Title: Comparative digestive morphology and physiology of five species of *Peromyscus* under controlled environment and diet

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Highlights:

- In individual organs, we identified a phylogenetic signal in relative foregut mass and small intestine length (Pagel's $\lambda = 1$). As proportions of total gut mass, we identified phylogenetic signals in relative foregut mass and relative small intestine mass ($\lambda = 1$) across five *Peromyscus* species.
- Several species exhibited distinct differences in gut morphology, such as species from arid regions (*P. californicus* and *P. eremicus*) exhibiting larger foregut chambers, and the oldfield mouse (*Peromyscus polionotus*) exhibiting a longer large intestine.
- We suggest that *Peromyscus* is a promising model to study interactions between the environment, genetics, morphology, physiology, and natural history.

Abstract: Digestive morphology and physiology differ across animal species, with many comparative studies uncovering relationships between animal ecology or diet, and the morphology and physiology of the gastrointestinal tract. However, many of these studies compare wild-caught animals feeding on uncontrolled diets and compare broadly related taxa. Thus, few studies have disentangled the phenotypic consequences of genetics from those potentially caused by the environment, especially across closely related species that occupy similar ecological niches. Here, we examined differences in digestive morphology and physiology of five closely related species of *Peromyscus* mice that were captive bred under identical environmental conditions and identical diets for multiple generations. Using phylogenetic generalized least squares (PGLS) of species means to control for body size, we identified a phylogenetic signal in the mass of the foregut and length of the small intestine across species. As proportions of total gut mass, we identified phylogenetic signals in relative foregut and small intestine masses, indicating that the sizes of these structures are more similar among closely related species. Finally, we detected differences in activities of the protease aminopeptidase-N enzyme across species. Overall, we demonstrate fine-scale differences in digestive morphology and physiology among closely related species. Our results suggest that *Peromyscus* could provide a system for future studies to explore the interplay between natural history, morphology, and physiology (e.g. ecomorphology and ecophysiology), and to investigate the genetic architecture that underlies gut anatomy.

Keywords: comparative anatomy, gut anatomy, aminopeptidase-N, gut length, evolutionary hotspot

INTRODUCTION

Digestive morphology and physiology differ widely across animal taxa, often to promote efficient nutrient extraction from foods and to support the overall fitness of animals. Generally, the digestive tracts of animals are adapted to their feeding strategy. For example, due to the presence of refractory material, plant tissue is often considerably more difficult to digest and extract nutrients from than animal tissue, and thus herbivores tend to have more voluminous or longer digestive anatomy than carnivores (Duque-Correa et al., 2021; Griffen and Mosblack, 2011; Karasov and Douglas, 2013); though this trend depends on the dataset and phylogenetic breadth used (Hoppe et al., 2021; Langer and Clauss, 2018; O'Grady et al., 2005). Additionally, it is generally hypothesized that because the digestive tract tissue is one of the most expensive to maintain in terms of energy and protein metabolism, there is selective pressure towards physiological efficiency and the avoidance of maintaining excess tissues (Cant et al., 1996). Although digestive morphology and physiology are well studied across animals with different feeding strategies, our understanding of the differences between closely related species, particularly when they occupy similar ecological niches, is lacking. Feeding and nutrition are critical components of an animal's niche and fitness, and understanding the dynamics and patterns that underlie the relationship between ecological variation, genetic control, and physiological and morphological specialization across species is a goal for the field of comparative physiology (Griffen and Mosblack, 2011; Mykles et al., 2010).

Most inquiries that aim to uncover digestive differences across species have been limited to observational interspecific studies that use wild-caught individuals (Naya et al., 2014; Spinks and Perrin, 1995). These studies have each correlated varying wild diets to differences in gut morphology and physiology among species. For example, one study examined wild-caught

individuals from nineteen species of Southern African myomorph rodents and found that the ratio of small to large intestine was smallest in herbivorous rodents compared to those with other feeding strategies (Perrin and Curtis, 1980). A similar study from Mongolia compared six species of syntopic rodents and also found the largest hindgut in the herbivorous species (Wang et al., 2003). While there are some benefits to conducting these studies in wild animals, these studies are limited in their ability to disentangle the effects of short-term variation in diet or environmental characteristics from the consequences of long-term evolutionary history across species and within populations. For example, intraspecific experimental studies show that insectivorous and herbivorous diets, fiber quantity, temperature, and water availability can all influence gastrointestinal morphology (Gorman et al., 1997; Green and Millar, 1987; Gross et al., 1985; Koteja, 1996; Spinks and Perrin, 1995; Woodall, 1987). Thus, captive-based studies may control for environmental variation and better highlight species-level differences in gut anatomy and physiology. However, animals in these studies are often wild-caught, and the environmental conditions experienced during development may yield life-long differences to digestive physiology via developmental plasticity (Kotrschal et al., 2014). Further, biomedical studies have demonstrated multigenerational effects of diet on metabolic phenotypes of offspring (Adedeji et al., 2019; Ng et al., 2010). Collectively, these studies highlight the complex set of factors that may impact gut anatomy and physiology across closely related species. Moving forward, the use of comparative animal models in captivity under identical or variable conditions may aid in understanding the genetic and molecular mechanisms underlying variation in gut anatomy and physiology.

Rodents offer enormous potential to understand the effects of the environment and genetics on digestive morphology and physiology (Cork, 1994; Hume, 1994). Rodents are the

most diverse mammalian order and from an evolutionary perspective, they have been a highly successful group of animals, as indicated by their vast ecological distribution, number of families (~30), number of species (~1700), and overall abundance/biomass of individuals (Stevens and Hume, 2004). Understandably, a small number of species have become very well studied in various fields of biology thanks to their overall tractability and easy maintenance in labs. Consequently, the huge diversity of rodent species is currently underutilized in biology, presenting both challenges and great potential to learn about morphological and physiological specialization across species.

In recent years, *Peromyscus* mice have become increasingly useful as model organisms. Species in this genus occupy similar ecological niches from a broad perspective, but also exhibit distinct adaptations to particular environments. Additionally, at least two of these species (*P. maniculatus* and *P. polionotus*) can produce hybrids, and have been used to identify the genetic loci involved in complex behaviors such as burrowing (Weber et al., 2013) and parental care (Bendesky et al., 2017). Thus, *Peromyscus* species may provide a promising opportunity and first step towards understanding the complex and interconnected influence of genetics and the environment on digestive morphology and physiology across species.

Here, we compare various metrics of gut morphology and physiology across five species of *Peromyscus* mice (Table 1, Fig. 1) while controlling environmental conditions and offering the same controlled diets. We investigated relative mass of the foregut, stomach, small intestine, cecum, and large intestine in each of our species, length and surface areaas of the small and large intestines. Most rodents have a pouch called the cecum that sits at the first part of the large intestine, where absorption of some nutrients begins and bacterial communities help to digest food material (Kohl et al., 2014). Some species also have an additional segment of their stomach,

known as the foregut, for which the overall function is poorly understood (Kohl et al., 2014; Langer and Clauss, 2018). We also investigated activities of the digestive enzymes maltase and sucrase, which are two of the most abundant intestinal disaccharidases, and aminopeptidase-N, an intestinal peptidase involved in the final stages of alanine digestion. Using these metrics, our results uncover the extent to which morphological and physiological phenotypes vary across closely related species in the absence of environmental variation, opening the door for future studies to investigate the genetic basis of this variation.

MATERIALS AND METHODS

Animals: We obtained female individuals of each of the following *Peromyscus* species (*P. polionotus* [n =6], *P. maniculatus* [n = 6], *P. leucopus* [n = 5], *P. eremicus* [n = 6], *P. californicus* [n = 6]) from the *Peromyscus* Genetic Stock Center at the University of South Carolina (Havighorst et al., 2019). This facility has maintained breeding colonies of all 5 *Peromyscus* species since 1993 or earlier for some species. (Havighorst et al., 2019). All individuals were shipped to the University of Utah, where we attempted to maintain husbandry conditions to those of the *Peromyscus* Genetic Stock Center. During the experiment, all animals were housed individually in shoebox cages with paper bedding and small plastic tubes as nests/shelters. Animals were held for a period of 5 weeks, under a 12h:12h light:dark cycle, 28°C ambient temperature and 20% humidity, and given *ad libitum* water from bottles with stainless-steel nipples. All the animals were adults and sexually mature at the time of our study (between 5 and 8 months of age) and were provided with *ad libitum* access to the same powdered laboratory rodent chow as used at the *Peromyscus* Genetic Stock Center (Formula 8904, Harlan Teklad,

Madison, WI), which is composed of 24.3% protein, 4.7% fat, 40.2% available carbohydrates, and 12.4% neutral detergent fiber (NDF) on an as-fed basis. Individuals were moved to a wire-bottom metabolic cages for 4 evenings before dissection as part of other experiments (Brooks et al., 2016), but did not lose significant body mass during this portion of the experiment.

Dissection Procedures: After measuring body mass (± 0.1 g), all animals were euthanized using a high dose of isoflurane. Immediately after being euthanized, we dissected each animal's abdominal cavity and collected their foregut (fornix ventricularis or fundus), acidic stomach, small intestine, caecum, and large intestine. For our purposes, the large intestine is defined as all hindgut tissue independent of the cecum (*i.e.* the colon and rectum). Contents of each gut region were placed into plastic tubes, and the pH of gut contents was immediately measured using an Omega Soil pH electrode (PHH-200). All tissues were then briefly cleaned of mesenteric and adipose tissues, rinsed with physiological saline solution, blotted dry, and masses of each region were measured. Next, total length of small and large intestines were measured to the nearest millimeter using a stainless-steel ruler. Then, small and large intestines were cut open longitudinally and opened flat on a metal tray with ice underneath. We used digital calipers to take measurements of the width of these flattened tissues, which essentially represent the circumference of the intestines. We collected 9 "width" measurements for the small intestine and 4 measurements for the large intestine. These values were averaged within a tissue and used to estimated surface area by multiplying length and width. We investigated for differential results depending on normalization to organ mass or surface area, but these methods produced consistent conclusions. Finally, tissues were frozen on dry ice and stored at -80°C. All

procedures involving rodents were approved under the University of Utah Institutional Animal Care and Use Committee protocol #12-12010.

Enzyme Assays (Maltase, Sucrase, Aminopeptidase-N)*:* Small intestine tissue samples were thawed over ice and immediately homogenized with cold homogenizing buffer (300 mM mannitol in 1 mM HEPES/ KOH, pH 7; HEPES came from Sigma-Aldrich Corp., St. Louis, Missouri, CAS number 7365-45-9) with an OMNI TH-01 tissue homogenizer (Omni International, Kennesaw, Georgia) set at ~20,000 rpm for 30 s. To avoid underestimating enzyme activity, whole tissue homogenates were utilized instead of isolated brush border membrane preparations (Martínez del Rio 1990). Each assay was performed in triplicate.

For disaccharidase activities (maltase and sucrase), we diluted tissue homogenates in the homogenizing buffer above (maltase: 2 µL of homogenate in 498 µL of homogenizing buffer; sucrase: 10 µL of homogenate in 90 µL) Then, we took 30 µL of the diluted tissue homogenate and incubated it for 20 min at 37°C in a 1.5 ml vial containing 30 µL of maleate/NaOH buffer (0.1 maleate/1 N NaOH, pH 7) containing 56 mM maltase or sucrase, depending on the enzyme of interest. The reactions were stopped with the addition of 400 µl of Glucose-Assay-Kit stop solution (GAGO-20 glucose assay kit; Sigma Aldrich, St. Louis, MO. USA) to each vial. All the replicates were incubated at 37°C for 30 min, after which 400 µL of 12 N H_2SO_4 was added to each tube, and the absorbances were measured at 540 nm using a plate reader (BioTek Synergy H1 , Winooski, VT, USA). Using a glucose standard curve, maltase and sucrase activities were calculated as the average of triplicate absorbances for each sample minus blanks and expressed as µmol min^{-1} g^{-1} wet tissue.

Aminopeptidase-N activity was assayed using L-alanine-p-nitroanilide (Sigma-Aldrich, item # A9325) as a substrate (Brzęk et al. 2013). Each reaction began in a 1.5 ml vial upon mixing 2.5 µL of tissue homogenate with 250 µl of assay solution (0.1 M phosphate buffer [NaH2 PO4 /Na2 HPO4 , pH = 7.0] containing 2.0 mM L-alanine-p-nitroanilide). After 20 min of incubation at 37°C, each reaction was stopped by adding 250 µl of chilled 2 N acetic acid to the vial. The absorbances were measured at 380 nm using a plate reader (as above). After subtracting background absorbance, we used a calibration standard curve made with dilutions of L-alanine-p-nitroanilide to calculate aminopeptidase-N activity in units of µmol min^{-1} g^{-1} wet tissue.

STATISTICS

We used analysis of variance (ANOVA) and post-hoc Tukey's HSD tests to compare body mass and pH differences and analysis of covariance (ANCOVA) to compare morphological and physiological metrics across species. For all ANCOVA results, the interaction between species and body mass was never significant (data not shown). We also investigated the proportion of each region in regards to total gut mass, a metric that is independent of body size. Enzyme activities are standardized to mass-specific values for tissue (µmol min−1 g−1 wet tissue), and were compared with ANOVA and post-hoc Tukey's HSD tests.

For each morphological or physiological metric, we tested for phylogenetic signals, or the tendency for more closely related species to resemble each other, as opposed to resembling species on a randomly drawn tree. We estimated phylogenetic signal by conducting a phylogenetic generalized least squares (PGLS) regression analysis of species means and by calculating lambda using maximum likelihood as implemented in the *caper* package using R

version 4.1.2 (Orme et al., 2012; Orme et al., 2013). Body mass was incorporated into the PGLS analyses for absolute organ masses, lengths, and surface areas. To determine the allometric scaling relationship for each morphometric trait, log-transformation was used. For other traits (pH of contents, organ masses standardized to total gut mass, enzyme activities), we simply tested for a phylogenetic signal of the trait values.

RESULTS

Peromyscus species exhibited body masses that were significantly different from one another (ANOVA: $F_{4,24} = 64.55$, $P < 0.0001$). Specifically, the body mass [g; mean ± standard error (S.E.)] of each species was as follows: *P. californicus*: 41.09 ± 1.72; *P. eremicus*: 19.18 ± 0.40; *P. leucopus*: 22.11 ± 2.76; *P. maniculatus*: 17.94 ± 0.46; *P. polionotus*: 14.59 ± 0.31. Using Tukey's HSD test, *P. californicus* was significantly heavier than all other species, and *P. polionotu*s weighed significantly less than all other species, with *P. eremicus*, *P. leucopus*, and *P. maniculatus* exhibiting intermediate body masses (with no significant differences based on Tukey's HSD). All individual measurements can be found in our supplemental materials.

The pH of gut contents varied significantly by gut region ($F_{4,23} = 353.88$, $P < 0.0001$). However, there were no significant differences by species, nor a significant species x region interaction. The mean ± S.E. values of pH for each region were as follows: foregut: 5.02 ± 0.11; stomach: 2.06 ± 0.13; small intestine: 7.23 ± 0.07; cecum: 6.79 ± 0.07; large intestine: 6.76 ± 0.09. There was no evidence of a phylogenetic signal for pH of any gut region ($\lambda = 0$).

We observed that the relative masses of individual gut compartments exhibited significant differences across *Peromyscus* species. For example, after accounting for body mass, *P. eremicus* exhibited a foregut chamber that was roughly 1.5 x heavier than several other

224 species (Fig. 2). Large intestine mass exhibited the largest effect size across rodent species, with
225 this being relatively heavier in *P. polionotus*. Specifically, *Peromyscus polionotus* exhibited a
226 1.41x greater relative large intestinal mass when compared to *P. maniculatus*, the most closely
227 related species (Fig. 1, 2). When investigating for phylogenetic signals, we only found a
228 significant relationship between phylogeny and relative foregut mass ($\lambda = 1$).
229 We also compared the relative masses of specific gut regions as a percentage of the total
230 gut mass, highlighting differential investment in various gut regions. Here, we found significant
231 differences in the relative masses of all gut regions across species (Fig. 3). Again, *P. eremicus*
232 exhibited a heavier foregut region as compared to most other species. Similarly, the relative mass
233 of the large intestine was greater in *P. polionotus* as compared to a closely related species, *P.*
234 *maniculatus*. Using phylogenetic analysis, we found that relative foregut mass and relative small
235 intestine mass (as a percentage of total gut mass) exhibited significant relationships with
236 phylogeny ($\lambda = 1$).
237
238 Next, we compared the relative lengths and surface areas of the animals' small and large
239 intestines. Across species, we identified significant differences in both relative small intestine
240 length and small intestine surface area. Two species, *P. polionotus* and *P. maniculatus,* exhibited
241 significantly greater small intestine surface areas than *P. eremicus* (Fig. 4). Similarly, we found
242 significant differences in relative large intestine length and surface area across the species.
243 Specifically, *P. polionotus* demonstrated a large intestine length of approximately 1.24 x longer
244 than *P. maniculatus* and a large intestine surface area about 1.3 x larger than *P. maniculatus*.
245 Estimates of phylogenetic signal suggest that small intestine length is highly dependent on the
246 phylogenetic relationship of species ($\lambda = 1$).

Finally, we investigated activities of digestive enzymes as a measurement of gut physiology and digestive function. There were no significant differences in mass specific maltase activities across our five different *Peromyscus* species ($P = 0.21$; Fig. 5). Similarly, sucrase activities did not vary across species (data not shown). However, we did observe species-specific differences in aminopeptidase activity (Fig. 5). *Peromyscus leucopus* and *P. maniculatus* demonstrated 1.96 x greater aminopeptidase-N than *P. californicus*. We did not observe any significant relationship between enzyme activities and phylogeny.

DISCUSSION

Thanks to the rich history of comparative anatomy, numerous studies have previously investigated differences in digestive form and function across species, often identifying morphological and physiological differences among species with different feeding strategies. However, we currently lack an understanding regarding how digestive morphology and physiology differ across closely related species that share similar broad feeding strategies and ecological niches, especially in controlled experimental settings, and thus in the absence of environmental variation. Here, we examined morphology and physiology of digestive structures in five closely related species of *Peromyscus* mice. We found several aspects of gut anatomy and physiology that vary across species, which demonstrate that *Peromyscus* is a promising model for future studies aimed at understanding genetic determination of gut anatomy and physiology.

First, we aimed to investigate for phylogenetic signals, which are statistical measurements that quantify how species' phenotypes differ from one another relative to their phylogenetic relationships. We used phylogenetic generalized least squares (PGLS), a statistical test that allowed us to test for phylogenetic signal across our phenotypes of interest using Pagel's

lambda. It is important to note that our study includes data for only five closely related species of *Peromyscus*, and some of our traits of interest may have an undetected phylogenetic signal due to our small sample size (Boettiger et al., 2012; Münkemüller et al., 2012). For both anatomical and physiological traits, the power of the test increases dramatically with an increased sample size of animals. It should be noted that because we are only examining traits in five different species of *Peromyscus*, we recognize that some of our traits of interest may have a phylogenetic signal that we were unable to detect because of our small sample size. Additionally, our study design and the phylogenetic distances across our species may preclude us from detecting traits under phylogenetic influence that are shared across *Peromyscus* species. For example, if we included more distantly related species into our study, we would be more likely to identify significant signals in *Peromyscus* than by just using the five closely related species alone. Thus, in our study, the lack of a phylogenetic signal indicates that there is no significant phylogenetic relationship in a given trait across our species, but it does not indicate whether they share a unique characteristic that might otherwise be identifiable as a phylogenetic signal if other species were incorporated in our analysis. For these reasons, we remained conservative with the conclusions we made regarding the phylogenetic signals that we identified.

Specifically, we identified a significant phylogenetic signal in small intestine length, relative small intestine mass as a proportion of total gut mass, and relative foregut mass (both accounting for body size and as a proportion of total gut mass). Thus, closely related *Peromyscus* species are more likely to resemble each other in these traits. Interestingly, other studies with wider taxonomic breadth have come to varied conclusions about phylogenetic influence on gut morphology. One study that analyzed previously published datasets on 493 species of birds and nonflying mammals found that small intestine length and surface area demonstrated phylogenetic

293 signals in birds, but not in mammals (Lavin et al., 2008). Another study that examined
294 relationships between diet and intestine length in 32 species of tropical cichlid fishes found that
295 intestine length demonstrated significant phylogenetic signal (Wagner et al., 2009), and a more
296 recent study examined published datasets for small intestine length in 397 mammalian species
297 also identified a strong phylogenetic signal (Duque-Correa et al., 2021). Notably, while these
298 studies have the benefit of large sample sizes, it impossible to standardize diets and environment
299 across so many different species. While smaller, our study accounts for these challenges by
300 feeding a single diet across species and individuals from long-term captive breeding lines.
301 Collectively, these findings underscore the importance of studying the digestive morphology and
302 physiology of closely related species using phylogenetically informed methods.
303 We also identified several morphological or physiological traits that differed significantly
304 across *Peromyscus* species. Given that our animals were bred in captivity and fed captive diets,
305 these differences represent evolutionary differences. We briefly discuss the potential functional
306 significance of these differences in relation to natural environments, though strongly
307 acknowledge that these are largely speculative given our limited sample size of species and life
308 history strategies.
309 In *P. polionotus,* we identified significantly larger large intestine in terms of mass, length,
310 and surface area, when combined to *P. maniculatus*. The oldfield mouse (*P. polionotus*) is found
311 in early successional habitat, which is often characterized by nutrient fluctuations, temporary
312 influxes of plant productivity, and high food web complexity (Swanson et al., 2011). Further,
313 population sizes of *P. polionotus* have been found to correlate with peak seed abundance
314 (Everett, 1985), suggesting strong selection on aspects of feeding, nutrition, and likely digestion.

While speculative, it could be that the enlarged hindgut of this species facilitates fermentation of low nutrient foods in an area of fluctuating resources.

Species of *Peromyscus* from the southwestern USA (*P. californicus* and *P. eremicus*) demonstrated significantly heavier relative foregut masses compared to the other species. The foregut chambers of omnivorous rodents seem to play a role in food storage prior to the material moving into the glandular and acidic stomach (Gärtner and Pfaff, 1979). A previous comparative study hypothesized that the enlarged foregut of another species, *Acomys spinosissimus*, may assist with temporary food storage to assist with foraging in arid habitats (Perrin and Curtis, 1980). Interestingly, cheek pouches are often used for food storage, and are more common in arid southwestern North America (Vander Wall and Dittel, 2021), the natural habitat of *P. eremicus*, though this species lacks cheek pouches. Thus, while it remains speculative, the enlarged foregut of southwestern *Peromyscus* species could play a role in temporary food storage, analogous to the enlarged cheek pouches in many other granivorous rodent species in these areas.

We also found that activities of a digestive peptidase (APN) showed interspecific variation, while there were no significant differences in maltase or sucrase activities across species. It has been previously shown that diet influences digestive enzyme activities in vertebrates over short-term time scales. For example, *Peromyscus leucopus* increases intestinal APN activities when fed increased dietary protein (Wang et al., 2019). The same phenotypic flexibility is also present in numerous avian species (Afik et al., 1995; Caviedes-Vidal et al., 2000; del Rio et al., 1995; Sabat et al., 1998). However, the interspecific differences that we observed between *P. leucopus, P. maniculatus, and P. californicus*, represent evolved differences in constitutive activity under controlled conditions, independent from the

consequences of variation in dietary composition. We might predict APN activities to be higher in species that typically consumer higher protein diets. Interestingly, comparative studies using individuals collected from the wild on phyllostomid bats (Schondube et al., 2001), minnows (German et al., 2010), and passerine birds (Kohl et al., 2011) each point to evolutionary relationships between an animals' natural diets and the activities of disaccharidases, but not APN activities. These trends are often explained by the essential need all animals have for dietary protein, and that enzyme levels may be regulated simply to ensure protein needs are met at a baseline level (Kohl et al., 2011). Thus, differences in constitutive APN activities across species may be somewhat idiosyncratic and represent the notion of "good enough", as opposed to the notion of optimal evolutionary matching between functional demands and physiological capacities (Dudley and Gans, 1991; Garland Jr, 1998).

While we did control the animals' environment and diet to isolate evolved differences across species, we cannot know whether our study treatments were truly equivalent for all species. In other words, the environmental conditions that the mice experienced throughout our study may have been a better representation of the wild environments for some species than for others. Even though one of our goals was to begin disentangling phenotypic plasticity from genetics in *Peromyscus*, it is impossible to quantify the extent to which plasticity may have influenced our results. Further, while we speculate some ecological interpretations of these data, it has previously been argued that species which appear to exhibit phenotypic specializations in morphology or physiology may, in actuality, be ecological generalists that consume preferred food items when available but maintain specialized phenotypes to be able to feed on unpreferred resources when needed (Robinson and Wilson, 1998). In the context of this study, our equal conditions could be phenotypic equalizers, but they also potentially make species differences

more evident, highlighting the difficulty of understanding the relative influence of phenotypic plasticity versus constitutively evolved differences in digestive morphology and physiology. Ideally, further studies will continue to build on our existing knowledge of *Peromyscus* natural history in the wild, along with captive studies under a variety of standardized conditions to understand the roles of phenotypic flexibility and evolved, genetic control of gut morphology and physiology.

Regardless, our results along with previous research suggest *Peromyscus* may be useful as a future model to study the genetic underpinnings of gut morphology. Understanding the molecular and genetic basis for morphological diversity is of interest to evolutionary biologists (Rebeiz et al., 2015), and has largely focused on external morphological traits. To date, a handful of genome-wide association studies have established a foundational understanding of genomic regions that are important for determining gut morphology and physiology. Nearly all of these studies, however, have focused on farmed animals, namely pigs, chickens, and other livestock animals (Cao et al., 2020; Li et al., 2018; Lu et al., 2014; Mabelebele et al., 2014; Moreira et al., 2019; Zhu et al., 2019). For example, one study found that a ~170 Mb region on the GGA1 gene is important for small intestine length determination in chickens, and that small intestine length demonstrated good SNP-based heritability (Li et al., 2018). However, livestock have experienced extensive artificial selection towards increased growth and production, and so the genomic regions identified in these species may be very different from those genomic changes yielding interspecific differences across species. *Peromyscus* mice are among the most abundant and well-studied of all small mammal species in North America (Bedford and Hoekstra, 2015), with the ability for some species to hybridize (Weber et al., 2013), and for the relative ease of raising them in laboratory settings. Thus, *Peromyscus* species represent a biologically promising and

logistically feasible system in which to study the underlying genetic mechanisms leading to these interspecies differences in gut anatomy. However, we encourage the development of other rodent and animal systems to address similar questions.

Our understanding of the nuanced evolution associated with digestive morphology and physiology among closely related species occupying similar niches is limited. Previous literature has not robustly disentangled genetics from environmental impacts on these systems. In this study, we found phylogenetic signals in relative foregut mass and small intestine length, as well as relative foregut and small intestine mass as a proportion of total gut mass. We also highlight several closely related species with marked differences in gut morphology. Our results build on our limited understanding of the specific ways that the environment and genome can interact to determine animal phenotypes, and highlight *Peromyscus* as a promising system for future studies in the realm of comparative morphology, physiology, and the genetic architectures that underlie environmental specialization across species.

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595 Table 1. Natural history and niche information of our five *Peromyscus* species

Species (Latin name; English name)	**Documented Range**	**Habitat**	**Diet**	**Notable Characteristics**	**References**
P. polionotus; oldfield mouse or beach mouse	Southeastern US	Open sandy areas or dunes in coastal areas; pine-hardwood forests with scant understory	Seeds and grains; opportunistic carnivores	Early succession species; Smallest species in the genus (body mass of 10-15 g)	(Dewsbury et al., 1980; Gentry and Smith, 1968; Moyers, 1996; Smith, 1971)
P. maniculatus; deer mouse	Abundant throughout all of North America	Preferences vary depending on subspecies, mainly grasslands	Generalists; arthropods, nuts, seeds, fruits, and fungi	Most abundant mammal in North America	(Hall and Kelson, 1959; King, 1968; Lawlor, 1982; Meserve, 1976; Whitaker, 1980)
P. leucopus; white-footed mouse	Eastern half of the US	Brushy fields, rocky woodlands, habitats with canopies and woody debris	Insects and arthropods, seasonal consumption of seeds and fruit		(Hamilton, 1941; Kamler and Pennock, 2004)
P. eremicus; cactus mouse	Southwestern US, north central Mexico	Deserts, rocky and sandy substrates, arid environments	Shrub fruit, seeds, flowers, opportunistic and seasonal	Likely acquires water by consuming succulents; Estivates during	(Cahalane, 1939; Dewsbury et al., 1980; Meserve,

			consumption of insects	driest months to save water	1976; Schmidly and Bradley, 2016; Veal and Caire, 1979);
P. californicus; California mouse	South of the San Francisco Bay and west of California's deserts	Chaparral and oak woodland, mesic laurel forests, redwood forests	Specialist on shrub fruits, seeds, flowers, particularly of California laurel	Largest *Peromyscus* species in the USA (body mass 33 – 54 g)	(Grinnell and Orr, 1934; Meserve, 1976)

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FIGURE LEGENDS

Figure 1. Phylogenetic relationships of our five focal *Peromyscus* species, from (Brooks et al., 2016).

Figure 2. Masses of different gut sections across *Peromyscus* species. Graphs depict least square means (to control for body mass) ± standard error. One-way analysis of covariance (ANCOVA) was used to determine statistical differences across species and relationships with body mass. Letters above points denote statistical significance within a gut region across rodent species, based on a Tukey's post-hoc test. Species not sharing letters are significantly different from one another.

Figure 3. Relative masses of different sections of the gut as percentages of total gut mass. One-way analysis of variance (ANOVA) was used to determine statistical differences across species. Error bars represent standard error of the mean. Letters inside colored regions denote statistical significance within a gut region across rodent species, based on a Tukey's post-hoc test. Species not sharing letters are significantly different from one another.

Figure 4. Relative lengths and surface areas of the small and large intestines across *Peromyscus* species. Graphs depict least square means (to control for body mass) ± standard error. One-way analysis of covariance (ANCOVA) was used to determine statistical differences across species and relationships with body mass. Letters above points denote statistical significance within a gut region across rodent species, based on a Tukey's post-hoc test. Species not sharing letters are significantly different from one another.

Figure 5. Activities of maltase and aminopeptidase-N across *Peromyscus* species. Graphs depict means ± standard error. One-way analysis of variance (ANOVA) was used to determine statistical differences across species. Letters above points denote statistical significance within a gut region across rodent species, based on a Tukey's post-hoc test. Species not sharing letters are significantly different from one another.