake to offset rapid flow of digesta. Yield maximizers, on the other hand, would have gut structures that facilitate digesta retention. In our findings, however, we observed no gut length differences, thus Pod Mrčaru lizards, acting as rate maximizers, may use cecal valves to slow their digesta instead.

Lizard Digestive Physiology

Digestive physiology in reptiles has been less studied than in other taxa (Kohl *et al.*, 2016a; Stevens and Hume, 2004), particularly in wild populations. We included the Zagreb population of *P. sicula* to give context to the magnitude of differences between the Pod Mrčaru and Pod Kopište populations, and identify which structural and physiological characteristics are unlikely to change (e.g.: gut length) even between distantly related populations. Based on the CRT, we predicted that lizard populations with different diets would have more differences in gut structure and physiology. Thus, we expected that the Pod Kopište and Zagreb populations, which both mostly consume invertebrates, would show the most similarity in gut form and function. However, insularity (i.e., island dwelling) appears to have greater effects on gut structure than diet.

Generally, the island populations differed from the mainland lizards and not from each other. Island effects often outweigh other factors in lizards, including effects on diet (Van Damme, 1999; Cooper and Vitt, 2002; Spiller *et al.*, 2010) and digestion (Sagonas *et al.*, 2015; Caviedes-Vidal and Sabat, 2010; Pafilis *et al.*, 2007). The more massive guts of the island lizards

compared to the Zagreb population suggest that the insular lizards allocate more tissue resources to digestion, perhaps because they are nutrient challenged. The Zagreb lizards live in an urban area with copious plant cover and potential food sources, whereas the island lizards live on small, densely populated islets that are likely challenging for all organisms living on there. Overall, the enzymes that were higher in the Zagreb population are brush-border enzymes. These brush-border enzymes show elevated activity along the gut and overall compared to the Pod Kopište and Pod Mrčaru lizards' digestive enzymes. Brush-border enzymes have higher substrate specificity, degrading smaller carbohydrates and peptides as one of the last steps of digestion before absorption. This may compensate for the Zagreb population's lower gut tissue mass, leading to equivocal nutrient acquisition outcomes in both mainland and island populations.

Still, as more than two-thirds of the enzymatic differences we identified were between island and mainland, and not between the rapidly evolved population and its source, it appears that endogenous enzyme activities are not the main response to this dietary shift. While we know some complex morphological traits are able to evolve in this short time span of <30 generations (Herrel *et al.*, 2008; Vervust *et al.*, 2007, 2010), feeding behavior and endosymbionts may dampen the selective pressures on the digestive system, as changes in behavior can offset evolutionary pressures leading to changes in physiology and morphology (Huey *et al.*, 2003; Clements and Raubenheimer, 2006; Sibley, 1981). By increasing food intake and through shifts in microbiome function (Vigliotti *et al.*, *in prep*), Pod Mrčaru lizards may not need to shift their gut form and function much beyond the addition of cecal valves.

Amylase, trypsin, and lipase activities were highest in the pancreas (Figure 1.9) and decreased distally along the gut (Fig. 1.2), patterns consistent with pancreatic enzymes (Clements and Raubenheimer, 2006; German *et al.*, 2015; Stevens and Hume, 2004).

Aminopeptidase, a brush-border enzyme, also fit the expected pattern with a spike of activity in the mid gut, whereas maltase and trehalase were more active proximally.

Perhaps most unexpected is the enzyme activity pattern of β-glucosidase (Fig. 1.6b). No non-avian reptile has been recorded to produce endogenous β-glucosidases in their digestive tracts (Stevens and Hume, 2004; Karasov and Douglas, 2013), and thus must rely on microbial endosymbionts to produce this enzyme for digesting cellulose. As predicted, *P. sicula* has a spike of β-glucosidase activity in the DIGC, consistent with microbial synthesis (Fig. 1.2; German *et al.*, 2015). However, the β-glucosidase activity is just as high in the PI as in the DIGC. Of interest, the high activity in the PI is most starkly found in the island populations, with present, but much diminished activity observed in the mainland lizards' proximal intestines. A β-glucosidase is present in the *Anolis carolinensis* genome (on chromosome 5; ensembl.org), but it remains unknown if this enzyme is expressed in the digestive system, or mainly liver, as in mammals (de Graaf *et al.*, 2001; Hayashi *et al.*, 2007). Overall, these patterns suggest that *P. sicula* may produce β-glucosidase endogenously or acquire it from microbial endosymbionts in the PI in addition to in the DIGC. However, Kohl and colleagues (2016b) propose that β-galactosidase, known to be endogenously produced in reptiles (on chromosome 1 in the *A. carolinensis* genome; ensebl.org), is active against β-glucosidase substrates. However, the β-galactosidase activity patterns we measured varied from the β-glucosidase activity patterns throughout the gut (Figure 1.8). Thus, there is some evidence that *P. sicula* may endogenously produce β-glucosidase or house β-glucosidase producing microbes in their PI.

In conclusion, the *P. sicula* system offers a rare opportunity to observe evolution in action in wild populations. Few studies investigate animals' digestive physiology on a natural diet, within their ecosystem. In this newly omnivorous population of lizards, changes in gut form

and function—including valves, enzyme activity, and microbial fermentation— start from the distal end. Yet many potential shifts in digestive morphology and physiology are potentially mitigated by increased food intake and thus a shift to a "rate-maximizing" strategy in the Pod Mrčaru lizards. While we know the Pod Mrčaru lizards have changed their morphology over ecological time, the evolution of their digestive physiology appears to be more constrained on this timescale, or the selective pressures dampened by behavior and ecology. Some dietary shifts may not be as limited by physiology as they are by these animals' ecology.

Figure 1.1 *Podarcis sicula* island collection sites showing Pod Kopište (source population) and Pod Mrčaru (newly omnivorous population). The box in the bottom map shows the area of the inset. Zagreb (mainland population) not pictured. Map credit: L. Dobson.

Table 1.1 Predictions of relative diet, gut morphology, enzyme activities, and fermentation products in Pod Mrčaru (new omnivore), Pod Kopište (source), and Zagreb (mainland) populations.

Characteristics	**Pod Mrčaru**	**Pod Kopište**	**Zagreb**
Diet: % plant matter	highest	low	low
Gut Length	long	short	short
Gut mass	heaviest	light	light
ESM	largest	least	least
Enzyme activities *(substrate)*			
pancreatic			
α-amylase *(starch[a])*	moderate	low	low
Trypsin *(protein)*	moderate/high	high	high
Lipase *(fats)*	moderate	moderate	moderate
intestinal			
N-acetyl-β-D-glucosaminidase *(chitins)*	low	moderate	moderate
Trehalase *(arthropod sugars)*	low	moderate	moderate
Maltase *(dissacharides[a])*	moderate	low	low
Aminopeptidase *(dipeptides)*	moderate/high	high	high
microbial			
β-glucosidase (*β-glucosides[b]*)	high	low	low
SCFAs			SCFAs not measured for this
acetate	high	low	
propionate	high	low	
butyrate	moderate	low	
isobutyrate	moderate	low	
valerate	low	moderate	
isovalerate	low	moderate	

[a]From plants, seeds, glycogen sources; [b]From plant cell wall sources

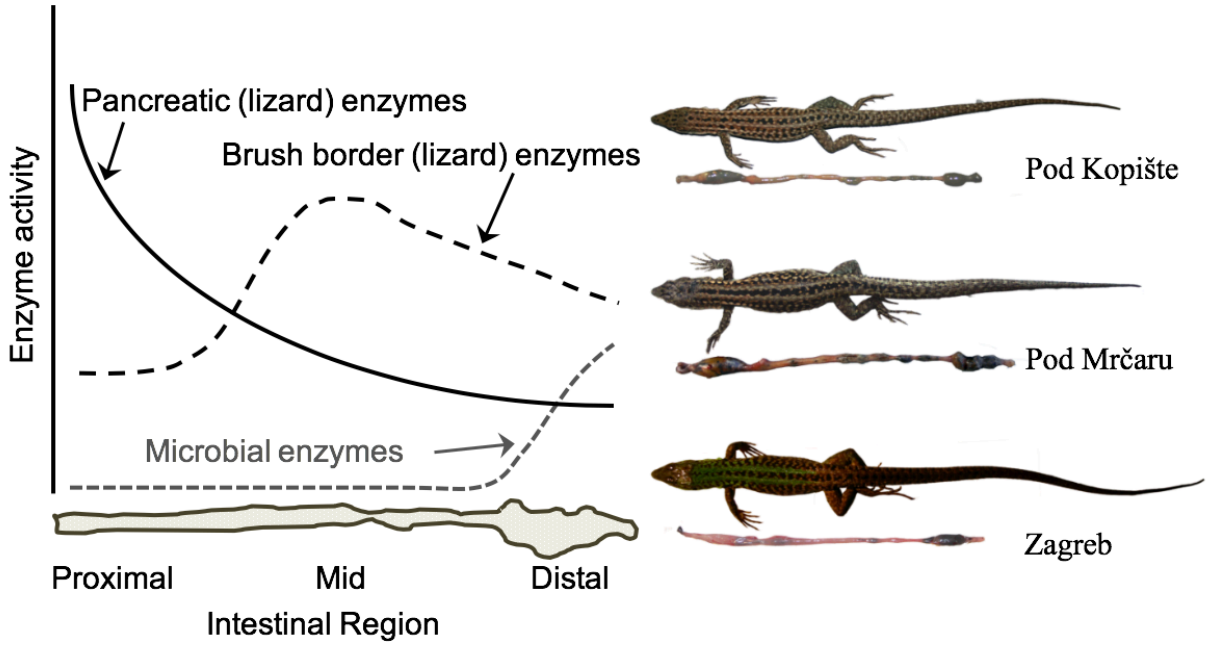

Figure 1.2 Potential patterns of digestive enzyme activities and representative examples of lizard guts from each population (with stomachs). Pancreatic digestive enzymes are secreted into the proximal intestine and expected to decrease along the gut and brush border enzymes peak in the mid intestine. Microbial enzymes tend to peak in the distal intestine where symbiotic microbes are housed. Modified from German *et al.,* 2015

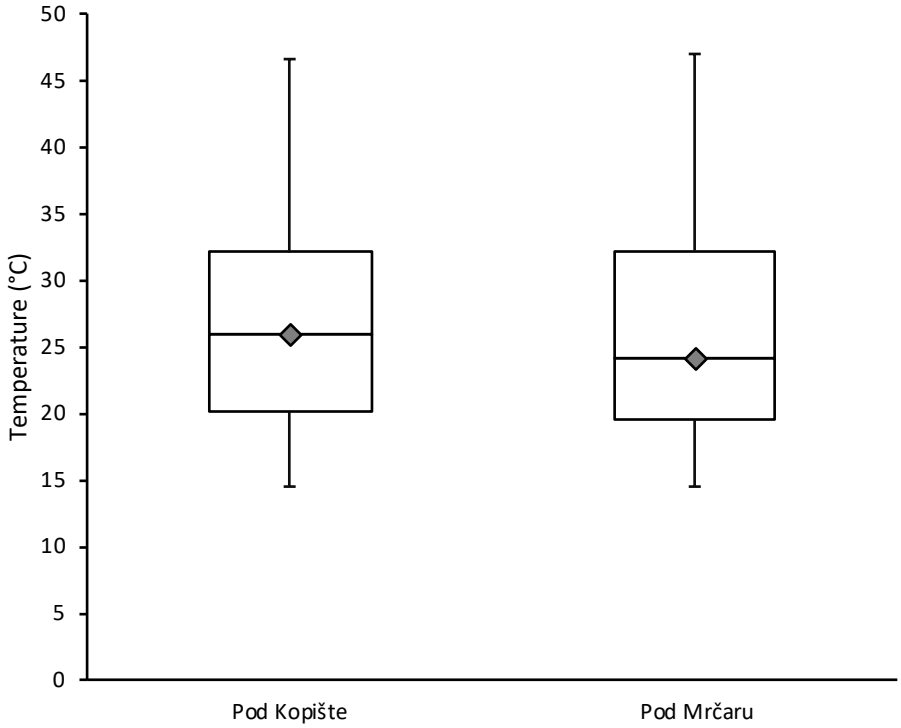

Figure 1.3. Box-plot of iButton temperature data collected on islands from May–September. Temperatures were collected from three loggers/ island every 1.5-2 hours, with both full sun and shade represented. Quartile data are represented by boxes and whiskers, with means denoted by ◆. Temperatures are not different by island.

Table 1.2 Average stomach contents (±SD) by mass of lizards from Pod Kopište (*N*=30), Pod Mrčaru (*N*=36), and Zagreb (*N*=4). We found empty stomachs in lizards from Pod Kopište (*N*=1) and Zagreb (*N*=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 ± 68.0 mg	207.41 ± 16.1 mg	98.79 ± 123.2 mg
Plant material %	24.49 ± 33.1	64.24 ± 30.8	2.37 ± 8.3
leaves	6.25 ± 25.0	7.65 ± 12.0	0
seeds	81.25 ± 40.3	91.18 ± 13.3	0
wood	12.50 ± 34.2	1.18 ± 3.0	0
fruit	0	0	100
Animal material %	75.36 ± 33.0	35.58 ± 30.9	70.48 ± 44.2
Other %	0.15 ± 0.8	0.19 ± 0.8	25.37 ± 22.8

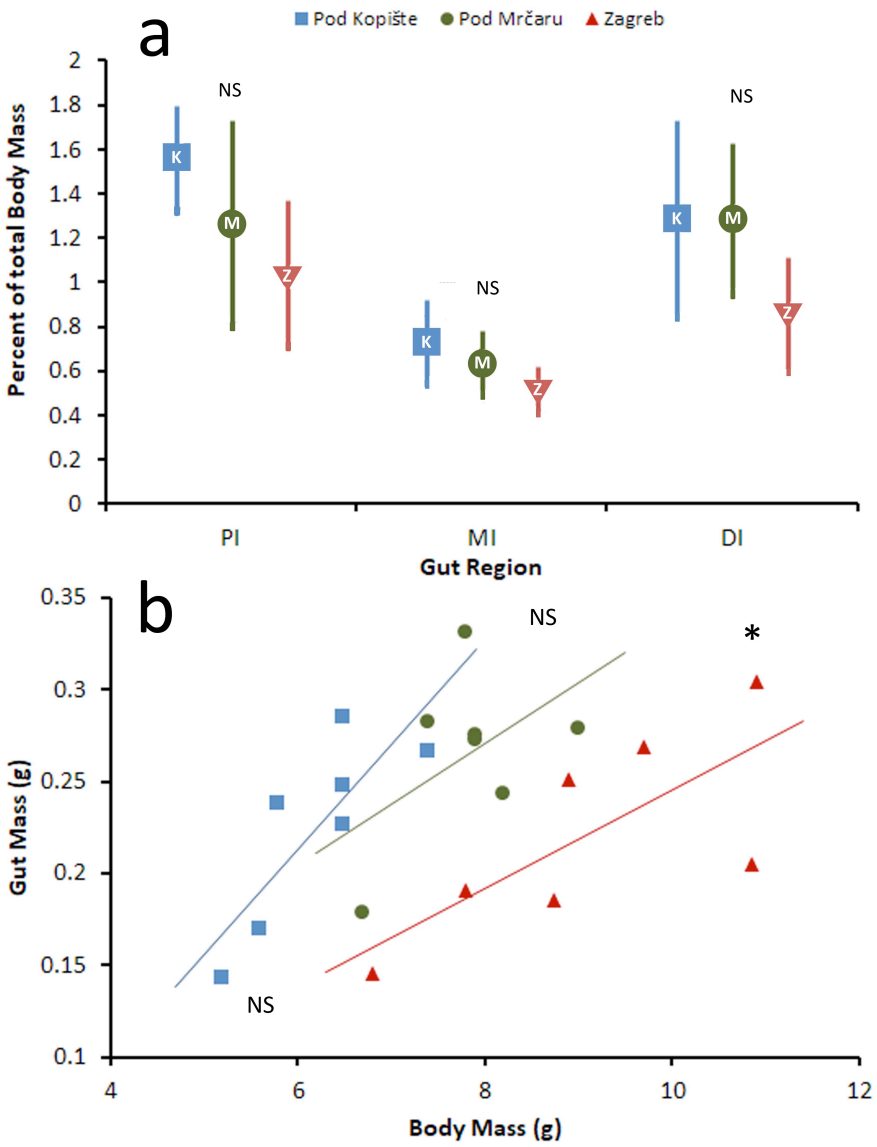

Figure 1.4 (a) Regional and (b) total intestinal mass (without contents) in Pod Kopište (source), Pod Mrčaru (new omnivore), and Zagreb (mainland) populations. Gut regions are proximal intestine (PI), mid intestine (MI) and distal intestine (DI) and presented as a percentage of body mass. Values are mean ± standard deviation. *n*=7 in all except *n*=6 in Pod Mrčaru PI. Comparisons of populations were done via ANCOVA with body mass as a covariate. No particular region showed differences in mass (a), but the Zagreb population had lower total gut masses than the Pod Mrčaru population (b) that were also significantly affected by body mass. Pod Kopište gut masses were not different from either population.

Table 1.3 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.

	Pod Kopište	**Pod Mrčaru**	**Zagreb**
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)

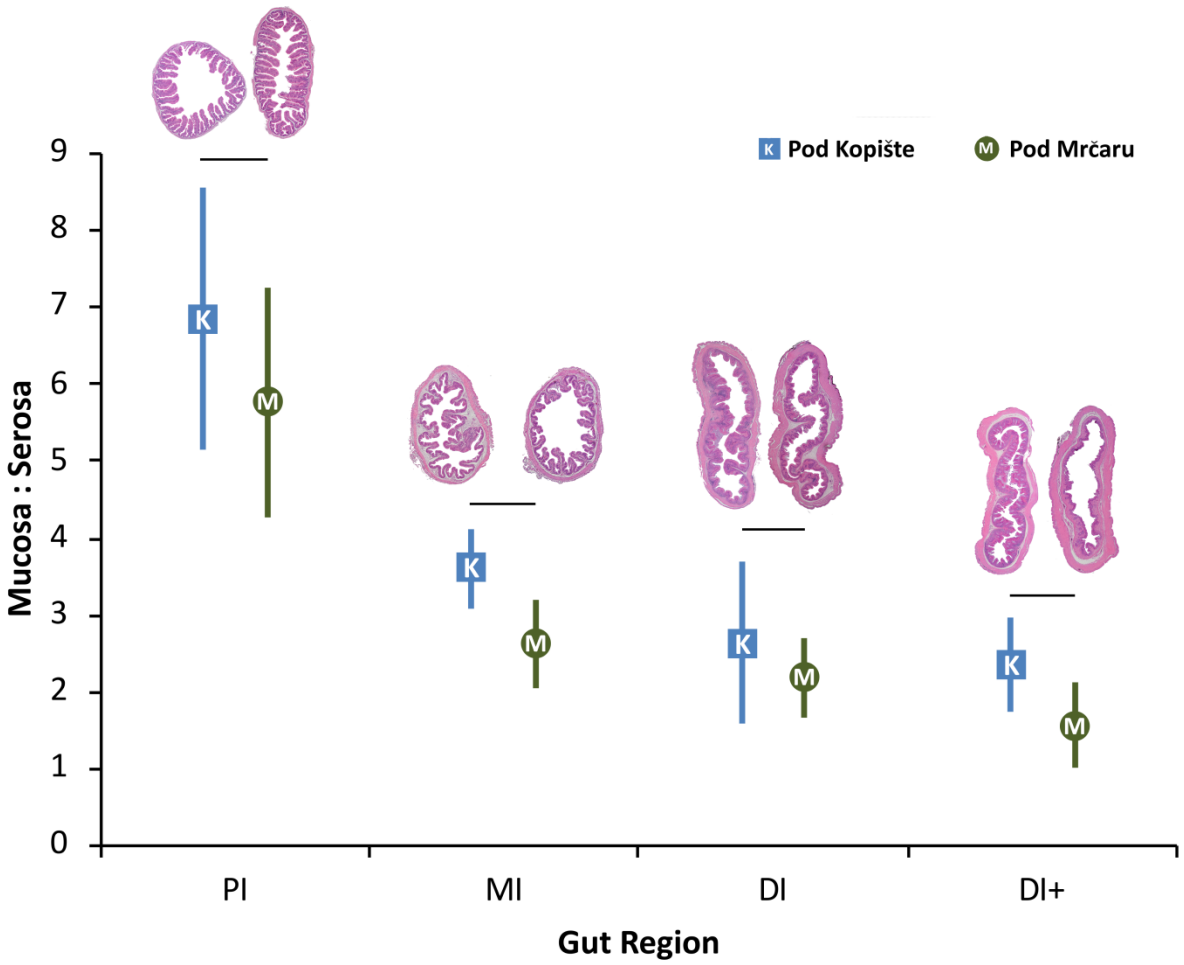

Figure 1.5 Ratio of inner perimeter length of mucosa to inner perimeter length of serosa in proximal intestine (PI), mid intestine (MI), proximal half of distal intestine (DI), distal half of distal intestine (DI+). Values are mean ± standard deviation, *n*=3. Comparisons of populations were done via equal variance t-test. No population differences. Cross section images are representative stained histological sections from each gut region of Pod Kopište (source) and Pod Mrčaru (new omnivore) populations (not to scale).

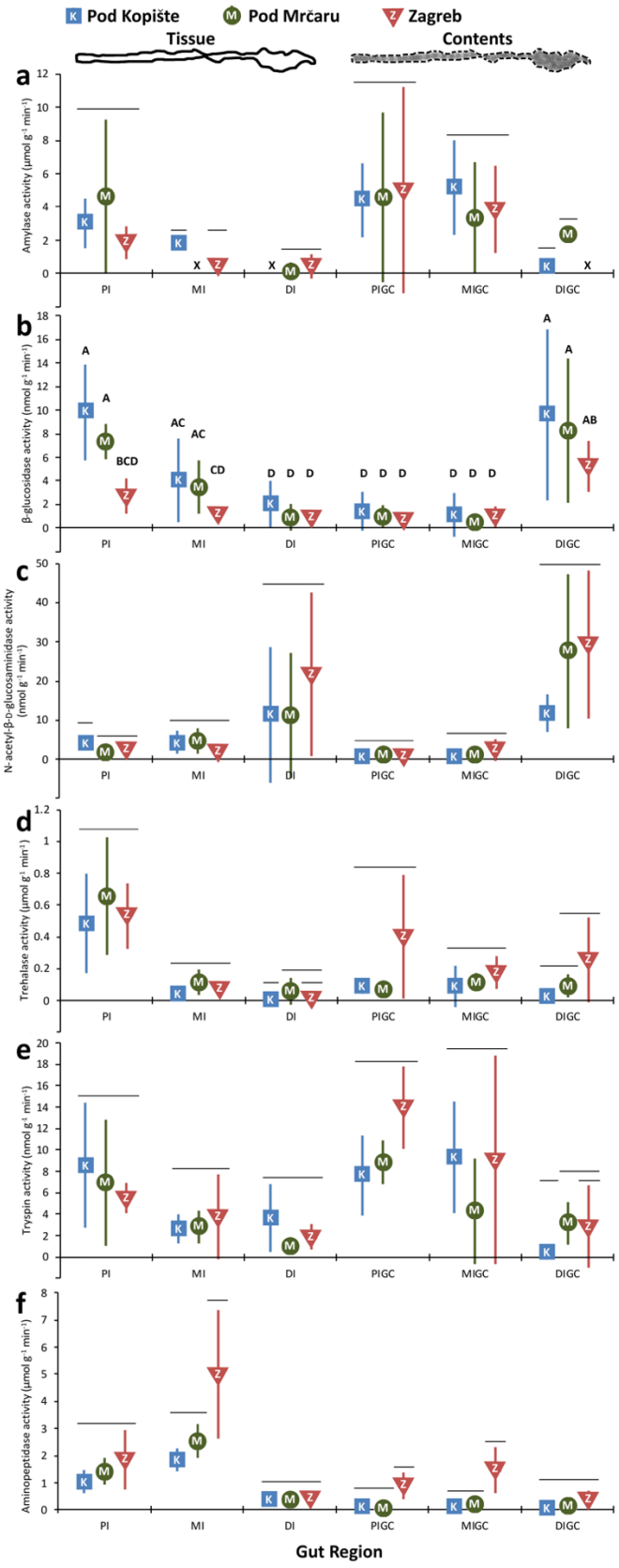

Figure 1.6 (a) amylase activity in µmol glucose liberated g^{-1} min^{-1}, "**X**" denotes undetectable activity. (b) β-glucosidase and (c) N-acetyl-β-D-glucosaminidase activities in nmol MUB liberated g^{-1} min^{-1}, (d) trehalase activity in µmol glucose liberated g^{-1} min^{-1}, and (e) trypsin in nmol and (f) aminopeptidase activities µmol of p-nitroaniline liberated g^{-1} min^{-1} throughout the gut in Pod Kopište (source), Pod Mrčaru (omnivore), and Zagreb (mainland) populations. Values are mean ± standard deviation, Pod Kopište *n*=4-7, Pod Mrčaru *n*=3-7, Zagreb *n*=3-6. In (b) populations by tissue and tissues within populations were compared via separate ANOVAs, shared letters above icons denote no differences. In (a, c-f) comparisons of populations were done via ANOVA where lines of a different elevation for a gut region indicate significant differences for that population and overlapping lines indicate no differences.

Table 1.4 Average enzyme activities ± standard deviation.

Enzyme, *units*	**Region**	**Pod Kopište**	**Pod Mrčaru**	**Zagreb**
Amylase, *µmol glucose liberated min^{-1} g^{-1}*	DIGC	0.400 ±0.30	2.387±1.99	undetectable
maltase, *µmol glucose liberated min^{-1}*	total	0.0380±0.035	0.0612±0.010	0.1549 ±0.051
trehalase, *µmol glucose liberated min^{-1}*	total	0.0447 ±0.046	0.0732 ±0.071	0.1283 ±0.076
β-glucosidase, *nmol MUB released min^{-1} g^{-1}*	PI	9.834 ±4.08	7.362 ±1.50	2.706 ±1.49
β-galactosidase, *nmol MUB released min^{-1} g^{-1}*	PI	22.461 ±8.51	14.647 ±6.82	17.332 ±8.57
N-acetyl-β-D-glucosaminidase, *nmol MUB released min^{-1} g^{-1}*	PI	4.022 ±1.43	1.845 ±0.70	2.076 ±1.50
Trypsin, *nmol p-nitroaniline released min^{-1} g^{-1}*	DIGC	0.545 ±0.23	3.150 ±2.01	2.852 ±3.85
aminopeptidase, *µmol p-nitroaniline released min^{-1}*	total	0.204 ±0.06	0.318 ±0.13	0.748±0.29
Lipase, *µmol p-nitrophenol released min^{-1}*	total	3.055 ±1.41	4.215 ±4.79	5.071 ±2.55

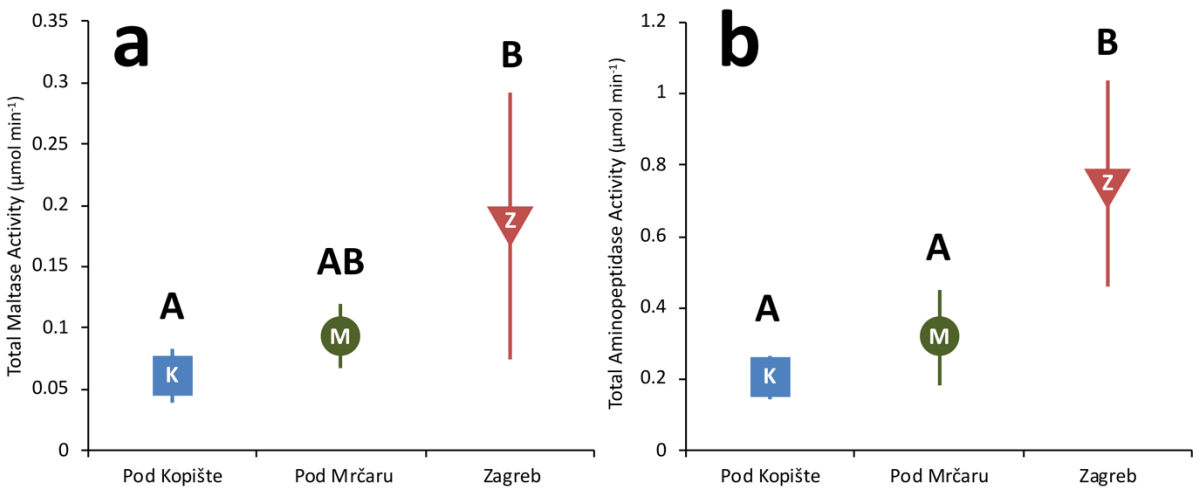

Figure 1.7 Total maltase activity (a) in µmol glucose liberated min^{-1} and total aminopeptidase activity (b) in µmol p-nitroaniline liberated min^{-1}. Values are mean ± standard deviation, *n*=7, Zagreb *n*=6. Populations compared via ANOVAs, different letters above icons denote significant differences.

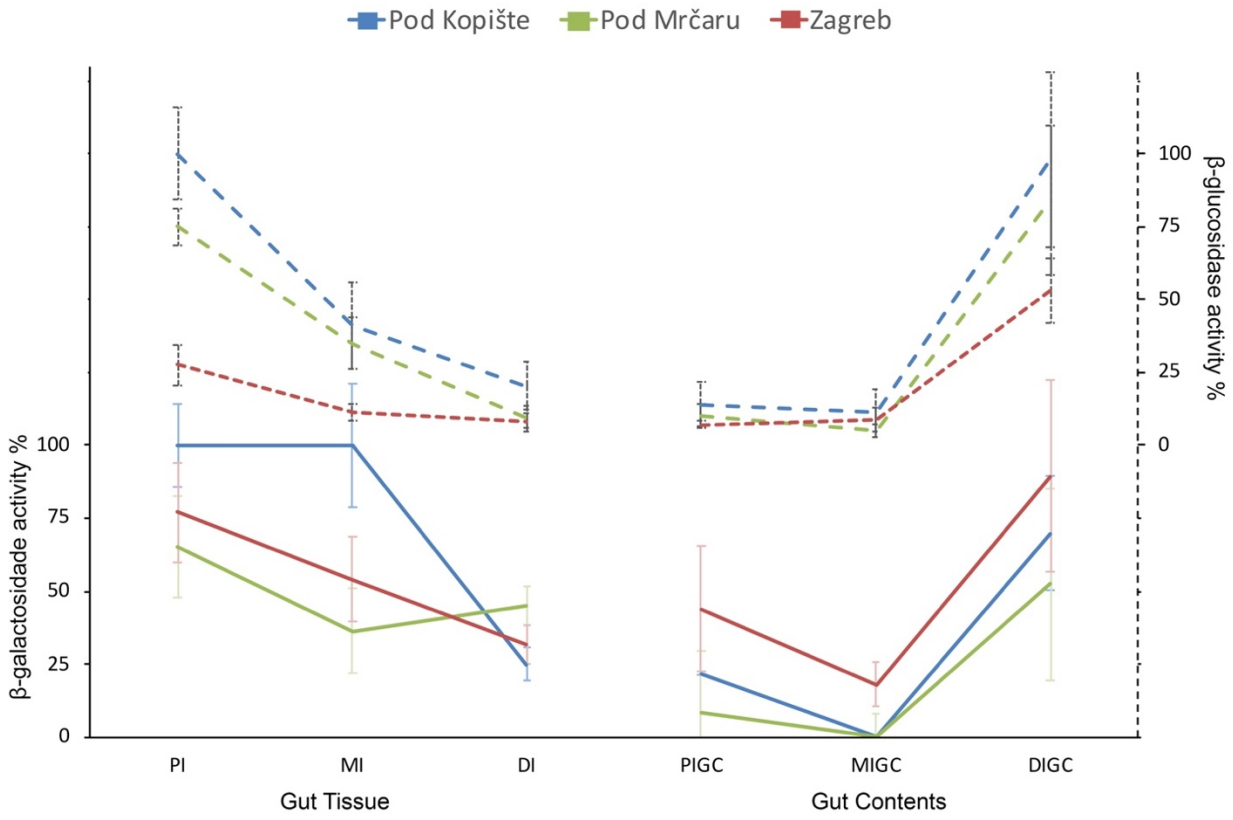

Figure 1.8 β-galactosidase (left axis, solid lines) and β-glucosidase (right axis, dashed lines) activities in each gut region as a percentage of the highest activity (i.e.: Pod Kopište PI activity is 100% for both enzymes as it has the highest activity of any gut region and lizard population). Points are averages ±SEM.

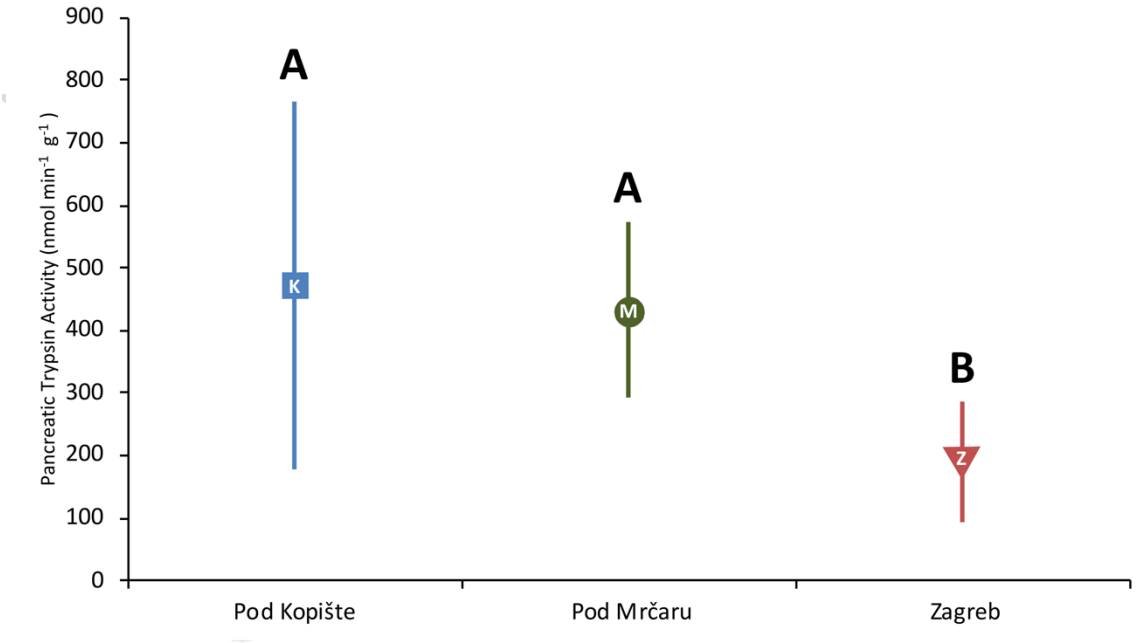

Figure 1.9 Trypsin activity in the pancreas in nmol p-nitroaniline liberated g^{-1} min^{-1} in Pod Kopište (source), Pod Mrčaru (new omnivore), and Zagreb (mainland) populations. Values are mean ± standard deviation, *n*=10, Zagreb *n*=6. Populations compared via ANOVA, different letters above icons denote significant differences.

Table 1.5 Total short-chain fatty acid (SCFA) concentrations and ratios of acetate: propionate: butyrate: isobutyrate: valerate: isovalerate to total SCFAs in distal intestines of Pod Kopište (source; *n*=4) and Pod Mrčaru (omnivore; *n*=3) populations. Values are mean ± standard deviation. Compared between populations using equal variance t-tests, * denotes significant differences between populations.

Population	**Total SCFA (mM)**	**Ratio**
Pod Kopište	61.86 ±3.95 mM *	65*:19: 8 :5*:2:1*
Pod Mrčaru	19.22 ±8.63 mM *	56*:16:16:7*:1:4*

Table 1.6 Comparison of digestive enzyme activities ranges between the *Podarcis sicula* 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. sicula*. [a]N-acetyl-β-D-glucosaminidase

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

Chapter 2

Nutrient digestibility of a novel diet is affected by feeding frequency and sex in a rapidly evolving lizard

Introduction

Although evolution is generally thought to happen over many generations and a different timescale than ecological changes (Hairston *et al.*, 2005), examples of rapid evolutionary change are being discovered with increasing frequency (Carroll, Hendry, Reznick, & Fox, 2007; Hendry & Kinnison, 1999; Lallensack, 2018). One such example, the dietary and morphological changes in a population of Italian Wall Lizards (Fig. 2.1; Herrel *et al*., 2008), has garnered considerable attention.

After an experimental transplantation of five pairs of Italian Wall lizards (*Podarcis sicula;* (Nevo *et al.*, 1972) from an insular source population (Pod Kopište, Croatia) to a new nearby island (Pod Mrčaru, Croatia), researchers have used this system to investigate population divergence over a known time-scale. Starting with an initial visit 33 years after this introduction, researchers found that the new population on Pod Mrčaru had shifted to a diet rich in plants (61% in 2004 Herrel *et al*., 2008, to 64% in 2013 Wehrle *et al*., *under review*), whereas their source population on Pod Kopište remained primarily insectivorous (consuming 7% plants in 2004 and 24% in 2013). Additionally, the mass of the contents in the stomachs of Pod Mrčaru lizards was greater than two-fold the stomach content mass found in the Pod Kopište lizards (Wehrle *et al.*, *under review*). The Pod Mrčaru lizards were larger and morphologically distinct from their source population and all dissected individuals across multiple years had valves in their hindguts (Herrel *et al*., 2008; pers. obs.), a characteristic absent in the Pod Kopište

population. Increased size, but especially hindgut valves are generally associated with highly derived herbivory in lizards, and are thought to slow digesta transit through the gut and allow for microbes to aid in the digestive process (Iverson, 1982). Hindgut valves can increase surface area for microbial attachments and provide microhabitats for diverse microbial communities (Troyer, 1984; McBee, 1971). Microbial endosymbionts are necessary to break down recalcitrant material (e.g.: cellulose and hemicellulose) for which the lizards do not produce their own digestive enzymes (e.g.: cellulase and xylanase endogenously. Thus, based on diet and morphology, we would expect the Pod Mrčaru lizards to outperform their Pod Kopište counterparts' ability to digest a plant diet. However, digestive biochemistry of the two populations did not clearly suggest higher plant digestive performance with respect to enzyme activities or the products of microbial fermentation in the Pod Mrčaru lizards (Wehrle *et al.*, *under review*) as would be expected for plant specialists. In fact, the insectivorous Pod Kopište lizards displayed greater evidence of microbial fermentation of ingested substrates, likely due to decreased intake, longer gut residence time, and more time afforded for microbial fermentation (Stevens and Hume, 1998; Karasov and Martinez del Rio, 2007; Wehrle *et al., under review*). To determine if the Pod Mrčaru lizards are indeed specialized to digest a plant rich diet, it is necessary to measure their ability to digest plant material, which would be a measure of digestive performance.

Herbivory is rare in lizards, occurring in <1-4% of species (Cooper and Vitt, 2002; Espinoza *et al.*, 2004), with insectivory as the ancestral state. Transitions to a primarily plant diet have occurred independently >30 times in the greater lizard phylogeny (Espinoza *et al.*, 2004). Although incidental plant eating is relatively common, what an animal ingests is not necessarily what is digested and assimilated, and thus, the source of nutriment for the animal. For example, wood eating catfishes have been found to digest little of the cellulose that makes up the wood,

but instead appear to gain their sustenance from microbes decomposing the wood (German, 2009; German and Bittong, 2009; German and Miles, 2010; Lujan *et al.*, 2011). To determine if the Pod Mrčaru population's switch to a plant-rich diet is indicative of a change in nutritional strategy, and if it is an indication of a dietary specialization, we must do more than examine stomach contents.

To be a dietary specialist, an animal must be able to acquire resources from that diet (Karasov & Douglas, 2013; Karasov *et al.*, 2011). This may mean it experiences a performance tradeoff, digesting other diets for which it is not specialized with less efficiency than its normal diet. Alternately, a dietary specialist may experience no performance tradeoffs and have a higher digestibility than a generalist would on the specialized diet. The Chemical Reactor Theory (CRT) of digestion (D. Penry & Jumars, 1987; D. L. Penry & Jumars, 1986)) posits that the goal of digestion is to optimize nutrient or energy gain. Thus, the digestive tract must be morphologically and physiologically optimized for the food that is being digested (Cant, McBride, & Croom, 1996; Ferraris & Diamond, 1989; W. Karasov & Diamond, 1983; W. H. Karasov & Douglas, 2013), in part because the digestive tract is metabolically expensive to maintain (Karasov and Diamond 1983). This leads to the following relationships:

$$Digestibility \propto \frac{enzyme\ activities}{substrate\ concentration} \propto \frac{gut\ size}{digesta\ transit\ rate} \propto time$$

(equation 1, modified from Sibly, 1981; Karasov and Douglas, 2013). Although there are morphological differences amongst the lizards from the two islands, there are only a few changes in digestive biochemistry that are localized to the hindgut in the Pod Mrčaru lizards, hardly representing specialization. Examining digestive function of the animals will illuminate what role these shifts in gut structure and physiology play in their whole biology.

Digestibility is a measure of how efficiently an animal metabolizes nutrients from its ingested diet, digestibility = $\frac{intake - feces}{intake}$ (equation 2). Many studies measure this as digestive or assimilation efficiencies, determining the proportion of energy available in the food is absorbed by the animal. Energy, however, addresses only one piece of an animal's nutritional needs. Thus, we more generally measure digestibility of organic matter, and the macronutrients that constitute it: carbohydrates, proteins, and lipids. By measuring digestibility of these specific nutrients, we can match digestibility to physiology.

Dietary intake plays a pivotal role in the dynamics of digestibility. Following equation 1, high intake can lead to increased substrate concentration, but it also directly increases digesta transit rate. By feeding more material into the gut, digesta flow increases, decreasing digesta time in the gut. This decrease in time decreases opportunity for digesta to be degraded by digestive enzymes and for absorption to occur. Understanding digestibility in the context of intake can also be explained in a "rate vs. yield" model (Fig. 2.2; Sibly, 1981; Clements and Raubenheimer, 2006; German *et al.*, 2015). "Rate maximizers" generally have high intake, rapid transit of digesta throughout the gut, and rely primarily on endogenous digestive enzymes to digest the more soluble components of their food. In contrast, "yield maximizers" tend to have measured intake, high fermentation, and slow digesta transit times, absorbing more of the nutrients within their diets and minimizing "wastage" lost in feces. Rate vs. yield maximizing strategies also predict differences in gut structure. Rate maximizers should have long digestive tracts to accommodate nutrient uptake to offset rapid flow of digesta. Yield maximizers, on the other hand, would have gut structures that facilitate digesta retention.

A diet that consists of low nutrient density food necessitates higher intake to meet an animal's nutritional needs (Simpson *et al.*, 2004; Montgomery and Baumgardt, 1965; Slansky

and Wheeler, 1992). As the Pod Mrčaru lizards eat more plant material and have more stomach contents than their Pod Kopište counterparts, it follows that they are employing this strategy to accommodate their low nutrient density diet (i.e.: plant material). Pod Mrčaru lizards appear to fit the model of rate maximizers. If this is the case, we hypothesize that Pod Mrčaru lizards will be better at digesting all diets compared to Pod Kopište lizards when fed with high frequency. When fed with lower frequency, we predict that Pod Kopište lizards will have a higher digestibility of all diets than the Pod Mrčaru population.

Alternately, however, it has been proposed that dietary intake is dependent on an animal's ability to process nutrients (Karasov *et al.*, 1986; Levey and Martínez del Rio, 1999). If the *P. sicula* system fits this model better, we would expect Pod Mrčaru lizards to better digest plant diets than their Pod Kopište source population counterparts, regardless of feeding frequency.

As illustrated in equation 1, digestibility is influenced by animal physiology and morphology, type of diet, dietary intake, and time over which digestion and retention occur. As such, these factors vary with the ecology and natural history of the animal (e.g.: diet, feeding frequency, season, sex, health, etc.). Neither population revealed dietary differences by sex (Fig. 2.1; Herrel *et al.*, 2008), and thus, based on the model of dietary intake as dependent on an animal's nutrient processing ability, this suggests that males and females in the same population should have equivalent digestibilities of their diets. Few digestive performance studies address sex differences beyond the effects of body mass. However, several studies on birds have found higher digestive (Kwieciński and Tryjanowski, 2009) and protein assimilation efficiencies (Stahlschmidt *et al.*, 2011) in females compared to males. Work on sunbirds found equivalent digestibility of diets by sex, but at faster transit times in females (Markman *et al.*, 2006). In fact,

these birds showed opposing patterns by sex, with greater transit time of sucrose vs. hexose based diets in females and the reverse in males. In the context of these findings, we anticipate that females will have higher digestive efficiency than males.

To understand the interplay of some of these factors as they apply to the *P. sicula* system, we conducted feeding trials with three experimental diets, comparing among populations, between males and females, and between two feeding regimens of different frequencies. We conducted a high frequency feeding trial with males from Pod Mrčaru and Pod Kopište. Based on chemical reactor theory (equation 1) and enzyme activity differences between the populations (i.e. carbohydrases and proteases; Wehrle *et al.*, *under review*), we expect the Pod Mrčaru lizards would have higher digestibility proteins of all diets and carbohydrates of plant material compared to Pod Kopište lizards. With this high frequency feeding, we expect that if gut length is plastic, lizards of both populations would have longer guts on the plant diet (low nutrient density) to increase digesta retention time and shorter guts on the insect diet (high nutrient density).

To match the lower intake per feeding (and the lower frequency of feeding; pers. obs.), we conducted low feeding frequency trials, matching an intake rate likely intermediate to that of the Pod Kopište and Pod Mrčaru populations in the wild. For the low frequency feeding trials, we included males and females of three populations, the two island populations introduced above and an outgroup of lizards from Split on the Croatian mainland (Fig. 2.3). (The Split lizards were from an urban population closely related to the two island populations. Split represents the geographically closest mainland, and nearest relative, of the Pod Kopište/Pod Mrčaru system, Fig. 2.4, (Podnar *et al.*, 2005). While the population in Vallo della Lucania, Italy, appears more closely related to this system, its much greater geographic distance and barriers make this more

likely the artifact of a gene tree vs. population tree. Nevertheless, the geographic distance to Vallo della Lucania made it an unfeasible population from which to sample.) Based on CRT, the lower frequency feeding trials should result in higher digestibilities than the high frequency feeding trial due to increased digesta transit time. As generalized insectivores, we expect Split lizards to have the same digestive patterns as the Pod Kopište population. The low frequency feeding should lead to less difference in gut length by diet because of the lowered digesta transit rate.

Methods

Feeding trials

We conducted two feeding trials using *P. sicula* from Pod Kopište (source population), Pod Mrčaru (newly omnivorous population), and in the low frequency experiments only, Split (mainland population).

High frequency—From September 1-5, 2013, we collected male *P. sicula* from both Pod Kopište and Pod Mrčaru (*N*=15 from each island), transported them in individual cloth bags to the University of Zagreb, where they were allowed to acclimate in individual plastic terraria 30 x 19 x 14 or 20 cm with rock substratum, a hide box, and a water dish for one week. During acclimation to the lab conditions, the lizards were offered live cockroaches (*Blatta sp.*) and finely chopped brussels sprouts (*Brassica oleracea*). Lizards had *ad lib* access to water. The lab was kept at 25-31°C on a 10:14 h light/dark schedule. Five lizards from each population were assigned to one of three diets: insectivore, omnivore, or herbivore. On the first day, each lizard was fed 1.56±0.07% of its body mass of its assigned diet (or ~0.33kJ/g), and thereafter 0.77±0.01% of its body mass of its assigned diet daily (~0.16kJ/g) for a duration of 11-32 days.

Low frequency—From August 27-Sepember 8, 2014, we collected male and female *P. sicula*, respectively, from Pod Kopište (N=27, 25), Pod Mrčaru (N=24, 26), and the southwest region of Split (N=24,20). We allowed the lizards to acclimate in the lab for at least 5 days in the same conditions as the high frequency trial and then assigned ~1/3 of each sex by population group to insectivore, omnivore, or herbivore diets. Each lizard was fed 0.89±0.03% of its body mass of its assigned diet (or ~0.19kJ/g) every other day over a duration of 6-28 days.

Lab conditions—Lizards were kept with ad lib access to water, live insect prey (high frequency trial: mealworms; or low frequency trial: cockroaches), and plant material in mesh enclosures ≤ 18 days, until transport to University of Zagreb in individual cloth bags. At the University of Zagreb, lizards were housed individually in plastic terraria with rock substratum, a hide box, and a water dish. Lizards had *ad lib* access to water and terraria were misted with water each morning. The lab was kept at 25-31°C on a 10:14 h light/dark schedule.

Diets— A sample of each diet and an empty gelatin capsule were combusted in a IKA c2000 calorimeter. The insectivore diet (24.7 kJ/g) was made of cockroaches, the omnivore diet (21.4 kJ/g) was a 50:50 by dry mass mixture of the insectivore and omnivore diets, and the herbivore diet (18.1 kJ/g) was 30% by mass dried plant material collected from Pod Mrčaru, including leaves, flowers, and seeds, and 70% commercial birdseed (primarily millet, flax, hemp seed, and barley), based on an average mass of 80% seeds found in the Pod Mrčaru population's stomachs during summer 2013 (Wehrle *et al.*, *under review*). All diets were dried for >2 days at 50°C, ground to ≤1 mm particle size, and supplemented with Herptivite multivitamins and calcium with vitamin D3 (Rep-Cal, Los Gatos, CA) per manufacturer instructions. Diets were weighed

out in approximately isocaloric ratios and packed into gelatin capsules (undetectable energy content). For the first feeding, the diet was mixed with trace amounts of powdered carmine dye to track passage time and mark the beginning of feces to be collected. We checked for red stained feces hourly.

Feeding— Each lizard was weighed, gently force fed the gelatin capsule of a known mass of experimental diet using a plunger from a syringe to push the pill into their esophagus. After the pill was in the esophagus or swallowed, we administered an equal mass of water into the lizard's mouth via pipette. We adjusted the mass of diet fed at each feeding to maintain lizard body mass ±10%. We collected all feces and urates daily and measured the SVL of each lizard weekly.

Digestibility Analyses— Compiled feces from each individual lizard were dried at 50°C for >1 week and weighed to the nearest 0.001 g.

We estimated the organic matter of each diet and of the dried feces by combusting a portion of the sample (Bjorndal 1989). Samples were dried at 105°C in a drying oven for at least 3 hours to remove all moisture, weighed, then combusted in a Lindburg/ Blue M combusting oven (Ashville, NC, USA) at 550°C for 3 hours. The combusted remains were considered non-organic ash and we subtracted that mass from the initial mass to determine the proportion of organic material in the original sample. We calculated organic matter digestive efficiency as $\frac{(mass\ food\ ingested - ash) - (feces\ mass - ash)}{mass\ food\ ingested - ash}$.

Nutrient Content Assays— For component nutrient digestibilities, we homogenized portions of the dried feces in 10 volumes dilutions of 25 mM Tris HCl buffer, pH 8.6 (hereafter referred to

as “buffer”) for carbohydrate and protein content analyses, or the same Tris buffer with 5.8 mM Sodium Cholate added (to ensure emulsification of the lipids) for the lipid content analyses. We chose a buffer at pH 8.6 as it was the average pH we measured in the intestinal fluids of the *P. sicula* (Wehrle *et al.*, *under review*). We used a Polytron homogenizer (Binkmann Instruments, Westbury, NY) with 12mm or 7mm generators set to 1100-3000 rpm for 3 x 30 s, with 30 s between pulses to homogenize tissues. For samples <300 µl after 10x dilution, we used a CL-18 Sonicator (Fisher Scientific, Waltham, Massachusetts, USA) at 5 W output for 30 s to break up the feces. All sonicating and homogenizing was done on ice. We centrifuged the homogenates at 4°C, 9400 x *g* for 2 min and recovered the supernatant. We flash froze homogenates in liquid nitrogen and stored them at -80° C until just before use in nutrient content assays.

For all assays, we prepared a standard curve to match nutrient content to measured absorbance. We measured nutrient content in duplicate or triplicate and read absorption in flat-bottomed 96-well microplates using a BioTek Synergy H1 Hybrid spectrophotometer equipped with a monochromator (BioTek, Winooski, VT, USA).

We used the Bicinchoninic acid assay (Smith *et al.*, 1985) to measure protein content of the samples using a bovine serum albumin as the standard. We thawed homogenates and further diluted them 1:10 volumes in buffer, combined them with 200 µL of BCA working reagent from a Pierce® BCA Protein Kit (Pierce Biotechnology, Rockford, IL, USA), and incubated the mixture at 37°C for 30 min. After the incubation, we read the absorbance at 562 nm.

We determined total soluble carbohydrate content of the samples via methods developed by Dubois and others (1956), wherein larger polysaccharides are hydrolyzed through boiling, and we measure the resulting reducing sugars colorimetrically through the reaction of phenol with sulfuric acid. Homogenates were boiled for 30 min in a water bath, cooled, then we added 5%

phenol and 18M sulfuric acid. We used a solution of glucose in buffer as the standard. We incubated samples at room temperature for 10 minutes then transferred them to a shaker/incubator to shake at ~100 rpm at 30°C for 40 min. After the shaking incubation, we read the absorbance at 490 nm.

We conducted lipid content analyses on the samples from the high frequency feeding trial only. We used a charring method in sulfuric acid for the determination of total lipid content (Marsh and Weinstein; 1966), extracting the lipids following the Bligh and Dyer (1959) solvent extraction method, using a standard of stearic acid mixed with 100% chloroform. We mixed sample with 2:1 Chloroform:methanol and vortexed for 30-sec on/off intervals for a total of 10 min, then vortexed again after adding 1M NaCl. We centrifuged the mixture at 6200 x g for 2 min and collected the liquid below the protein disc. The liquid was baked at 60°C for 60 min to evaporate the solvent. We added 36 M sulfuric acid and combusted the solution in a Lindburg/ Blue M combusting oven at 200°C for 15 min. We cooled the charred solution and added nanopure water, then read the absorbance at 375 nm.

We calculated each component nutrient digestibility as

$$\frac{(total\ nutrient\ in food\ ingested) - (total\ nutrient\ present\ in\ feces)}{total\ nutrient\ in food\ ingested}.$$

Gut Length

At the end of the feeding experiments, all lizards were sacrificed and dissected. In addition to the lab animals described above, we dissected lizards (*N*=10-13) in the field from each represented group in each of the two time periods of our sampling (i.e.: males from Pod Kopište and Pod Mrčaru in 2013, males and females from Pod Kopište, Pod Mrčaru, and Split in 2014). Lizards were weighed to the nearest 0.1-g and euthanized via intramuscular injections of sodium pentobarbital (~0.1mg/g-tissue). We measured snout-vent length (SVL) and dissected the

lizards on sterilized, chilled dissecting trays (~4°C). We removed the entire gut from esophagus to cloaca and measured the whole gut length. For wild lizards, we squeezed the contents out of their stomachs and weighed them to the nearest 0.1-mg to use as a proxy for daily intake.

Statistical Analyses

We compared digestibility and gut length among diet treatments (and for gut length only, wild individuals) within the same population and across experimental lizards of each population, within sexes. For the low frequency fed lizards, we also compared sexes within a population and diet treatment. All analyses were initially performed with ANCOVAs to check for covariance between our independent variables and body mass, SVL, total intake over the course of the study as a proportion of body mass, and for digestibility only, number of days in the study. If the ANCOVA, potential covariate, and/or independent variable- covariate interaction were non-significant, we report the results of ANOVAs. All analyses were done in R (version 3.4.3).

Results

High Frequency Feeding Trial

All lizards gained body mass over the course of the feeding trials.

Digestive Efficiency—The Pod Mrčaru lizards had a >1.7x (>16%) higher organic matter digestibility than the Pod Kopište lizards on the herbivore diet (Fig. 2.5a; $F_{1,8}$=7.495, P=0.0255). They also trended towards a higher organic matter digestibility of the omnivore diet than their Pod Kopište counterparts (~7.5% difference; $F_{1,8}$=4.92, P=0.0574), however this effect was just shy of significant. The two populations did not differ in digestibility of an all insect diet. When

comparing organic matter digestibility across the three diets within each population, both populations showed the pattern of higher organic matter digestibilities on insectivore and omnivore diets than herbivore diets (Pod Mrčaru: ANOVA $F_{2,12}$=27.761, P<<0.0001), although in the Pod Kopište lizards, diet and total mass of food ingested over the course of the study covaried (Pod Kopište: ANCOVA diet: $F_{2,10}$=,42.86, P<<0.0001; total mass of food ingested over the course of the study: $F_{1,10}$=10.40, P=0.0091).

On the herbivore diet, the Pod Mrčaru lizards were 20x better at digesting plant proteins than the Pod Kopište lizards (Fig. 2.6a; $F_{1,8}$=7.059, P=0.0289). Protein digestibility did not differ by population on any other diet. Neither carbohydrate (Fig. 2.7) nor lipid digestive efficiency differed by population.

In all cases, nutrients from the insectivore diets were more digestible than from the herbivore diets. Whether the nutrients from the omnivore diets were equally digestible compared to the other two diets or distinctly intermediate varied by population. For protein and carbohydrate digestibility, the omnivore diet was more digestible than the herbivore diet in both populations (protein: Pod Kopište: ANCOVA diet: $F_{2,9}$=215.12, P<<0.0001, body mass: $F_{1,9}$=14.98, P<0.0038, diet*body mass: $F_{2,9}$=26.87, P<0.0002; Pod Mrčaru: ANOVA $F_{2,11}$=16.3, P<0.0006; carbohydrate: Pod Kopište: ANOVA $F_{2,12}$=134.12 P<<0.0001; Pod Mrčaru: ANOVA $F_{2,11}$=48.14, P<<0.0001). (In fact, protein digestibility was negative-- meaning the lizards were losing protein--on the herbivore diet for both populations.) Yet in the Pod Mrčaru population, the protein and carbohydrates from the insect and mixed diets were equally digestible whereas in the Pod Kopište lizards, the protein and carbohydrates of the omnivore diet was less digestible than the insect diet. In addition, in Pod Kopište only, more massive lizards were more efficient at digesting protein on the herbivore diet, whereas there was no effect of body mass on the

insectivore nor omnivore diets. Carbohydrate digestibility was not affected by lizard body mass. Lipid digestibility was equivalent between the insectivore and omnivore diets in both populations, but in the Pod Mrčaru lizards, lipid digestibility was also equivalent between the omnivore and herbivore diets, in contrast to the decreased lipid digestibility on the all plant diet for the Pod Kopište lizards (ANOVAs: Pod Kopište $F_{2,12}$=32.29, P<<0.0001; Pod Mrčaru $F_{2,11}$=7.428, P<0.0091).

Gut length—Lizards dissected in the field that were collected at the same time (summer 2013) as the lizards used for the high frequency feeding trial did not have different gut lengths by population (Fig. 2.10a), nor were their gut lengths different from the lizards from the lab feeding trial (Fig. 2.11a). At the end of the high frequency trial, lizards did not have different gut lengths by population (Fig. 2.10a) nor by experimental diet (Fig. 2.11a).

Low Frequency Feeding Trial

By the end of the experiment, 73% of the low-frequency trial lizards had lost weight (12±8% loss from their original body mass), 6% had no change in body mass, and 21% gained weight (5±4% gain from their original body mass). Pod Kopište males were more likely to gain weight on an herbivore diet (Fisher's exact test P<0.0338) and Pod Mrčaru males were more likely to lose weight on an omnivore diet (Fisher's exact test P<0.0149) than were males of other populations on those same diets. Pod Kopište and Split males were less likely to lose weight on insectivore diets than any other diet (Fisher's exact test: Pod Kopište P<0.0096; Split P=0.00667), but Pod Mrčaru lizards did not show this pattern. Females gained and lost body

mass equally across populations, diets, and compared to males. Change in body mass was not correlated with digestive efficiency.

Digestive Efficiency—The male lizards did not differ in their organic matter digestibility by population. The females of Pod Mrčaru and Split, however, had a >1.2x higher organic matter digestibility than the Pod Kopište females on the insectivore diet (Fig. 2.5c; ANOVA $F_{2,8}$=22.01, P<0.0006) but their digestibility on the other diets did not differ by population. Like in the high-frequency feeding trial, organic matter digestibility was higher on insectivore diets than herbivore diets (Pod Kopište: males ANOVA $F_{2,19}$=4.14, P<0.0323; Pod Mrčaru: males ANCOVA diet $F_{2,14}$=13.060, P<0.0015, days in study $F_{1,14}$=7.749, P=0.0146, females ANOVA $F_{2,10}$=20.66, P<0.0003; Split ANOVAs: male $F_{2,18}$=19.98, P<<0.0001; females $F_{2,11}$=46.1, P<<0.0001) excepting in the Pod Kopište females that had equivalent organic matter digestibility on all three experimental diets. Each other population and sex combination showed a different relationship between the organic matter digestibility of the omnivore diet and the other two diets (Table 2.1).

On the insectivore diet, the Pod Mrčaru and Split female lizards were >1.7x better at digesting protein (Fig. 2.6c, Fig. 2.7; ANCOVA population: $F_{2,5}$=59.396, P<0.0004; body mass: $F_{1,5}$=17.050, P<0.0091; population*body mass: $F_{2,5}$=7.117, P<0.0345) than the Pod Kopište lizards. Protein digestibility did not differ by population on the herbivore and omnivore diets, nor were the males of any population different from each other (Fig. 2.8b).

Once again on the insectivore diet, the Pod Mrčaru and Split female lizards were better at digesting carbohydrates (Fig. 2.8c ANOVA $F_{2,8}$=26.81, P<0.0003) than the Pod Kopište lizards. When the interaction of population and number of days each individual was in the digestibility

study was taken into consideration, this same pattern appeared for the females on the herbivore diet (Fig. 2.8c, Fig. 2.9 ANCOVA population: $F_{2,9}$=8.806, P< 0.0077; days: $F_{1,9}$=3.683, P=0.0872; population*days: $F_{2,9}$=6.946, P<0.0150). However, only the Pod Kopište population increased carbohydrate digestibility as individuals were on the herbivore diet longer. The males did not differ by population (Fig. 2.8b).

We did not measure lipid digestibility for any of the low-frequency feeding trials due to insufficient feces to conduct this analysis.

Gut length— Male lizards dissected in the field at the same time (summer 2014) that we collected the lizards used for the low frequency trial had longer guts in the Pod Kopište population compared to the Pod Mrčaru population (ANOVA $F_{2,27}$ =4.567, *P*=0.0196), but neither differed from the Split population (Fig. 2.10b). On the omnivore experimental diet, however, Pod Mrčaru males fed a low frequency of the experimental omnivore diet had longer guts than their Pod Kopište counterparts (ANOVA $F_{2,11}$ =7.488, *P*<0.0089). Yet, on pure plant or insect diets, no male differed in gut length with respect to population. Females dissected in the field, on the other hand, had longer guts in the Pod Mrčaru population compared to the Split population (ANOVA $F_{2,27}$=3.89, P=0.0328) and neither differed from the wild Pod Kopište females (Fig. 2.10c). While our data suggests that the Pod Kopište females on the low frequency insectivore diet had shorter guts than the other two populations when controlled for SVL (ANCOVA population: $F_{2,6}$=7.44, P<0.0238; SVL: $F_{1,6}$=14.44, P<0.009), we were only able to measure N≤2 Pod Kopište females for each experimental diet due to low survivorship in the lab.

Among experimental diets within a population, no lizards had different gut lengths. However, several experimental diets led to shorter guts than we measured in populations in the

wild. In males, only Pod Kopište omnivores had shorter guts than their wild counterparts (Fig. 2.8b; ANCOVA diet: $F_{3,21}$=4.23, P<0.0174; SVL: $F_{1,21}$=8.88, P<0.0072). In females (Fig. 2.11c), each population had shorter gut lengths on insectivore (Pod Mrčaru: diet: $F_{3,14}$=3.921, P=0.0318; SVL: $F_{1,14}$=4.805, P=0.0458), omnivore (Split: $F_{3,18}$=3.367, P=0.0416), or both of those experimental diets (Pod Kopište: diet: $F_{2,10}$=5.802, P<0.0213; SVL: $F_{1,10}$=14.191, P<0.0037) compared to individuals measured in the field. Once again, we are not confident about our Pod Kopište female gut lengths due to low sample sizes.

High vs. Low Frequency Feeding

In the males of Pod Kopište and Pod Mrčaru, we were able to compare digestibility and gut lengths on the high and low frequency feeding trials.

Digestibility— Organic matter digestibility was higher in the low frequency feeding trials for Pod Mrčaru lizards on all diets, and for the Pod Kopište lizards on the herbivore diet (however there was also a significant effect of total food intake amount over the course of the study for this diet and population combination). The insectivore and omnivore diets were 18% and 15% more digestible, respectively, for the Pod Mrčaru males when fed at the lower frequency (ANOVAs insectivore: $F_{1,9}$=6.617, P=0.0301; omnivore: $F_{1,9}$=8.672, P=0.0164). The herbivore diet was 58% and 148% more digestible for the Pod Mrčaru and Pod Kopište lizards, respectively, when fed at the lower frequency (Pod Mrčaru: ANOVA $F_{1,8}$=30.27, P<0.0006; Pod Kopište: ANCOVA trial: $F_{1,10}$=33.424, P<0.0002; total intake: $F_{1,10}$=6.599, P=0.0279). The higher Pod Kopište digestibility result is partially due to a considerably lower total food intake over the

course of the entire feeding trial in the low-frequency experiment that could not be avoided due to food rejection (via vomiting).

Gut length— Male lizards dissected in the field at the same time that we collected the lizards used for the high frequency (summer 2013) and low frequency (summer 2014) trial had different gut lengths in each year in the Pod Mrčaru population but stayed constant in the Pod Kopište lizards. The Pod Mrčaru lizards measured in the field in the low frequency year (summer 2014) had longer guts than the previous year (ANOVA $F_{1,21}$=27.33, P<<0.0001). However, on the experimental diets, Pod Mrčaru lizards' gut length did not vary between frequency trials/years. The opposite was true of Pod Kopište lizards—while their gut lengths did not vary in the field, on the omnivore diet, the low frequency trial lizards had longer guts than their high frequency counterparts (ANCOVA trial: $F_{1,9}$=6.879, P<0.0277; SVL: $F_{1,9}$=13.441, P<0.0052)

Discussion

When challenged with the high frequency feeding of daily meals, the new omnivores of Pod Mrčaru were better at digesting plant proteins than were their source population counterparts, matching a rate maximizing strategy. This daily feeding was likely similar to the Pod Mrčaru lizards' behavior in the wild where they are observed actively foraging throughout the day (A. Herrel, *pers. obs*.). The Pod Kopište lizards, on the other hand, are less active outside of refugia and likely feed less frequently than their Pod Mrčaru counterparts. With frequent, high volume feeding, digesta flow is increased, leading to decreased digesta transit time. Indeed, per equation 1, $Digestibility \propto \frac{enzyme\ activities}{substrate\ concentration} \propto \frac{gut\ size}{digesta\ transit\ rate} \propto time$, decreased transit time of digesta decreases digestibility if all other factors are unchanged. With a decrease in transit

time comes decreased opportunities for digesta to interact with digestive enzymes and gut microbes and decreases time for nutrient absorption. The valves in the hindguts of Pod Mrčaru lizards may slow the digesta down to a maximum velocity at which digesting plant material is still possible. Pod Kopište lizards do not have means by which to slow their digesta transit to enough to use plant material at this high an intake rate. In concert with organic matter digestibility, wild-caught Pod Mrčaru lizards show increased carbohydrase (i.e.: amylase) and protease (i.e.: trypsin) activities in their hindguts than the Pod Kopište lizards (Wehrle *et al.*, *under review*), providing a potential mechanism for differences in digestibility. Furthermore, the population differences we found in digestibility were revealed only with high intake, which supports the hindgut valves as an accommodation for a plant diet. By increasing transit time in the hindgut, an area where Pod Mrčaru lizards have the biochemical advantage over the Pod Kopište population, the Pod Mrčaru lizards are able to have a compounded digestibility advantage.

On the high frequency intake treatment, the Pod Mrčaru lizards trended strongly towards a 7.4% increased digestibility of the omnivore experimental diet compared to the Pod Kopište lizards. This may be an additive effect (Bjorndal, 1991; Bouchard and Bjorndal, 2006), showing the digestibility of the mixed plant/insect diet as an intermediate effect. While both populations had lower digestibility of the herbivore diet, the clear lack of population differences in digestibility of the insectivore diet was missing for the omnivore diet.

It is not surprising that the males of the two populations show no differences in digestibility of an insect diet. Animal material is generally more digestible than plant material (Pough, 1973; McKinon and Alexander, 1999). It is more nutrient dense and, bite-for-bite, requires less intake to meet energetic needs (Bowen *et al.*, 1995). As such, the male lizards of

two island populations and the mainland population were not challenged by the insectivorous diet and thus were equally successful at digesting it.

Our hypothesis that less frequent intake would lead to higher digestibility due to increased digesta transit time was supported in the Pod Mrčaru population for each of the three diets. The Pod Kopište lizards, however, only showed this pattern on the herbivore diet. When fed the insectivore or omnivore diet, the frequency of intake did not change the digestibility. This suggests that the Pod Kopište lizards had already reached their digestive maximum, perhaps due to their ease of digestion of insect material. This supports the Pod Kopište lizards as yield maximizers, using time to acquire as much nutrient from their food as possible.

The female lizards from Pod Kopište had a lower digestibility (of organic matter, protein, and carbohydrates) on the insectivore diet than did their Pod Mrčaru and Split counterparts. Considering population and sex as explanatory variables, this finding is unexpected for several reasons. Firstly, our expectations of the three populations place the Pod Mrčaru and Split populations as the most different ecologically and evolutionarily (based on the premise that the Pod Kopište lizards have not evolved in the past ~40 years). The Split population is from a semi-urban area on the mainland. While they have been found to eat some plant material (A. Herrel, B. Wehrle, *unpub. data*), this is mostly easily digestible fruits supplementing a predominantly carnivorous diet. Thus, as both populations are insectivorous, we expected the Split lizards to be most similar to the Pod Kopište population. If digestive performance were most similar among closely related populations, the Pod Kopište and Pod Mrčaru populations should have the same patterns of digestibility.

Secondly, as the Pod Kopište lizards are insectivores, we would expect them to be most adept at digesting insect material compared to the newly omnivorous Pod Mrčaru population, or

for the two populations to be equivocal. If the Pod Mrčaru lizards had an adaptation that made them more efficient at digesting overall so as to get more nutrients from their more recalcitrant plant diet in the wild, we would expect to see higher digestibility on each of the three experimental diets (Vervust *et al*., 2010). When controlled for number of days each lizard was in the study, we do find the Pod Mrčaru and Split females have higher carbohydrate digestibility on the herbivore diet than do the Pod Kopište lizards. Thus, it appears the Pod Kopište females started out with lower ability to digest plant carbohydrates, but were acclimating to the plant diet while in the lab.

Lastly, this of pattern digestibility is different than what we found for the males, both in the high and low frequency feeding regimens. This is not unprecedented in other taxa as Markman and colleagues (2006) found sunbird females had a higher digestive performance of one sugar substrate over another, whereas the males showed the reverse. In this study, the high frequency fed males of Pod Mrčaru were more efficient at digesting a plant diet due to more efficient protein digestion. However, the females of Pod Mrčaru and Split derived their higher digestibility of an insect diet from both higher protein digestion and higher carbohydrate digestion compared to their Pod Kopište counterparts. The Pod Kopište females increase protein digestibility considerably as the lizards themselves increase in body mass (Fig. 2.7), a pattern only slightly seen in the Split females, and absent in the Pod Mrčaru population.

While we do not have an ultimate explanation for the unexpected high insect digestibility in the females of Pod Mrčaru and Split, we can draw some conclusions. As the males and females of these populations generally exhibit different patterns by population, this supports that there are sex*population interactions in digestive strategies. In their description of this system, Herrel and colleagues (2008) found no differences in diet between male and female lizards.

Thus, the different patterns of digestive performance we observed show different strategies to meet the same goals—those goals being to subsist off of their insectivore or omnivore diets. Indeed, whether the omnivore diet was as digestible as the insectivore or herbivore diets was different by sex in each population, suggesting some physiological niche partitioning by sex. This may be due to different thermal ecologies of males and females (Liwanag *et al.*, 2016), different energy uses (e.g.: social and reproductive activities, energy storage; for example: Baird *et al.*, 2003; Beaupre *et al.*, 1993; Jackson *et al.*, 2015; Derickson, 1976). It is important to note that the female lizards from Pod Kopište had a low survivability (16%) in the lab, though this was not significantly different from the other two populations ($\chi^2 = 2.696$, df = 2, P = 0.2598). Each group's response to captivity also played a role in their digestive performance in ways that we are unable to control for.

Gut length did not show a pattern with digestibility as would be expected based on equation 1 with a larger gut size (i.e.: length) allowing for higher digestibility. On the high frequency intake trial, we found no differences in gut length by diet or by population. The lizards fed less frequently did show some differences in gut length by population and by experimental diet. However, these data are confounded by being collected in different years. We had expected patterns of gut length to remain constant from year to year in the same season. The gut lengths of wild lizards collected in tandem with the high frequency feeders did not vary by population. Those collected with the low frequency fed lizards did differ by population, but once again, these patterns were different by sex. As much as we would like to attribute gut length to dietary strategy and examine its relationship to digestibility, this year, population, and sex interplay shows more complexity at work than we can untangle in this study. Indeed, the low survivorship of the Pod Kopište females particularly affected our gut length sample sizes. We do feel

confident concluding that gut length did not show clear patterns of increasing in the lab with increased plant material in the diet, as has been seen in other animals such as fish (German *et al.*, 2015; Leigh *et al.*, 2018a), birds (Savory and Gentle, 1976; Dykstra and Karasov, 1992), beetles (Bounoure, 1919), and mammals (Selman *et al.*, 2001; Stevens and Hume, 2004).

Few studies on digestion in reptiles have fed plant material to insectivores (Ruppert, 1980), as even omnivores and herbivores are often unwilling to freely eat plant material in captivity. We recognize that modulating intake is one of the ways in which animals eating a plant-based diet regulate their nutrient acquisition. However, in this common garden experiment, we were able to successfully force feed each population the same three diets. By choosing a plant diet rich in seeds and with fibrous plant material from Pod Mrčaru, we did our best to recreate the diet of the Pod Mrčaru lizards in the wild. Thus, using the Pod Kopište lizards, we simulated the experiences the founder population of *P. sicula* on Pod Mrčaru after their transplantation. On the high frequency feeding regimen, none of the lizards digested plant protein well. All lost more protein than they were able to digest, although the Pod Mrčaru lizards lost less. However, with the supplementation of a little insect material, the two populations were able to achieve equivalent digestibilities of their diets and stay in positive protein balance.

Because our experimental plant diet and the Pod Mrčaru population's natural diet was primarily seeds, we would have expected to find differences in lipid digestibility. While we did not find this generally, the lizards may have metabolized different lipids and developed affinities to use different types of fats found in different sources. However, looking into that is outside of the scope of this study. We appreciate, too, that a higher fiber diet (i.e.: primarily leaves) may have shown different patterns of digestibility in our lizards, especially concerning carbohydrate

and lipid digestibility, but we were more interested in diets that the new omnivores of Pod Mrčaru are actually consuming and using for their nutritional needs.

As the males and females in our system showed very different patterns of digestive performance, this solidifies the importance of including females in physiological studies. We tested digestibility at a time of year when the lizards are not reproductively active, thus sex effects are likely not due to reproductive status. Had we just included the males, we would have likely concluded that sex does not play a role in digestion. However, it is apparent that males and females employ different strategies for digestion and thus we must consider the diversity of the population in order to understand what kind of evolutionary pressures and shifts are contributing to this case of rapid evolution.

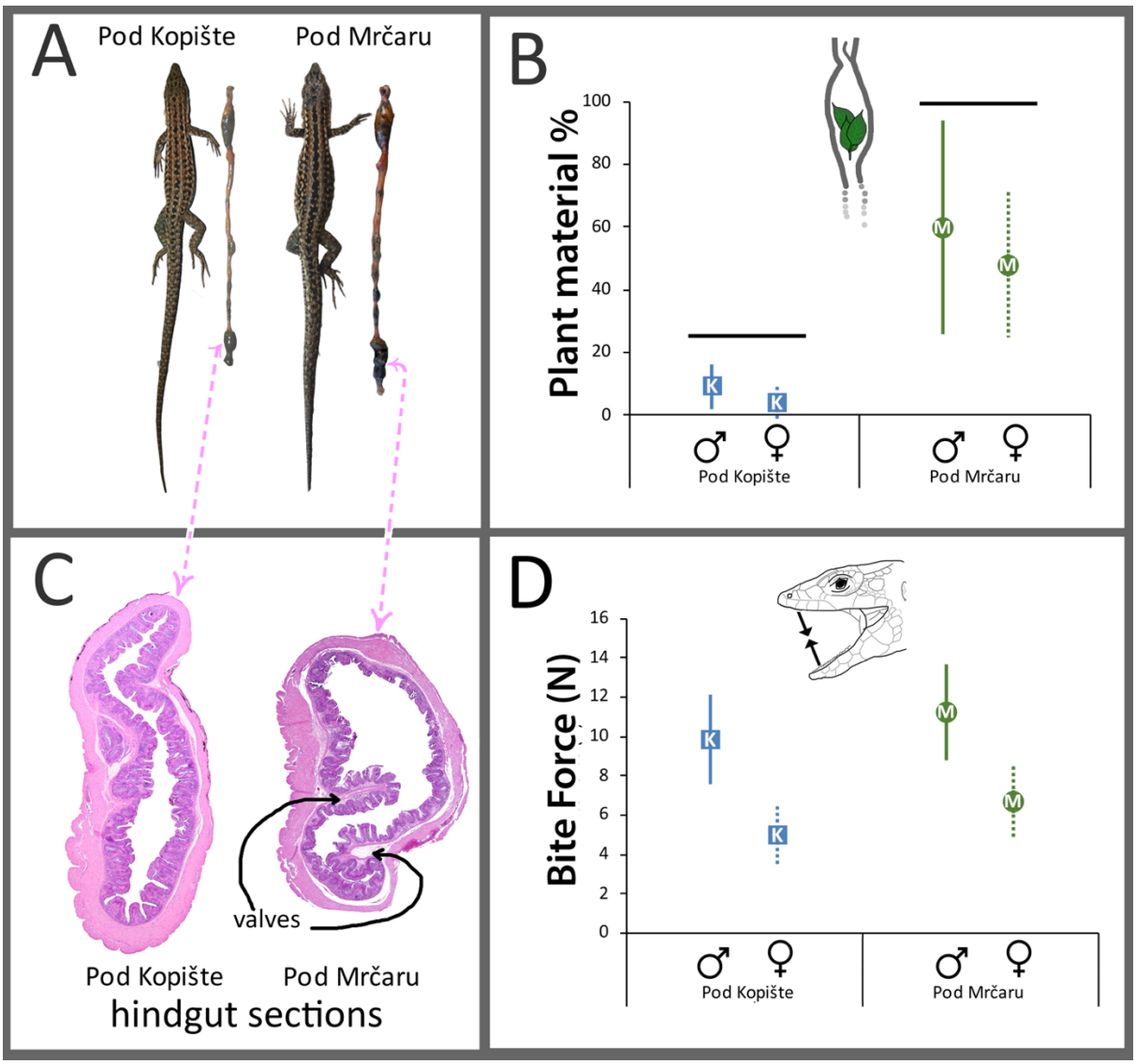

Figure 2.1 Selected differences between Pod Kopište (source population) and Pod Mrčaru (new omnivores) lizards found by Herrel *et al.* (2008). Pod Mrčaru lizards were (A) more massive and longer and have different head morphometrics (to scale). (B) The Pod Mrčaru population had a higher percentage of plant material in their stomachs. Values are means ± standard deviation and lines of different elevations above markers denote differences. (C) Representative microscopy images of hindgut cross sections (not to scale). Valves were present in hindguts of Pod Mrčaru lizards and absent in Pod Kopište lizards. (D) Bite forces in newtons. Pod Mrčaru males and females had higher bite forces than Pod Kopište males and females, respectively. Values are means ± standard deviation. Each group was significant different from all others.

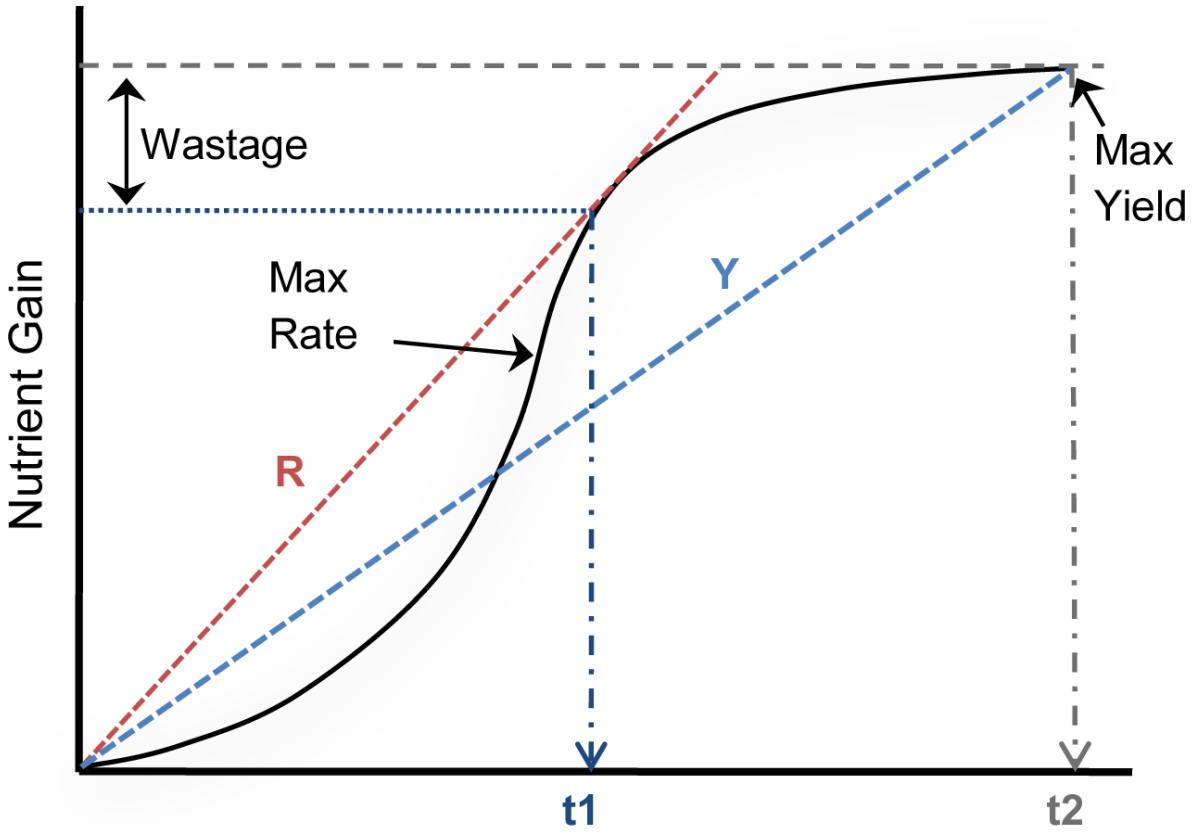

Figure 2.2 Cumulative nutrient gradient by a lizard as a function of time (from German *et al.*, 2015; modified from Clements and Raubenheimer, 2006). The slope of the black line at the point labeled "Max Rate" is the maximum rate that a nutrient can be absorbed from the meal. The value of the point labeled "Max Yield" is the maximum amount of a nutrient that a lizard can absorb from the meal. An ideal rate maximizing strategy (line R) is tangent to the curve with defecation at time 1 (t1). Some of the nutrient is lost in the feces ("wastage"), but at t1 the lizard can refill its gut. A max yield (line Y) strategy retains the meal until time 2 (t2) to absorb the maximum nutrient from the meal and minimize wastage. However, a yield maximizing strategy sacrifices high digestive rate for high digestive efficiency.

Figure 2.3 *Podarcis sicula* island collection sites showing Pod Kopište (source population) and Pod Mrčaru (newly omnivorous population). In the bottom map, Split collection site (mainland population) is marked with a black circle and the box shows the area of the inset.

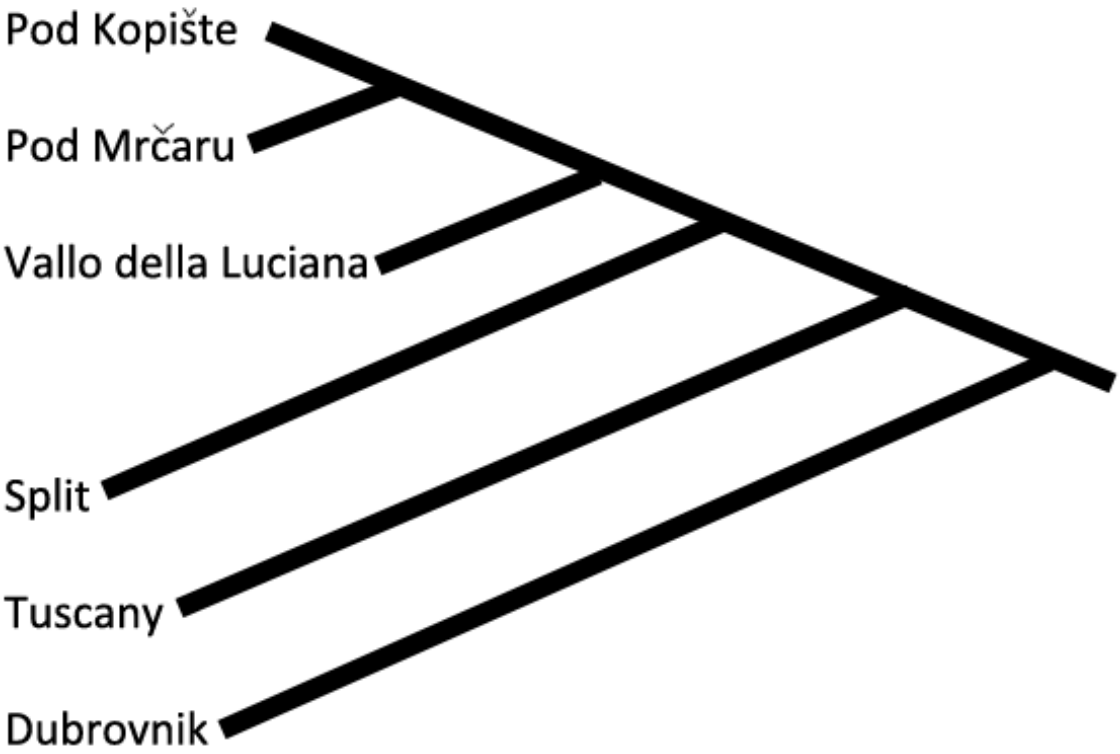

Figure 2.4 Phylogeny of cytochrome b and *16s r*RNA haplotypes in *Podarcis sicula* for this system and most closely related mainland populations, modified from Podnar *et al*. (2005). Each name is a locale in Croatia or Italy.

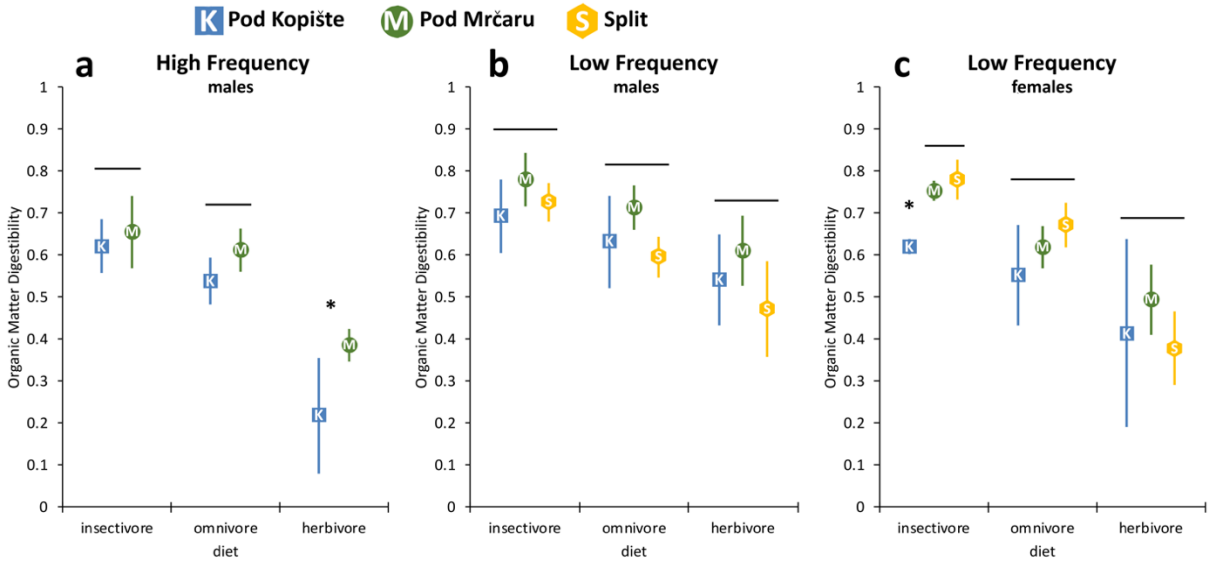

Figure 2.5 Organic matter digestibility as a proportion of total ingested food by experimental diet and population in the (a) high frequency trial, (b) males of the low frequency trial, and (c) females of the low frequency trial. Values are mean ± standard deviation, *N*=3-8, mean *N*=5.4. Populations compared within diets via ANOVAs, lines above icons denote no differences, whereas * denotes significant differences. Note in (a) Pod Mrčaru lizards trend towards higher digestibility of an omnivore diet than Pod Kopište, *P*=0.057.

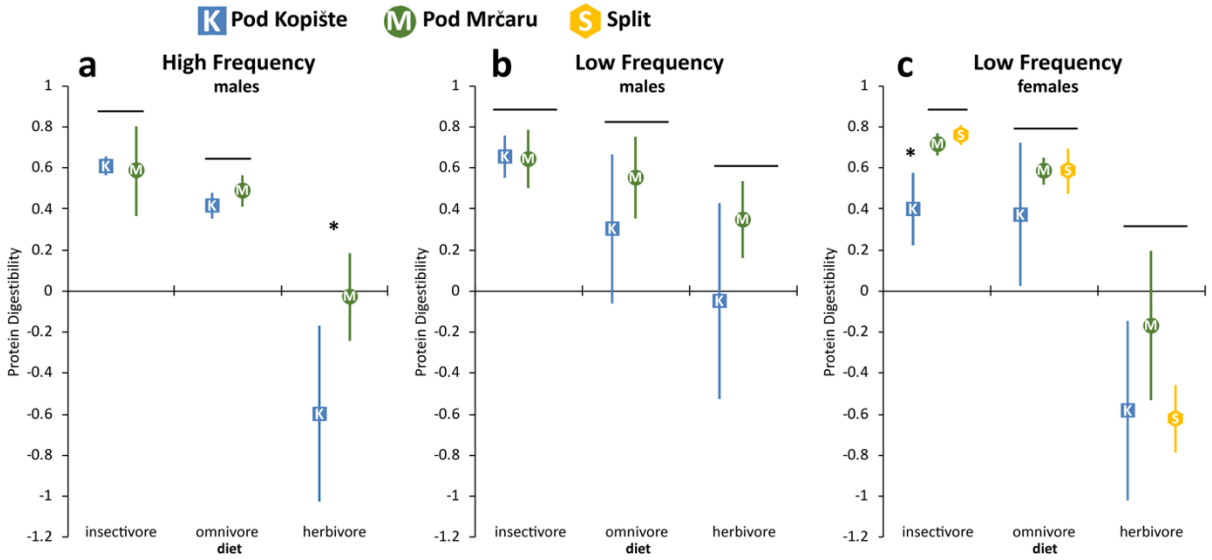

Figure 2.6 Protein digestibility as a proportion of total ingested food by experimental diet and population in the (a) high frequency trial, (b) males of the low frequency trial, and (c) females of the low frequency trial. Values are mean ± standard deviation, *N*=3-8, mean *N*=5.1. Populations compared within diets via ANOVAs, lines above icons denote no differences, whereas * denotes significant differences.

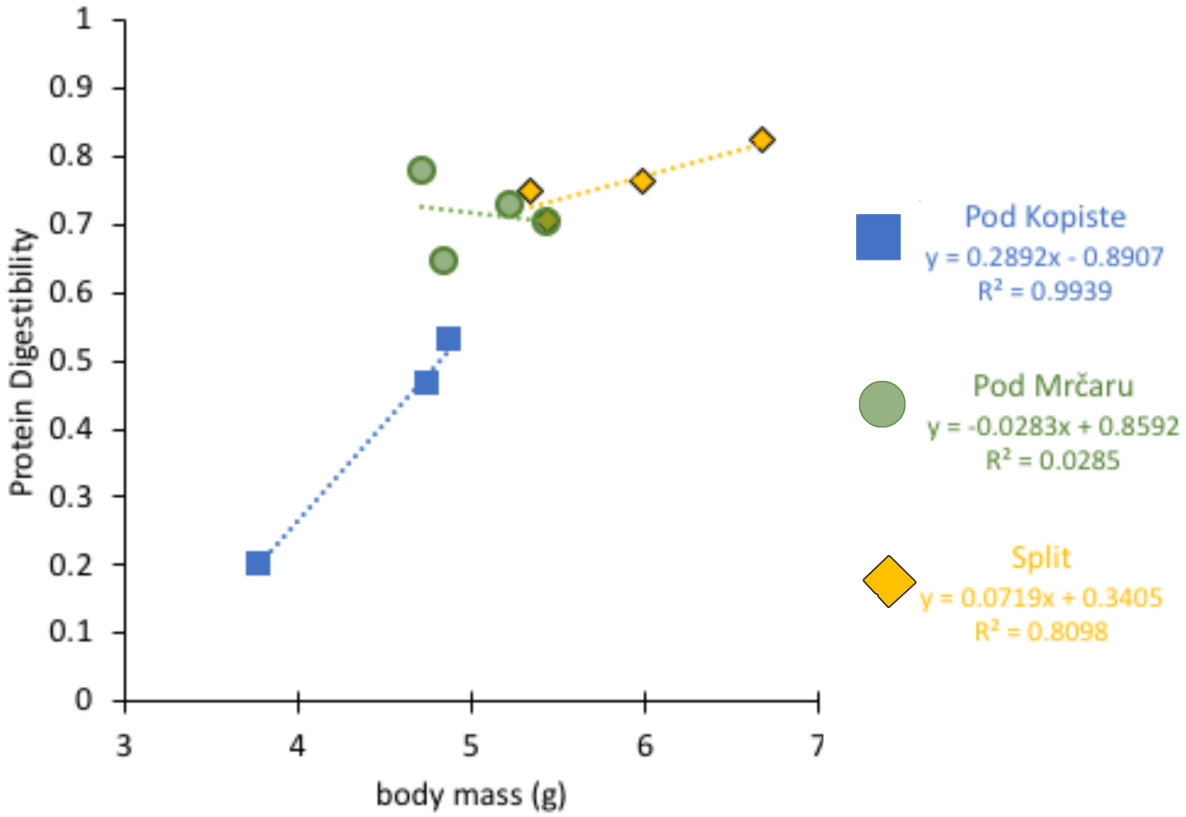

Figure 2.7 Protein digestibility by body mass in female lizards on the insectivore experimental diet. An ANCOVA of protein digestibility by population with body mass as a covariate showed Pod Kopište digestibility as different than that of Pod Mrčaru and Split, with significant effects of body mass and population*body mass.

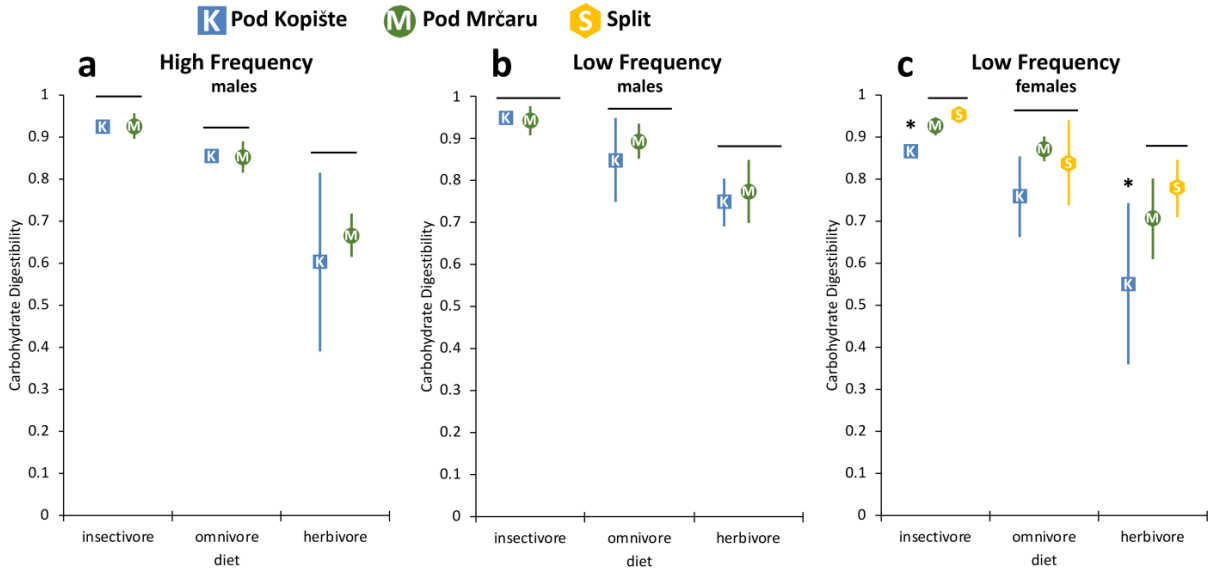

Figure 2.8 Carbohydrate digestibility as a proportion of total ingested food by experimental diet and population in the (a) high frequency trial, (b) males of the low frequency trial, and (c) females of the low frequency trial. Values are mean ± standard deviation, *N*=3-8, mean *N*=5.2. Populations compared within diets via ANOVAs or, for (c) the herbivore groups was compared via ANCOVA with population covaried with number days each lizard was in the study. Lines above icons denote no differences, whereas * denotes significant differences.

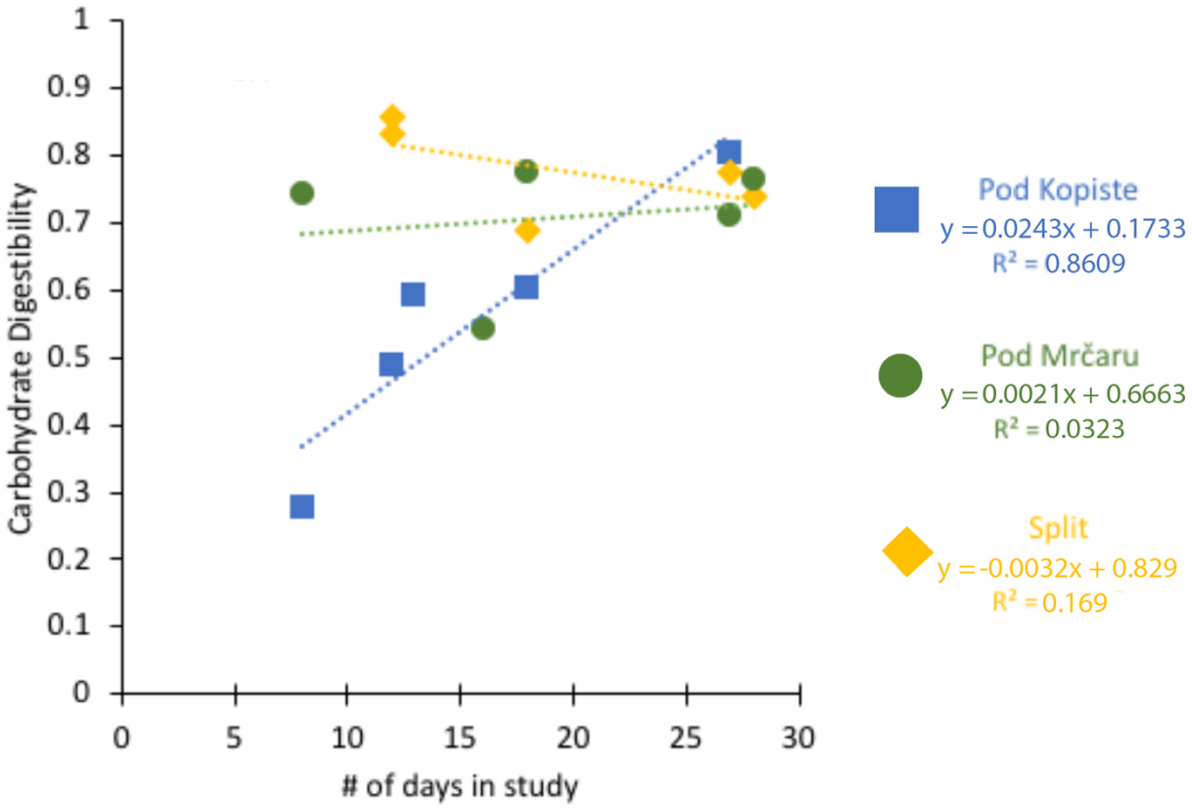

Figure 2.9 Carbohydrate digestibility by number of days in the study in female lizards on the herbivore experimental diet. An ANCOVA of carbohydrate digestibility by population with number of days in study (days) as a covariate showed Pod Kopište digestibility as different than that of Pod Mrčaru and Split, with significant effects of days and population*days.

Table 2.1 Low frequency diet trial: quantitative relationship of organic matter digestibility between experimental diets within a sex within a population. Diets are I = insectivore, O = omnivore, H = herbivore and relationship is denoted as greater than (>), equivalent (=), or lesser than (<).

Population	Sex	Relationship of OM Digestibility
Pod Kopište	Male	I > H \\ // O
	Female	I = O = H
Pod Mrčaru	Male	I = O > H
	Female	I > O = H
Split	Male	I > O = H
	Female	I = O > H

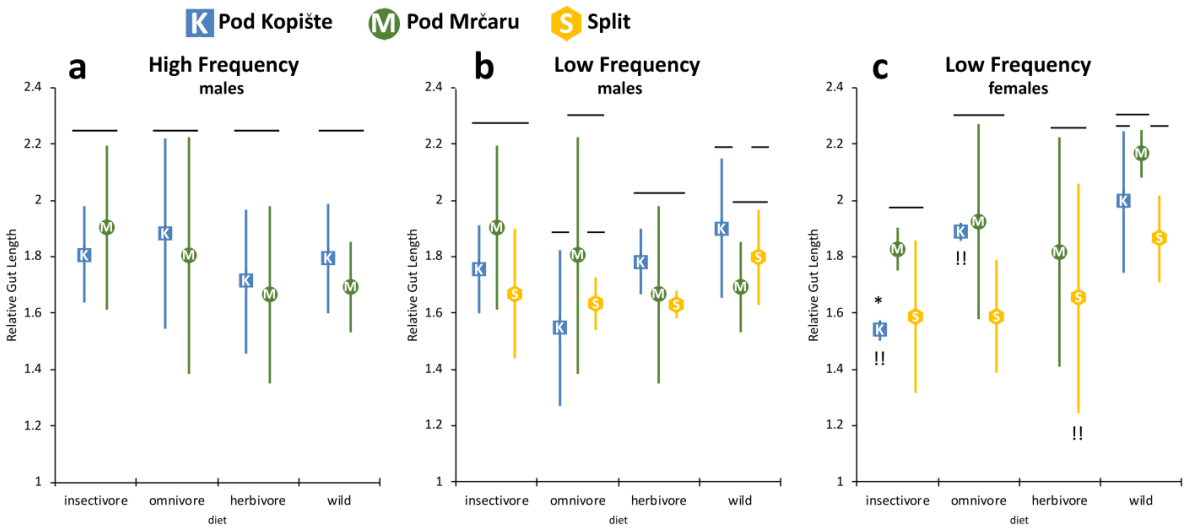

Figure 2.10 Gut length standardized to SVL compared by population on each experimental diet and in wild individuals in the (a) high frequency trial, (b) males of the low frequency trial, and (c) females of the low frequency trial. Values are mean ± standard deviation. Note we have no measurements for Pod Kopište females on the herbivore diet, and several other groups have *N*=2 (denoted with ‼), otherwise *N*=3-13, average *N*=6.4. Populations compared within diets via ANOVAs and ANCOVAs with SVL as a covariate, lines of the same elevations above icons denote no differences, whereas * denotes significant differences.

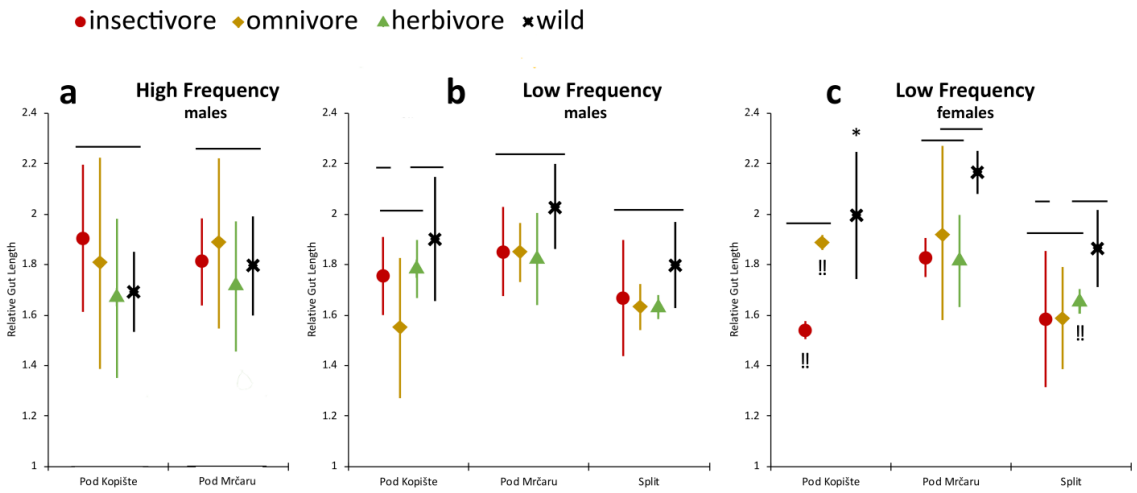

Figure 2.11 Gut length standardized to SVL compared by diet in the (a) high frequency trial, (b) males of the low frequency trial, and (c) females of the low frequency trial. Values are mean ± standard deviation. Note we have no measurements for Pod Kopište females on the herbivore diet, and several other groups have *N*=2 (denoted with ‼), otherwise *N*=3-13, average *N*=6.4. Populations compared within populations via ANOVAs and ANCOVAs with SVL as a covariate, lines of the same elevations above icons denote no differences, whereas * denotes significant differences.

Chapter 3

Increased differences in spring and oppositional effects by sex on the digestive physiology and gut structure of a newly omnivorous lizard

Introduction

Studies of digestive tract structure and function in ecological and evolutionary contexts are limited, particularly in ectothermic animals (German *et al.*, 2010; Karasov *et al.*, 2011). This is surprising given that an animal's diet and digestive physiology influence its resource acquisition, behavior, and ecological and trophic interactions (Karasov and Martínez del Rio, 2007). What an animal eats is not necessarily what it digests (e.g.: in fish, German and Miles, 2010; German, 2009; Lujan *et al.*, 2011). For example, *Salvator meriange*, a culturally and economically important lizard in South America, had been considered omnivorous until researchers found that these lizards have very little ability to digest the plant matter they may ingest (Vega Parry et al, 2009). Misidentifying how an animal uses resources may have consequences in estimating its niche and ecosystem contribution, highlighting the importance of investigating nutritional physiology (Karasov *et al.*, 2011; Tracy *et al.*, 2006; Leigh *et al.*, 2018b). But even if we get a "snapshot" of a population's nutritional physiology, how static is this through time? What role do ecological and life history factors play in nutrient acquisition?

We investigate these factors in Italian Wall lizards (*Podarcis sicula*) from two islets in the Adriatic Sea of Croatia, a system known as an example of rapid evolution (Herrel *et al.*, 2008; Vervust *et al.*, 2010). Prior to 1970, *P. sicula* were not present on the tiny island of Pod Mrčaru (Nevo *et al.*, 1972). Less than 40 years after an experimental relocation of five male-female pairs of lizards from the source population of Pod Kopište to Pod Mrčaru (Nevo *et al.*,

1972), *P. sicula* were not only established at a high density on the new island, but had diverged in morphology and diet (Herrel *et al.*, 2008). The new population on Pod Mrčaru had become omnivorous and consumed more plant material in summer (61% plant material) than in spring (34% plant material). The Pod Kopište lizards maintained a primarily insectivorous diet, eating only 7% and 4% plant material in summer and spring, respectively, significantly less than their Pod Mrčaru counterparts. A decade later in summer 2013, the Pod Mrčaru lizards consumed a similar proportion of plant material (64%), while the Pod Kopište lizards, still more insectivorous than the Pod Mrčaru lizards, had increased plants in their diets to 24% (Wehrle *et al.*, *under review*). Although the Pod Mrčaru lizards showed seasonal differences in diet and the Pod Kopište lizards did not, Herrel and colleagues (2008) found neither population exhibited dietary differences between males and females.

Overall, Herrel and colleagues (2008) found the Pod Mrčaru lizards were larger and morphologically distinct from their source population. Additionally, both adult and neonate lizards from Pod Mrčaru had developed valves in their hindguts, a feature not found in the Pod Kopište population. Hindgut valves in lizards are generally associated with highly derived herbivory (Iverson, 1982; Bjorndal, 1997; Stevens & Hume, 2004). These valves can slow the passage of digesta to allow more time for chemical processing and increase surface area for nutrient absorption and endosymbiotic microbial attachment.

Determining what an animal eats and how it digests its food requires a multi-faceted approach that considers not only the diet, but also digestive tract structure and function. Theoretical models such as the Chemical Reactor Theory (CRT; Penry & Jumars, 1986, 1987) and the related Adaptive Modulation Hypothesis (AMH; Ferraris & Diamond, 1989; Karasov & Diamond, 1983) postulate that an increase in a dietary substrate will lead to changes in gut

structure, digestive biochemistry, or overall intake of food to maintain overall digestibility of the new diet, and ostensibly, energy balance. Another model, the Nutrient Balancing Hypothesis (NBH; Clissold *et al.*, 2010, 2013), proposes that essential nutrients found scarcely in a diet (e.g. low protein intake) will be prioritized for digestion and metabolism (e.g. via increased proteases, increased amino acid transporters). Although these models are not mutually exclusive, they predict weighting of different nutritional needs (i.e. energy vs. specific nutrients). All three models predict changes in the physiology and morphology of the gut towards digesting the derived diet, as has been confirmed in experimental (Leigh *et al.*, 2018a; Buddington *et al.*, 1987; German *et al.*, 2004) and phylogenetic (German *et al.*, 2010; Schondube *et al.*, 2001; Kohl *et al.*, 2011) contexts. These models provide the "ultimate" reasons for why digestive innovations should arise. Comparative studies, on the other hand, provide opportunities to test the "proximate" mechanisms through which innovation arises, and the consequences of these changes on organismal performance.

As the Pod Mrčaru *P. sicula* ingests far more plant material than their Pod Kopište source population, we would expect them to accommodate their plant-rich diet with morphological and biochemical shifts to their digestive tracts (see Table 3.1 for predictions). Based on the theoretical models connecting diet to digestive physiology, we would expect these omnivorous lizards to: have longer, more massive guts with more surface area and structures to slow digesta, have increased activities of enzymes for breaking down plant material (e.g.: amylase, maltase, and perhaps lipase due to high seed content of the plant diet), equivalent activities of enzymes for digesting nutrients abundant in both diets (e.g. aminopeptidase to degrade proteins, lipase to degrade lipids) and decreased activities for degrading insect material (based on CRT and AMH) or have increased enzyme activity associated with acquisition of essential nutrients infrequently

found in the diet (i.e.: protein in a plant fiber rich diet; based on NBH). However, a study of males from these two populations in summer of 2013 (Wehrle *et al.*, *under review*) found that this system does not entirely fit these models. Researchers found no differences in gut length or Epithelial Surface Magnification (ESM) between the two populations. Pod Mrčaru lizards had increased amylase (degrades starch), trypsin (degrades protein), and trehalase (degrades an arthropod specific disaccharide) in their hindguts compared to their Pod Kopište counterparts. Additionally, another protease (aminopeptidase) was higher throughout the gut in the Pod Mrčaru lizards. As the morphological and physiological differences are not as stark as expected based upon the ingested diet, this may indicate that, like in *S. meriange* (Vega Parry *et al.*, 2009), the digested diet is not matched to what the lizards eat.

To account for this possible mismatch between ingested and digested diet, we compare the similarity of assimilated nutrients in the lizard tissues via stable isotope analyses (SIA). More similar isotopic niches in the lizard tissue across populations, sexes, and/or seasons would suggest more similar digested diet. For example, if the two populations have different isotopic signatures than each other, this supports that their different ingested diets are aligned with what they digest. However, if the two populations' isotopic signatures are not different, occupying the same isotopic niches, this suggests the two populations are not different in the diet they digest and assimilate. In particular, higher $\delta^{15}N$ signatures are associated with higher trophic levels (Martínez del Rio *et al.*, 2009; Vidal and Sabat, 2010). Based on stomach contents (Herrel *et al.*, 2008; Wehrle et al., *under review*), we expect isotopic niche to be different between the populations. Specifically, we expect higher $\delta^{15}N$ signatures in Pod Kopište lizards to align with the greater insect material in their stomachs, and distinctly different $\delta^{13}C$ signatures between

populations. But in addition to just population differences, do males and females on the same island occupy the same niche space? Does niche space change across seasons?

As Pod Mrčaru lizards consume more plant material in summer and thus their diets diverge more from the Pod Kopište source population's compared to spring, we would expect decreased differences in gut structure and function in spring compared to summer and signals of these dietary patterns in the biochemistry of their tissues. We expect to find greater differences in isotopic niche space between the populations in summer than in spring to reflect these ingested diet differences. With higher plant consumption in the Pod Mrčaru lizards in summer compared to spring, we expect their $\delta^{15}N$ signatures to be lower in summer than in spring. We hypothesize that males in spring will have no differences in gut length between populations, and perhaps, due to their lower plant diet in spring, Pod Mrčaru lizards will show decreased gut lengths then compared to lizards from summer. We would anticipate that any population differences in digestive enzymes would also be localized to the hindgut and would show the same general trends in the same enzymes in spring as was found in summer (i.e.: higher in Pod Mrčaru lizards: amylase, trypsin, and trehalase in the hindgut, and aminopeptidase throughout the gut).

As there were no differences found between diets of males and females in either population, we would expect females' gut form and function and isotopic signatures to match that of males of their population from the same season. However, despite the lack of sex differences in diet, researchers (Herrel *et al*., 2008) calculated higher phenotypic divergence rates in females in this system for >70% of the characters they measured associated with dietary switches. Taking this into consideration, it is likely that females will show greater differences between populations than are present in males.

In the present study, we compare the nutritional physiology and morphology in male lizards across seasons and between males and females in a single season in a system where a dietary shift rapidly occurred. Season can affect digestive physiology through temperature, food type, and food availability, in addition to variation in seasonal behavior and ecological factors not directly driven by nutrition (e.g.: social interactions, reproduction, predation pressures). Both gut size (Piersma & Lindstrom, 1997) and digestive biochemistry (Kofuji *et al.*, 2005; Naya *et al.*, 2006; Naya *et al.*, 2009, 2011; Schweitz *et al.*, 1973) have been found to vary with season in vertebrates. Females, however, have often been left out of physiological studies due to assumptions that females act as modified males when controlled for reproductive effects. Even if true, omission of females still erases part of the variation of the population when reproduction is a factor. Sex differences are often considered in the ecological and physiological studies in the context of reproduction, but not unrelated processes.

The island populations of *P. sicula* offer the rare opportunity to examine evolution in action in a vertebrate under natural conditions. In our study, we expand the scope of the natural conditions to include sexes, seasons, and years to determine if a "snapshot" of a system undergoing rapid evolution can inform us of evolutionary processes.

Materials and Methods

Gross morphology and Stable isotope analysis

We collected *P. sicula* from the Croatian islets of Pod Kopište and Pod Mrčaru in two spring (late April- early May) and two summer (late August- early September) field seasons spanning 2013-2015. In each season we collected 10-13 of each male and female lizards from each population, excepting in summer 2013 when we collected males only. We captured all lizards in

the morning after they became active. Lizards were kept individually in cloth bags and were euthanized and dissected upon returning to the laboratory (within four hours).

We weighed each lizard to the nearest 0.1-g and euthanized it via an intramuscular injection of sodium pentobarbital (~0.1mg/g-tissue). We measured snout-vent length (SVL) and dissected the lizards on sterilized, chilled dissecting trays (~4°C). We removed the entire gut from esophagus to cloaca and measured the whole gut length to the nearest 1-mm. For ≥3 from each available combination of population, sex, season, we removed the distal intestine (DI) and fixed it in McDowell Trump's fixative (4% formaldehyde, 1% glutaraldehyde, McDowell & Trump, 1976). We examined the fixed DI sampled with a blunt probe to confirm the presence or absence of hindgut valves.

To compare stable isotopic signatures between populations, males and females, and among seasons, we used elemental analysis of carbon and nitrogen from *P. sicula* livers. We used stable isotopic analyses (SIA) to examine integrated, longer-term (~20 days, Warne *et al.*, 2010) trophic level signals than can be obtained from stomach flushing. We removed a portion of the liver and flash froze it in liquid nitrogen and stored it at -80°C until it was dried for >48hrs at 60°C. Liver samples underwent elemental analysis in duplicate using a Thermofinnigan Delta Plus, Delta Plus XP, Delta V, or MAT 252 elemental analyzer at the UC Irvine Stable Isotope Ratio Mass Spectrometry Facility to determine $\delta^{13}C$ and $\delta^{15}N$ signatures.

Gut structure and Biochemistry

Dissections

We further dissected the guts of lizards collected in summer 2013 (N= 10 males) and spring 2014 (N= 10 males, 10 females). (Hereafter when referencing these comparisons, we will refer to

"summer" and "spring" without a year designation.) We divided the gut into stomach, proximal intestine (PI), mid intestine (MI), and distal intestine (DI) and removed the pancreas (Fig. 3.1). The distal intestine was easily identifiable and the proximal and mid intestine portions were separated by dividing the remaining intestine in half. In seven individuals from each population, we removed the gut contents from the proximal, mid, and distal sections (Fig. 3.1; e.g., Proximal Intestine Gut Contents, PIGC) and flushed out the DI with chilled 25 mM tris-HCl, pH 7.5. Gut tissues and contents from each gut region and pancreases were frozen separately in 1.5 mL vials in liquid nitrogen for storage and transport. Vials were transported on dry ice to the University of California, Irvine, where they were stored at -80°C until used.

For the remaining three lizards from each population, we preserved the PI, MI, and DI in McDowell Trump's fixative (4% formaldehyde, 1% glutaraldehyde, McDowell & Trump, 1976) for subsequent histological analyses.

Gut Mass

We weighed frozen gut sections and gut contents (excluding stomachs) to the nearest 0.001 g. We summed the masses of the gut tissues and of the contents for each individual lizard.

Histology and Transmission Electron Microscopy

Gut sections preserved in Trump's Solution were further sectioned into 3-10 mm sections with a razorblade and rinsed in phosphate buffer pH 7.5 (PBS) 3x for 20 min. and overnight in PBS at 4°C. The PBS rinsed tissues were flushed with running deionized water 2x for 20 min. The 2nd most proximal section of the DI gut region was set aside for Transmission Electron Microscopy (TEM) and stained for two hours in a 1:1 solution of 4% osmium tetroxide and deionized water.

These TEM samples were flushed with running deionized water for 40 min. We then subjected all histology and TEM samples to serial ethanol dilutions of 30%, 50%, and 75%. We selected the proximal portions of the PI, MI, and DI from Pod Mrčaru and Pod Kopište lizards, and portions starting at the half way point of the distal intestine (DI+, Fig. 3.1) from all three populations. Tissue portions for histology were placed in tissue cassettes wrapped in ethanol-soaked cheesecloth, sealed in plastic bags, and were sent to Mass Histology Services (Worcester, MA, USA) for embedding in paraffin wax. We stained 7-µm sectioned samples with hematoxylin and eosin and imaged them with a Zeiss Axioplan 2 epifluorescence microscope and Zeiss and Cannon cameras. Tiled images were assembled using the Photomerge function of Adobe Photoshop CS3.

Starting at the proximal end of each region (PI, MI, DI, DI+, see Fig. 3.1) ,we analyzed 1-25 sections of each sample by measuring the perimeters of mucosa and serosa using imageJ. We then calculated the epithelial surface magnification (ESM) as the ratio of mucosal to serosal perimeters (German, 2009; Hall & Bellwood, 1995) to observe how much the mucosal folds increase the inner surface area of the intestine.

To determine microvilli length and more finely estimate ESM, we observed a region of the DI via TEM. Note that the DI samples we chose for TEM were from lizards collected in spring and summer 2014 (not summer 2013, as is referenced for other procedures in the rest of this section). We embedded the osmium tetroxide stained DI gut samples in resin and cut a 100 nm ultrathin section of a representative area where the tissue interacted with the lumen, between the crypt and the apex of the villi. We stained the sections with uranyl acetate and observed with a HITACHI H-7100 microscope at the Muséum national d'Histoire naturelle in Paris, France. We

used imageJ to quantify and measure the lengths of microvilli along the lumen, and to count bacterial cells present in the lumen within 3-µm of gut tissue.

Homogenate Preparation

We homogenized frozen gut tissues following German and Bittong (2009) and Wehrle (chapter 1). We diluted the tissues in the following chilled buffers: pancreases (P) diluted 38-385 volumes and gut contents (PIGC, MIGC, DIGC pellet) diluted 8-565 volumes in 25 mM tris-HCl buffer, pH 8.6 and, intestinal wall tissues (PI, MI, or DI) diluted 10-99 volumes in 350 mM mannitol in 1 mM Tris-HCl, pH 8.6. We chose buffers at pH 8.6 as it was the average pH we measured throughout the gut contents in our field measurements (Wehrle *et al.*, under review).

To ensure the rupture of the microbial cells and the release of all enzymes, we sonicated the gut contents (CL-18 Sonicator, Fisher Scientific, Waltham, Massachusetts, USA) at 5 W output for 3 x 30 s, with 30 s intervals between pulses. For all tissues, we used a Polytron homogenizer (Binkmann Instruments, Westbury, NY) with a 12 mm generator set to 1100-3000 rpm for 3 x 30 s, with 30 s between pulses to homogenize tissues. All sonicating and homogenizing was done on ice. We centrifuged the homogenates at 4°C: pancreas and gut tissues at 9400 x *g* for 2 min, gut contents at 12000 x *g* for 10 min. We recovered the supernatant and stored the homogenates in 100-200µl aliquots at -80° C until just before use in digestive enzyme assays.

Enzyme Assays

We conducted digestive enzyme assays at 25°C, following protocols outlined in German and Bittong (2009), German *et al.* (2015), and chapter 1 of this dissertation. We measured enzyme

activities in duplicate or triplicate and read absorption or fluorescence in flat-bottomed 96-well microplates using a BioTek Synergy H1 Hybrid spectrophotometer/ fluorometer equipped with a monochromator (BioTek, Winooski, VT, USA). Our primary buffer was 25mM Tris-HCl, pH 8.6 (referred to henceforth as "buffer," any deviations are noted), measured at room temperature (22°C). Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). We optimized each assay for duration and homogenate volume. Amylase, maltase, trehalase, trypsin, and aminopeptidase activities were measured in each gut region (PI, MI, DI, PIGC, MIGC, DIGC) for each lizard. Pancreatic tissue was only used for measuring pancreatic enzyme activity (i.e.: amylase, trypsin, and lipase). We simultaneously conducted control experiments using homogenate or substrate blanks in buffer to check for endogenous substrate and/or product in the substrate solutions. For all kinetic assays, we determined the slope of the longest linear section of absorbance vs. time and used the standard curve of the product to calculate enzymatic activity U per gram wet mass of tissue.

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

Assays of protein and lipid degrading enzymes— Following Wehrle (chapter 1), modified from German and Bittong (2009) and German *et. al* (2015), we measured trypsin, aminopeptidase, and

lipase activities as kinetic assays. To measure trypsin activity, we used 2mM Nα-benzoyl-L-arginine-p-nitroanilide hydrochloride (BAPNA) substrate dissolved in 100 mM Tris-HCl buffer. For pancreatic tissue homogenates, we pre-incubated the homogenates with 15 µl enterokinase (4 U mL-1 in 40 mM succinate buffer, pH 5.6)/ 100 µl homogenate for 15 min to change trypsinogen from its zymogen form to active trypsin enzyme, then proceeded with the assay as with the other tissues. For aminopeptidase activity, we used 2.04 mM L-alanine-p-nitroanilide in buffer. These protease assays were read at 410 nm absorbance for 30 min to detect a p-nitroaniline product as U (µmol p-nitroaniline released min^{-1}) per gram of tissue.

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

In addition to the regional enzyme activities (U x g^{-1}), we calculated the total gut enzyme activities as the sum of mass-specific activity for each region multiplied by the tissue mass to yield total U (µmol product released min^{-1}) for α-amylase, maltase, trehalase, trypsin, and aminopeptidase. We did not include pancreatic samples in total gut enzyme activities as this region does not interact directly with nutrients.

Statistical Analyses

We preformed all statistical analyses in R (version 3.3.2). Data were screened for equal variances using a Bartlett's test and normality of residuals using a Shapiro-Wilk's test. If the data were not naturally parametric, we employed transformations. Data distributions that could not be

transformed to homoscedascity or normality were instead compared with unequal variance t-tests or Wilcoxin / Kruskal tests, respectively. We used Tukey's HSD test with a family error rate of P=0.05 to identify pairwise differences following any ANOVAs that indicated significant differences. We performed the following comparisons with all data: between populations (within a single sex and field season), males vs. females (within a population during one field season), and field season (within a single population and sex).

We used ANCOVAs to check for covariance. If there was no interaction of factor and covariance, we report the ANCOVA without interaction. If there was no covariance, nor change in significance with covariance controlled for, we reported the results of the ANOVA.

Results

Elemental Analysis

Generally, the ^{13}C and ^{15}N signatures patterns of the *P. sicula* livers did not show clear patterns to discriminate between populations throughout time (Fig. 3.2). While the $\delta^{13}C$ signature was less depleted in the Pod Kopište males in summer 2013 ($F_{1,18}$=6.246, P=0.0223) and males and females spring 2015 (males: $F_{1,21}$=48.3, P<<0.0001; females: $F_{1,21}$=26.4, P<0.0001) compared to their Pod Mrčaru counterparts, we found no such pattern in either season of 2014. In summer 2013 only, $\delta^{15}N$ was more enriched in males from Pod Mrčaru than from Pod Kopište ($F_{1,18}$=6.246, P=0.0223), however in spring 2015, females showed the opposite effect with Pod Kopište lizards revealing greater $\delta^{15}N$ enrichment than the Pod Mrčaru counterparts ($F_{1,21}$=26.4, P<0.0001).

Stable isotope signatures in the liver showed a strong association with field season. All lizards had higher $\delta^{15}N$ signatures in spring 2015 than any other season and year (Pod Kopište,

male: $F_{3,23}$=25.35, P<<0.001, female: $F_{2,14}$=13.81, P<0.0005; Pod Mrčaru, male: $F_{3,25}$=8.298, P<0.0006, female: $F_{2,15}$=5.85, P=0.0132). For Pod Mrčaru females, $\delta^{13}C$ in the liver did not vary by season and year, but for all other groups, $\delta^{13}C$ was lowest in only spring 2014 for the Pod Kopište population (males: $F_{3,23}$=5.997, P<0.0046; females: $F_{2,14}$=5.096, P=0.0217) and springs of both 2014 and 2015 for Pod Mrčaru males ($F_{3,25}$=7.042, P<0.0014).

Gross morphology

Pod Mrčaru lizards were larger than Pod Kopište lizards with respect to both mass (Fig. 3.3; Summer 2013 males: $F_{1,23}$=9.937, P<0.0042; Spring 2014 males: $F_{1,21}$=25.9, P<<0.0001, females: $F_{1,20}$=6.201, P=0.0217; summer 2014 males: $F_{1,19}$=30.25, P<<0.0001, females: $F_{1,19}$=6.238, P=0.0219; spring 2015 males: $F_{1,20}$=49.73, P<<0.0001, females: $F_{1,20}$=8.1, P<0.0010) and SVL (summer 2013 males: $F_{1,24}$=22.11, P<0.0002; spring 2014 males: $F_{1,21}$=14.29, P=0.0011, females: $F_{1,20}$=8.1, P<0.0010; summer 2014 males: $F_{1,19}$=50.38, P<<0.0001, females: NS; spring 2015 males: $F_{1,20}$=41.77, P<<0.0001, females: $F_{1,19}$=7.107, P=0.0153) with the exception of females in summer 2014. In both populations, males were more massive (spring 2014 Pod Kopište: $F_{1,23}$=18.14, P<0.0003, Pod Mrčaru: $F_{1,18}$=54.78, P<<0.0001; summer 2014 Pod Kopište: $F_{1,19}$=34.61, P<0.0001, Pod Mrčaru: Wilcoxin test W=0, P<0.0002; spring 2015 Pod Kopište: NS, Pod Mrčaru: $F_{1,20}$=18.98, P<0.0003) and had longer SVLs (spring 2014 Pod Kopište: $F_{1,23}$=36.43, P<<0.0001, Pod Mrčaru: $F_{1,18}$=23.78, P<0.0002; summer 2014 Pod Kopište: $F_{1,19}$=14.19, P<0.002, Pod Mrčaru: $F_{1,19}$=36.73, P<<0.0001; spring 2015 Pod Kopište: NS, Pod Mrčaru: $F_{1,20}$=20.36, P<0.0003) than females, in this case excepting the lack of sex differences in the Pod Kopište population in spring 2015.

Gut morphology

The Pod Mrčaru lizards generally had longer guts than the Pod Kopište lizards (Fig. 3.4; summer 2013: NS; spring 2014 males: $F_{1,21}$=5.494, P=0.0290, females: pop $F_{1,19}$=46.34, P<<0.0001, mass $F_{1,19}$=11.98, P<0.0027; summer 2014 males: $F_{1,18}$=8.876, P<0.0081, females: $F_{population1,16}$=11.395, $P_{population}$<0.0039, $F_{mass1,16}$=27.754, P_{mass}<0.0001, $F_{population*mass1,16}$=7.492, $P_{population*mass}$<0.0147; spring 2015 males: $F_{1,20}$=21.19, P<0.0002, females: $F_{population1,17}$=5.491, $P_{population}$=0.0315, $F_{SVL1,17}$=3.967, P_{SVL}=0.0267). In several instances, this was partially due to increased overall size of Pod Mrčaru lizards. Yet in females of summer it was apparent that gut length increased with body mass in the Pod Kopište lizards but had minimal covariance with body mass in the Pod Mrčaru lizards (Fig. 3.5). Instead, the Pod Mrčaru population maintained long guts regardless of lizard size with a shallow slope and a low R^2-value. Since the Pod Kopište lizards had a 7x greater effect of body mass on gut length and a strong correlation between these variables, ANCOVA analyses revealed a significant interaction between population and body mass. It was only in the Pod Mrčaru population that we observed any sex differences in gut length (longer guts in females, spring 2014: $F_{population1,17}$=6.736, $P_{population}$=0.0189, $F_{mass1,17}$=4.626, P_{mass}=0.0462) or any differences through time (Pod Mrčaru males: $F_{population3,39}$=12.021, $P_{population}$<0.0001, $F_{mass1,39}$=5.306, P_{mass}=0.0267).

With the exception of individuals from summer 2013 (N=6), all other Pod Mrčaru lizards (N=28) had a clearly identifiable fold in the proximal to mid portion of their hindgut, dividing the gut in the transverse plane. The Pod Kopište lizards (N=29) lacked this valve entirely. One Pod Kopište male from spring 2015 had a fold in the same area of the hindgut but running in the dorsal plane. This anomaly was clearly distinct from the valves we observed in the Pod Mrčaru lizards, and in fact, this lizard had one of the shortest guts we measured for a Pod Kopište male,

79.17 mm, nearly 2x the standard deviation below the average gut length of 109.76 mm for Pod Kopište males that season.

The starkest differences in gut mass were in the PI where Pod Mrčaru males from summer had the most massive PI region (summer males: $F_{population1,10}$=5.395, $P_{population}$=0.0426, mass $F_{mass1,10}$=6.825, $P_{population}$=0.0259; Pod Mrčaru males: $F_{1,11}$=5.375, P=0.0407) followed by Pod Kopište males from summer (Pod Kopište males: $F_{1,11}$=5.375, P=0.0407), both of which more massive than PIs from spring lizards (Fig. 3.6A). Additionally, in spring, Pod Kopište females had not only less massive PIs compared to their Pod Mrčaru counterparts ($F_{population1,10}$=20.62, $P_{population}$<0.0011, $F_{gutlength1,10}$=10.97, $P_{gutlength}$<0.0079), but also less massive MI ($F_{1,10}$=39.34, P<0.0001) and DI ($F_{1,11}$=11.4, P<0.0062) regions as well. Gut content mass differed in the spring lizards, but not summer (Fig. 3.6B). The Pod Kopište males had higher PIGC (spring males: $F_{1,11}$=47.38, P<0.0001; spring Pod Kopište: $F_{1,10}$=11.65, P<0.0067) and MIGC masses (spring males: $F_{1,12}$=11.71, P<0.0051; spring Pod Kopište: $F_{sex1,10}$=13.131, P_{sex}<0.0047, $F_{total\ gut\ content\ mass1,10}$=5.595, $P_{total\ gut\ content\ mass}$<0.0396) than the other spring groups. Distal intestinal gut content (DIGC) mass was higher in summer in Pod Kopište than in spring (F $F_{1,11}$=8.123, P=0.0158) and more massive in Pod Mrčaru females compared to Pod Kopište ($F_{1,12}$=6.434, P=0.0261). Overall, these gut regional differences translate to a lighter total gut tissue masse in Pod Kopište females than the other groups (Fig. 3.7.; Pod Kopište spring: $F_{sex1,10}$=5.545, P_{sex}=0.0403, $F_{mass1,10}$=2.856, P_{mass}=0.1219; $F_{sex1,10}$=6.117, P_{sex}=0.0329, $F_{gutlength1,10}$=4.184, $P_{gutlength}$=0.0680; $F_{sex1,10}$=9.276, P_{sex}=0.01234, $F_{total\ gut\ contents\ mass1,10}$=11.508, $P_{total\ gut\ content\ mass}$<0.0069; spring females: $F_{population1,10}$=38.406, $P_{population}$<0.0002, $F_{gutlength1,10}$=5.616, $P_{gutlength}$<0.0393; $F_{population1,10}$=84.39, $P_{population}$<<0.0001, $F_{total\ gut\ contents\ mass1,10}$=24.32, $P_{total\ gut\ contents\ mass}$<0.0006). Total gut content did not differ in summer, but in

spring showed opposing patterns by population and sex (Fig. 3.7). Pod Kopište males and Pod Mrčaru females had more overall gut contents compared to their respective populations and sexes (spring males: $F_{population1,11}$=4.966, P=0.0477, $F_{total\ gut\ tissue\ mass1,11}$=4.766, P=0.0516; spring Pod Kopište: $F_{sex1,10}$=12.98, P_{sex}=0.0049, $F_{total\ gut\ tissue\ mass1,10}$=11.51, $P_{total\ gut\ tissue\ mass}$=0.00686; spring females: $F_{1,11}$=7.243, P=0.021; spring Pod Mrčaru: $F_{1,12}$=5.357, P=0.0392).

Epithelial Surface Magnification (ESM) only differed by population in the hindgut, but showed different patterns depending on how distal the section was (Fig. 3.8). The DI sections at the proximal portion of the hindgut had more magnification in Pod Mrčaru females compared to Pod Kopište females ($F_{1,4}$=22.28, P<0.0092). In this section, the Pod Mrčaru females had a higher magnification than their spring male counterparts ($F_{1,3}$=13.73, P=0.0341).However, the Pod Kopište females had a lower ESM than their corresponding males from spring in both the DI ($F_{1,4}$=9.531, P=0.0367) and the mid-section of the hindgut, or DI+ ($F_{1,4}$=8.956, P=0.0427). The DI+ had a higher ESM in Pod Kopište males in both summer and spring than in Pod Mrčaru males (summer: $F_{1,7}$=7.668, P=0.0277; spring: $F_{1,4}$=31.84, P<0.0049). In spring, the Pod Mrčaru males had a higher magnification of their MI region than the summer Pod Mrčaru males ($F_{1,4}$=247, P<0.0001). The Pod Kopište population appears not to show seasonal differences in the MI region but have only one measurement for the Pod Kopište spring MI region and thus cannot make claims with any confidence.

Through TEM, we found no differences in microvilli length or number by population, sex, or season (Fig. 3.9). While we did observe microbes close to the microvilli in most of the sections, we did not find any difference in microbial quantity.

Enzyme activity

Carbohydrases

Amylase— In the pancreas, amylase activity only differed between summer and spring in the Pod Mrčaru males, with slightly higher activity expressing in spring ($F_{1,18}$=4.79, P=0.0421). Mass-specific amylase activity in the intestines and their contents varied by population, sex, and season, but did so in different regions (Fig. 3.10). Generally, when we did observe differences, amylase activity was higher in Pod Mrčaru lizards, in females, and in the spring season. An exception was in summer males, whose amylase activity was detectable in the MI Pod Kopište lizards, but undetectable in that region for Pod Mrčaru lizards. In spring, Pod Mrčaru females had >5x higher activity in the MI than did males ($F_{1,11}$=5.937, P=0.033). The Pod Kopište males of both seasons had low amylase activity in the DI region; in summer activity was undetectable, whereas in spring, Pod Kopište males had > 3x lower amylase activity than their Pod Mrčaru counterparts ($F_{1,9}$=23.53, P<0.0010). Amylase activity in the DIGC of Pod Kopište summer males was <6x lower than their summer Pod Mrčaru counterparts (also reported in Wehrle *et al.*, under review; $F_{1,6}$=6.759, P=0.0407) and <13x lower than Pod Kopište males in spring ($F_{1,7}$=11.05, P=0.0127).

The total amylase activities throughout the gut were fairly consistent across lizard categories. However, total amylase activity in the Pod Kopište lizards increased with gut length, with nearly twice the increase in males than in females (Fig. 3.11A; $F_{sex1,9}$=6.011, P_{sex}=0.0367, $F_{gutlength1,9}$=11.305, $P_{gutlength}$=0.0084). While Pod Kopište females saw increased total amylase activity with increased gut lengths, Pod Mrčaru females had a lower amylase activity in relation to gut length (Fig. 3.11A; $F_{population1,10}$=5.281, $P_{population}$=0.0444, gut length $F_{gutlength1,10}$=2.477, $P_{gutlength}$=0.1466). However, when controlled for mass, Pod Mrčaru females had a higher amylase

activity per gram of lizard than did the Pod Kopište females (Fig. 3.11B; $F_{population1,10}$=5.896, $P_{population}$=0.0356, $F_{body\ mass1,10}$=3.930, $P_{body\ mass}$=0.0756).

Maltase—In spring, Pod Mrčaru males had higher mass-specific maltase activities than Pod Kopište males in more proximal regions of the gut (Fig. 3.12; PI: $F_{1,11}$=6.128, P=0.0308; MI: $F_{1,12}$=8.637, P=0.0124; MIGC: $F_{1,9}$=14.26, P<0.0044). In summer, this difference was only present in the MIGC (Fig. 3.12; $F_{1,8}$=10.97, P=0.0107), and was only ~2x higher in the Pod Mrčaru lizards (compared to the >19-fold, but highly variable, difference observed in spring). Pod Kopište and Pod Mrčaru females in spring differed maltase activity in every gut region compared to each other (Fig. 3.12). However, this pattern was quite unexpected. While the Pod Mrčaru females had higher maltase activity in their intestinal tissues than did their Pod Kopište counterparts (PI: $F_{1,10}$=12.28, P<0.0057; MI: $F_{1,9}$=5.418, P=0.0449; DI: $F_{1,10}$=7.555, P=0.0205), the reverse was true of the maltase activity in their gut contents (PIGC: $F_{1,8}$=6.965, P=0.0298; MIGC: $F_{1,11}$=7.317, P=0.0205; DIGC: $F_{1,8}$=9.204, P=0.0162). Indeed, in spring, maltase activity was higher in the gut contents of Pod Kopište females than in males (PIGC: $F_{1,8}$=9.024, P=0.017; MIGC: $F_{1,11}$=26.29, P<0.0004; DIGC: $F_{1,8}$=11.1, P=0.0104).

Total maltase activity in the entire intestine differed by season, population, and sex, but only when the lizards' body mass or the mass of digesta was considered. The only instance when total maltase activity did not differ between comparisons was by population in summer (also reported in Wehrle *et al.*, under review). Males had higher total maltase activity in spring than in summer (Fig. 3.13; Pod Kopište: $F_{season1,10}$=18.696, P_{season}=0.0015, $F_{mass1,10}$=12.017, P_{mass}<0.0061, $F_{season*mass1,10}$=6.598, $P_{season*mass}$<0.0280; Pod Mrčaru: $F_{1,12}$=71.4, P<<0.0001). In spring, Pod Mrčaru lizards had higher total maltase than Pod Kopište lizards (Fig. 3.13; spring males: $F_{population1,11}$=25.94, $P_{population}$<0.0004, $F_{mass1,11}$=11.74. P_{mass}<0.0057; spring females:

$F_{population1,10}$=43.796, $P_{population}$<<0.0001, $F_{gutlength1,10}$=5.271, $P_{gutlength}$=0.0446, $F_{population1,10}$=44.224, $P_{population}$<<0.0001, $F_{gut\ content\ mass1,10}$=5.726, $P_{gut\ content\ mass}$=0.0378), however these differences may be partially explained by differences in mass, gut length, or amount of digesta in the intestines (Fig.11). Males had higher total maltase activity per gram of lizard than females (Fig. 3.14; Pod Kopište: $F_{sex1,10}$=7.734, P_{sex}<0.0194, $F_{mass1,10}$=28.897, P_{mass}<0.0004; Pod Mrčaru: $F_{sex1,11}$=6.822, P_{sex}<0.0242, $F_{mass1,11}$=10.091, P_{mass}<0.0089).

Trehalase— The most pronounced pattern in trehalase activity differences was the high activity for Pod Kopište females in the gut contents (Fig. 3.15). In the PIGC, Pod Kopište females had higher activity than Pod Mrčaru females ($F_{1,9}$=15.87, P<0.0032) and a strong trend towards higher activity than Pod Kopište males (P=0.0507). In the rest of the gut contents, Pod Kopište females had >5-19x higher trehalase activity than other groups (MIGC females: $F_{1,9}$=7.521, P<0.0023; MIGC Pod Kopište: $F_{1,6}$=7.61, P=0.0329; DIGC females: $F_{1,8}$=13.94, P<0.0058; DIGC Pod Kopište: $F_{1,10}$=30.18, P<0.0003). The Pod Kopište males had a >80-fold higher PIGC trehalase activity compared to the practically nonexistent activity in this region for Pod Mrčaru males in spring ($F_{1,9}$=37.36, P<0.0002), yet by the MIGC, the Pod Mrčaru population had overshot them and the Pod Mrčaru females by ~8x (males: $F_{1,4}$=10.34, P=0.0324; Pod Mrčaru: $F_{1,7}$=25.74, P<0.0015). Consistent with this pattern, the Pod Mrčaru males had less trehalase activity in their PIGC in the spring than in summer ($F_{1,10}$=12.85, P<0.0050), but the opposite seasonal pattern in their MIGC ($F_{1,8}$=22.89, P<0.0014).

Pod Kopište females had higher trehalase activity in the MI compared to their Pod Mrčaru counterparts ($F_{1,9}$=7.017, P=0.0265) and higher activity in the DI compared to males ($F_{1,4}$=15.55, P=0.0169). Summer populations only differed in the hindgut, with higher trehalase activity in the DI and DIGC of Pod Mrčaru males (DI: $F_{1,10}$=11.07, P<0.0076; DIGC: $F_{1,9}$=6.296,

P=0.0334). In fact, Pod Kopište males had lower trehalase in the DIGC in summer than in spring ($F_{1,11}$=12.61, P<0.0046).

Despite these numerous regional differences, total trehalase activity did not differ by population, sex, or season. Males from Pod Kopište had marginally higher total trehalase activity per mm of lizard in spring than in summer ($F_{season1,11}$=6.053, P_{season}=0.0317, $F_{SVL1,11}$=7.787, P_{SVL}=0.0176), but this was not a strong relationship.

Proteases

Trypsin—We measured no differences in pancreatic trypsin for any comparison. Pod Mrčaru females had >4x higher MI trypsin activity compared to Pod Kopište females (Fig. 3.16; $F_{1,10}$=13.04, P<0.0049). Trypsin activity in the DIGC was >5x higher in the summer Pod Mrčaru males compared both within summer to Pod Kopište ($F_{1,10}$=16.5, P<0.0023) and between seasons with spring Pod Mrčaru activities ($F_{1,9}$=10.71, P<0.0097). Gut content enzyme activities, specifically PIGC and MIGC, were especially variable compared to other gut regions, perhaps contributing to lack of differences for these regions.

There were no differences in total trypsin activity in the entire lizard guts.

Aminopeptidase— In males, aminopeptidase activity was higher in Pod Mrčaru lizards than in Pod Kopište, however the region of this difference varied by season. In summer, aminopeptidase was nearly 7x higher in the DIGC of Pod Mrčaru lizards compared to Pod Kopište (also see Wehrle *et al.*, under review; Fig. 3.17; $F_{1,9}$=8.16, P=0.0189). In Pod Mrčaru males in spring, it was >4x more active in the MI ($F_{1,10}$=22.61, P=0.0008). When we observed seasonal differences, these same two tissues were implicated and aminopeptidase activity was higher in spring. In Pod Kopište males, activity was >3x higher in the DIGC in spring than in summer ($F_{1,11}$=5.885,

P=0.0336). In Pod Mrčaru males, activity in the MI was >1.8x higher in spring compared to summer ($F_{1,10}$=8.995, P=0.0134).

In females, this pattern was reversed. Pod Kopište females had roughly twice the PI aminopeptidase activity of any other group, including Pod Mrčaru females (females: $F_{1,11}$=14.34, P=0.00301; Pod Kopište: $F_{1,11}$=14.36, P=0.003). In addition to Pod Kopište females in spring having higher PI aminopeptidase activity than their male counterparts, they also had higher activity in the MI ($F_{1,10}$=7.834, P=0.0188) and MIGC ($F_{1,10}$=7.475, P=0.021). The Pod Mrčaru lizards showed the opposite pattern with spring males having higher enzyme activity in the MI than their counterpart females ($F_{1,9}$=11.93, P<0.0073).

Total aminopeptidase activity in the entire gut (Fig. 3.18) was higher in Pod Mrčaru males in both seasons compared to Pod Kopište males (summer: $F_{population1,11}$=23.330, $P_{population}$<0.0006, $F_{mass1,11}$=6.898, P_{mass}<0.0236; spring: $F_{1,12}$=9.861, P<0.0086). Females were not different from each other nor were they different from males.

Lipase

We measured lipase in two tissues: pancreases and DIGC. We found no differences in lipase activity excepting >3x higher pancreatic activity in Pod Mrčaru males in spring compared to summer ($F_{1,15}$=14.7, P<0.0017).

Meta-Analysis

Since many of the patterns we observed between males and females appeared to be in opposite directions, we applied a Chi^2 test to test for independence of enzyme data. We used counts of how many regions enzymes were higher in in Pod Kopište or Pod Mrčaru or not different

between the populations. Females were significantly more likely to have differences in enzyme activity between populations than were males of either season (χ^2=13.868, df=4, P<0.0078). Half of the population differences in enzyme activity between females were higher in Pod Mrčaru, half higher in Pod Kopište. Males, on the other hand, had higher enzyme activities in the Pod Mrčaru population when differences were present, excepting trehalase in the PIGC in spring.

Discussion

Diet

Assimilated diet varied more by year than by season or sex. For example, males and females from both populations were most ^{15}N enriched in spring 2015 and all but Pod Mrčaru females had their lowest ^{15}N and ^{13}C signatures in spring 2014. Both spring and summer 2014 showed no population differences in assimilated diet. As these patterns were not consistent between springs or summers of different years, nor consistent between populations, we conclude that environmental factors play a bigger role than population, sex, or season. That we found no differences in isotopic signatures between males and females supports Herrel and collegues' (2008) observation of no sex differences in stomach contents. However, several results were particularly surprising. In summer 2013, Pod Mrčaru lizards had livers more enriched with ^{15}N. This signal generally comes from a diet higher on the trophic scale. As Pod Mrčaru lizards had a very high proportion of plant material in their stomachs and little animal prey and Pod Kopište lizards had the opposite, this is surprising. Insectivorous lizards from mainland populations have much lower $\delta^{15}N$ values (range: 8.3-10.4‰, unpublished data), suggesting that the $\delta^{15}N$ in the island lizards (range: 12.2-14.5‰) are likely due to marine nitrogen enrichment of diet items on

both islands. These unexpected results from summer 2013 are the first evidence that this season, while it was our baseline for our predictions about this system, was an anomaly in itself.

It is possible that the higher $\delta^{15}N$ in Pod Mrčaru may reveal a cryptic nutrient source (e.g. nematodes or microbes already living in the gut; (German, 2009). Overall, these patterns of stable isotope signatures suggest that the nutrients both populations of lizards acquire are not as dissimilar as their stomach contents suggest.

Gut content masses were higher in females vs. males in Pod Mrčaru and in males vs. females in Pod Kopište. Once again, this points to an interaction between population and sex, with intake and digesta retention strategies differing by demographic. Gut content masses varied considerably by population, season, and sex, but also by which regions differed. In spring males, Pod Kopište lizards had higher content masses in the PIGC, MIGC, and overall, whereas Pod Mrčaru males had a low content masses except for DIGC. In contrast, in spring females, Pod Mrčaru lizards had higher DIGC and total content masses. That the Pod Mrčaru population has a spike in DIGC mass suggests that (1) they experience considerable microbially aided digestion (Choat et al, 2002, 2004), (2) digesta moves through the more proximal regions of the gut quickly, and (3) hindgut valves are slowing digesta flow. The higher mass of gut contents in proximal gut regions in the Pod Kopište spring males is consistent with lower intake, and thus longer digesta retention in gut regions with high digestive and absorptive capability.

Morphology and gut structure

In line with previous findings (Herrel *et al.*, 2008), Pod Mrčaru lizards were always larger than Pod Kopište lizards. Males were always larger than females, with the exception of no size differences by sex in the Pod Kopište lizards during spring 2015. (As this was the same season of

the global high $\delta^{15}N$, sex differences may have been washed out due to high protein intake from high prey abundance.)

Longer gut lengths and hindgut valves in the Pod Mrčaru lizards serve as evidence of adaptation for eating plant material (Dearing, 1993; Iverson 1982, O'Grady 2005; Stevens & Hume 2004). The more massive gut regions in summer males and in spring females from Pod Mrčaru compared to Pod Kopište suggests a more active gut and more tissue resources allocated to digestion. As the spring males from Pod Mrčaru had longer guts than their summer counterparts, it appears there may have been a seasonal tradeoff between gut mass and gut length. A shorter gut could be more active, or could have greater smooth muscle mass. If the Pod Mrčaru lizards ate more insect material and less fibrous plant material in spring, they would not need to consume as much since, bite for bite, insects are more energetically dense than plant material (Bowen *et al.*, 1995).

Pod Mrčaru lizards had their lowest gut length in summer 2013, thus producing the only field season when we did not observe longer guts in Pod Mrčaru lizards. Summer 2013 was also the only season when we did not find valves in the hindguts. The combination of shorter guts, less massive proximal intestines and proximal intestinal contents, and no hindgut valves once again paints summer 2013 as an anomalous season for the Pod Mrčaru lizards.

Based on expectations that valves in the hindguts would increase surface area in the gut region, it is surprising that males from Pod Kopište had greater epithelial magnification (ESM) in the mid portion of the distal intestine (DI+) in summer and spring than their Pod Mrčaru counterparts. This may be due to increased microbial fermentation in the hindguts of the Pod Kopište population (Wehrle *et al.*, under review). Short chain fatty-acids (SCFAs) can promote the growth of gut tissue (Scheppach, 1994; Scheppach *et al.*, 1997). However, the opposite

pattern is apparent in females in the more proximal region of the distal intestine—Pod Mrčaru had greater ESM. If microbial products are driving the ESM pattern in males, why would the same not be true of females? Or conversely, if the increased ESM in the DI is an acclimation for a plant based diet, why would this be absent in males? Many of the differences among the sexes are perplexing and strongly suggest that sex-differences should be a focus of nutritional physiology (as a field, not just in *P. sicula*) moving forward.

With the differences in ESM (albeit in two opposing directions by sex) and the hindgut as the source of increased enzyme activities in Pod Mrčaru in summer, we expected to also find differences in ultrastructure. Microvilli length can change very quickly and has been found to even increase when food is first sensed, let alone digested (German *et al.*, 2010; Secor, 2008). Longer microvilli could lead to exponentially more surface area when combined with increased ESM (Karasov and Hume, 1997). On the other hand the higher ESM in Pod Kopište males could be made equivalent to Pod Mrčaru hindgut surface area had microvilli length been increased in Pod Mrčaru. However, as absorption is generally higher in more proximal portions of the vertebrate gut, the microvilli length in the DI may not change rapidly. There are no data that we know of on nutrient transport rates in the hindguts of lizards, so we can not conclude this for sure.

Enzyme activities

Enzyme activities were generally higher in spring than in summer, but no enzyme was different throughout all gut regions. In Pod Mrčaru, amylase, maltase, trehalase, and aminopeptidase were higher in spring in more proximal regions compared to the seasonal differences in Pod Kopište lizards for those same enzymes. Greater enzyme activity in more proximal gut regions would

allow for more digestive function. In more proximal regions of the gut, nutrient substrates are higher, giving the enzymes more opportunity to degrade the digesta into absorbable units. Nutrient absorption potential also should be increased as there is more gut left over through which the nutrient can be absorbed. Thus, the proximal gut loaded seasonal differences in Pod Mrčaru should lead to a greater nutrient acquisition difference between seasons that would be found in the Pod Kopište population. Although we do not have intake mass data for spring, a previous study (Wehrle *et al*., under review) found that Pod Mrčaru lizards had >2.2x the stomach content mass that Pod Kopište lizards did. With this higher intake, Pod Mrčaru lizards should have a shorter digesta transit time, leading to less opportunity for nutrient digestion and absorption. Thus, this difference in seasonal patterns between the two populations may indeed just be a way for the Pod Mrčaru lizards to mitigate their high intake (Sibly, 1981, Clemments & Raubenheimer, 2006; German *et al.*, 2015).

When fed a high intake diet, Pod Mrčaru males from 2013 digested plant proteins better than their Pod Kopište counterparts (Wehrle, *Chapter 2*). However, in the same study, when fed a low intake diet in 2014, the males showed no digestibility differences by population. This suggests that specializations in gut form and biochemistry are optimized for a high intake diet, with any differences being washed out when the lizards are not digestively challenged. While this finding is confounded by the evidence that summer 2013 Pod Mrčaru males had different patterns of gut length and structure than we observed in any other field season, that population differences in digestive enzyme activities in males were almost always higher in Pod Mrčaru is informative. A high intake diet with the resulting rapid digesta transit rate would require increased enzyme activity to mitigate the short interaction of digesta with enzymes and the absorptive surface of the gut. However, with low intake and thus low flow of digesta, digestive

performance may then be limited by absorptive capacity, not enzyme activity. These enzyme patterns combined with digestibility findings (Wehrle, *Chapter 2*) suggest that variation in nutrient transport rates may be implicated in understanding the differences between these populations.

In Pod Mrčaru males, trypsin was higher in the DIGC in summer. We do not have evidence that greater long polypeptide substrates would have been present in the hindgut in summer rather than spring. This suggests a difference in microbial function between seasons, but we do not have further explanation for this pattern.

Trehalase was also higher in summer compared to spring for Pod Mrčaru males. This again is surprising as we expect the Pod Mrčaru population to eat fewer insects in summer. However, activity jumps up in the MIGC in spring, appearing that by season, the site of trehalase production may shift—encompassing more length of the gut in spring and then retreating proximally in summer. That in males, regardless of season, trehalase was more active in the Pod Mrčaru population is curious. While this evidence may point to the NBH (Clissold *et al.*, 2010) as we expect arthropod consumption to be lower in Pod Mrčaru lizards and thus trehalose to be a rare substrate in this population. However, trehalose is a disaccharide that is broken down by trehalase into glucose. Glucose is not limited in this population as it can easily come from plant sources. Plant material degrading carbohydrases (i.e. amylase and maltase) are relatively high with comparable amylase activities and >10x the maltase activity in some herbivorous fishes (German *et al.*, 2015), though half the level of maltase activity found in agamid lizards (Iglesias *et al.*, 2009). As such, this trehalase pattern, combined with the higher $\delta^{15}N$ signature than expected, suggests that the Pod Mrčaru lizards subsist off of insect material more than previous data suggests (Herrel *et al.*, 2018; Wehrle *et al.*, under review).

Female lizards had very different patterns of enzyme activities than their male counterparts. Firstly, females displayed more population differences in enzyme activities than did males. Secondly, half of those differences were higher in Pod Mrčaru, half higher in Pod Kopište. Amylase and trypsin, enzymes produced in the pancreas and secreted into the gut, were consistently higher in Pod Mrčaru when differences occurred, regardless of sex. In females, maltase, trehalase, and aminopeptidase, enzymes that are produced in the brush border of the intestine, were higher in Pod Kopište for one or more gut regions. Maltase activity in the gut tissue matched within population regardless of sex and was higher in Pod Mrčaru than Pod Kopište. However, gut content maltase activity was higher in Pod Kopište female lizards. As mentioned above, this is the opposite pattern we would expect based on diet data from previous studies (Herrel *et al*., 2008, Wehrle *et al*., under review). Both maltase and trehalase show these spikes of activity in the gut contents for Pod Kopište females compared to most other groups. Might this be due to low digesta flow in Pod Kopište females? Pod Kopište females had less massive gut contents than Pod Mrčaru females and Pod Kopište males. As these enzymes are produces in the gut tissues, this suggests that the production of the brush border enzymes do no differ. However, once secreted, a high flow of digesta would carry the enzymes distally through the gut. With a low digesta flow, the Pod Kopište females would be able to retain the digestive services of these enzymes for longer.

The higher activity of aminopeptidase in the proximal intestine in Pod Kopište females compared to other lizard groups appears once again to be a shift in the site of enzyme production. While the Pod Mrčaru lizards and the Pod Kopište females appear to produce much of their aminopeptidase in the mid intestinal region, the Pod Kopište females have a longer stretch of gut over which this peptidase is produced. While this would suggest that Pod Kopište females would

have higher digestibility of equivalent meals than Pod Mrčaru females, on experimental diets I observed the opposite (Wehrle, *Chapter 2*). In fact, in Pod Kopište, even with higher carbohydrases and peptidase in females than males in the wild, males in the lab had higher carbohydrate and protein digestibility of their experimental diets (Wehrle, *Chapter 2*).

Conclusions

This rapidly evolving system is ecologically, morphologically, and physiologically dynamic. While it is tempting to consider the information we have so far explains acclimations to a dietary switch (Herrel *et al*., 2008; Wehrle *et al*., under review), this study adds complexity to that understanding. General trends such as increased gut length, hindgut valves, and increased amylase activity in the Pod Mrčaru lizards are promising directions for canalization of acclimation. However, even these mostly consistent differences have not been the rule throughout the time period we sampled.

The conclusions we would have drawn with females omitted from this study are very different than we have found with female data. As Herrel and others (2008) found, increased differences in females compared to males may be driving overall evolutionary patterns. We stress the importance of including females in physiological studies. Males and females appear to use different strategies to meet the same goals of subsisting on a novel plant based diet. These sex based differences may be due to differences in social structure and resource use. In fact, female *Podarcis sicula* from an invasive population were found to have cooler thermal niches compared to males (Liwanag *et al.*, 2016) which may have effects on multiple aspects of digestion (e.g. Dandifross, 1974; Troyer, 1987, Sun *et al.*, 2007)

With this variation of diet, morphological, and digestive physiology patterns by season and by sex, we may be observing a highly plastic system. This may be the drunkard's walk of evolution that we are watching unfold.

Table 3.1 Predictions of gut morphology and enzyme activity shifts as increased (↑), decreased (↓), or no change (=) with an increase of plant material in the diet under Chemical Reactor Theory (CRT), the Adaptive Modulation Hypothesis (AMH), and the Nutrient Balancing Hypothesis (NBH). Lipase predictions are split into spring and summer seasons as, in the Pod Mrčaru *P. sicula* system, plant material consumed in summer is mostly seeds (presumed to have high fat content) compared to more prevalent leaves, flowers, pollen, etc. consumed in spring.

character	CRT	AMH	NBH
Gut length	↑	↑	=
Gut mass	↑	↑	=
Hindgut valves	↑	↑	=
Enzymes for degrading:			
Plant-material	↑	↑	=
Arthropod-material	=	↓	=
proteases	=	=	↑
carbohydrases	=	=	=
Lipase, spring	=	↓	↑
Lipase, summer	↑	↑	=

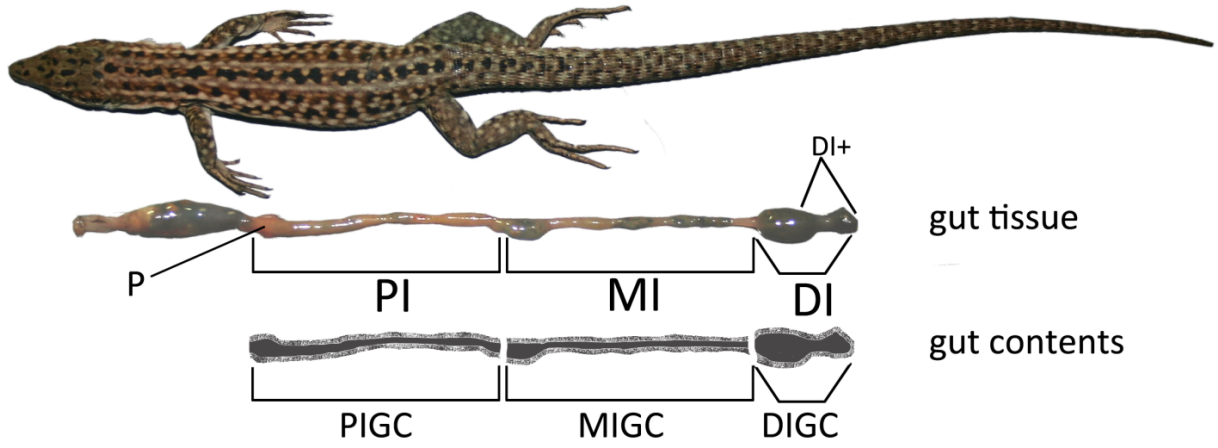

Figure 3.1 Representative lizard gut including stomach. We divided the gut into proximal intestine (PI), mid intestine (MI), and distal intestine (DI). For enzyme assays only, we collected the pancreas (P) and contents: proximal intestinal gut contents (PIGC), mid intestinal gut contents (MIGC), and distal intestinal gut contents (DIGC). For histology only, we separated the DI+ from the rest of the DI sample.

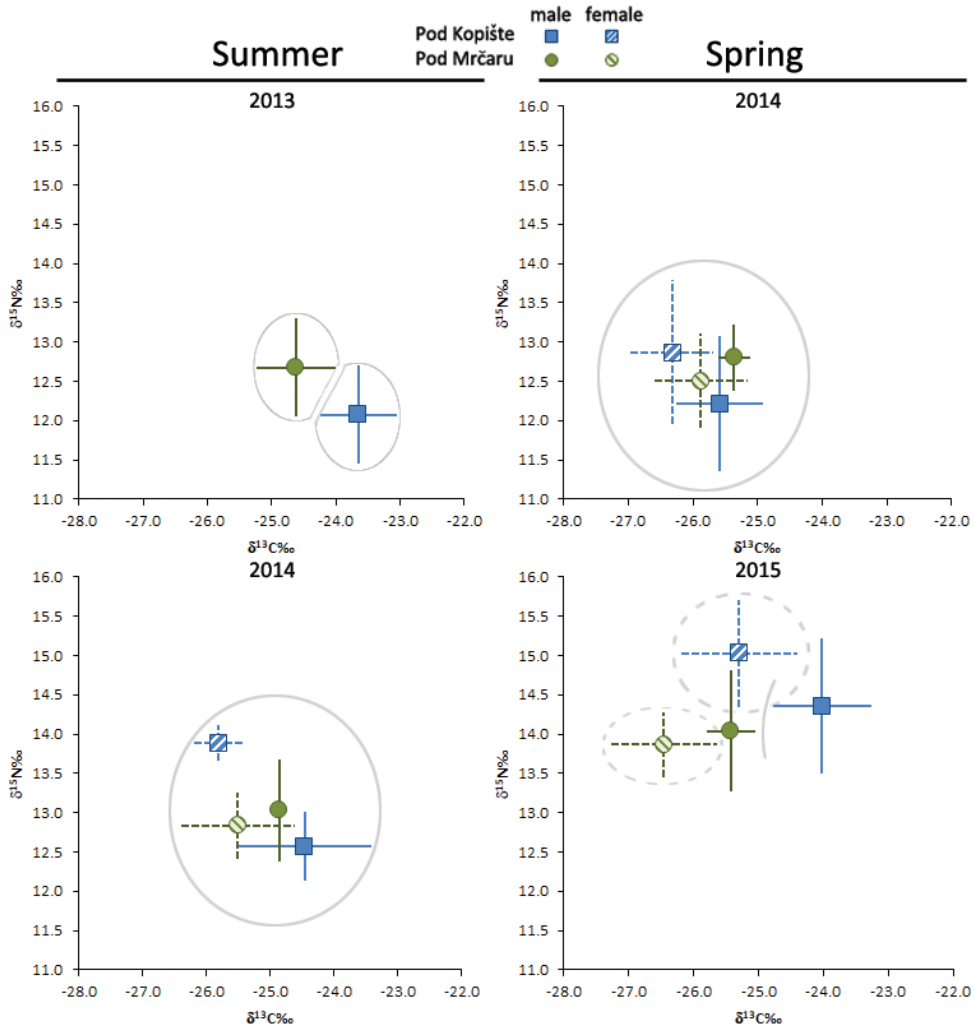

Figure 3.2 Liver $\delta^{13}C$ and $\delta^{15}N$ stable isotopic signatures from *P. sicula* separated by sex from Pod Kopište (source) and Pod Mrčaru (newly omnivorous) populations, across two summers (2013, 2014) and two springs (2014, 2015). Values are means ± standard deviation. Separate gray outlines on the same plot denote differences: summer 2013 males differ in $\delta^{13}C$ ($P<0.002$) and $\delta^{15}N$ ($P<0.023$), spring 2015 males differ in $\delta^{13}C$ ($P<<0.001$) and females differ in $\delta^{13}C$ ($P<<0.001$) and $\delta^{15}N$ ($P<<0.001$).

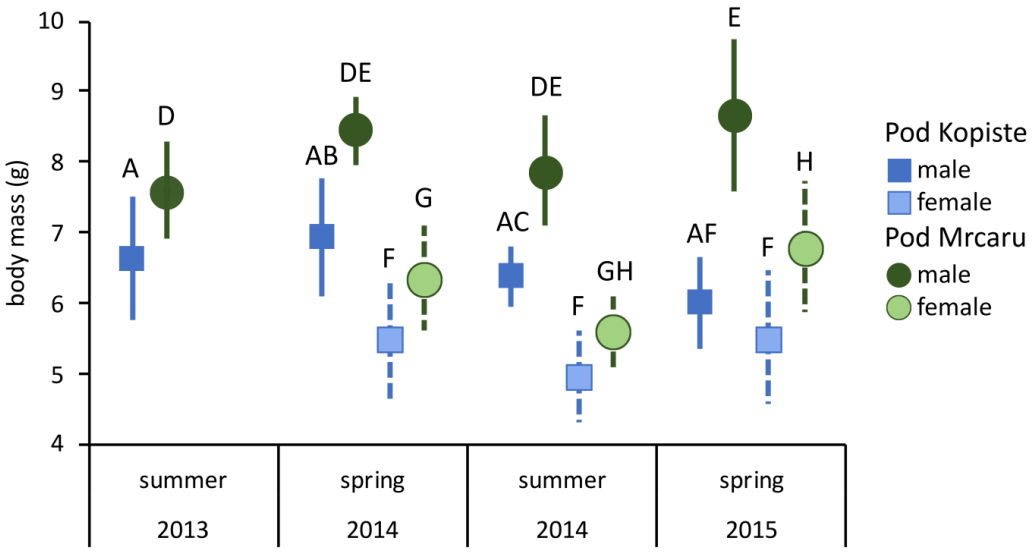

Figure 3.3 *P. sicula* body mass of males and females from Pod Kopište (source) and Pod Mrčaru (newly omnivorous) populations, across two summers (2013, 2014) and two springs (2014, 2015). Values are means ± standard deviation. The same letter above markers denotes no differences among those body masses.

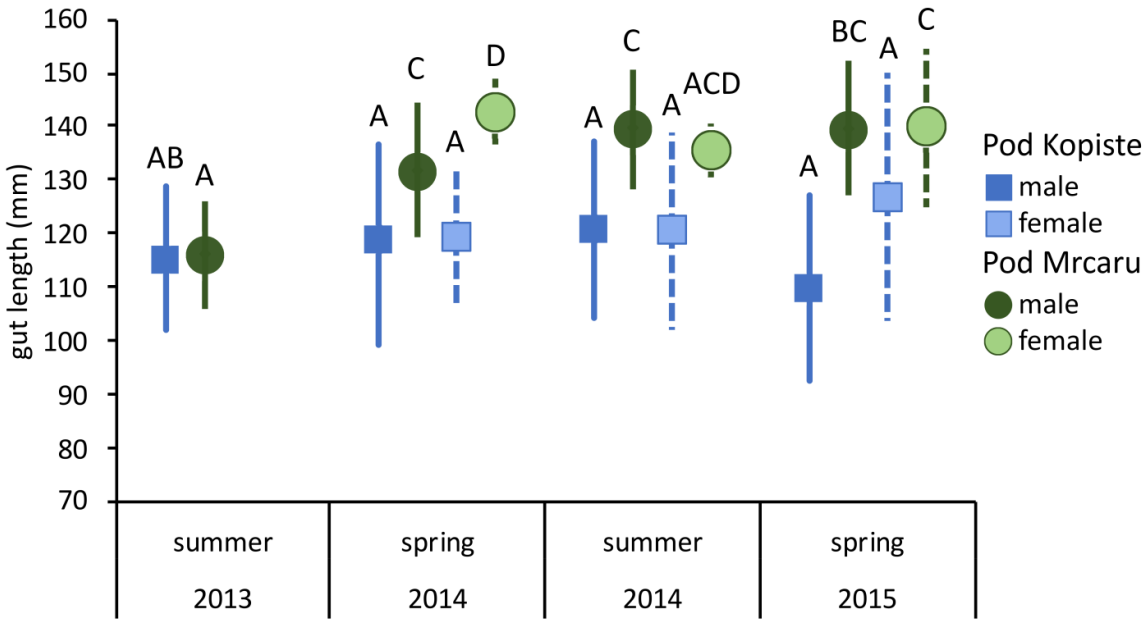

Figure 3.4 *P. sicula* gut lengths of males and females from Pod Kopište (source) and Pod Mrčaru (newly omnivorous) populations, across two summers (2013, 2014) and two springs (2014, 2015). Values are means ± standard deviation. The same letter above markers denotes no differences among those gut lengths.

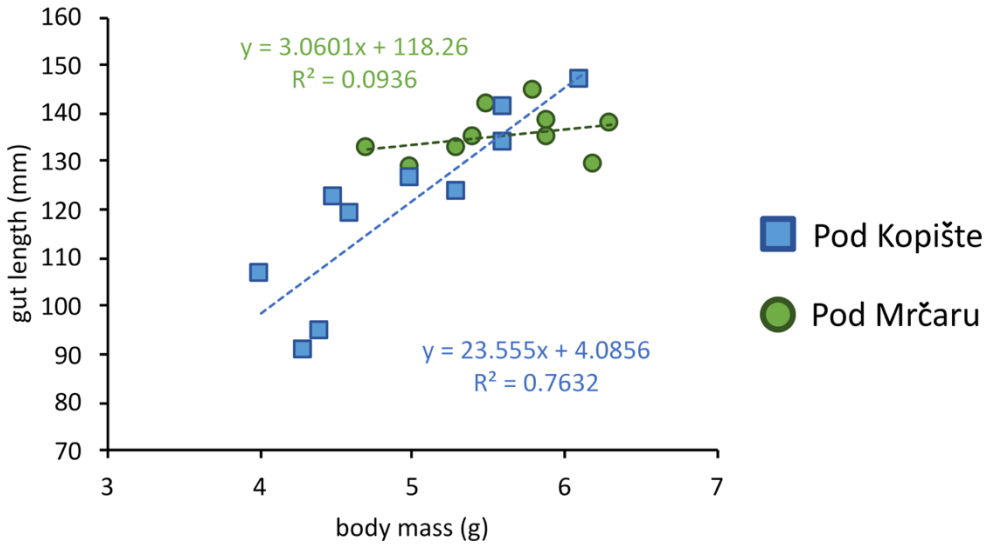

Figure 3.5 Gut length vs. body mass in Pod Mrčaru and Pod Kopište females from summer 2014.

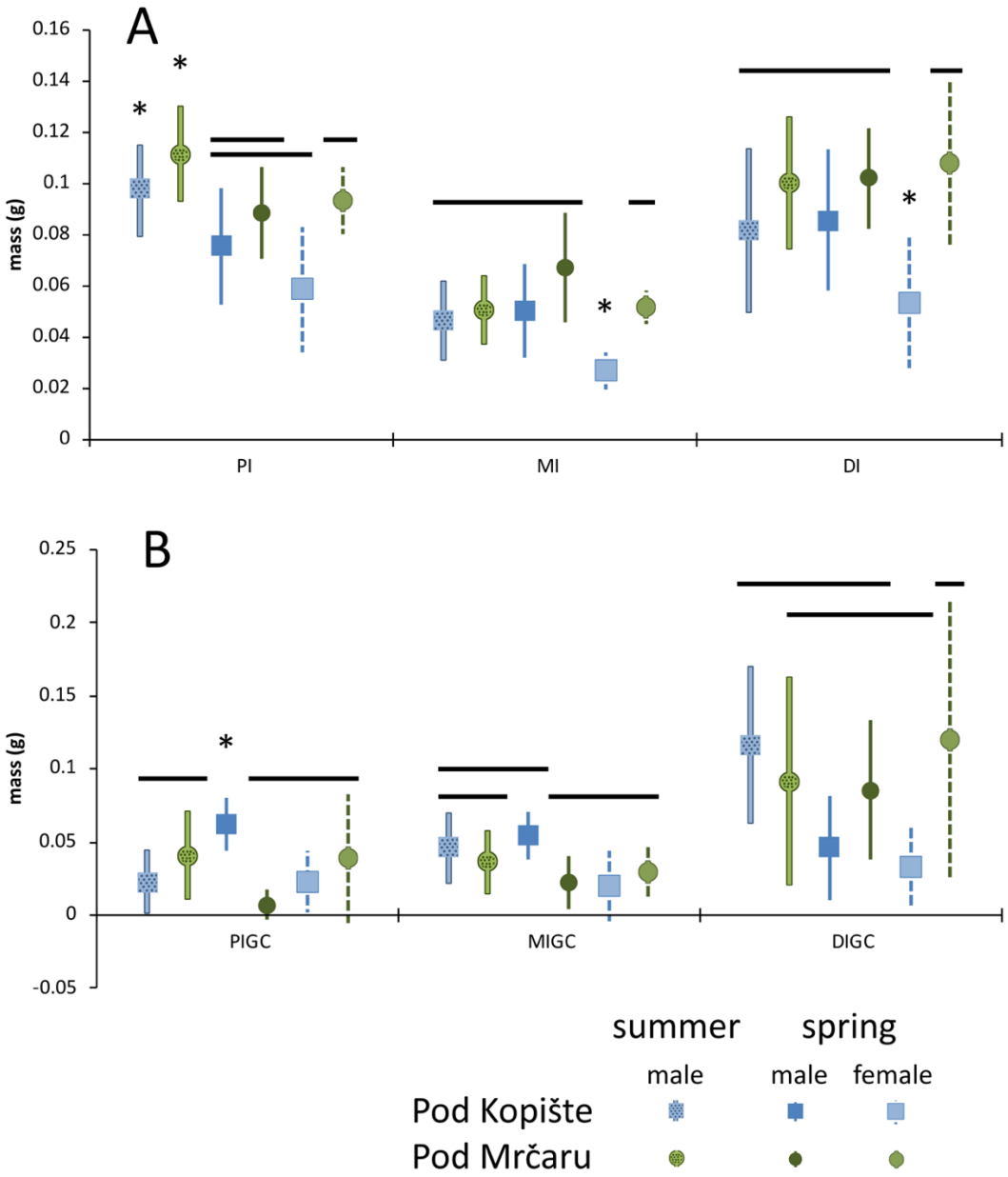

Figure 3.6 Regional mass of gut (A) tissues and (B) contents. Values are mean ± standard deviation. Comparisons are within a tissue type between populations, seasons, and sexes. Lines of the same elevation indicate no differences, as do overlapping lines, whereas an * marks that mass is different than all others of that tissue type.

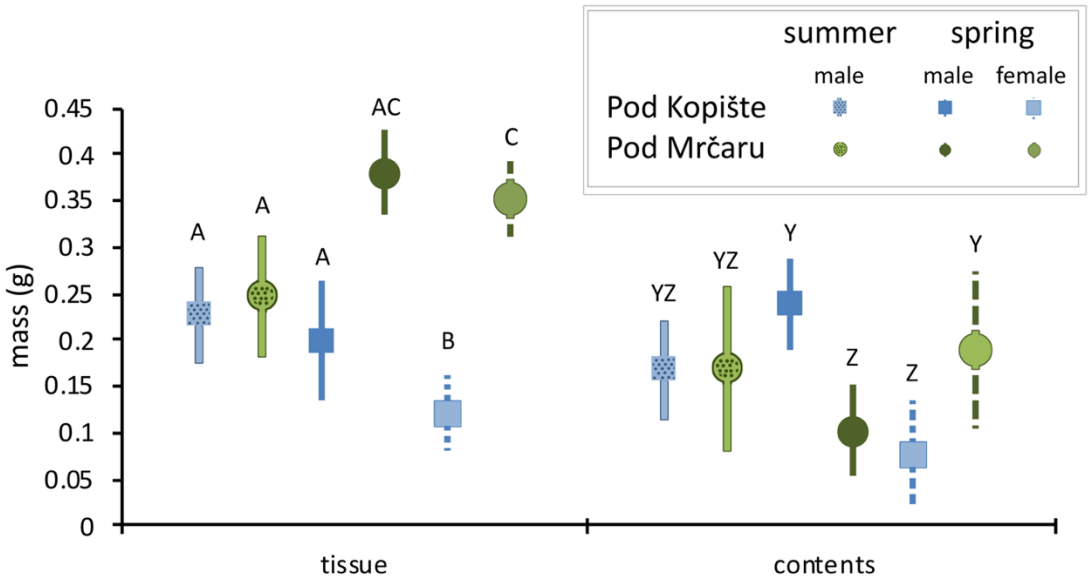

Figure 3.7 Total gut tissue mass and total gut content mass by population, season, and sex. Gut tissue and contents were analyzed separately. Values are mean ± standard deviation. The same letter above the value indicates no differences in that comparison, whereas different letters denote differences.

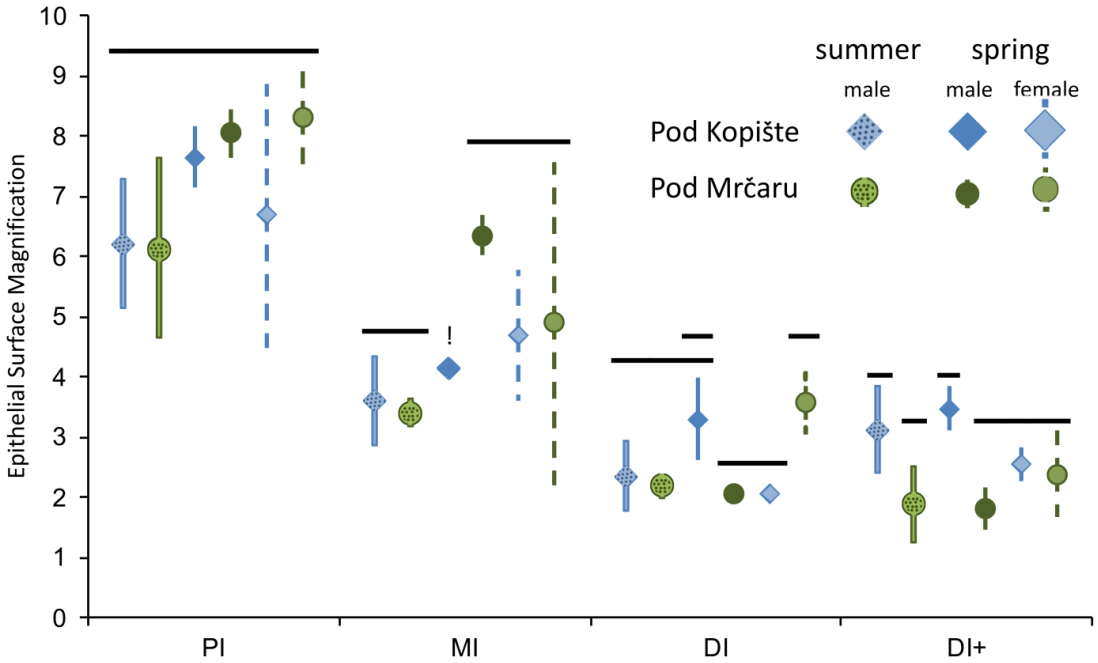

Figure 3.8 Epithelial Surface Magnification (ESM), the ratio of mucosa to serosa length in proximal end of three gut regions (PI, MI, DI) and the mid-point of the DI (DI+) in Pod Kopište (source) and Pod Mrčaru (omnivore) summer males, spring males, and spring females. Values are mean ± standard deviation, excepting the MI of the Pod Kopište spring male (marked with "!"). This point had only had one observation and thus could not be included in any statistical analyses. Comparisons are within a tissue type between populations, seasons, and sexes. Lines of the same elevation indicate no differences.

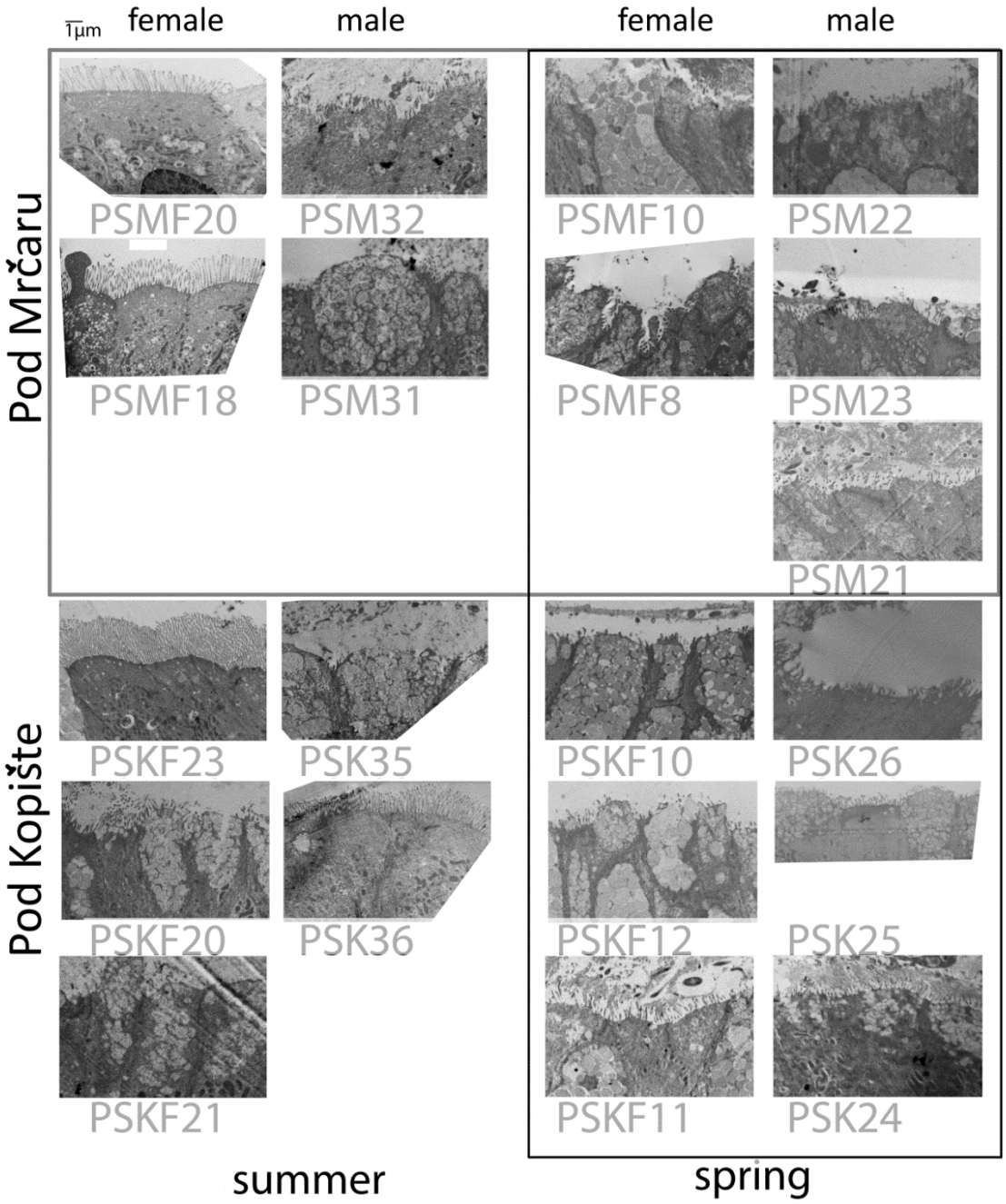

Figure 3.9 Transmission Electron Microscopy (TEM) images of DI sections in Pod Kopište (source) and Pod Mrčaru (omnivore) males and females in spring and summer. Images to scale.

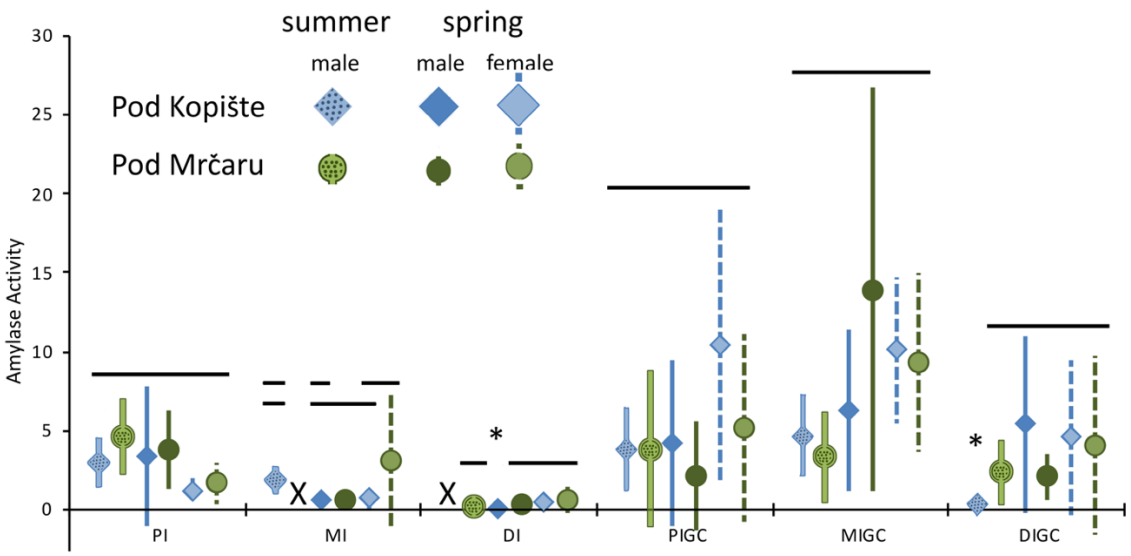

Figure 3.10 Amylase activity in µmol glucose liberated g^{-1} min^{-1} throughout the gut in Pod Kopište (source) and Pod Mrčaru (omnivore) summer males, spring males, and spring females. Values are mean ± standard deviation. Lines of a different elevation for a gut region indicate significant differences for that population/sex/season combination in that gut region and overlapping lines indicate no differences and an ***** marks that enzyme activity is different than all others of that tissue type. "**X**" denotes undetectable activity.

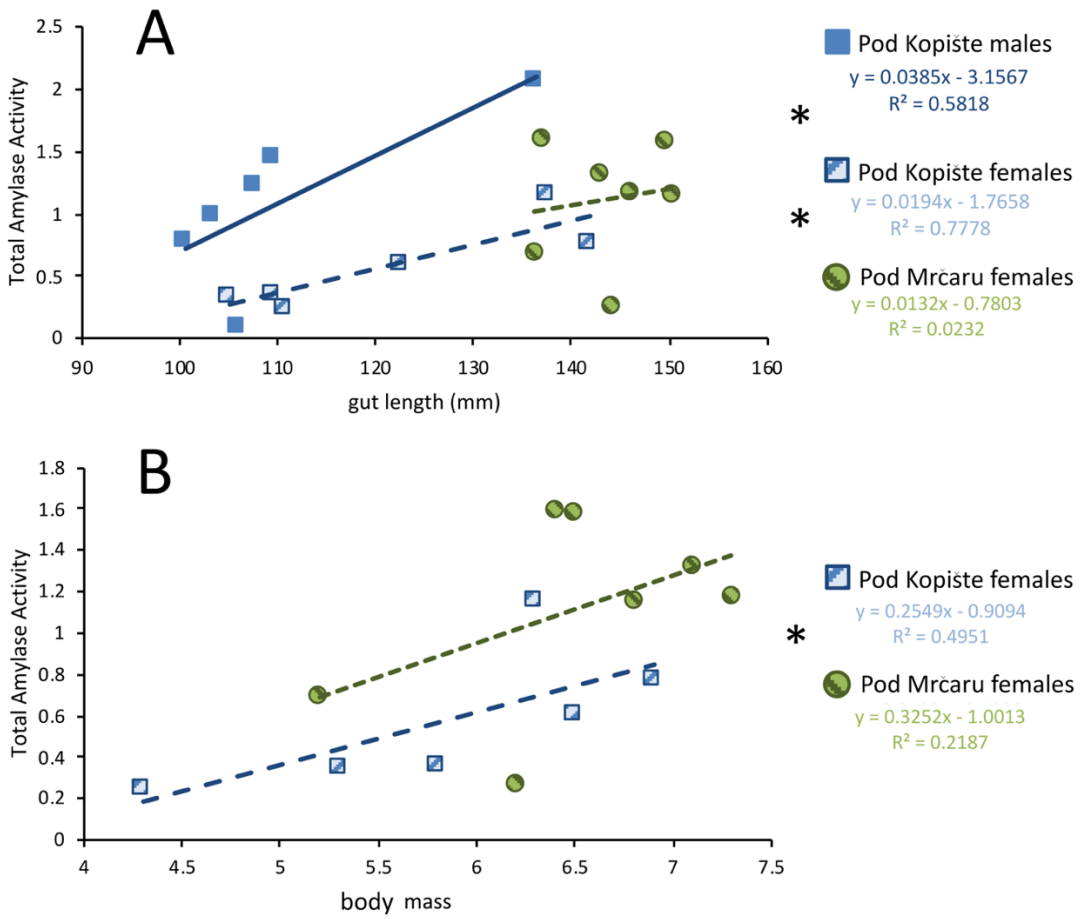

Figure 3.11 Spring total amylase activity in µmol glucose liberated min^{-1} as compared to (A) gut length and (B) lizard body mass in males (A only) and females from Pod Kopište and females from Pod Mrčaru Values are individual measurements regressed against (A) gut length or (B) body mass. Comparisons were done with ANCOVAS. All comparisons within a plot were significantly different, but there were no interactions of variables and covariants.

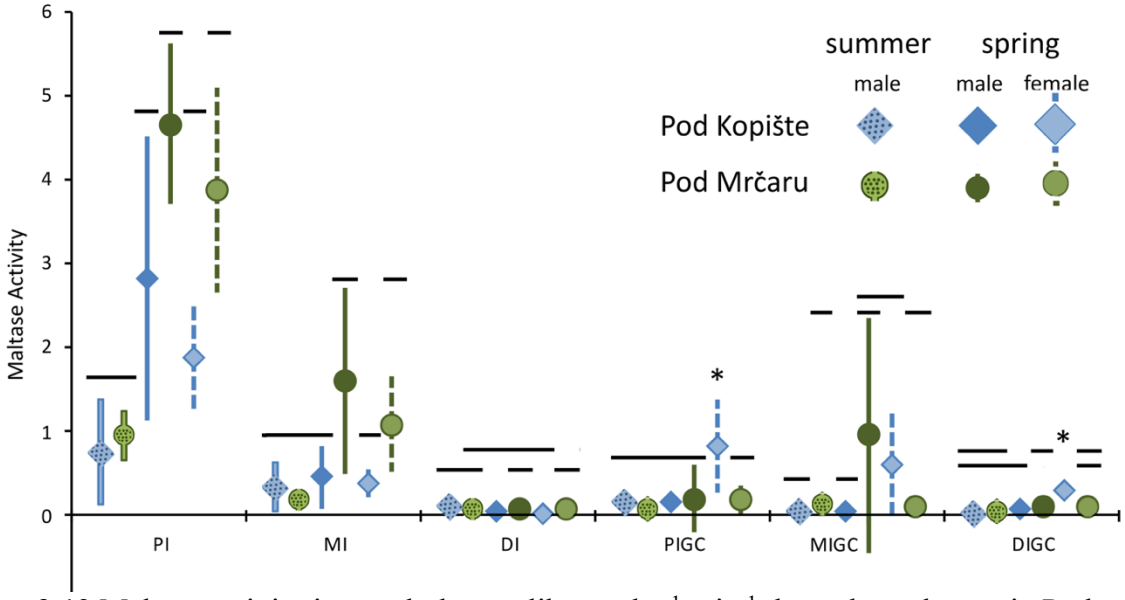

Figure 3.12 Maltase activity in µmol glucose liberated g^{-1} min^{-1} throughout the gut in Pod Kopište (source) and Pod Mrčaru (omnivore) summer males, spring males, and spring females. Values are mean ± standard deviation. Lines of a different elevation for a gut region indicate significant differences for that population/sex/season combination and overlapping lines indicate no differences and an * marks that enzyme activity is different than all others of that tissue type.

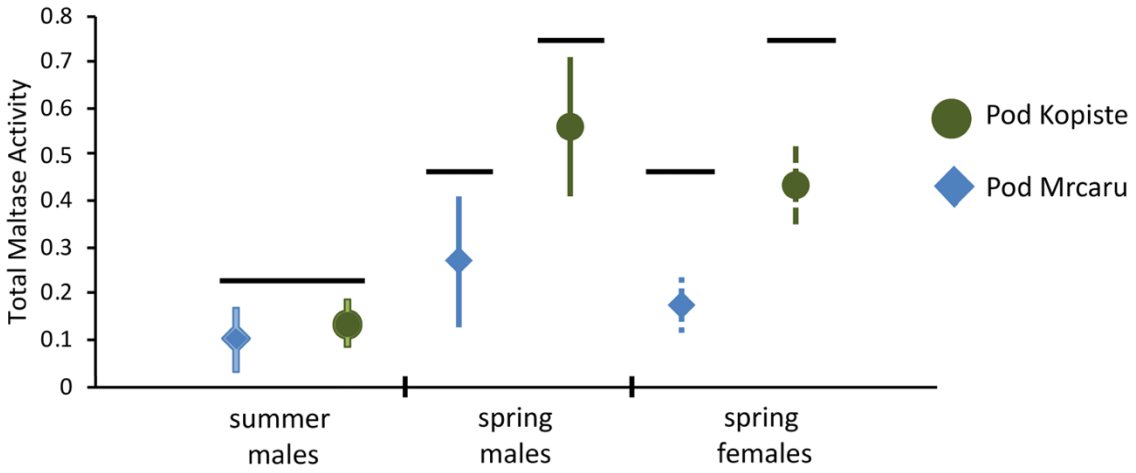

Figure 3.13 Total maltase activity in µmol glucose liberated min^{-1} in the entire intestine in Pod Kopište (source) and Pod Mrčaru (omnivore) summer males, spring males, and spring females. Values are mean ± standard deviation. Lines of a different elevation indicate significant differences.

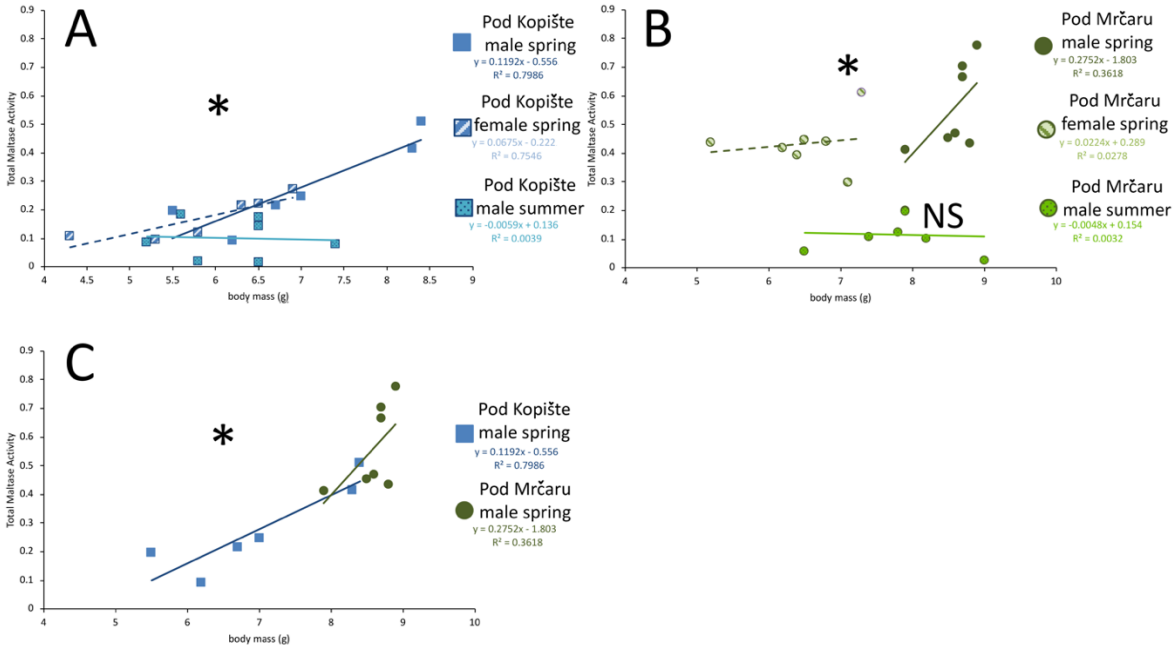

Figure 3.14 Total maltase activity in µmol glucose liberated min^{-1} regressed with body mass in lizards from each sex and season in (A) Pod Kopište and (B) Pod Mrčaru, and in males from spring between populations (C). Values are individual measurements and comparisons were done with ANCOVAS. All comparisons within a plot were significantly different, excepting Pod Mrčaru males from summer (marked with NS). Only the comparison between Pod Kopište males by season (A) yielded a significant interaction term between season and mass.

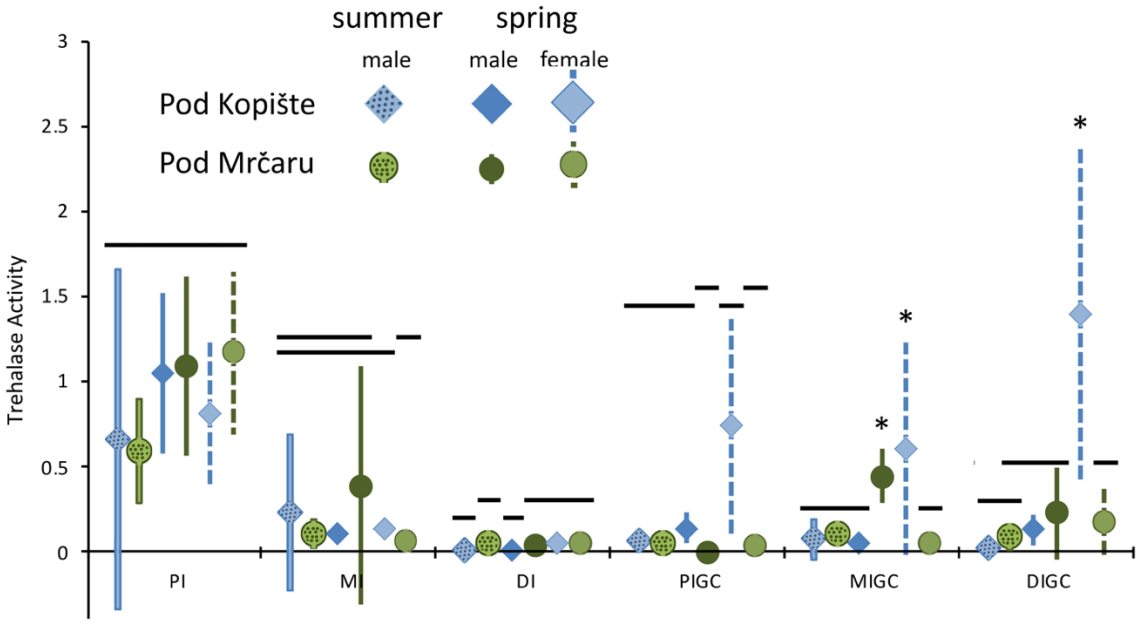

Figure 3.15 Trehalase activity in µmol glucose liberated g^{-1} min^{-1} throughout the gut in Pod Kopište (source) and Pod Mrčaru (omnivore) summer males, spring males, and spring females. Values are mean ± standard deviation. Lines of a different elevation for a gut region indicate significant differences for that population/sex/season combination in that gut region, overlapping lines indicate no differences, and an * marks that enzyme activity is different than all others of that tissue type.

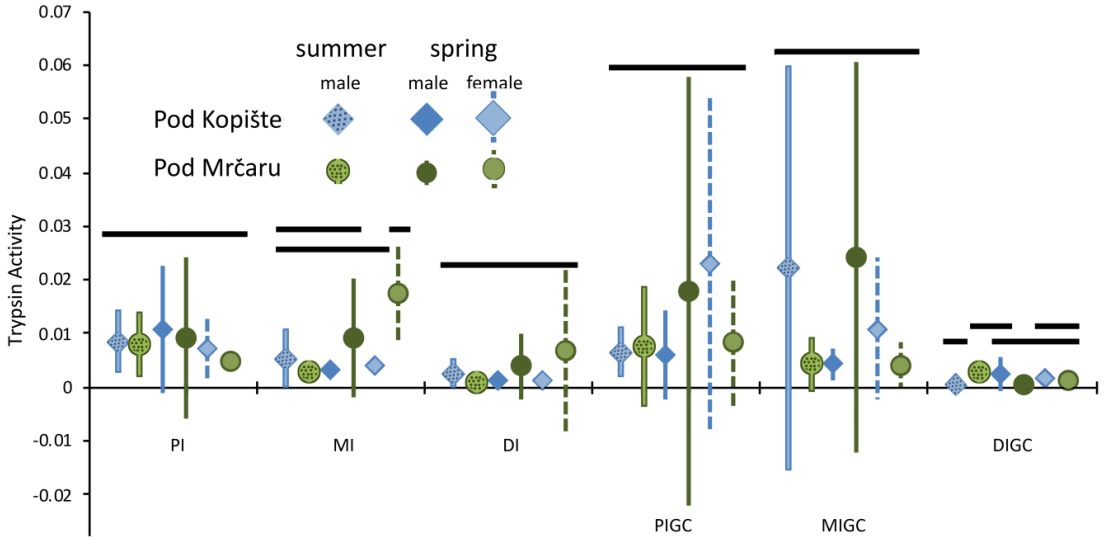

Figure 3.16 Trypsin activity in µmol of p-nitroaniline liberated g^{-1} min^{-1} throughout the gut in Pod Kopište (source) and Pod Mrčaru (omnivore) summer males, spring males, and spring females. Values are mean ± standard deviation. Lines of a different elevation for a gut region indicate significant differences for that population/sex/season combination in that gut region and overlapping lines indicate no differences.

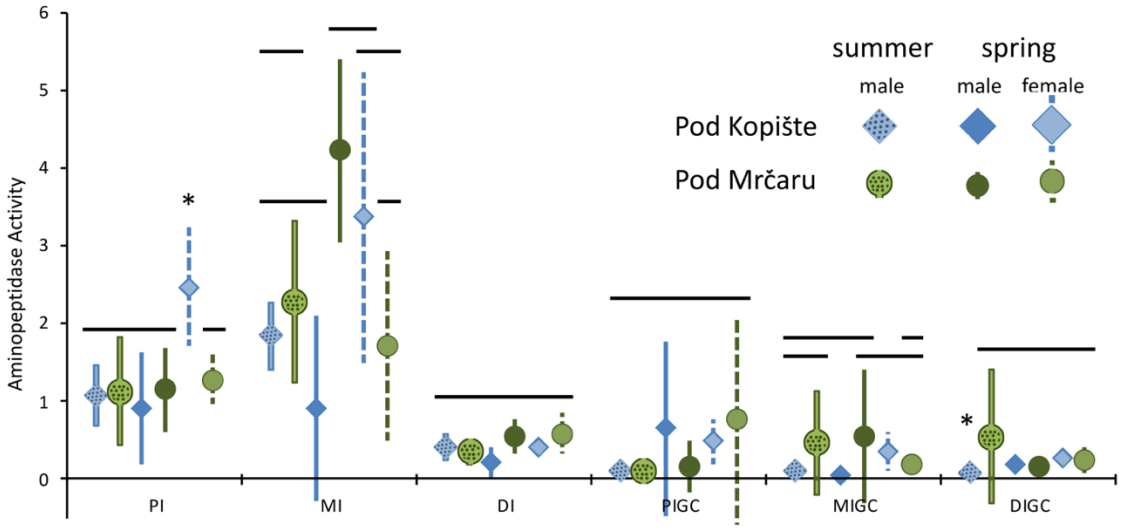

Figure 3.17 Aminopeptidase activity in µmol of p-nitroaniline liberated g^{-1} min^{-1} throughout the gut in Pod Kopište (source) and Pod Mrčaru (omnivore) summer males, spring males, and spring females. Values are mean ± standard deviation. Lines of a different elevation for a gut region indicate significant differences for that population/sex/season combination in that gut region, overlapping lines indicate no differences, and an * marks that enzyme activity is different than all others of that tissue type.

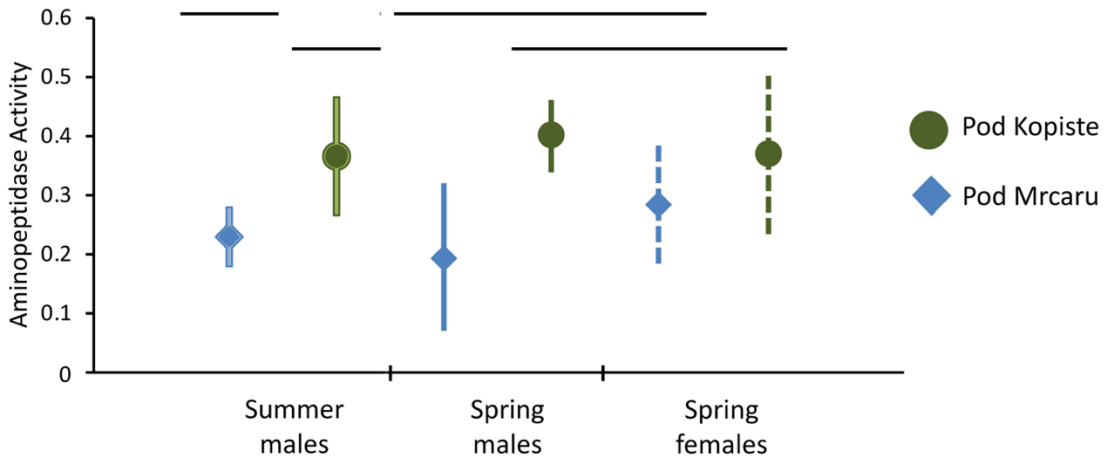

Figure 3.18 Total aminopeptidase activity in µmol of p-nitroaniline liberated min^{-1} in Pod Kopište (source) and Pod Mrčaru (omnivore) summer males, spring males, and spring females. Values are mean ± standard deviation. Lines of a different elevation indicate significant differences for that population/sex/season combination and overlapping lines indicate no differences.

DISCUSSION

Despite ingesting more plant material than their Pod Kopište counterparts, the lizards of Pod Mrčaru do not show consistent evidence of specialization for a plant-based diet. Increased gut length and mass and the presence of hindgut valves aligned with expectations of Pod Mrčaru lizards as specialists, but. However, the patterns of enzyme activities I would expect to support this varied so considerably by season, sex, and gut region that it is doubtful type of diet alone is what drives these patterns. Yet, as I have stressed throughout this dissertation, the lizards of these systems are not long diverged. The ways in which Pod Mrčaru lizards have departed from their source population may be the first steps of dietary specialization.

I had intended the data collected in summer 2013 to serve as a baseline for subsequent data sets. This field season is the subject of Chapter 1 and the high frequency feeding trial of Chapter 2. Compared to our other data, those collected in summer 2013 appear anomalous and I do not know why this is. We performed our collections over one to two-week periods, once or twice a year. I cannot say what the environmental conditions such as weather or seabird nesting patterns were outside of those visits nor what other modifications may have occurred in our absences. For example, in summer 2016 (not included in this dissertation), we encountered goats on Pod Mrčaru that had been brought to the 0.03 km^2 islet to graze by the local community. While these goats were novel to us, some locals claimed the goats resided on the island regularly, whereas others communicated that the goats inhabited the islet for short periods, years apart. Certainly, all of these abiotic, biotic, and anthropogenic forces may have had drastic effects on the lizards of this system. I hope we will have the ability to monitor this system in the future to build our confidence in the preliminary patterns we have observed. However, this highlights the importance of linger-term data sets. Based on our findings, I am compelled to question the

robustness of ecological data collected in one context and applied on broader time-scales or across population diversities not directly measured.

As I call for in Chapters 2 and 3, we need to be collecting data on female animals outside of the contexts of reproduction. While evidence is mounting that females use different physiological strategies than males across a range of body systems (e.g. Shavdahl *et al.*, 2005; Harver *et al*., 1993) there is still comparatively little data explicitly collected from females with respect to the quantity collected about males, or often when included, pooled with males. In this system, the differing patterns of digestibility, gut histology, enzyme activity, and isotopic niche space by the interaction of sex and population put these lizards in an interesting evolutionary position. How is selection on gut form and function and digestive performance acting upon these lizards considering that strategies used by males and by females may be different or even oppositional. Do these sex differences in phenotypic patterns dampen the evolutionary changes in these populations? Does this imply that phenotypic plasticity is the major mechanism of variation, not heritability?

The small, targeted changes we observed in the digestive biochemistry of the hindgut is matched with the similar finding of small, targeted changes in gut microbiome community assembly in the two lizard populations (Vigliotti *et al.*, in prep). Additionally, the presence of hindgut valves and the histological differences we observed in the hindgut support idea that microbes are important to the shifts we observe in this system. We even observed microbes in the TEM images of the hindguts of both populations. While some work has been done on the gut microbiome of plant eating lizards (Bjorndal, 1997; Hong *et al.*, 2011; Mackie *et al.*, 2004; Troyer, 1982, 1984b, 1984c; Foley *et al.*, 1992), there is little consideration of other microbial contributions to digestion. While in this system, microbial fermentation is higher in the Pod

Kopište source population, this appears to have little bearing on digestibility of plant material and does not explain the Pod Mrčaru population's higher performance. However, the function of the lizards' gut microbiome may in fact be useful in vitimin synthesis and metabolism (e.g. Sugahara *et al.*, 2015) in addition to nutrient sources other than fermentation products. Additionally, neurotransmitters produced by the lizards' microbiomes may promote plant-eating or any number of metabolic functions. Matching the gut microbiome data to functional data will be necessary to understand how this system works both mechanistically and with respect to performance. While the ~30 generations that divided the Pod Mrčaru and Pod Kopište lizards at the time of Herrel and colleagues' (2008) study is considerably short with respect to traditional concepts of evolution, the generations of divergence for their gut microbiomes would have been orders of magnitude higher. If many of these differences in the lizards' digestive physiology are due to differences in the assembly or function of the microbiome, the evolutionary change we are observing is not particularly rapid. The interaction of the lizards and their microbes likely allow for greater change than in lizard tissues alone.

One piece that is yet unclear is if the hindgut is an important site of nutrient absorption. With the microbial activity, increased digestive performances in Pod Mrčaru lizards, hindgut histological and gross morphological differences by population, and enzyme differences, it follows that nutrient digestion and absorption may be more important in the hindgut than previously thought. I am unaware of any work measuring nutrient transport rates in the hindgut of lizards, yet these data may be essential to understanding how this system works.

With all the variation in this lizard system, the most surprising pattern of all may be that of the isotopic niche space. Overall, these results vary more with particular field season than with any other factor we measured. I had expected to see clear trophic differences between the

populations, supporting Pod Mrčaru lizards as primarily plant eaters and Pod Kopište lizards are virtually entirely insectivorous. However, this was not the case. The arthropod supplementation the Pod Mrčaru lizards appear to do in the wild (supported by a performance on a mixed diet nearly as digestible as an insect only diet) appears to play a more pronounced trophic role than I had thought based on stomach content data, but also the morphological shifts we have observed in the lizards. As digestive enzyme and isotopic data show few differences, this supports that the two lizard populations likely have a much more similar to each other effective diet than found in Herrel and colleagues' (2008) descritions and Wehrle and others' (*under review*) follow up data.

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