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RESEARCH ARTICLE

Physiological and microbial adjustments to diet quality permit facultative herbivory in an omnivorous lizard

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ABSTRACT

While herbivory is a common feeding strategy in a number of vertebrate classes, less than 4% of squamate reptiles feed primarily on plant material. It has been hypothesized that physiological or microbial limitations may constrain the evolution of herbivory in lizards. Herbivorous lizards exhibit adaptations in digestive morphology and function that allow them to better assimilate plant material. However, it is unknown whether these traits are fixed or perhaps phenotypically flexible as a result of diet. Here, we maintained a naturally omnivorous lizard, *Liolaemus ruibali*, on a mixed diet of 50% insects and 50% plant material, or a plant-rich diet of 90% plant material. We compared parameters of digestive performance, gut morphology and function, and gut microbial community structure between the two groups. We found that lizards fed the plant-rich diet maintained nitrogen balance and exhibited low minimum nitrogen requirements. Additionally, lizards fed the plant-rich diet exhibited significantly longer small intestines and larger hindguts, demonstrating that gut morphology is phenotypically flexible. Lizards fed the plant-rich diet harbored small intestinal communities that were more diverse and enriched in Melainabacteria and *Oscillospira* compared with mixed diet-fed lizards. Additionally, the relative abundance of sulfate-reducing bacteria in the small intestine significantly correlated with whole-animal fiber digestibility. Thus, we suggest that physiological and microbial limitations do not *sensu stricto* constrain the evolution of herbivory in lizards. Rather, ecological context and fitness consequences may be more important in driving the evolution of this feeding strategy.

KEY WORDS: Digestion, Gut microbiome, Host–microbe interactions, Phenotypic flexibility, Plant–animal interactions

INTRODUCTION

Herbivory is a difficult feeding strategy. Plant material is generally high in indigestible fiber, low in essential nutrients, such as proteins, and often defended with toxic defensive compounds (Karasov and Martínez del Rio, 2007). Despite this, some vertebrate classes evolved adaptations that allow them to feed primarily on plant material. For example, herbivory is the most common feeding strategy among mammals (~43% of mammalian species; Price et al., 2012). This success is largely attributed to changes in gut morphology and associations with symbiotic microbes that enhance the digestion of plant material (Stevens and Hume, 2004). However, herbivory is extremely rare in squamate reptiles. While a considerable proportion of species incorporate some plant material into their diets, less than 4% of species are considered truly herbivorous (Pough, 1973).

Numerous hypotheses explain the scarcity of herbivorous lizards (Cooper and Vitt, 2002; King, 1996; Pough, 1973). It is often assumed that ecological contexts might drive the evolution of herbivory. Insectivory is likely a more successful feeding strategy for lizards, and herbivory may only evolve in areas with low insect abundance (Van Damme, 1999). Conversely, intrinsic physiological limitations may impede the evolution of herbivory in lizards. In other vertebrate classes, carnivorous or insectivorous species are unable to down-regulate rates of endogenous protein catabolism when feeding on low-protein diets, often resulting in negative nitrogen balance (Green et al., 2008; Robbins, 1983; Rumsey, 1981; Walton, 1986), whereas herbivores generally have physiological adaptations that allow them to maintain nitrogen balance on low-protein foods (Dearing et al., 2005; Robbins, 1983). Thus, lizards might be limited in their ability to feed on low-nitrogen foods and maintain nitrogen balance. Additionally, low digestive efficiency may limit the ability of lizards to feed on high-fiber food (Karasov et al., 1986; Ruppert, 1980). Last, it has also been suggested that limitations in the ability for the gut microbial communities of lizards to aid in digestion may constrain the evolution of herbivory (Karasov et al., 1986; Sokol, 1967; Szarski, 1962), though some herbivorous lizards have well-developed associations with gut microbes (Mackie et al., 2004). While research has demonstrated that physiological limitations do not preclude the rapid evolution of omnivory from insectivory in the Italian wall lizard (*Podarcis sicula*; Herrel et al., 2008; Vervust et al., 2010), we have less of an understanding of whether physiology might limit the evolution of herbivory, or feeding primarily on plant material.

The lizard family Liolaemidae evolved herbivory roughly 18 times independently, which is greater than the number of times herbivory evolved in all other squamate reptiles (Espinoza et al., 2004). Adaptations in the digestive tracts of these animals are thought to underlie their success as herbivores. For example, herbivorous members of Liolaemidae have longer small intestines compared with omnivorous and insectivorous species (O'Grady et al., 2005). Similar morphological differences have been demonstrated across feeding habits in a number of other lizard groups (Dearing, 1993; Herrel et al., 2004). Additionally, *Phymaturus punae*, a herbivorous species within Liolaemidae, exhibits intestinal disaccharidase

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activities that are tenfold higher than those in omnivorous and insectivorous lizards, which may allow them to more efficiently digest plant material (Brigada et al., 2004).

It is currently unknown whether the differences in gut anatomy and function across feeding groups of lizards are fixed or whether they might be phenotypically flexible. A number of other vertebrate taxa exhibit significant flexibility when feeding on high-fiber diets. For example, when fed high-fiber diets, voles (*Microtus ochrogaster*) increase the size of their ceca (Gross et al., 1985), and Japanese quail (*Coturnix japonica*) increase gizzard mass and small intestine length (Starck and Rahmaan, 2003). The capacity for phenotypic flexibility is thought to be especially important for organisms to adapt to their environments (Piersma and Drent, 2003). Specifically, phenotypic flexibility of the gastrointestinal tract is important for allowing animals to cope with changing energy demands and variations in food quality (McWilliams and Karasov, 2014). It is also unclear how the gut microbiome of lizards responds to changes in diet quality. The addition of fiber results in higher abundances of cellulolytic microbes in mammals (Saro et al., 2012) and birds (Bedbury and Duke, 1983). However, the gut microbiome of lizards, especially in terms of varying diet quality, has been understudied.

Here, we investigated whether an omnivorous species in the family Liolaemidae could maintain nitrogen balance on a plant-rich diet, and whether this diet induced phenotypic changes in the morphology and function of the gastrointestinal tract and changes in the community structure of the resident gut microbiota. We focused our study on *Liolaemus ruibali*, a small omnivorous lizard from the Southern Andes in Argentina. In nature, plant material comprises roughly 16–20% of the diet of *L. ruibali* (S. A. Castro, Comparación de la dieta de las especies sintópicas *Liolaemus* cf. *ruibali* y *Phymaturus* cf. *palluma*: variación intrapoblacional y estacional en los Andes central de San Juan, Argentina, BSc thesis, Universidad Nacional de San Juan, 2013; Villavicencio et al., 2005). Evolutionarily, *L. ruibali* falls within a clade of lizards where omnivory is the ancestral feeding strategy (Espinoza et al., 2004). We maintained animals in the laboratory on either a diet of mixed insects and plant material or a diet of primarily plant material. We hypothesized that physiological limitations do not constrain herbivory in lizards. Therefore, we predicted that lizards fed the herbivorous diet would be able to maintain nitrogen balance despite the low nitrogen content. Additionally, we predicted that lizards fed the herbivorous diet would exhibit digestive adjustments such as longer small intestines and higher disaccharidase activities, traits that have been observed in naturally herbivorous lizards. Last, we predicted that the plant-rich diet would result in changes in gut microbial community structure, including increases in the abundance of microbes associated with cellulolytic fermentation.

MATERIALS AND METHODS

Animals and maintenance

Individuals of *L. ruibali* Donoso-Barros 1961 were collected under permission of Secretaría de Medio Ambiente and Dirección de Conservación y Áreas Protegidas, Provincia de San Juan (exp. no. 13004047, J.C.A.) from Quebrada Vallecito, located in the Andes Mountains, 40 km west of Calingasta town, San Juan province, Argentina (31°11′21″S; 69°42′15″W, ∼3000 m above sea level) in December 2014 (summer in the Southern Hemisphere). Animals were transported to the animal facility at the University of San Luis, Argentina, and housed individually in small plastic cages (10×20×30 cm) with mesh tops. Animal rooms were maintained at ∼28°C on a 12 h:12 h light:dark cycle with Reptistar lights (Sylvania Company, London, UK) to provide UV radiation. Animals were provided with water *ad libitum*. Upon entering captivity, all lizards were fed a liquefied 'mixed' diet with a 50:50 mixture (dry mass) of alfalfa-based rabbit chow and ground mealworms. Lizards were fed ∼9.4 mg dry food g^{-1} body mass with a syringe every other day for 12 days. All methods were approved by the Institutional Committee of Animal Care and Use of the Universidad de San Luis under protocol number 13185/14.

Experiments and laboratory samples

The lizards were then divided into two groups. Sexes of animals were balanced across groups. All animals were adults, and females were not reproductive. One group remained on the mixed diet [50:50 mixture (dry mass) of alfalfa-based rabbit chow and ground mealworms], while the other was placed on a 'plant-rich' diet with a 90:10 mixture (dry mass) of alfalfa-based rabbit chow and ground mealworms. Details of diet composition can be found in Table 1. Lizards were fed ∼9.4 mg dry food g^{-1} body mass with a syringe every other day for 40 days.

Lizards were fed by placing liquefied diet (roughly 80% water content; Table 1) into syringes. The body mass of a lizard was measured, an increment of food was fed to the animal, and the animal was reweighed. This process was repeated until the animal had consumed an adequate amount of food. Food intake was determined by recording the mass of lizards before and after feeding, with the difference being the amount of wet food ingested. Food samples were collected at each feeding to determine the water content of food, and food intake is presented as dry matter intake. Additionally, from days 24 to 34 of the experiment, we collected feces and urine pellets, which were later separated, dried and weighed. These samples were used to determine dry matter digestibility, fiber digestibility and nitrogen balance using methods described below. On day 35, dirt was removed from the cages and they were sterilized with ethanol. Cages were checked daily for the production of feces, which were collected and frozen for microbial inventories (described below).

Utilization efficiency

Dry matter digestibility was calculated as (g dry food ingested−g dry feces produced)/g dry food ingested. We measured total fiber content (neutral detergent fiber, NDF) and cellulose/lignin content (acid detergent fiber, ADF) of food and feces using an Ankom fiber analyzer 200/200 (Ankom, Fairport, NY, USA). Fiber digestibility (of both NDF and ADF) was calculated as (g fiber ingested−g fiber excreted in feces)/g fiber ingested.

We also compared two metrics of nitrogen utilization. Food samples, feces and urine pellets were dried and ground with a mortar and pestle to pass through a 1 mm mesh screen. We used an EA 1110 elemental analyzer (CE Instruments, Wigan, UK) coupled with a DELTAplus Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) to measure the percentage nitrogen in each sample. Apparent nitrogen digestibility was calculated as (g N ingested−g N excreted in the feces)/g N ingested (Dearing et al., 2005; Robbins, 1983). We also calculated

Table 1. Details of diet composition

	Plant-rich diet	Mixed diet
Water content (%)	78.8	80.6
Nitrogen (% dry matter)	3.2	4.4
Neutral detergent fiber (% dry matter)	26.6	19.9
Acid detergent fiber (% dry matter)	18.1	12.9

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nitrogen balance by calculating the difference between nitrogen intake and nitrogen output (in mg N $kg^{-0.75}$ day^{-1}), where output included urine pellet nitrogen (Dearing et al., 2005; Robbins, 1983). No animals shed their skin during the course of this experiment.

Gut morphology

After feeding on different diets for 40 days, lizards were killed using isoflurane. The entire gastrointestinal tract was immediately removed from the animal and further dissected on a metal board over ice. The stomach, small intestine and hindgut were separated and the contents were removed and saved for microbial inventories (described below). The mass and the total length of each section were measured. A small section (~0.5 cm) was removed from the middle of the small intestine and preserved in buffered formalin for histological analysis (described below). Before freezing, the remaining sections of the entire small intestine and the hindgut were cut longitudinally and opened, and length and width dimensions were measured using calipers to the nearest 0.01 mm. With these values we calculated the surface area and volume of each region (assuming a rectangular shape for surface area and a cylinder for volume).

Histology

Intestinal sections were fixed in a 10% buffered formalin solution. Tissue samples were then dehydrated through a graded series of 70%, 96% and 100% ethanol solution, then clarified two times in xylene, and embedded in paraffin at 56°C for 2 h. We obtained cross-sections using a universal rotary microtome. Samples were then mounted on slides, stained with hematoxylin and eosin, and covered with cover glasses. Microphotographs were taken using an Olympus BX50 microscope connected to a video camera HDCE-30C and a PC-based image analysis system using Image J software (Schneider et al., 2012). We measured the epithelial surface magnification, calculated as the epithelial surface perimeter divided by a baseline (defined by the perimeter of the inner circular muscle layer), expressed as a dimensionless ratio.

Digestive enzyme activity

We measured the activity of several digestive enzymes. Frozen small intestines (minus the small piece removed for histology) were thawed at 4°C and individually homogenized for 30 s using a FSH-G 7/107 basic homogenizer (Fisher Scientific, Waltham, MA, USA) in 350 mmol l^{-1} mannitol buffer (pH 7), using 10 ml g^{-1} tissue.

Disaccharidase activities (maltase and sucrase) were measured using a colorimetric method (Dahlqvist, 1984). Briefly, tissue homogenate was diluted using mannitol buffer, and 40 µl of the resulting tissue homogenate was incubated with 40 µl of substrate (56 mmol l^{-1} sugar, either maltose or sucrose) in a 0.1 mol l^{-1} maleate/NaOH buffer, pH 6.5, at 30°C for 20 min, after which the reaction was arrested with 1 ml of Glucosa Liquid Plus (GT Laboratorio, Rosario, Argentina). The reaction mixture was kept at room temperature for 20 min and the absorbance was then measured at 505 nm. Blank tubes were run in parallel to account for endogenous glucose in tissues. These tubes contained all the same solutions, but the substrate solution was added after the arresting reagent to prevent the reaction from occurring. Enzyme activity was determined using a glucose standard curve.

We assayed aminopeptidase-N (EC 3.4.11.2) activity using L-alanine-*p*-nitroanilide as a substrate following previously established techniques (Maroux et al., 1973; Roncari and Zuber, 1969). We started the reaction by adding aliquots of 10 µl of tissue homogenate to 1 ml of assay solution containing 2.0 mmol l^{-1} L-alanine-*p*-nitroanilide in 0.2 mol l^{-1} phosphate buffer (NaH_2PO_4: Na_2HPO_4, pH 7). The reaction was incubated for 20 min at 30°C and then arrested with 3 ml of chilled 2 mol l^{-1} acetic acid. The absorbance was measured at 384 nm, and activity was determined using a *p*-nitroanilide standard curve. Similar to the disaccharidase assays, blank tubes were run in parallel where substrate was not added until after the arresting agent.

Enzyme activity is expressed as µmol min^{-1} g^{-1} tissue, normalized to measured wet tissue mass. Calculated enzymatic activities per gram tissue represent an estimation of the mean hydrolysis capacity of the entire intestine. We also calculated the summed hydrolysis activity of the entire small intestine, an index of the total hydrolysis capacity, by multiplying activity per gram of tissue by mass of the entire small intestine.

Statistics of physiological measurements

We compared body mass over the course of the trial with a repeated measures ANOVA using the start and end body mass, and by calculating the percentage change in body mass as (start body mass −end body mass)/start body mass, and using a *t*-test to compare this with a value of zero. Performance parameters (body mass, food intake, digestibility, nitrogen balance, etc.) were compared between the two diet treatments using Student's *t*-tests.

Gut morphological measurements were compared with ANCOVA with diet as a main effect and snout–vent length (SVL) as a covariate. We used SVL as a covariate rather than body mass so that we could compare our results with those of the interspecific study conducted by O'Grady et al. (2005). We also calculated the surface area:volume ratios for the small intestine and hindgut, and compared the effect of diet using a Student's *t*-test. Intestinal surface magnification factors were compared using Student's *t*-test, and we also conducted an ANCOVA with villus perimeter as a dependent variable, diet as a main effect and inner muscle perimeter as a covariate. Mass-specific enzyme activities were compared using Student's *t*-tests, and summed digestive enzyme activities were compared using ANCOVA with SVL as a covariate.

Microbial inventories

We extracted total DNA from feces, gut contents and three samples of each diet using a MoBio PowerFecal DNA isolation kit. We also conducted nine 'blank' extractions to correct for contaminants found in DNA extraction kits (Salter et al., 2014). Extracted DNA was sent to Argonne National Laboratory (US Department of Energy, Chicago, IL, USA) for amplification of the V4 region of the 16S rRNA gene with primers *515F* and *806R* (Caporaso et al., 2012). The reverse primer also contained a 12 base barcode sequence, which allowed pooling of samples. PCR reactions were conducted in triplicate and the resulting products were pooled within a single sample. DNA was quantified using PicoGreen (Invitrogen) and a plate reader and cleaned using the UltraClean PCR Clean-Up Kit (MoBIO). Amplicons were sequenced on the Illumina MiSeq platform.

Microbial sequences were analyzed using QIIME v1.9.1 (Caporaso et al., 2010b). We applied standard quality control settings and split sequences into libraries using default parameters in QIIME. We grouped sequences into operational taxonomic units (OTUs) using an open reference method and a minimum sequence identity of 97% (He et al., 2015). The most abundant sequences within each OTU were designated as a 'representative sequence' and aligned against the Greengenes core set (DeSantis et al., 2006) using PyNAST (Caporaso et al., 2010a) with default parameters set by

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QIIME. A PH Lane mask supplied by QIIME was used to remove hypervariable regions from aligned sequences. A phylogenetic tree of representative sequences was built using FastTree (Price et al., 2009). OTUs were classified using UCLUST (Edgar, 2010). Singleton OTUs and sequences identified as chloroplasts or mitochondria were removed from the analysis. Additionally, any OTUs present in the 'blank samples' were considered contaminants and were removed from all other samples (Salter et al., 2014). We used SourceTracker (Knights et al., 2011) to compare the proportion of the communities that were composed of allochthonous, or transient, microbes present in the diet.

We compared several aspects of gut microbial community diversity and structure. We calculated Faith's phylogenetic diversity (Faith, 1992), which measures the cumulative branch lengths from randomly sampling OTUs from each sample. For each sample, we calculated the mean of 20 iterations for a sub-sampling of 1000 sequences, a depth that is sufficient for capturing a majority of the community diversity (Caporaso et al., 2012). Three small intestinal samples from lizards fed the mixed diet did not have 1000 sequences, and so were not included in this or further analyses described below. Faith's phylogenetic diversity was compared using a mixed effects model including diet, gut region (here we consider feces to be a 'gut region') and individual as factors. We also compared diversity within each gut region using Student's *t*-test.

Community membership and structure were compared by conducting principal coordinates analysis (PCoA) on unweighted and weighted UniFrac distances (Lozupone and Knight, 2005). The effect of diet on community membership or structure was compared within each gut region using the ANOSIM function within QIIME with 999 permutations (Clarke, 1993). Additionally, we compared UniFrac distances across gut regions to investigate which gut region exhibited the largest changes in community membership or structure. Distances were calculated by taking each sample from the plant-fed lizards, calculating the distance to samples collected from the same gut region of mixed diet-fed lizards, and averaging these distances within samples from plant-fed lizards to generate one measurement per sample.

Last, we investigated the effects of diet on the relative abundance of microbial taxa. Relative abundances were transformed using a variance stabilizing transformation of $\arcsin(\text{abundance}^{0.5})$ (Kumar et al., 2012; Shchipkova et al., 2010). We used JMP 12.0 to compare relative abundances of bacterial phyla and genera using the Response Screening function with the robust fit option to conduct multiple *t*-tests and correct *P*-values with the false discovery rate (FDR) correction. We then searched for abundances of microbial genera that were correlated with fiber digestibility of the host. We conducted this analysis only in the plant-fed group, and only used NDF digestibility as NDF digestibility and ADF digestibility were highly correlated (R^2=0.96, P<0.0001). Again, we used the Response Screening function in JMP 12.0 to conduct multiple regressions and correct *P*-values with the FDR correction.

The 16S rRNA sequences have been deposited in the Sequence Read Archive.

RESULTS

Body mass, digestibility and nitrogen balance

The average body mass of lizards in our experiment was 3.15±0.18 g. Body mass remained unchanged over the course of the experiment (repeated measures ANOVA using the start and end body mass; time effect: P=0.88; diet effect: P=0.77; interaction: P=0.73). Additionally, the percentage change in body mass was not significantly different from zero (P>0.6 for both diets).

Table 2. Performance parameters of lizards fed a plant-rich or mixed diet

	Plant-rich diet	Mixed diet	*P*
End body mass (g)	3.08±0.35	3.22±0.25	0.76
Food intake (mg day^{-1})	14.55±1.18	14.30±1.14	0.88
Fecal output (mg day^{-1})	5.32±0.46	3.73±0.46	**0.028**
Urine pellet output (mg day^{-1})	1.21±0.07	1.50±0.18	0.15
Dry matter digestibility	0.63±0.01	0.74±0.01	**<0.0001**
Digestibility of NDF	0.22±0.05	0.24±0.05	0.80
Digestibility of ADF	0.15±0.05	0.13±0.04	0.82
N content of feces (%)	1.93±0.21	2.61±0.31	0.08
N content of urine pellets (%)	25.50±0.27	26.74±0.21	**0.003**
Apparent N digestibility	0.78±0.02	0.85±0.02	0.056

NDF, neutral detergent fiber; ADF, acid detergent fiber. Values represent means±s.e.m. N=8 for the plant-rich diet and N=9 for the mixed diet. *P*-values correspond to Student's *t*-tests between the two groups; significant values are in bold.

Food intake did not differ between the two groups (Table 2). Lizards fed the herbivorous diet produced a greater amount of feces, which translated into lower dry matter digestibility in this group (Table 2). Fiber digestibility of both NDF and ADF did not differ between the two groups (Table 2).

Lizards fed the plant-rich diet produced feces and urine pellets with lower concentrations of nitrogen compared with those on the mixed diet (Table 2). Additionally, when accounting only for fecal nitrogen, lizards fed the plant-rich diet exhibited a lower apparent nitrogen digestibility (Table 2). After accounting for urinary nitrogen losses, most animals were still in positive nitrogen balance (Fig. 1). While animals fed the mixed diet consumed more nitrogen (Fig. 1; P=0.002), there was no significant difference in nitrogen balance between the two diet treatments (Fig. 1; P=0.14). There was one lizard in the plant-fed group that was largely in negative nitrogen balance (Fig. 1). This lizard was a small animal (1.95 g) and had the greatest urine pellet output and second highest urine nitrogen content of the animals in the plant-fed group. Though it was not an outlier in these measurements by any means, these factors combined resulted in a largely negative nitrogen balance.

We estimated the minimum nitrogen requirements of lizards using only data from the plant-fed animals (excluding the one animal that was largely in negative nitrogen balance). There was a significant correlation between nitrogen intake and nitrogen balance

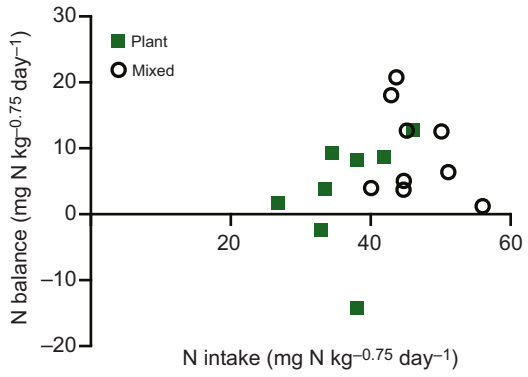

Fig. 1. Relationship between nitrogen intake and nitrogen balance in lizards fed a plant-rich diet or a mixed diet of plant material and insects. N=8 for the plant-rich diet and N=9 for the mixed diet.

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(R^2=0.61, P=0.038). The minimum nitrogen requirement was estimated at 26.75 mg N $kg^{-0.75}$ day^{-1}.

Gut morphology

The mass of stomach, small intestine or hindgut tissues did not significantly differ between diet treatments (ANCOVA: diet effect: P>0.15 for all organs, SVL covariate: P<0.001 for all; interactions: P>0.2 for all). Lizards fed the plant-rich diet had small intestines that were roughly 17% longer than those fed the mixed diet (P=0.013), though there were no differences in small intestine surface area or volume (Table 3, Fig. 2). Lizards fed the plant-rich diet tended to have small intestines with a greater surface area:volume ratios (Fig. 2; P=0.065), and these surface area:volume ratios significantly correlated with apparent nitrogen digestibility (Fig. 3; diet effect: P=0.001, surface area:volume ratio effect: P=0.004, interaction: P=0.77). Additionally, lizards fed the plant-rich diet had 38% greater hindgut surface area and 63% greater hindgut volume compared with lizards fed the mixed diet (Table 3, Fig. 2). These increases in hindgut surface area and volume were more pronounced in larger lizards, given significant or near-significant interaction terms between diet and SVL (Table 3). The surface area:volume ratio of the hindgut chamber tended to be smaller in lizards fed the plant-rich diet (Fig. 2; P=0.078). Importantly, all differences (longer small intestines, larger hindgut surface area and volume) remained statistically significant even when the the largest lizard fed the plant-rich diet was removed from the analysis. The intestinal surface magnification factors did not differ between diet treatments (P=0.1), with the average being 3.17±0.16. Similarly, we observed no effect of diet on the villus perimeter of the intestine (ANCOVA: diet effect: P=0.50, inner muscle layer perimeter covariate: P=0.055, interaction: P=0.73).

Digestive enzyme activities (normalized to wet mass or summed activity) did not differ between the two diet treatments (P>0.05 for all comparisons). Mean±s.e.m. enzyme activity was as follows: maltase: 34.55±2.68 μmol min^{-1} g^{-1} tissue, sucrase: 2.69±0.18 μmol min^{-1} g^{-1} tissue, aminopeptidase-N: 2.13±0.30 μmol min^{-1} g^{-1} tissue.

Table 3. Details of ANCOVA of various measurements of the gastrointestinal tract

	F	d.f.	P
Small intestine length			
Diet	8.26	1,14	**0.013**
SVL	23.86	1,14	**0.0003**
Diet×SVL	0.40	1,14	0.54
Small intestine surface area			
Diet	1.53	1,14	0.24
SVL	33.69	1,14	**<0.0001**
Diet×SVL	0.94	1,14	0.35
Small intestine volume			
Diet	0.10	1,14	0.76
SVL	10.51	1,14	**0.006**
Diet×SVL	0.68	1,14	0.42
Hindgut length			
Diet	2.81	1,14	0.12
SVL	8.50	1,14	**0.012**
Diet×SVL	0.25	1,14	0.63
Hindgut surface area			
Diet	39.06	1,14	**<0.0001**
SVL	65.48	1,14	**<0.0001**
Diet×SVL	4.21	1,14	0.06
Hindgut volume			
Diet	27.67	1,14	**0.0002**
SVL	34.21	1,14	**<0.0001**
Diet×SVL	5.08	1,14	**0.04**

SVL, snout–vent length. Significant P-values are in bold.

Microbial inventories

After quality control, we obtained 471,978 high-quality 16S rRNA sequences that were used in subsequent analyses. The number of sequences differed significantly across gut regions (mixed effects model: gut region effect: $F_{3,43.6}$=30.20, P<0.001), but did not differ between diets (diet effect: $F_{1,14.2}$=0.34, P=0.57; gut region×diet effect: $F_{3,43.6}$=1.57, P=0.21). The mean±s.e.m. number of sequences per sample for each gut region and for feces was as

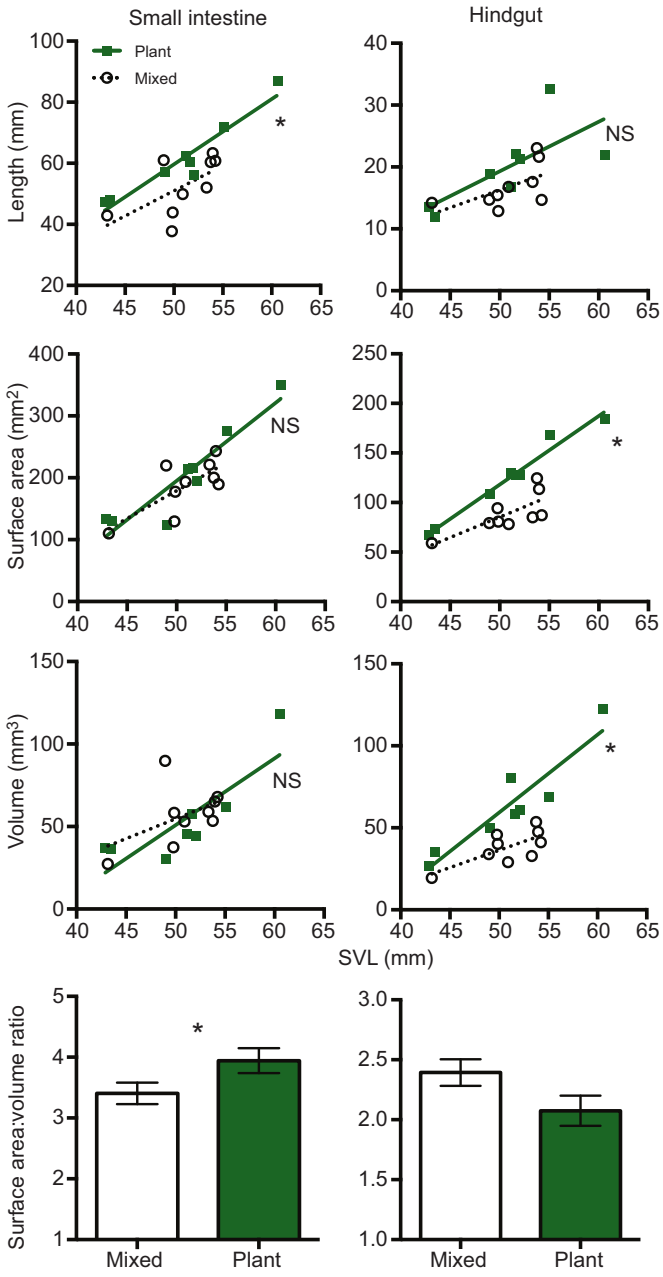

Fig. 2. Effect of diet quality on various morphological measurements of the gastrointestinal tract. SVL, snout–vent length. Asterisks in the panel indicate a significant effect of diet; NS, not significant. N=8 for the plant-rich diet and N=9 for the mixed diet. Statistics for length, surface area and volume can be found in Table 3. Statistics regarding surface area:volume ratios can be found in the Results.

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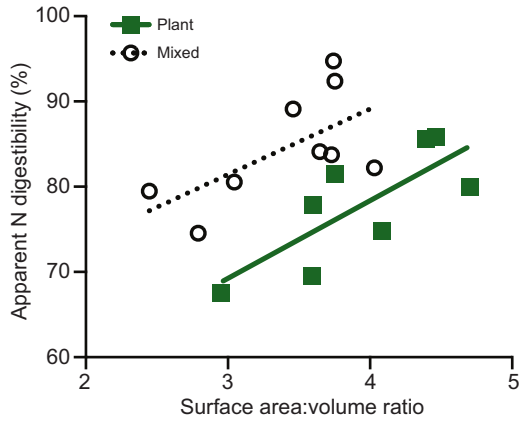

Fig. 3. Relationship between small intestinal surface area:volume ratio and apparent nitrogen digestibility. *N*=8 for the plant-rich diet and *N*=9 for the mixed diet.

follows: stomach 4903±458; small intestine 2361±703; hindgut 11,231±884; feces 9406±920.

The results presented below are unlikely to be driven by allochthonous, or transient, microbes present in the diets. First, both diets were mixtures of rabbit chow and ground mealworms, and thus any dietary microbes would be present in both diets. When comparing the microbial communities of the two diets, we only detected a single microbial genus that was present in differential abundances. The plant-rich diet contained a higher relative abundance of *Agrobacterium* (0.103±0.026%) when compared with the mixed diet (0.012±0.012%; FDR-corrected P=0.008). Using SourceTracker, we found that the percentage of communities that may have come from dietary sources varied across gut regions (Fig. S1; mixed effects model: gut region effect: $F_{3,44.2}$=8.71, P=0.0001), such that the stomach and small intestinal communities were composed of roughly 20% allochthonous dietary microbes, while the hindgut and feces only contained ∼2% transient microbes (those also present in food samples). However, the contribution of transient microbes to gut communities did not differ between the diet treatments (Fig. S1; diet effect: $F_{1,15.4}$=0.04, P=0.85; gut region×diet effect: $F_{3,44.2}$=0.14, P=0.94).

Feeding on a plant-rich diet significantly altered the gut microbial communities of lizards. Microbial diversity as measured by Faith's phylogenetic diversity varied significantly across gut regions, and as a result of diet (Fig. 4; mixed effects model: gut region effect: $F_{3,40.9}$=44.25, P<0.0001; diet effect: $F_{1,12.9}$=8.47, P=0.01; gut region×diet effect: $F_{3,40.9}$=1.47, P=0.24). Within gut regions, lizards fed the plant-rich diet harbored significantly higher phylogenetic diversity in the small intestine (*t*-test; P=0.02) and feces (P=0.05), but not in the stomach or hindgut (P>0.5 for both). Diet significantly influenced the community membership (presence and absence of microbial lineages) of the small intestine (Fig. 5A; ANOSIM: P=0.008), hindgut (P=0.049) and feces (P=0.004), but not the stomach (P=0.52). Diet did not significantly alter the community structure (as measured by weighted UniFrac distance metrics, which takes relative abundance into account) in any gut region (ANOSIM: P>0.15 for all). We compared unweighted distances within gut regions and feces between diets (for example, the distance metrics from plant-fed lizard small intestinal samples to mixed diet-fed small intestinal samples). Larger distances are indicative of greater changes in community membership. Unweighted distances varied significantly across gut regions ($F_{3,21}$=62.48; P<0.0001), and were significantly higher in the small intestine (Fig. 5B).

A number of microbial phyla and genera were present in differential abundances as a result of diet, with the small intestine exhibiting the greatest number of taxa that were differentially abundant (Table 4). We also observed a number of significant

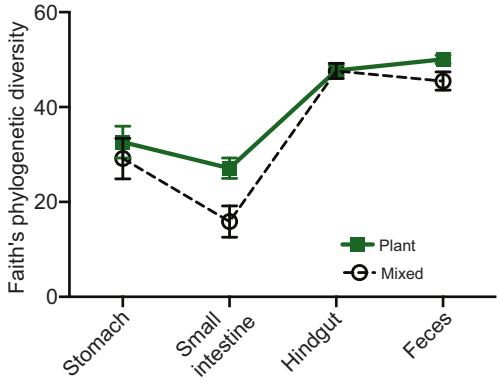

Fig. 4. Microbial diversity across gut regions and in feces for lizards fed a plant-rich diet or a mixed diet of plant material and insects. Data are means±s.e.m. *N*=8 for the plant-rich diet and *N*=9 for the mixed diet.

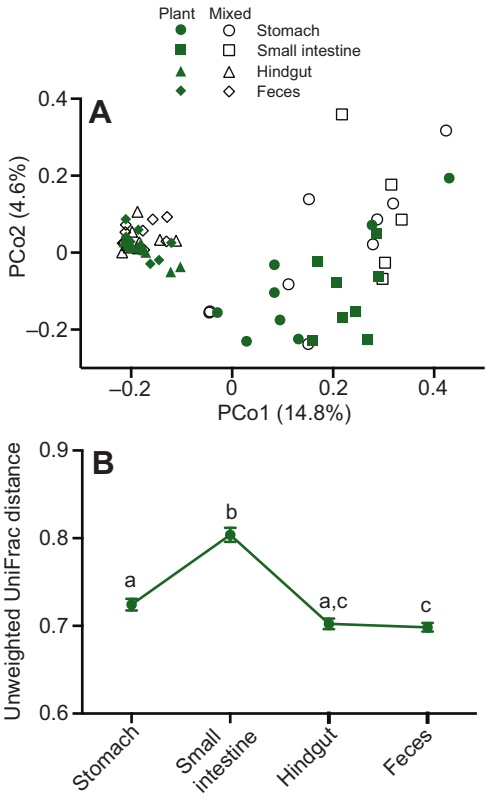

Fig. 5. Community membership of the microbiota of lizards fed a plant-rich diet or a mixed diet of plant material and insects. (A) Principal coordinate analysis of community membership and (B) unweighted UniFrac distances, both of which only investigate the presence or absence of microbial lineages. Distances were calculated by taking each sample from the plant-fed lizards, calculating the distance to samples collected from the same gut region (or feces) of lizards fed a mixed diet, averaging these distances within plant-fed samples to generate one measurement, and then averaging within each gut region (or feces). Points represent means±s.e.m. Points that do not share letters are significantly different (Tukey's HSD test). *N*=8 for the plant-rich diet and *N*=9 for the mixed diet.

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relationships between the abundance of microbial genera and host performance in the digestibility of NDF (Table 5). Most notably, individuals that had a higher abundance of *Desulfovibrio* in the small intestine and *Oscillospira* in the hindgut and feces exhibited higher fiber digestibility.

DISCUSSION

Ecologists have attempted for decades to explain the paucity of herbivorous lizards compared with other vertebrate classes, with physiological and microbial limitations being often-cited reasons (Cooper and Vitt, 2002; King, 1996; Pough, 1973; Sokol, 1967; Szarski, 1962). However, the gut and the resident microbiota are highly dynamic and can undergo flexible adjustments to meet the demands of organisms (Karasov et al., 2011). Here, we found that an omnivorous lizard that consumes an average of 16% plant material in the wild (Villavicencio et al., 2005) was able to maintain nitrogen balance and change its gut morphology and microbial community structure when fed a plant-rich diet of up to 90% plant material. Our results suggest that physiological and microbial limitations do not strictly constrain the evolution of herbivory from omnivorous ancestors. Thus, we propose that ecological contexts are potentially more important than physiological limitations for the evolution of herbivory. However, it is also worth noting that reproduction is an extremely energetically expensive life history stage, and that our diet trials were conducted on non-reproducing lizards with no foraging costs. Therefore, the physiological capacities demonstrated in our work may not be relevant for lizards under different energetic states.

Individuals of the omnivorous species *L. ruibali* exhibited digestive performance on a par with that of small, herbivorous lizards in previous studies. In our study, lizards fed the plant-rich diet exhibited a dry matter digestibility of 63%. This is similar to measurements of dry matter digestibility in two species of herbivorous lizards on high-fiber diets: 47–66% in the chuckwalla (*Sauromalus obesus*; Karasov et al., 1986; Ruppert, 1980) and 45–66% in the desert iguana (*Dipsosaurus dorsalis*; Harlow et al., 1976; Karasov et al., 1986). Further, *L. ruibali* digested 24% of total fiber, with no differences between diet treatments. This value is consistent with measurements taken in chuckwallas, which digest 21% of total fiber (Karasov et al., 1986). Some large-bodied lizards are more efficient at fiber digestion and are able to digest 46–76% of total fiber (Durtsche, 2004; Troyer, 1984), though this is likely due to a longer retention time in the gut that allows for more microbial fermentation.

Additionally, individuals of *L. ruibali* were able to maintain nitrogen balance when fed on the herbivorous diet. The rabbit chow used in our experiment was roughly 3% nitrogen, which is within the range of nitrogen content of plants that herbivorous lizards feed on in the wild (Dearing and Schall, 1992; Nagy and Shoemaker, 1975). Even on this low-nitrogen diet, *L. ruibali* was able to maintain body mass and nitrogen balance over the course of the trial. The estimated nitrogen requirement of our animals (26.8 mg N $kg^{-0.75}$ day^{-1}) is relatively low compared with that of other herbivorous lizards (109 mg N $kg^{-0.75}$ day^{-1} in *Sauromalus obesus*; Nagy, 1975), though similar to that of herbivorous desert tortoises (14.4 mg N $kg^{-0.75}$ day^{-1} in *Gopherus agassizii* Barboza, 1995).

The gastrointestinal tracts of *L. ruibali* were phenotypically flexible in response to diet. Lizards fed the plant-rich diet had longer small intestines and larger hindguts. The increase in small intestine length resulted in a higher surface area:volume ratio for this tissue. Contact between food material and the gut lining is important for digestion by brush-border enzymes and absorption of nutrients (Karasov and Martínez del Rio, 2007). Lengthening of the small intestine with an increase in surface area:volume ratio would result in increased interaction between digesta and the gut lining, potentially increasing the digestion and assimilation of nutrients (Lassuy, 1984). Indeed, lizards with larger small intestinal surface area:volume ratios exhibited higher apparent nitrogen digestibility. The larger volume of the hindgut is likely important for holding material longer to allow for microbial fermentation of fiber (Stevens and Hume, 2004). It could be argued that the changes in gut morphology do not represent physiological adjustments, but rather are a result of the mechanical forces of fibrous food material moving through the gut. However, an intriguing study in quail suggests otherwise; here, researchers maintained birds on a low-fiber diet, a nutrient-poor high-fiber diet or an energy-enriched high-fiber diet. They found that while food intake did not differ across these groups, only the animals fed the nutrient-poor high-fiber diet had larger gut morphology and higher fiber digestibility (Williamson et al., 2014). Their results suggest that energy dilution may be more important in the reshaping of gut anatomy than simply the mechanical or

Table 4. Relative abundance of bacterial phyla and genera that exhibited significant differences between diet groups

	Relative abundance (%)		
Bacterial taxa	Plant diet	Mixed diet	*P*
Small intestine			
Phylum Melainabacteria	0.21±0.08	ND	0.007
Phylum Proteobacteria	13.7±3.1	23.9±2.2	0.007
Genus *Bacillus*	1.62±0.55	0.05±0.03	0.006
Genus *Lactobacillus*	0.18±0.06	ND	<0.001
Genus *Oscillospira*	1.08±0.29	0.12±0.09	0.003
Genus *Pediococcus*	0.27±0.07	0.03±0.02	0.023
Hindgut			
Genus *Lactobacillus*	0.04±0.01	0.002±0.001	<0.001
Feces			
Genus *Helicobacter*	0.03±0.01	0.33±0.09	0.041
Genus *Leuconostoc*	0.06±0.02	0.01±0.006	0.007

Data (means±s.e.m.) are separated into gut regions and feces. No significant differences were observed in the stomach. Statistics were conducted on transformed abundances. *P*-values are false discovery rate (FDR) corrected.

Table 5. Microbial genera that correlate with whole-animal digestibility of NDF in lizards fed the plant-rich diet

Microbial genera	Direction	R^2	*P*
Stomach			
Anaerotruncus	Negative	0.41	0.007
Bacteroides	Negative	0.31	0.007
Coprococcus	Positive	0.37	0.026
Desulfovibrio	Negative	0.53	0.0005
Sporanaerobacter	Positive	0.72	0.012
Staphylococcus	Negative	0.37	<0.0001
Small intestine			
Desulfovibrio	Positive	0.82	<0.0001
Serratia	Negative	0.54	0.0007
Hindgut			
Coprobacillus	Positive	0.72	<0.0001
Lactobacillus	Positive	0.17	0.005
Lawsonia	Negative	0.32	0.022
Oscillospira	Positive	0.65	0.003
Feces			
Coprobacillus	Positive	0.83	<0.0001
Oscillospira	Positive	0.56	0.028

Statistics were conducted on transformed abundances. *P*-values are FDR corrected.

physical effects of high fiber diets (Williamson et al., 2014). Thus, we hypothesize that the changes in gut morphology observed in our lizards truly represent physiological responses and phenotypic flexibility.

Our results regarding changes in gut morphology are consistent, to some extent, with a comparative study across 22 species in Liolaemidae, which demonstrated that herbivorous species have small intestines that are 70% longer than those of omnivores (O'Grady et al., 2005). However, our results do not fully explain the magnitude of the differences exhibited across feeding groups in that study (O'Grady et al., 2005), given that the plant-rich diet only lengthened the small intestine by 17% compared with that of lizards fed the mixed diet. Similarly, though some evidence suggests that herbivorous lizards have higher enzymatic digestive capacity (Brigada et al., 2004), the diet treatments in our experiment did not result in differences in enzyme activity. Thus, we conclude that some aspects of lizard digestive physiology may be flexible enough to allow for herbivory, and that natural selection may act further to exaggerate these differences over evolutionary time. It would be interesting to compare the digestive performance of naturally insectivorous, omnivorous and herbivorous lizards when all are fed high-fiber diets.

The gut microbial communities of *L. ruibali* were also responsive to changes in diet quality. Lizards fed the plant-rich diet harbored more diverse small intestinal and fecal communities. Additionally, the small intestinal, hindgut and fecal communities of plant-fed lizards all differed in community membership from the microbiota of lizards fed the mixed diet. These changes in community membership are likely driven by differential detection of microbes that are truly present in the guts of both groups. For example, some fiber-degrading microbes may be present in the guts of lizards fed the mixed diet, but only increase in abundance when lizards are feeding on high-fiber diets. Harboring these fiber-degrading microbes, even at low levels, may enhance the capacity for lizards to switch to high-fiber diets temporarily, especially given that the gut microbiota can rapidly respond to large dietary changes (David et al., 2014). Overall, our results are consistent with a comparative study demonstrating that mammalian herbivores maintain distinct and more diverse microbiota than omnivores and carnivores (Ley et al., 2008).

Diet induced a number of changes in the relative abundance of microbial taxa. The small intestine of lizards fed the plant-rich diet harbored a higher abundance of the candidate phylum Melainabacteria, which are obligate anaerobic fermenters based on genome reconstruction (Di Rienzi et al., 2013). Additionally, the small intestine and hindgut of lizards fed the plant-rich diet exhibited a higher relative abundance of *Lactobacillus*. While *Lactobacillus* is not considered a highly cellulolytic microbial genus, members of this genus may be digesting simple sugars in the lizard gut. Last, we observed a higher abundance of *Oscillospira* in the small intestine of lizards fed the plant-rich diet. While the functions of the genus *Oscillospira* are unknown, it is likely that it plays a role in fiber fermentation given its presence in the in the guts of many ruminants and other herbivores (Kohl et al., 2014; Mackie et al., 2003). Additionally, ruminants fed fresh forage exhibit a higher abundance of *Oscillospira* compared with those fed low-fiber grain, and microscopy reveals that these bacteria associate with the surfaces of plant material in the gut (Mackie et al., 2003). From our study, it is unclear whether these changes are driven by the increase in fiber content of the diet or perhaps by a decrease in protein content.

Our analyses also highlighted several microbial genera that may be important for fiber digestion in lizards. Individuals that exhibited a higher abundance of *Oscillospira* in the hindgut and feces displayed higher fiber digestibility, consistent with the notion that this genus may play a role in fiber degradation (Mackie et al., 2003). Additionally, individuals that harbored a higher abundance of *Desulfovibrio* in the small intestine exhibited higher fiber digestibility. This genus is known to reduce sulfate (Huisingh et al., 1974), an important process in reducing the H_2 byproducts associated with anaerobic fermentation (Morvan et al., 1996a; Rey et al., 2013). Further, the presence of sulfate-reducing bacteria enhances the fermentation performance of some cellulolytic bacteria *in vitro* (Morvan et al., 1996b). Interestingly, sulfate-reducing bacteria are present in the guts of herbivorous marine iguanas (*Amblyrhynchus cristatus*), and are thought to explain the lack of methanogenic archaea in these animals (Hong et al., 2011). We did not investigate the presence of methanogenic archaea in our experiment. The importance of sulfur-reducing bacteria and other hydrogenotrophic microbes in the gut of herbivorous reptiles remains unclear and demands further attention.

Overall, it seems that physiological and microbial limitations do not stringently constrain feeding on a plant-rich diet in the omnivorous lizard *L. ruibali*. Thus, we hypothesize that ecological context and the likely fitness benefits of feeding on energy-dense insects may be more critical in constraining the evolution of herbivory in Liolaemid lizards. However, it should be recognized that *L. ruibali* falls within a family of lizards that has repeatedly evolved herbivory (Espinoza et al., 2004). The capacity for phenotypic flexibility in the gut can have a strong phylogenetic component (Karasov et al., 2011). It could be that the family Liolaemidae has an inherent capacity for phenotypic flexibility, which allowed the evolution of herbivory. Indeed, it has been argued that studies relating to flexibility of the digestive system in lizards have been biased towards more flexible species (Vervust et al., 2010). It would be interesting to investigate physiological limits and phenotypic flexibility in other groups of lizards that have not evolved herbivory. Together, these studies will elucidate the ultimate factors constraining herbivory in lizards.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
K.D.K. conceived the study, coordinated the study and wrote the manuscript. A.B., M.M. and J.B. assisted with data collection and revision of the manuscript. A.L. and J.C.A. were instrumental to obtaining lizards and assisted with manuscript revisions. S.R.B. and E.C.-V. oversaw the study and data analysis, and revised the manuscript. All authors gave final approval for publication.

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Data availability
All 16S rRNA sequences have been deposited in the Sequence Read Archive (SRA): accession number PRJNA293117.

Supplementary information
Supplementary information available online at
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