Universidad Autónoma de Tlaxcala

Postgraduate in Biological Sciences

Ecological and Evolutionary Factors that Modulate the Intestinal Bacterial Communities in Different Lizard Species of the Genus *Sceloporus*

Doctoral Thesis

TO OBTAIN THE DEGREE OF

DOCTOR IN BIOLOGICAL SCIENCES

By

Delmer Mauricio Hernández Espinal

Director:
Yendi Ebenezer Navarro Noya, Ph.D.

Co-director:
Sergio Iván Ancona Martínez, Ph.D.

Tlaxcala, Tlax. August, 2023

Universidad Autónoma de Tlaxcala

Postgraduate in Biological Sciences

Ecological and Evolutionary Factors that Modulate the Intestinal Bacterial Communities in Different Lizard Species of the Genus *Sceloporus*

Doctoral Thesis

TO OBTAIN THE DEGREE OF

DOCTOR IN BIOLOGICAL SCIENCES

By

Delmer Mauricio Hernández Espinal

Committee Members:

Yendi Ebenezer Navarro Noya, Ph.D.
Sergio Iván Ancona Martínez, Ph.D.
Aníbal Helios Díaz de la Vega-Pérez, Ph.D.
Arturo Estrada Torres, Ph.D.
Sean Rovito, Ph.D.

Tlaxcala, Tlax. August, 2023


UNIVERSIDAD AUTÓNOMA DE TLAXCALA
POR LA CULTURA Y LA JUSTICIA SOCIAL
POSGRADO EN CIENCIAS BIOLÓGICAS
CTBC
Centro Tlaxcala de Biología de la Conducta
estación científica la malinche.
CONAHCYT
CONSEJO NACIONAL DE HUMANIDADES CIENCIAS Y TECNOLOGÍAS
INSTITUTO DE ECOLOGIA
UNAM

1. Preface

The work was carried out at the Centro Tlaxcala de Biología de la Conducta (CTBC), Universidad Autónoma de Tlaxcala, Mexico, under the supervision of Dr. Yendi Ebenezer Navarro Noya and Dr. Sergio Iván Ancona Martínez. This doctoral project was funded by the National Council of Humanities, Science and Technology (CONAHCyT), under the scholarship number: 967648. The thesis was also supported by Ciencia de Frontera project number: 137748, Infraestructura project number: 205945 and Cátedras CONACyT project number: 883.

I hereby declare that this thesis represents my own work which has been done following the research ethics guidelines for scientific research and proper animal handling in Mexico.

Signature: 

Date: August 2023

Asunto: Análisis de similitud de tesis

COMITÉ ACADÉMICO

POSGRADO EN CIENCIAS BIOLÓGICAS

Sirva este medio para describir el proceso de revisión de la tesis realizada por el estudiante **Delmer Mauricio Hernández Espinal** titulada "**Ecological and Evolutionary Factors that Modulate the Intestinal Bacterial Communities in Different Lizard Species of the Genus *Sceloporus***" para obtener su grado de **Doctor en Ciencias Biológicas**.

La tesis de **Delmer Mauricio Hernández Espinal** fue revisada por todos los miembros del comité tutoral y por los miembros del comité de examen de grado. La versión final del documento de tesis se sometió a un análisis de similitud en el programa Turnitin, LLC el día 10 de agosto de 2023. Se analizaron un total de 7,551 palabras y 44,566 caracteres, excluyéndose la portada y la sección de Bibliografía, y se encontró una similitud general del 16%. Todas las coincidencias encontradas no representaron >2% del total del texto y en la inspección manual de las coincidencias se encontró que la mayoría era por "Fraseología de uso común" o menciones debidamente referenciadas.

Por lo anterior, confirmamos que **el estudiante no incurrió en ninguna práctica no deseable** en la escritura de la tesis.

Sin más por el momento, reciban atentos saludos.

A T E N T A M E N T E
"POR LA CULTURA A LA JUSTICIA SOCIAL"
TLAXCALA DE XICOTÉNCATL A 10 DE AGOSTO DE 2023

DRA. YENDI E. NAVARRO NOYA
DIRECTORA DE TESIS

COORDINACIÓN DOCTORADO EN CIENCIAS BIOLÓGICAS
UNIVERSIDAD AUTÓNOMA DE TLAXCALA
P R E S E N T E

Los abajo firmantes, miembros del jurado evaluador del proyecto de tesis que **Delmer Mauricio Hernández Espinal** realiza para la obtención del grado de **Doctor en Ciencias Biológicas**, expresamos que, habiendo revisado la versión final del documento de tesis, damos la aprobación para que ésta sea impresa y defendida en el examen correspondiente. El título que llevará es **"Factores ecológicos y evolutivos que modulan las comunidades bacterianas del intestino en diferentes especies de lagartijas del género *Sceloporus*".**

Sin otro particular, aprovechamos para enviarle un cordial saludo.

A T E N T A M E N T E
TLAXCALA, TLAX., A 10 DE AGOSTO DEL 2023

DR. ARTURO ESTRADA TORRES

DR. ANÍBAL HELIOS DÍAZ DE LA VEGA PÉREZ

DR. JORGE ISRAEL AYALA BERDÓN

DRA. STEPHANIE ELIZABETH HEREIRA PACHECO

DRA. ELIZABETH SELENE GÓMEZ ACATA

2. Acknowledgments

I would first like to thank the CTBC and La Malinche Scientific Station for infrastructure, logistical and administrative support, and CONAHCyT for my Ph.D. scholarship number 967648.

I would like to express my greatest appreciation to my two supervisors, Dr. Yendi Navarro and Dr. Sergio Ancona, for introducing me to this fantastic and complex research area of microbial ecology. Their support, counsel, guidance and encouragement were invaluable, since the completion of this project would not have been possible without them.

Thanks to all committee members, including my two supervisors, as well as Dr. Aníbal Díaz de la Vega-Pérez, Dr. Arturo Estrada and Dr. Sean Rovito for their constructive comments and suggestions which helped me to improve the clarity of this work. I learned a lot from their experience as researchers.

I am enormously grateful to Dr. Stephanie Hereira, who provided me vital guidance to acquire new knowledge into the bioinformatics and statistical analysis, thanks for the opportunity to learn from you. Thank you also to Dr. Ligia Muñoz, Dr. Alejandra Miranda and BS. Ana Lilia Toriz for their great help and expertise regarding laboratory analysis. My sincere gratitude to Dr. Luc Dendooven and Dr. Antton Alberdi for their valuable comments on the manuscripts.

I am thankful to Erick Gómez, Dr. Miguel Domínguez-Godoy, Raúl López, Aramis Meraz and Laura Del Sampedro for their support during fieldwork.

Lastly, I would like to thank all of my family members, particularly to my mother for her consistent motivation during my Ph.D. journey. Your support gave me the strength to keep going, even in the worst of times.

Dedication

In Memory of my Father and Sister, May they Rest in Peace

3. Abstract

The gut microbiota comprises a complex microbial community influenced by a diverse range of extrinsic and intrinsic factors. In vertebrate hosts, the major richness and abundance of microbial taxa resides in the gastrointestinal tract (GIT), which exerts a central role in nutrient absorption, intestinal homeostasis, immune system, protection against pathogens, hormone and vitamin production, among others. However, to date, most microbial studies have been performed on human and laboratory animals and little is known about the gut microbiota composition in wild populations, particularly in reptilian species, one of the most diverse and successful groups of vertebrates. Therefore, understanding how the gut microbiota contributes to host fitness is a major goal of microbial research. Moreover, to avoid lethal procedures in wild populations is necessary to estimate which non-lethal method (fecal samples or cloacal swabs) is more accurate to assess GIT microbiota. Here, through next-generation sequencing technology, we examined the variation of gut bacterial communities among four lizard species of the genus *Sceloporus* (*S. aeneus*, *S. bicanthalis*, *S. grammicus* and *S. spinosus*) inhabiting a mountainous region in central Mexico (La Malinche volcano). The bacterial 16S rRNA gene was PCR-amplified to assess the gut bacterial communities and investigate how environmental conditions, phylogenetic relatedness, seasonal dynamics and diet composition influence their diversity and composition. Furthermore, by amplifying the mitochondrial cytochrome c oxidase subunit I (COI) gene, we characterized the diet of these *Sceloporus* lizard species during the dry and rainy seasons and explored whether seasonal dietary changes promote seasonal variation in gut microbiota composition.

Chapter I. Non-lethal sampling methods are frequently used to investigate the GIT microbiota of vertebrate hosts. However, which non-lethal method is more appropriate to study lizard gut microbiota remains largely unknown. To validate which method better represents the gut microbiota in wild lizards, we compared the bacterial communities retrieved by fecal samples and cloacal swabs to those obtained by directly sampling the dissected GIT segments (i.e. stomach, small intestine and rectum) in the mesquite lizard *S. grammicus*. Our results revealed that bacterial communities from fecal samples and cloacal swabs were highly correlated with

bacterial communities from different GIT segments. However, at Amplicon Sequencing Variants (ASVs) level, fecal samples reflected more accurately bacterial communities of GIT segments, particularly with the small intestine and rectum compared to stomach, suggesting that fecal samples comprise a reliable non-lethal method for monitoring gut bacterial communities in *Sceloporus* populations.

Chapter II. After confirming the suitability of fecal samples to study lizard gut microbiota, we compared the fecal microbiota (hereafter, gut microbiota) of two closely related species, *S. aeneus* and *S. bicanthalis*, inhabiting contrasting environments (i.e. i.e. cornfields and human-induced grasslands versus alpine grasslands) within La Malinche volcano to discern how species identity and ecological conditions promote shifts in their gut microbiota. Furthermore, to investigate whether the bacterial composition is primarily driven by environmental conditions or phylogenetic relatedness, we compared the core gut microbiota from two coexisting lizard species at ~2600 m above sea level "m a.s.l." (i.e. *S. aeneus* and *S. grammicus*) and two coexisting lizard species at ~4150 m a.s.l. (i.e. *S. bicanthalis* and *S. grammicus*). Our results indicated that bacterial alpha diversity and community composition varied significantly between lizard species. Strikingly, *S. bicanthalis* living at ~4150 m a.s.l. showed a higher taxonomic, phylogenetic and functional alpha diversity compared to *S. aeneus* living at ~2600 m a.s.l. Moreover, we detected differences in core microbial community structure between lizards *S. aeneus* and *S. grammicus* coexisting at ~2600 m a.s.l. as well as between *S. bicanthalis* and *S. grammicus* coexisting at ~4150 m a.s.l., indicating that despite inhabiting the same area their differences may be associated to variation in life history traits, microhabitat use and divergence time. Additionally, the core microbial community did not differ between closely related species, *S. aeneus* and *S. bicanthalis* inhabiting two different sites, suggesting that core microbial taxa remain stable over time.

Chapter III. There is increasing evidence that diet greatly modulates gut microbiota, however, it is necessary to first investigate how the dietary composition of wild populations varies over time to better understand whether this variability may affect temporal dynamics of gut microbiota. We applied DNA metabarcoding analysis to characterize the diet composition of *S.*

aeneus, *S. bicanthalis*, *S. grammicus* and *S. spinosus* during the dry and rainy seasons. According to the results, Hemiptera, Araneae, Hymenoptera and Coleoptera were the most frequently consumed orders in both seasons. Among lizard species, we found a greater taxonomic (arthropod genera) and phylogenetic (arthropod lineages) diversity during the dry season compared to the rainy season, which probably implies that during the dry season exists a scarcity of food resources leading to individuals exploiting alternative prey, resulting in more diverse diets. Furthermore, we observed significant differences in dietary and phylogenetic composition between seasons, and seasonal dietary turnover was higher in *S. spinosus* than *S. bicanthalis*, two species living in contrasting environments in the study area. Lastly, *S. bicanthalis* was the only species that showed changes in seasonal diet breadth, being greater in the dry season than in the rainy season. Due to their broad dietary spectrum, these lizard species can be considered generalist predators, and diet could be a key factor influencing their gut microbiota.

Chapter IV. In the last decade, descriptive and comparative studies have shown that gut microbiota composition is shaped by a wide variety of factors. Here, we quantified seasonal shifts in gut bacterial communities across four *Sceloporus* species (*S. aeneus*, *S. bicanthalis*, *S. grammicus* and *S. spinosus*). We also evaluated whether lizard gut microbiota vary synchronously with temporal changes in diet composition. Our results showed that the most abundant phyla were Firmicutes, Bacteroidota and Proteobacteria, and the closely related species *S. aeneus* and *S. bicanthalis* shared a great number of ASVs. The interactive effect species*season greatly influenced bacterial diversity and composition. Gut bacterial alpha diversity varied by season depending on the species, being higher during the dry season than the rainy season for *S. bicanthalis*, whereas *S. aeneus* showed opposite trends and the two other species did not exhibit seasonal differences. Additionally, bacterial community assembly was more similar between *S. aeneus* and *S. bicanthalis*, as well as between *S. grammicus* and *S. spinosus*. Changes in gut microbiota composition were associated with shifts in dietary composition, but dietary richness did not influence gut bacterial diversity. According to our expectations, lizard gut microbial communities exhibit seasonal dynamics that are linked to seasonal dietary changes. In addition to diet composition, other key factors that vary seasonally

such as temperature, humidity, microbial inoculums and seasonal physiological shifts may induce temporal dynamics of lizard gut microbiota.

For this fourth chapter, we do not show formal results **(i.e.** ***images, figures, tables, etc.*****)** ***because they are presented in a manuscript that will be submitted to an international journal, and copyright conflicts should be avoided***.

Table of Contents

4. General Introduction

4.1. The Importance of Gut Microbiota in Vertebrate Populations

The terms "Microbiota" and "microbiome" are often used interchangeably, but they denote distinct meanings. Microbiota properly refers to the community of microorganisms themselves, whereas microbiome is defined as the collective genome from all the microorganisms inhabiting a specific environment (Marchesi and Ravel 2015). The animal microbiota is a complex microbial community that includes bacteria, archaea, viruses, protozoans and fungi (Wu and Wu 2012). These microbes can reside within and outside of animal bodies, however, the gastrointestinal tract (GIT) contains the greatest richness and abundance of microbial taxa (Colston and Jackson 2016). The host-microbiota relationship comprises a bidirectional interaction in which microbial communities are important for diverse hosts' functions, such as energy metabolism through the production of short-chain fatty acids (Bäckhed and cols. 2005). In addition, gut microbiota stimulates the development and function of the immune system (Turnbaugh and cols. 2007), restrains pathogen growth by competitive exclusion (i.e. habitat and dietary resources) (Khan and cols. 2021) and, in a wider context, can contribute to environmental or ecological adaptation (Alberdi and cols. 2016). At the same time, host provides different microhabitats with high input of nutrients for the microbial communities. Nevertheless, when host-microbiota symbiosis is altered, detrimental effects can occur on host health, giving rise to a phenomenon known as dysbiosis, which is characterized by a reduced microbial diversity (Carding and cols. 2015).

During the last decades, next-generation sequencing (NGS) technologies have revolutionized the biological sciences, allowing the scientific community to sequence hundreds of samples in parallel at much-reduced cost, and evaluate different aspects related to diversity and function of microbial communities. However, most studies using NGS have focused their efforts to understand how GIT microbial communities influence on human health (Méndez-Salazar and cols. 2018), laboratory animals (Zhang and cols. 2019) and captive populations such as birds (Jacobs and cols. 2019) and mammals (Prabhu and cols. 2020). While individuals under controlled settings constitute a valuable tool, it is questionable to make inferences about their

wild counterparts (Colston and Jackson 2016), since they do not experience shifts in dietary patterns and other environmental variables, and are exposed to fewer microbial inoculum sources (soil, plant material, arthropods). As such, captive animals often have less diverse microbial communities than their wild counterparts (McKenzie and cols. 2017). For instance, it has been reported that diversity, composition (i.e. taxonomic richness) and structure (i.e. relative abundance of each taxon) of the gut microbiota vary according to nonspecific and specific host factors (Figure 1A). Interestingly, vertebrate studies have revealed that gut microbiota composition is shaped by intrinsic (e.g. host genetics, age, sex and reproductive status), environmental (e.g. dietary habits, seasonality, habitat use and geographical distance) and evolutionary (e.g. phylogenetic relatedness) factors (Colston and Jackson 2016; Grond and cols. 2018; Ingala and cols. 2018; Baniel and cols. 2021). However, dietary habits and phylogenetic relatedness have been considered the major drivers of gut microbiota variation (Kartzinel and cols. 2019; Youngblut and cols. 2019). Under this context, it is crucial to assess the relative influence of different factors on gut bacterial communities, particularly in wild reptile populations, the second most diverse vertebrate group after birds (Pincheira-Donoso and cols. 2013).

Non-avian reptiles have been considered among the most diverse radiations of terrestrial vertebrates (Pyron and cols. 2013). There are currently over 10,000 species of extant reptiles, distributed in all continents except Antarctica. They include lizards (59%), snakes (35%), turtles (3.4%), amphisbaenids (2%), crocodiles (0.3%) and tuataras (0.01%) (Pincheira-Donoso and cols. 2013; Uetz and cols. 2022). In particular, the lizard family Phrynosomatidae (order Squamata) comprises a diverse group with 10 genera and more than 150 species (Wiens and cols. 2010). However, most phrynosomatids belong to the genus *Sceloporus* with approximately 116 species distributed from southern Canada to western Panama (Leaché 2010; Wiens and cols. 2013; Uetz and cols. 2022), ranging from sea level to about 4600 m (Lemos-Espinal and Ballinger 1995). They are considered as active thermoregulators (Andrews 1998) and commonly use the sit-and-wait foraging strategy (Weiss 2001). Furthermore, *Sceloporus* members exhibit differences in sexual-size dimorphism, display different reproductive modes (i.e. oviparous and viviparous), occur in a wide variety of habitats including deserts, semiarid regions, shrublands,

forests and mountainous ecosystems, and are taxonomically diverse (Wiens and Reeder 1997; Wiens and cols. 2010). In addition, some spiny lizards can live from one year up to five years (Rodríguez-Romero and cols. 2002; Méndez de la Cruz and cols. 2018). These unique features make them a promising model system for studying gut microbial communities in relation with seasonal dietary variation. But these associations are just beginning to be explored. A better understanding of their diet and microbial dynamics may help explain their successful adaptation and occupancy of multiple habitats.

Based on the above listed criteria, this doctoral thesis integrates a descriptive approach to characterize the gut bacterial communities, and evaluate how different extrinsic (e.g. seasonal dynamics, diet composition and environmental conditions) and intrinsic (e.g. species identity and evolutionary history) factors influence gut microbiota variation, using as model study four *Sceloporus* lizard species (*S. aeneus, S. bicanthalis, S. grammicus* and *S. spinosus*) inhabiting in the La Malinche volcano (Figure 1B), central Mexico, a protected area characterized by its high biodiversity in a deforested region (Díaz de la Vega-Pérez and cols. 2019). Here, we used NGS of bacterial 16S rRNA gene and Cytochrome c Oxidase Subunit I (COI) gene to gain insight into gut microbiota and diet of studied lizards under natural conditions.

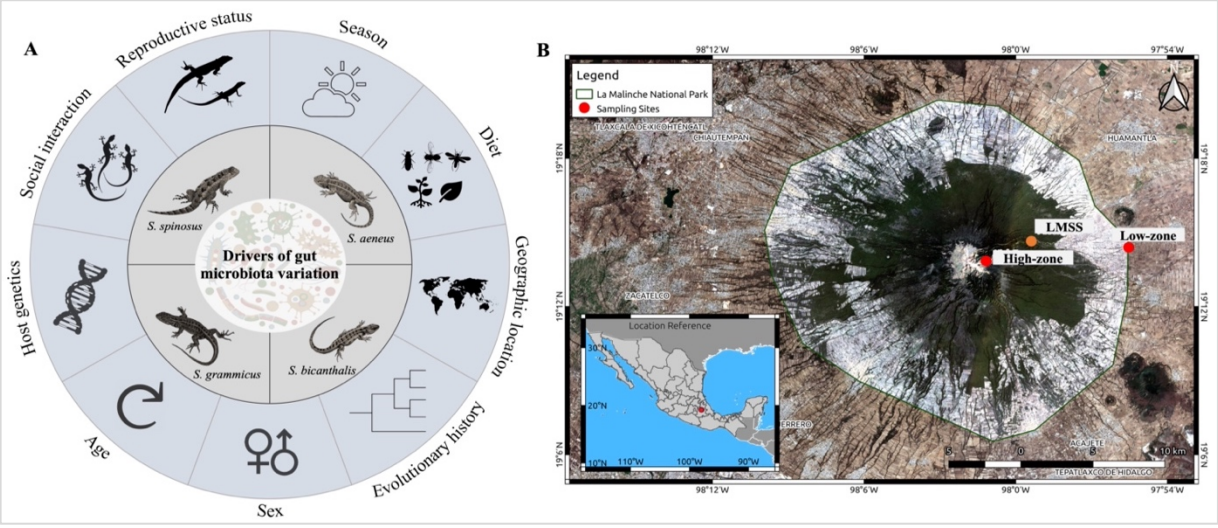

Figure 1. (A) The first circle displays different extrinsic and intrinsic factors influencing gut microbiota composition in animal populations. The four *Sceloporus* lizard species under study have been included in the second circle. (B) Map of the study area. Individuals were collected in two different sites along the La Malinche volcano, at ~2600 m above sea level "m a.s.l." (Low-zone) and ~4150 m a.s.l. (High-zone). Lizards were transported to La Malinche Scientific Station (LMSS) at ~3100 m a.s.l. to collect fecal samples.

5. Chapter I: Comparative analysis of two nonlethal methods for the study of the gut bacterial communities in wild lizards

Mauricio Hernández, Sergio Ancona, Stephanie Hereira-Pacheco, Aníbal H. Díaz de la Vega-Pérez and Yendi E. Navarro-Noya. *Integrative Zoology* 2023; 00: 1-16. DOI:10.1111/1749-4877.12711. [IF: 3.3]

Currently, sample collection, storage and DNA extraction are key steps when investigating the interaction between host and microbiota. Historically, most vertebrate microbiota studies have focused on GIT samples, fecal samples and rectal or cloacal swabs to evaluate microbial community composition. However, to avoid euthanasia procedures in threatened species or populations with a small number of individuals non-lethal sampling procedures, specifically rectal/cloacal swabs have been used. However, few studies have assessed whether non-lethal methods (i.e. fecal samples or cloacal swabs) can accurately represent the intestinal microbial communities. In the **First Chapter**, we characterized the bacterial communities of three GIT segments (i.e. stomach, small intestine and rectum) and compared them with the fecal and cloacal bacterial communities using the mesquite lizard (*S. grammicus*) as a model system. Since fecal samples or cloacal swabs can retrieve distinct microbial communities, the goal of this chapter was to estimate which non-invasive method is more accurate to study lizard gut microbiota. Given that previous studies have found a significant association between fecal and intestinal microbial assembly (Kohl and cols. 2017; Videvall and cols. 2017; Montoya-Ciriaco and cols. 2020), we hypothesized that fecal bacterial communities best resemble the intestinal microbiota. Briefly, our results revealed that bacterial communities of the three different GIT segments were correlated to those retrieved from the fecal and cloacal samples (Spearmans' rank correlations > 0.84). However, at the level of Amplicon Sequence Variant (ASVs: sequences differing from each other by a single nucleotide), feces were more accurate than cloacal swabs, supporting our hypothesis that fecal samples comprise a good proxy to study lizard gut microbiota. The following paper published in *Integrative Zoology* contains more detailed information of the study (Hernández and cols. 2023).

Integrative Zoology 2023; 0: 1–16 doi: 10.1111/1749-4877.12711

ORIGINAL ARTICLE

INTEGRATIVE ZOOLOGY

Comparative analysis of two nonlethal methods for the study of the gut bacterial communities in wild lizards

Mauricio HERNÁNDEZ,[1] Sergio ANCONA,[2] Stephanie HEREIRA-PACHECO,[3] Aníbal H. DÍAZ DE LA VEGA-PÉREZ[4] and Yendi E. NAVARRO-NOYA[5]

[1]Doctorado en Ciencias Biológicas, Centro de Tlaxcala de Biología de la Conducta, Universidad Autónoma de Tlaxcala, Tlaxcala, Mexico, [2]Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, Mexico City, Mexico, [3]Estación Científica la Malinche, Centro de Tlaxcala de Biología de la Conducta, Universidad Autónoma de Tlaxcala, Tlaxcala, Mexico, [4]Consejo Nacional de Ciencia y Tecnología—Centro Tlaxcala de Biología de la Conducta, Universidad Autónoma de Tlaxcala, Tlaxcala, Mexico and [5]Laboratorio de Interacciones Bióticas, Centro de Investigación en Ciencias Biológicas, Universidad Autónoma de Tlaxcala, Tlaxcala, Mexico

Abstract

Fecal samples or cloacal swabs are preferred over lethal dissections to study vertebrate gut microbiota for ethical reasons, but it remains unclear which nonlethal methods provide more accurate information about gut microbiota. We compared the bacterial communities of three gastrointestinal tract (GIT) segments, that is, stomach, small intestine (midgut), and rectum (hindgut) with the bacterial communities of the cloaca and feces in the mesquite lizard *Sceloporus grammicus*. The hindgut had the highest taxonomic and functional alpha diversity, followed by midgut and feces, whereas the stomach and cloaca showed the lowest diversities. The taxonomic assemblages of the GIT segments at the phylum level were strongly correlated with those retrieved from feces and cloacal swabs ($r_s > 0.84$ in all cases). The turnover ratio of Amplicon Sequence Variants (ASVs) between midgut and hindgut and the feces was lower than the ratio between these segments and the cloaca. More than half of the core-ASVs in the midgut (24 of 32) and hindgut (58 of 97) were also found in feces, while less than 5 were found in the cloaca. At the ASVs level, however, the structure of the bacterial communities of the midgut and hindgut were similar to those detected in feces and cloaca. Our findings suggest that fecal samples and cloacal swabs of spiny lizards provide a good approximation of the taxonomic assemblages and beta diversity of midgut and hindgut microbiota, while feces better represent the bacterial communities of the intestinal segments at a single nucleotide variation level than cloacal swabs.

Key words: animal microbiome, cloacal swabs, fecal samples, intestinal tract, reptile gut microbiome

Correspondence: Yendi Navarro-Noya, Laboratorio de Interacciones Bióticas, Centro de Investigación en Ciencias Biológicas, Universidad Autónoma de Tlaxcala, Carretera San Martín Texmelucan km 10.5, 90120 San Felipe Ixtacuixtla, Tlaxcala, Mexico.
Email: nyendi@hotmail.com, yendiebenezer.navarro.n@uatx.mx

INTRODUCTION

The microbiota of the gastrointestinal tract (GIT) is richer and more abundant than in other body parts, and includes bacteria, fungi, protozoa, archaea, and viruses (Belkaid & Hand 2014). Composition and diversity of bacterial communities can vary considerably along the GIT, raising the possibility of multiple bacterial community assemblies in the intestine of vertebrates, including mammals (Lkhagva *et al.* 2021), birds (Videvall *et al.* 2017; Grond *et al.* 2020), amphibians (Zhou *et al.* 2020), and reptiles (Colston *et al.* 2015; Tang *et al.* 2019). Spatial heterogeneity of bacterial communities along GIT segments have been attributed to differences in pH, oxygen concentration, water content, and nutrient availability, which impose differential selective pressures on microorganisms (Donaldson *et al.* 2016; Grond *et al.* 2018). Variation in the bacterial community composition along the GIT confers specific functions to gut segments (Miller *et al.* 2021), which are essential for physiological processes in the hosts, such as metabolism of carbohydrates and the subsequent production of short-chain fatty acids that are used for signaling and as a source of energy (Parada-Venegas *et al.* 2019). Gut bacterial communities also play a vital role in immune functioning and protection against pathogens (Turnbaugh *et al.* 2007; Belkaid & Hand 2014), contribute to survival and reproduction of their hosts, and are thus critical for ecological adaptation (Gilbert *et al.* 2015; Alberdi *et al.* 2016). In return, hosts provide nutrients and adequate microhabitats for the microbial communities in their GIT.

Most studies in laboratory and wild animals rely on rectal or cloacal swabs for monitoring gut microbial communities, since they can be easily taken. However, there is no standardized sample collection protocol to study the gut microbiota of wild animal populations (Ingala *et al.* 2018; Borreli *et al.* 2020), which limits comparison of data from different studies. Vertebrate gut microbial communities have been characterized using methods that are lethal or nonlethal to the hosts. Lethal methods imply dissection of the GIT, whereas nonlethal methods include collection of fecal samples and rectal or cloacal swabs. Each method has its advantages and disadvantages. Lethal methods give an accurate characterization of the diversity and structure of the gut microbiota, but the death of the hosts is the major drawback. Although dissection of the GIT provides more information as it gives a complete picture of the microbial communities, it is unacceptable in endangered species or small populations. Alternatively, nonlethal methods allow the study of individuals over time (e.g. from birth to death), increase sample size, and do not affect rare and endangered populations. However, nonlethal methods may yield biased estimations of the diversity and structure of the gut microbial communities since they can retrieve microorganisms that live in the lower or external areas of the GIT, which may differ from microorganisms that live in the upper GIT (Kohl *et al.* 2017; Tang *et al.* 2019).

The variation in biochemical and physiological characteristics along the GIT may affect the occurrence of specific microbial taxa (Grond *et al.* 2018). Therefore, cloacal or fecal samples may not resemble the bacterial communities in the gut, but as far as we know, this variation has not been investigated in detail. Comprehensive examination of the reliability of nonlethal methods is important because some of these methods could be more accurate than others (Stanley *et al.* 2015). In ostrich birds (*Struthio camelus* L.), feces provided a more accurate determination of the composition of the bacterial communities from the large intestine than cloacal samples (Videvall *et al.* 2017). Particularly, within the reptile group, Colston *et al.* (2015) and Tang *et al.* (2019) considered solely cloacal swabs, whereas Kohl *et al.* (2017) and Montoya-Ciriaco *et al.* (2020) only studied fecal samples to compare the microbiota profiles between lethal and nonlethal methods. To date, only one study has considered both fecal and cloacal samples and compared them to the gastrointestinal microbiota of the lizard *Sceloporus virgatus* Smith, 1938 obtained by dissection of the GIT (Bunker *et al.* 2022). The latter study reported that although cloacal swabs recovered bacterial communities that were similar to those found in the lower intestine, the cloacal community showed extreme specialization, and feces and cloacal swabs exhibit different communities (Bunker *et al.* 2022). However, it does not provide clear indications of which sampling method can be used as a good indicator of the structure and diversity of the microbial communities along the GIT. Still, we lack a general appreciation of the accuracy of fecal and cloacal samples regarding different traits of microbial communities such as alpha taxonomic and functional diversity, taxonomic assemblage, differential abundance of bacterial taxa and species turnover, in comparison to lethal methods.

We used 16S rRNA gene sequencing to compare bacterial community profiles of the stomach, small intestine, rectum, feces, and cloacal swabs (hereafter cloaca) of the mesquite lizard *Sceloporus grammicus* Wiegmann, 1828 (Fig. 1b). *Sceloporus grammicus* is an insectivorous lizard (Leyte-Manrique & Ramírez-Bautista 2010; Montoya-Ciriaco *et al.* 2020) that can be found from southern Texas in the United States to southern Oaxaca in Mexico (Lemos-Espinal & Ballinger 1995). The mesquite

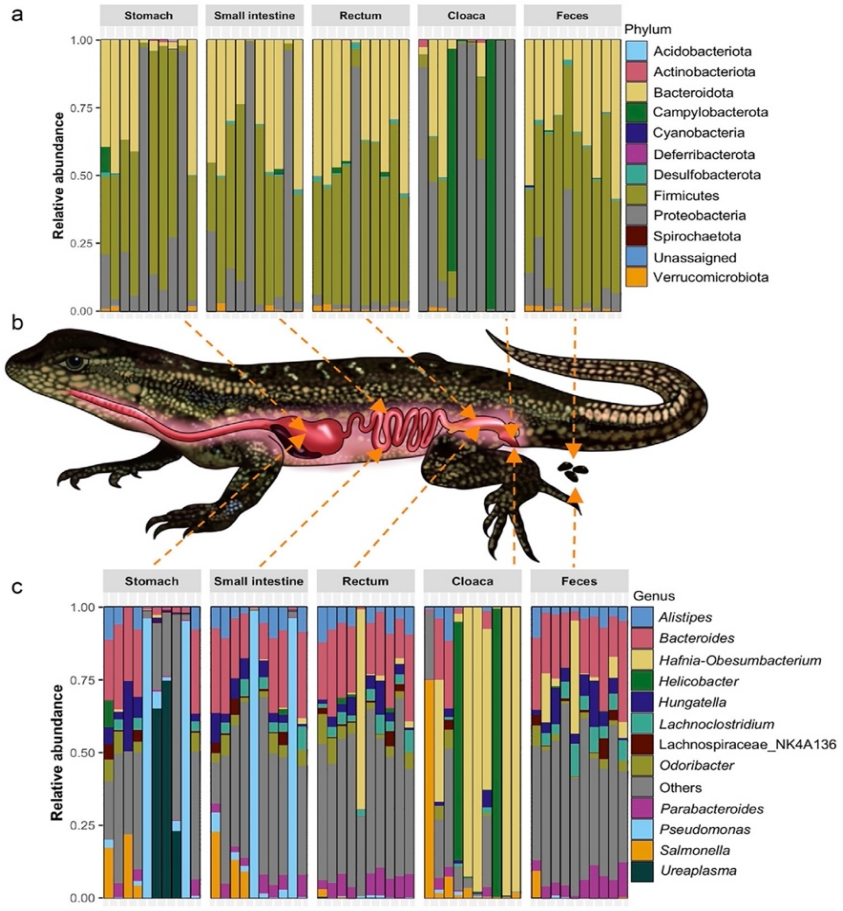

Figure 1 Taxonomic bacterial composition among compartments of the gastrointestinal tract (i.e. stomach, small intestine, and rectum), feces, and cloaca of the mesquite lizard (*Sceloporus grammicus*). (a) Relative abundance at the phylum level, (b) illustration showing the sampling sites, and (c) relative abundance at the genus level.

lizard is the most widely distributed species complex of lizards in Mexico, so its natural history is well known (Zúñiga-Vega *et al.* 2008; Díaz de la Vega-Pérez *et al.* 2019), and it is considered as a least-concern species according to *the Red List of Threatened Species* (Hammerson *et al.* 2007). As such, we considered this species as an excellent model organism to compare the accuracy of different nonlethal methods to characterize gut bacterial communities of lizards. Specifically, we aimed to answer (i) how different is the taxonomic and functional diversity and the structure of the bacterial communities of different GIT segments compared to bacterial communities retrieved from fecal and cloacal samples? and (ii) which nonlethal method, fecal or cloacal sampling, provides a bacterial community that best resembles the bacterial communities of the GIT?

MATERIALS AND METHODS

Ethics statement

The study has been approved by the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT, Mexico), under the collecting permit: SGPA/DGVS/007736/20. All procedures have been conducted following the Official Mexican Norm NOM-126-ECOL-2000 as a guideline to handle the reptiles.

Study area and sample collection

To prevent sex differences and age-related variation in gut bacterial communities of lizards (Martin *et al.* 2010; Kohl *et al.* 2017) from affecting our inferences, we only included adult males in this study (snout–vent length [SVL] > 44.1 mm, Jiménez-Cruz *et al.* 2005). Our sample consisted of 10 male adult lizards (mean SVL 56.5 ± 3.7 mm, mean total length 117.5 ± 9.7 mm, mean weight 5.6 ± 1.7 g; Table S1, Supporting Information), which were collected near the municipality of Ixtenco (19°13′39.5″N, 97°54′44.1″W, Tlaxcala, Mexico), at 2600 m above sea level (m a.s.l.) in October 2020. Specimens were captured by hand or noose during their daytime activity period (from 0900 to 1600 hour). Upon capture, lizards were transported individually to La Malinche Scientific Station at 3100 m a.s.l. (19°14′39.2″N, 97°59′25.1″W). At the research station, each individual was housed separately in plastic containers (20 × 30 × 15 cm), previously sterilized with Lysol disinfectant and 70% ethanol. The plastic containers had a sterile sheet of paper placed on the floor to collect fecal samples. Fecal samples were collected the day after each lizard was captured, 1–3 min after the first defecation of every lizard in captivity, using sterile forceps and transferred to 1.5 mL sterile polypropylene tubes. Lizards were exposed to sunlight to raise their body temperature and stimulate their natural defecation, which occurred within 10–15 min. As such, the variation of defecation time among individuals was controlled to prevent possible changes in the fecal bacterial communities that might occur over time and bias our results. Cloacal samples were collected after defecation using sterile rayon swabs (COPAN, Italy). The swabs were gently inserted ∼10 mm into the cloaca, rotated ∼360°, retrieved, and added to 1.5 mL sterile polypropylene tubes. Both fecal samples and cloacal swabs were kept at 4°C in a coolbox while being transported to the laboratory. Fecal samples were stored at −20°C and cloacal samples at −70°C prior to DNA extraction. Feces were processed first the next day and cloacal samples thereafter, that is, after approximately 3 days. Storage of the swabs at −70°C avoided possible DNA degradation. Approximately 20 h elapsed between capturing the lizards and collecting the fecal samples and cloacal swabs, and less than 4 h between fecal and cloacal data collection and GIT dissection.

Lizard individuals were placed into a chamber which contained cotton balls saturated with chloroform for euthanasia and death occurred within 20 s. Death was confirmed by examining the lack of heartbeat and breath. Dissection of each specimen was performed under sterile conditions in a laminar flow cabinet. Immediately postmortem, the entire GIT was removed from the body cavity. Three different gut segments were collected (stomach, small intestine, and rectum) (Fig. 1b), including both luminal and mucosal microbiotas. Dissecting the GIT after defecation (3–4 h) allowed the stomach and other GIT segments to discharge all or most of their contents before sampling, since lizards usually defecate only once a day (Kohl *et al.* 2017). Samples were placed in 1.5 mL sterile polypropylene tubes and stored at −20°C until DNA extraction on the next day. The work tools were disinfected with 70% ethanol and burned every time as a different GIT section was taken to reduce cross-contamination.

DNA extraction, library preparation, and amplicon sequencing

DNA extraction protocol from the different GIT segments and fecal and cloacal samples is detailed in the Supporting Information. In all cases, a negative control was run in parallel to verify any contamination during DNA extraction and no contamination was detected. The V3–V4 hypervariable regions of the 16S rRNA gene was PCR-amplified in triplicate using the primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-ACHVGGGTATCTAATCC-3′) modified with adapters for the Illumina sequencing platform (Herlemann *et al.* 2011). All PCRs were done in 20 μL final volume reaction containing reaction buffer 1×, $MgCl_2$ 1 mM, dNTPs mix 200 μM, bovine serum albumin 500 μg μL^{-1}, 2 units of Dream*Taq* DNA polymerase (Thermo Fisher Scientific, Waltham, MA USA), 25 μM of each primer, and 10 ng of DNA template. A negative control was included in each batch of reactions and no contamination was found. Thermal cycling consisted of an initial denaturation at 95°C for 2 min, followed by 28 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. The final elongation was done at 72°C for 5 min. Amplicon products were quan-

tified, combined in equimolar amounts, and purified for sequencing. The quantification of the PCR products was determined using a fluorospectrometer NanoDrop® 3300 (Thermo Fisher Scientific, MA, USA) with PicoGreen dsDNA assay (Invitrogen, CA, USA), and purifications were done using QIAquick PCR purification kit according to the manufacturer's instructions (QIAGEN, Germany). Sequencing was done by Macrogen Inc. (Seoul, South Korea) with 300-bp PE MiSeq runs (Illumina, CA, USA). The GIT segments from three individuals gave no reads after sequencing. To recover the samples, all the samples of those individuals were re-sequenced (i.e. the three GIT segments, feces, and cloaca). Raw sequence databases are available at Sequence Read Archive from the NCBI under accession number PRJNA808744.

Taxonomic classification of the gut bacterial communities

Sequencing data analysis was done using the open-source software Quantitative Insights Into Microbial Ecology (QIIME). Raw sequences were imported into QIIME v1.9.1 to extract barcodes using the "*extract_barcodes.py*" script (Caporaso *et al.* 2010). Then, sequences were imported to QIIME2 v2021.4.0 (Bolyen *et al.* 2019). Forward and reverse Illumina adapters were removed from the sequences and demultiplexed raw sequences were quality-filtered (i.e. by standard filtering parameters: maxEE = 2, truncQ = 2), denoised, trimmed (i.e. forward reads were trimmed to 260 bp and reverse reads to 200 bp), merged, and dereplicated into amplicon sequence variants (ASVs) by DADA2 (Divisive Amplicon Denoising Algorithm 2) plugin (Callahan *et al.* 2016). The two sequencing batches were analyzed with the same quality parameters, and the feature-tables and representative sequences were merged with the "*q2-feature-table*" plugin (https://github.com/qiime2/q2-feature-table). The taxonomy to each ASV was assigned using the "*classify-sklearn*" classifier trained against the SILVA 16S rRNA gene database version v.138.1. Finally, sequences classified as chloroplast and mitochondria were removed from the dataset for further analyses.

Statistical analysis

All downstream statistical analyses were done using the software R v.4.1.2 (R Core Team 2021). To visualize the relative frequency at the phylum and genus level of each sampling section, a barplot was constructed using the *phyloseq* v.1.34.0 R package (McMurdie & Holmes 2013). Due to the compositional nature of the microbiome datasets (Gloor *et al.* 2017), a centered log-ratio transformation (*clr*) was applied to the frequency table of ASVs for community composition analyses. This compositional approach simultaneously accounts for library size differences and biological variability. Differentially abundant ASVs among GIT segments and fecal and cloacal samples were determined with ANOVA-like differential expression analysis (ALDEx) with the function "*aldex.ttest*," which performed a Wilcoxon-test, using the *ALDEx2* v.1.25.1 R package (Fernandes *et al.* 2013).

Following Videvall *et al.* (2017), we examined the potential relationship of the bacterial communities at the phylum level between both nonlethal methods and the three GIT segments. Raw counts from the frequency table of phyla were *clr* transformed with *cmultRepl* and *codaSeq.clr* functions from the *zCompositions* v.1.4.0 (Palarea-Albaladejo & Martín-Fernández 2015) and *CoDaSeq* v.0.99.6 R packages (https://github.com/ggloor/CoDaSeq). To quantify the association between the relative frequency of bacterial phyla in the feces and cloaca with those in the GIT segments, a Spearman's rank correlation was computed.

A principal component analysis (PCA) was done with the *clr* transformed ASVs table as calculated by the *prcomp* function in R. Differences between the bacterial community structure in the fecal and cloacal samples and those in the GIT segments were determined with a perMANOVA based on Aitchison distances applying the *adonis2* function with 999 permutations using the *vegan* v.2.5-7 R package (Oksanen *et al.* 2020). Individual identity was included as a random factor with the *setblocks* function. A pairwise-perMANOVA was done to determine differences between sampling sites using the *pairwise.perm.manova* function from the *RVAideMemoire* v.0.9-81 R package (Hervé 2022) with a Benjamini–Hochberg correction of the *P*-value. The relative species turnover rate per community, which represents the proportion of a typical community that changes from one community to another, was quantified with the *hilldiv* v.1.5.4 R package (Alberdi & Gilbert 2019). Pairwise comparisons between nonlethal methods of sampling and GIT segments were selected as occurring within the same individual. A Venn diagram was generated to show unique and shared ASVs among GIT segments, feces, and cloaca based on core bacterial communities (i.e. bacterial ASVs that are shared by at least 50% of the samples).

Hill numbers were calculated to determine alpha taxonomic and functional diversity. This ecological metric measures diversity in terms of effective number of ASVs and considers different diversity orders (*q*D) which

weighs common species to a greater or lesser extent. The Hill numbers at $q = 0$ is the species richness as it does not consider the relative abundance, $q = 1$ consider both richness and evenness and is equivalent to the exponential of Shannon's entropy index and represent typical ASVs, whereas $q = 2$ considers dominant ASVs; that is, the relative abundances of ASVs are proportionally overweighted and are equivalent to the inverse Simpson's index (Chao *et al.* 2014). Alpha taxonomic diversity was computed with the frequency table of ASVs using the *hillR* v.0.5.1 R package (Li 2018). Functional diversity was determined as the mean functional diversity per species (MD_q), which considers pairwise distances of the ASVs calculated with the functional traits. Functional traits were predicted using the taxonomic assignments and computed using PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille *et al.* 2013). The MD_q was calculated within the *hillR* R package using the frequency table of ASVs and the predicted Enzyme Commission Numbers associated with the taxonomic assignment of each ASV. The Wilcoxon signed-rank test was used to test differences between groups, that is, differences between fecal samples and GIT segments, and between cloacal samples and GIT segments. In all cases, a P-value < 0.05 was considered as statistically significant. All scripts and R programming for the analysis are available at the GitHub repository: https://github.com/Steph0522/Sceloporus_grammicus.

RESULTS

Overall, 50 samples were obtained, that is, five samples from 10 adult males. After denoising and quality filtering, 740 562 high-quality sequences were acquired from 740 939 raw reads, with an average of 14 811 reads per sample (Table S2, Supporting Information). A total of 1296 ASVs were identified, representing 11 bacterial phyla, 16 classes, 52 orders, 83 families, 136 genera, and 85 species.

Taxonomic assemblies of bacterial communities along the gastrointestinal tract, feces, and cloaca

Gut bacterial communities of the mesquite lizard was dominated by Firmicutes (38.4% ± 24.5%), Bacteroidota (28.6% ± 20.8%), Proteobacteria (27.5% ± 36.9%), and Campylobacterota (4.0% ± 18.0%) (Fig. 1a; Fig. S1a, Supporting Information). All other bacterial phyla represented less than 1%. The relative abundance of some bacterial phyla varied among GIT segments, feces, and cloaca. Proteobacteria was the dominant phylum in cloaca (60%); Firmicutes dominated in the feces (51%), stomach (46%), rectum (46%), and small intestine (39%). Bacteroidota showed a large relative frequency in the rectum (40%), feces (36%), small intestine (33%), and stomach (22%).

At the genus level, *Bacteroides* (15.8% ± 11.4%), *Hafnia-Obesumbacterium* (12.8% ± 28.9%), *Pseudomonas* (8.5% ± 26.3%), *Alistipes* (4.2% ± 3.9%), *Helicobacter* (4.0% ± 18.0%), *Hungatella* (3.9% ± 4.6%), *Salmonella* (3.8% ± 11.6%), *Parabacteroides* (3.6% ± 3.2%), *Ureaplasma* (3.3% ± 14.2%), *Odoribacter* (2.9% ± 2.8%), *Lachnoclostridium* (2.8% ± 2.8%), and Laschospiraceae_NK4A136 (1.2 ± 1.9%) were the most abundant genera across all GIT segments, feces, and cloaca (Fig. 1c; Fig. S1b, Supporting Information). The most abundant bacterial genera had different relative abundances in the studied sections. For instance, the stomach was dominated by *Pseudomonas* (20.7%) and *Ureaplasma* (16.3%). In the small intestine, *Pseudomonas* (21%), *Bacteroides* (18.6%), and *Alistipes* (5.6%) showed a high abundance. The rectum harbored a high relative abundance of *Bacteroides* (20.8%), *Hafnia-Obesumbacterium* (7.5%), and *Alistipes* (6.1%). The feces were dominated by *Bacteroides* (20.7%), followed by *Hafnia-Obesumbacterium* (7.2%), and *Hungatella* (6.1%). Last, *Hafnia-Obesumbacterium* (48.8%), *Helicobacter* (18.3%), *Salmonella* (9.1%), and *Bacteroides* (5.3%) were the most abundant bacterial genera in cloaca. The analysis with ALDEx2 showed a larger number of differentially abundant ASVs between cloaca and rectum, and between feces and stomach than among other segments (Fig. 2). Overall, differential abundance analysis revealed that Enterobacterial ASVs were more frequent in cloaca (*Hafnia-Obesumbacterium* and *Salmonella*) and feces (Enterobacteriaceae). Strict anaerobes, such as Lachnospiraceae, were more frequent in the feces compared to stomach or small intestine, while these bacteria were more common in the rectum than in cloaca. In addition, some bacterial genera, such as *Bacteroides*, *Pseudomonas*, and *Hungatella*, increased significantly in the GIT segments.

Are relative frequencies of bacterial phyla in the feces and cloaca related to those observed in the gut segments?

We determined the association of the relative frequency of bacterial phyla between feces or cloaca with GIT segments using Spearman's rank correlations. All the

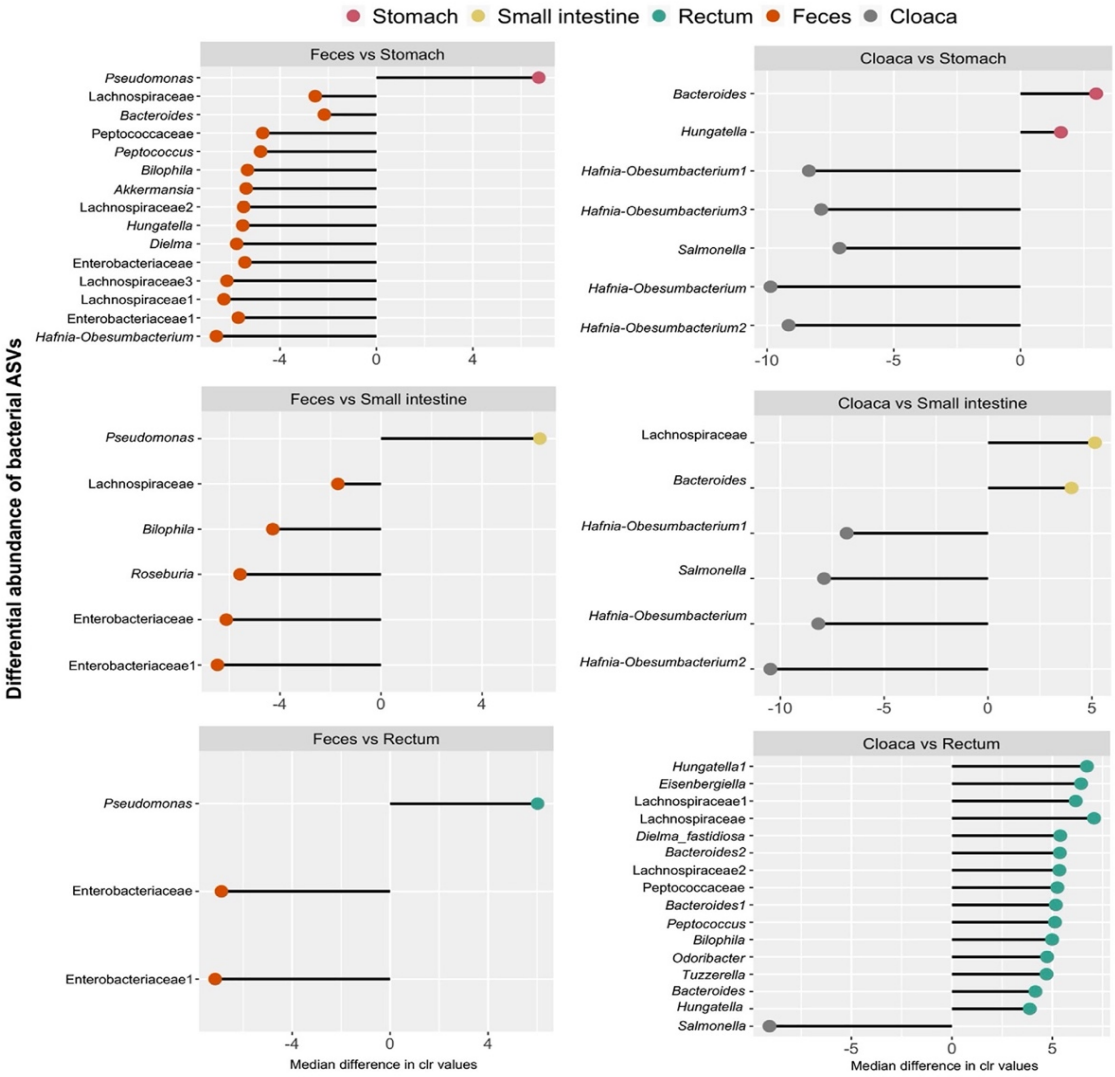

Figure 2 Differentially abundant amplicon sequence variants (ASVs) found among the gastrointestinal tract segments and the non-lethal sampling methods (i.e. feces and cloaca) of *Sceloporus grammicus*. Lines represent the median difference in center-log transformed (*clr*) values between groups where the direction of the line means higher values in that group. Differences were determined using ANOVA-like differential gene expression analysis (ALDEx2) for compositional data. Only ASVs with <0.05 Benjamini–Hochberg corrected *P*-values are given. The name on the left corresponds to the taxonomic assignment of the ASVs.

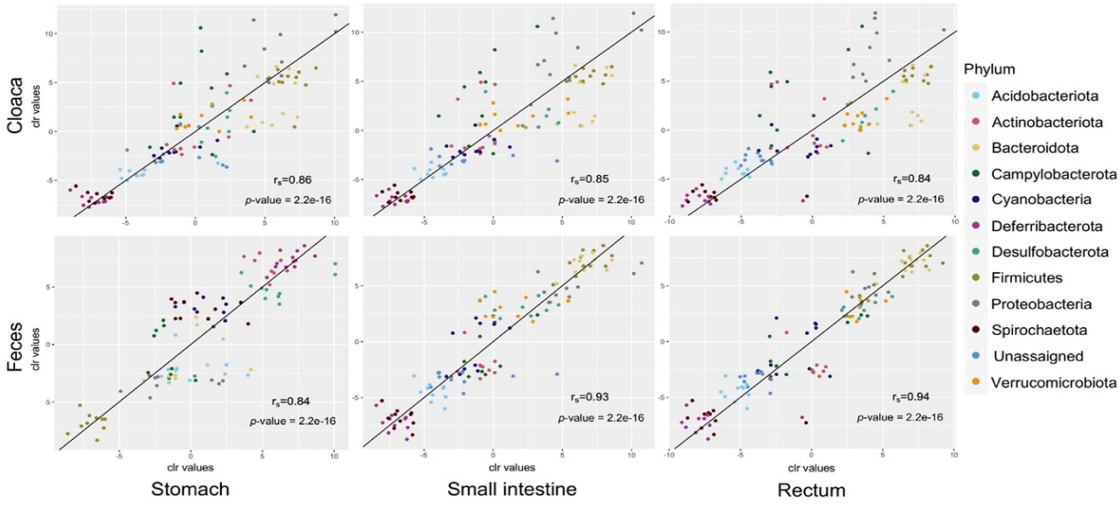

Figure 3 Spearman rank correlations between the relative frequency of bacterial phyla in gastrointestinal tract segments of *Sceloporus grammicus* and nonlethal sampling methods (i.e. feces and cloaca). The correlation coefficient (r_s) indicates the extent to which two variables fit on a straight line.

associations were strong and highly significant. The relative frequencies of bacterial phyla were strongly correlated between the feces and the stomach ($r_s = 0.84$), small intestine ($r_s = 0.93$), and rectum ($r_s = 0.94$) (Fig. 3). The correlations between the relative frequencies of bacterial phyla retrieved by cloacal samples and stomach ($r_s = 0.86$), cloacal samples and small intestine ($r_s = 0.85$), and cloacal samples and rectum ($r_s = 0.84$) were also strong.

ASVs turnover and shared diversity among gastrointestinal tract segments, feces, and cloaca

Overall, turnover ratio was higher among gut segments and cloaca than among gut segments and feces at $q = 0$, 1, and 2 orders. For instance, considering typical ASVs (i.e. $q = 1$), a turnover ratio of >80% was observed between cloaca and the three GIT segments; feces showed an ~60% turnover ratio compared to the rectum, ~68% compared to the small intestine, and ~80% compared to the stomach (Fig. 4a). A similar pattern was observed at $q = 0$ and $q = 2$. Similarly, pairwise-perMANOVA showed a statistically significant difference between cloaca and all GIT segments, while the bacterial communities in the feces only differed statistically with those bacterial communities of the stomach (Table S3, Supporting Information). The PCA of the bacterial community structure at ASVs level did not separate the sample sections, that is, GIT segments, feces, and cloaca, but grouped them per individual (Fig. 4b). The perMANOVA indicated a statistically significant difference in the microbiota structure between GIT segments, and the feces and cloaca (df = 4, $F = 1.102$, adjusted $R^2 = 0.090$, $P = 0.028$).

The core bacterial community, defined as those present in ≥50% of the samples, was represented by a total of 12 ASVs in the stomach, 32 in the small intestine, 97 in the rectum, 71 in feces, and 9 in cloaca. Venn diagram showed that rectum and feces shared 33 ASVs exclusively between them, only three were shared between the rectum and small intestine, and 20 were shared between feces, rectum, and small intestine (Fig. 4c). The core microbiota of the rectum had the largest number of unique ASVs (35), followed by stomach (12), feces (11), small intestine (5), and cloaca (2). Feces and small intestine, cloaca and feces, and cloaca and rectum shared one ASV, whereas stomach and rectum, stomach and cloaca, and stomach and feces did not share any ASV.

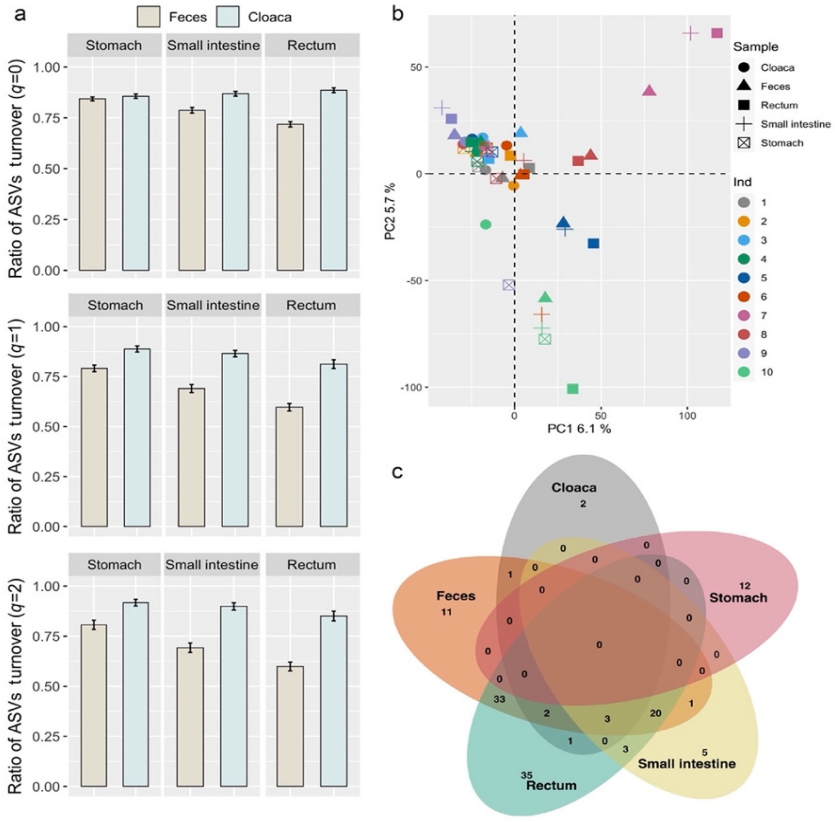

Figure 4 Turnover rate and shared amplicon sequence variants (ASVs) among gastrointestinal tract segments of *Sceloporus grammicus* and nonlethal sampling methods (i.e. feces and cloaca). (a) Relative turnover rate of richness ASVs ($q = 0$), typical ASVs ($q = 1$), and dominant ASVs ($q = 2$) and (b) principal component analysis (PCA) ordination plot of the bacterial composition at ASV level of the gastrointestinal tract segments, feces, and cloaca. Gastrointestinal tract segments, feces, and cloaca are indicated by symbols and colors. (c) Venn diagram illustrating the number of unique and shared ASVs (≥ 50% prevalence) in the gastrointestinal tract segments (i.e. stomach, small intestine, and rectum), feces, and cloaca. Values within intersections represent shared ASVs, while values outside intersections represent unique ASVs.

Alpha and functional diversity of the bacterial communities of the gastrointestinal tract, feces, and cloaca

The bacterial alpha diversity was statistically different among GIT segments, feces, and cloaca ($P < 0.05$) (Fig. 5a–c). The highest taxonomic diversity at all q diversity orders was observed in the rectum followed by small intestine and feces. The smallest diversity was found in the stomach and cloaca. Considering total ($q = 0$), frequent ($q = 1$), and dominant ($q = 2$) ASVs, the rectum had a higher diversity than cloaca, and feces had higher diversity than stomach (Fig. 5a–c, Wilcoxon signed-rank test, $P < 0.05$; Table S4, Supporting Information). Considering frequent and dominant ASVs, rectum displayed a higher diversity than feces (Fig. 5b,c). Functional alpha diversity, which considers the differences in functional traits between ASVs, showed differences

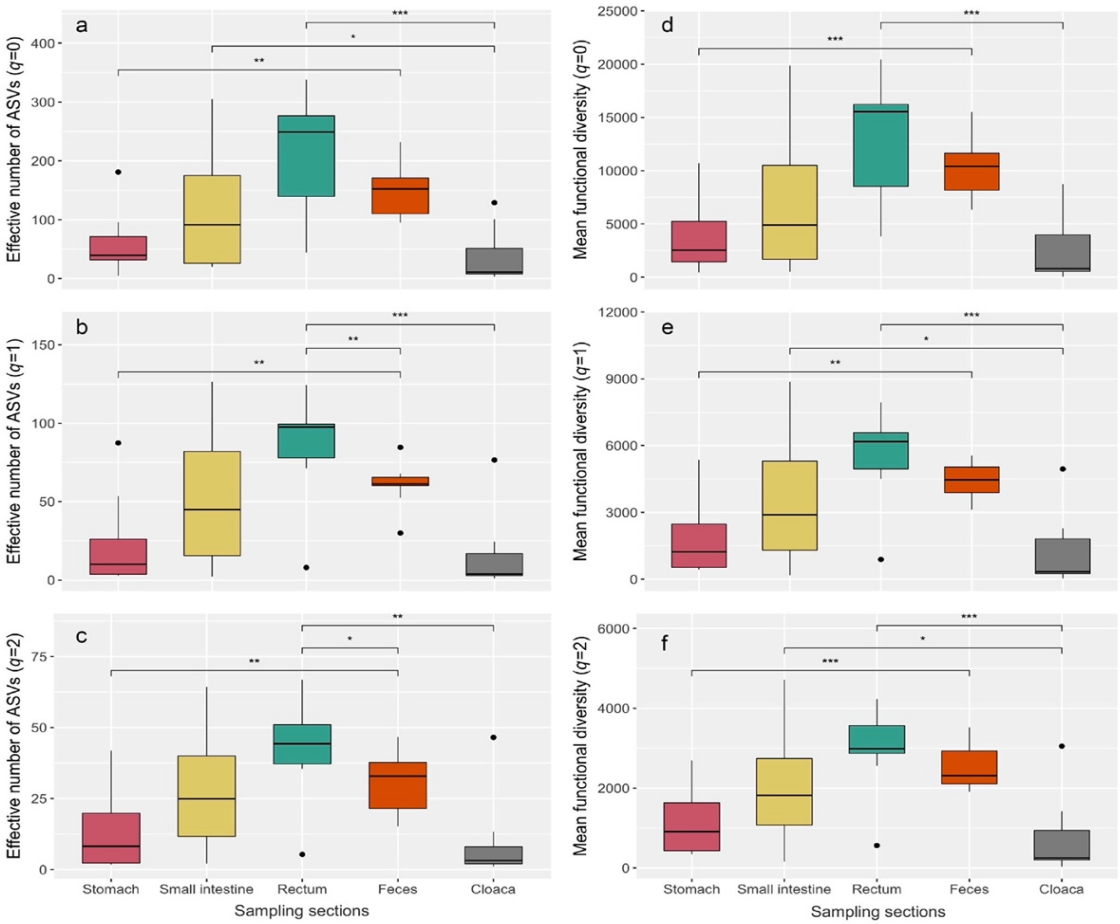

Figure 5 Boxplots of the alpha diversity estimated as Hill numbers of the gut bacterial communities along different gastrointestinal tract segments, feces, and cloaca of *Sceloporus grammicus*. Taxonomic alpha diversity (a–c) and functional alpha diversity (d–f) were calculated at diversity orders $q = 0$ (a,d), $q = 1$ (b,e), and $q = 2$ (c,f). Wilcoxon signed-rank test was used to test the significant differences ($* P < 0.05$, $** P < 0.01$, $*** P < 0.001$).

among GIT segments, feces, and cloaca (Fig. 5d–f). The greatest functional diversity at all q orders was found in the rectum, feces, and small intestine, followed by the stomach and cloaca (Wilcoxon signed-rank test, $P < 0.05$; Table S5, Supporting Information). No significant correlation was found between the alpha diversity of GIT segments with that of feces and cloaca (data not shown).

DISCUSSION

Bacterial communities retrieved by fecal and cloacal samples were compared with the bacterial communities of three different GIT segments, that is, stomach, small intestine (midgut), and rectum (hindgut), of the mesquite lizard to determine which nonlethal method is more accurate to study gut microbial communities. Based on our results, both fecal and cloacal samples comprise two

nonlethal methods that provide a good approximation of the gut microbial communities. However, fecal samples outperform at ASVs level as the turnover ratio of ASVs was higher among gut segments and cloaca compared to feces, and rectum and feces shared more core bacterial communities than rectum and cloaca. We did not find a significant correlation between the taxonomic and functional alpha diversity of the bacterial communities of feces and cloaca with those in the GIT segments.

Gut bacterial composition showed that Firmicutes, Bacteroidota, and Proteobacteria were the most dominant bacterial phyla of *S. grammicus*. Previous studies with the lizard species *Sceloporus undulatus* (Bosc and Daudin, 1801) (Trevelline *et al.* 2019), *Sceloporus occidentalis* Baird and Girard, 1852 (Moeller *et al.* 2020), and *S. virgatus* (Bunker *et al.* 2022) found that the same bacterial lineages dominated their GIT. We found some differences of microbial taxa along the GIT segments, feces, and cloaca. For instance, Proteobacteria showed a higher abundance in the cloaca than in other GIT segments. This result was consistent with those obtained in birds (Grond *et al.* 2020), oviparous lizards (Bunker *et al.* 2022), and amphibians (Zhou *et al.* 2020), where the cloaca had a higher frequency of this bacterial phylum. Given that Proteobacteria are considered facultative anaerobes (Moon *et al.* 2018), their high abundance in the cloaca could be associated with its semi-aerobic conditions (Grond *et al.* 2018). In the small intestine, rectum, and feces, the most abundant phyla were Firmicutes and Bacteroidota, similar to previous studies in birds (Videvall *et al.* 2017; Grond *et al.* 2020), snakes (Tang *et al.* 2019), and amphibians (Zhou *et al.* 2020). It is widely established that the GIT of most vertebrates is dominated by Firmicutes and Bacteroidota, and these bacterial phyla are capable of degrading complex molecules such as polysaccharides and proteins (Flint *et al.* 2012; Colston & Jackson 2016; Grond *et al.* 2018).

At the genus level, *Bacteroides, Hafnia-Obesumbacterium*, *Pseudomonas*, *Alistipes*, and *Helicobacter* were the most abundant bacterial genera in the mesquite lizard gut, as reported for other reptiles (Hong *et al.* 2011; Colston *et al.* 2015; Tang *et al.* 2019; Fong *et al.* 2020; Montoya-Ciriaco *et al.* 2020). The frequency of *Pseudomonas* was higher in the stomach and small intestine, which is consistent with other studies in snakes (Tang *et al.* 2019) and birds (Zhang *et al.* 2017). In snakes, for instance, it has been argued that *Pseudomonas* bacteria can break down proteins (Tang *et al.* 2019). The genus *Bacteroides* was found in all sampled sections, as reported along the GIT in other vertebrate groups (Tang *et al.* 2019; Zhou *et al.* 2020; Lkhagva *et al.* 2021). *Bacteroides* species are known to degrade complex polysaccharides (Flint *et al.* 2012). Their high abundance along the GIT in this insectivorous lizard could be related to the degradation of chitin, a polysaccharide found in the exoskeletons of insects. The genus *Lachnoclostridium* was well represented in the small intestine, rectum, and feces. Members of the Clostridia class and Clostridiaceae family were detected in insectivorous bats (Banskar *et al.* 2016; Ingala *et al.* 2018) and birds (Sottas *et al.* 2021). It has been reported that members of *Clostridium*, for example, *C. beijerinckii*, have the potential to convert N-acetylglucosamine, an essential structural component of the arthropod exoskeleton, into simple molecules (Al Makishah & Mitchell 2013).

The cloacal bacterial assemblages are different from those obtained in the GIT segments. In this study, cloacal bacterial communities were dominated by Proteobacteria and *Hafnia-Obesumbacterium*, *Helicobacter, Salmonella*, and *Bacteroides* genera. The cloaca is a complex structure that collects excretory products from the digestive, urinary, and reproductive systems (Díaz-Figueroa & Mitchell 2006), which may reflect a mixture of bacteria coming from these systems (Escallón *et al.* 2019). A recent study in *S. virgatus* (Bunker *et al.* 2022) found similar cloacal bacterial communities. For instance, they reported that at the family level, Enterobacteriaceae and Helicobacteraceae displayed a high abundance in the cloacal tissue and cloacal swabs. While our study and previous works have sampled cloaca to investigate reptile bacterial communities, the role of these bacterial members in the lizard's microbiome is still largely unknown. Therefore, metagenomic analyses are needed to reveal the influence of cloacal microbiota on lizard communities.

The structure of the bacterial communities at the phylum level in the fecal and cloacal samples showed a strong relationship with those in the GIT segments ($r_s > 0.84$). The strong correlation between the bacterial communities of the feces and cloaca, and the GIT segments despite differences in composition (Fig. 1a) might be due to the fact that cloacal swabs were taken just after defecating or that the factors affecting the composition of the bacterial communities in the gut are acting similarly on the bacterial communities in feces and cloaca in spiny lizards. A recent work on ostriches (*Struthio camelus*) found that the correlations of both fecal and cloacal samples with ileal and cecal samples were weak, whereas the correlations with colon were stronger and even more so with the fecal samples ($r_s = 0.56$) (Videvall *et al.* 2017). A possible explanation for the strong correlation between feces and rectum is that fecal matter formation and temporary storage prior to elimination takes place in the large intestine

(Zaher *et al.* 2012). Thus, in terms of composition, the fecal bacterial communities might resemble the actual hindgut microbiota. We cannot discard fecal contamination in the rectum, since we collected mucosal and luminal microbiota from the GIT segments. Given that reptiles often defecate once a day (Kohl *et al.* 2017), the digestive tract was empty or nearly empty after defecation. As such, the gut microbiota analyzed belonged mostly to the mucosal and not the luminal environment. Luminal and mucosal-associated microbiota are different as reported in previous studies (e.g. Kohl *et al.* 2019). In bats, the mucosal microbiota is closely related to intrinsic factors, such as the immune system, whereas the luminal microbiota is shaped primarily by dietary intake (e.g. Ingala *et al.* 2018). More studies are needed to elucidate how microbial communities might differ in the two intestinal environments of lizards.

At the ASVs level, the lowest turnover ratio was found between the bacterial communities of the rectum and feces, followed by the ratio between small intestine and feces, whereas turnover ratio between gut segments and cloaca outweighed more than 80%. Our results indicated that approximately 40% of the typical ASVs found in the rectum and 30% found in the small intestine were observed in the feces. Considering the core bacterial biota, the rectum and feces also shared more bacterial ASVs than the small intestine and feces. It is important to notice that although several genera were shared among the cloaca, rectum, and small intestine, only a few ASVs were shared among these structures. Hence, we cannot discard the possibility that the precipitation method used with the swabs to increase the yields of DNA might have affected this result. Multivariate analysis grouped bacterial communities from the same individual rather than sampling sections. This finding is interesting since previous studies in wild house mice have demonstrated that host-specific differences influence gut microbiota composition (Linnenbrink *et al.* 2013; Suzuki *et al.* 2019), which could be related to little genetic variation among individuals. Further genetic analyses are probably needed to test this hypothesis in the mesquite lizard.

The alpha diversity of the gut bacterial communities varied among GIT segments, feces, and cloaca. For instance, the stomach and cloaca had a lower effective number of total, frequent, and dominant ASVs than the feces, whereas the rectum and small intestine had a higher diversity. These differences in microbial alpha diversity were also reported for other vertebrate organisms. In ostriches, the caecum, colon, and feces had a higher alpha diversity than the cloaca and ileum (Videvall *et al.* 2017). Our findings also showed a reduced diversity and specialized microbiota in the cloaca, and the same phenomenon has been observed in birds (Zhang *et al.* 2017) and lizards (Martin *et al.* 2010; Bunker *et al.* 2022). A likely explanation is that in vertebrates, mucus secretion with immune cells produced by the mucosal surface (e.g. cloacal mucosa) can alter and select the most appropriate microbial communities for the host (Zarepour *et al.* 2013), which may reduce cloacal bacterial diversity. Furthermore, the complexity of the physiological functions that converge in the cloaca exposes its microbiota to different selective pressures such as a semi-aerobic environment, antimicrobial molecules, and exogenous microorganisms transmitted during copulation (White *et al.* 2011; Grond *et al.* 2018). Although cloaca displayed low bacterial diversity, some commensal bacteria detected in the cloaca of *S. grammicus* (e.g. *Hafnia-Obesumbacterium* and *Alistipes*) could provide beneficial effects to their hosts. For instance, in wild birds, it has been proven that cloacal bacterial assemblage was associated with phenotypic quality of hosts (Ruiz-Rodríguez *et al.* 2009). The diversity of the bacterial communities in the stomach was lower than in the two intestinal segments and no ASVs were shared between the stomach, and the small intestine and rectum. This might be explained by the conditions in the stomach, such as a low pH that chemically digests food and a higher oxygen content, that differ from those in the intestine segments (Grond *et al.* 2018). Notably, we did not find a relationship between the alpha diversity of bacterial communities of the GIT segments and feces and cloaca. This implies that the factors defining the diversity of the gut microbial communities in the spiny lizards do not affect the local diversity in the feces and cloaca. Hence, studies attempting to test hypotheses regarding changes in alpha diversity may not adequately be tested with fecal or cloacal samples.

In summary, our findings indicate that nonlethal sampling of the feces and cloaca reflect changes in the bacterial taxonomic assemblages while feces better represent the bacterial communities of the intestinal segments at a single nucleotide variation level in the spiny lizards than cloacal swabs. However, when possible, we recommend the validation of these nonlethal methods independently in other species of lizards. Our results provide valuable information, and further studies are required to elucidate whether fecal and cloacal microbial communities fully represent gut microbiota in the intestinal mucosa or in other GIT segments, such as mouth and esophagus, two semi-aerobic environments which may affect gut microbiota composition.

ACKNOWLEDGMENTS

The authors thank Dr. Luc Dendooven for his valuable comments on the manuscript, and Estación Científica La Malinche and Centro Tlaxcala de Biología de la Conducta for access and logistic support. This research was funded by Consejo Nacional de Ciencia y Tecnología (CONACyT), Ciencia de Frontera (project number: 137748), Infraestructura (project number: 205945), and the Cátedras CONACyT program (project number: 883). M.H. received a Ph.D. scholarship number: 967648 and S.H-.P. a postdoctoral grant number: 929602 by CONACyT. This article is a requirement for obtaining a Ph.D. degree of the first author.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Alberdi A, Aizpurua O, Bohmann K, Zepeda-Mendoza ML, Gilbert MTP (2016). Do vertebrate gut metagenomes confer rapid ecological adaptation? *Trends in Ecology and Evolution* **31**, 689–99.

Alberdi A, Gilbert MTP (2019). Hilldiv: An R package for the integral analysis of diversity based on Hill numbers. *BioRxiv*, https://doi.org/10.1101/545665.

Al Makishah NH, Mitchell WJ (2013). Dual substrate specificity of an N-acetylglucosamine phosphotransferase system in *Clostridium beijerinckii*. *Applied and Environmental Microbiology* **79**, 6712–8.

Banskar S, Mourya DT, Shouche YS (2016). Bacterial diversity indicates dietary overlap among bats of different feeding habits. *Microbiological Research* **182**, 99–108.

Belkaid Y, Hand TW (2014). Role of the microbiota in immunity and inflammation. *Cell* **157**, 121–41.

Bolyen E, Rideout JR, Dillon MR *et al.* (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology* **37**, 852–7.

Borreli L, Minichino A, Pace A, Dipineto A, Fioretti A (2020). Fecal sample collection method for wild birds-associated microbiome research: perspectives for wildlife studies. *Animals (Basel)* **10**, 1349.

Bunker ME, Martin MO, Weiss SL (2022). Recovered microbiome of an oviparous lizard differs across gut and reproductive tissues, cloacal swabs, and feces. *Molecular Ecology Resources* **22**, 1693–705.

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AM, Holmes SP (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods* **13**, 581–3.

Caporaso JG, Kuczynski J, Stombaugh J *et al.* (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**, 335–6.

Chao A, Gotelli NJ, Hsieh TC *et al.* (2014). Rarefaction and extrapolation with Hill numbers: A framework for sampling and estimation in species diversity studies. *Ecological Monographs* **84**, 45–67.

Colston TJ, Noonan BP, Jackson CR (2015). Phylogenetic analysis of bacterial communities in different regions of the gastrointestinal tract of *Agkistrodon piscivorus*, the cottonmouth snake. *PLoS ONE* **10**, e0128793.

Colston TJ, Jackson CR (2016). Microbiome evolution along divergent branches of the vertebrate tree of life: what is known and unknown. *Molecular Ecology* **25**, 3776–800.

Díaz de la Vega-Pérez AH, Barrios-Montiel R, Jiménez-Arcos VH, Bautista A, Bastiaans E (2019). High-mountain altitudinal gradient influences thermal ecology of the mesquite lizard (*Sceloporus grammicus*). *Canadian Journal of Zoology* **97**, 659–68.

Díaz-Figueroa O, Mitchell MA (2006). Gastrointestinal anatomy and physiology. In: Mader DR, ed. *Reptile Medicine and Surgery*, 2nd edn. Saunders Elsevier, St. Louis, MO, pp. 145–62.

Donaldson GP, Lee SM, Mazmanian SK (2016). Gut biogeography of the bacterial microbiota. *Nature Reviews Microbiology* **14**, 20–32.

Escallón C, Belden LK, Moore IT (2019). The cloacal microbiome changes with the breeding season in a wild bird. *Integrative Organismal Biology* **1**, oby009.

Fernandes AD, Macklaim JM, Linn TG, Reid G, Gloor GB (2013). ANOVA-like differential expression (ALDEx) analysis for mixed population RNA-seq. *PLoS ONE* **8**, e67019.

Flint HJ, Scott KP, Duncan SH, Louis P, Forano E (2012). Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* **3**, 289–306.

Fong JJ, Sung YH, Ding L (2020). Comparative analysis of the fecal microbiota of wild and captive beal's eye turtle (*Sacalia bealei*) by 16S rRNA gene sequencing. *Frontiers in Microbiology* **11**, 570890.

Gilbert SF, Bosch TC, Ledón-Rettig C (2015). Eco-Evo-Devo: developmental symbiosis and developmental

plasticity as evolutionary agents. *Nature Reviews Genetics* **16**, 611–22.

Gloor GB, Macklaim JM, Pawlowsky-Glahn V, Egozcue JJ (2017). Microbiome datasets are compositional: And this is not optional. *Frontiers in Microbiology* **8**, 2224.

Grond K, Guilani H, Hird SM (2020). Spatial heterogeneity of the shorebird gastrointestinal microbiome. *Royal Society Open Science* **7**, 191609.

Grond K, Sandercock, BK, Jumpponen A, Zeglin LH (2018). The avian gut microbiota: Community, physiology and function in wild birds. *Journal of Avian Biology* **49**, e01788.

Hammerson GA, Vazquez-Díaz J, Quintero-Díaz GE (2007). *Sceloporus grammicus*. In: *The IUCN Red List of Threatened Species*. https://doi.org/10.2305/IUCN.UK.2007.RLTS.T64107A12744035.en.

Herlemann DPR, Labrenz M, Jürgens K, Bertilsson S, Waniek JJ, Andersson AF (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *The ISME Journal* **5**, 1571–9.

Hervé H (2022). RVAideMemoire: Testing and Plotting Procedures for Biostatistics. R Package Version 0.9-80. https://CRAN.Rproject.org/package=RVAideMemoire.

Hong PY, Wheeler E, Cann IKO, Mackie RI (2011). Phylogenetic analysis of the fecal microbial community in herbivorous land and marine iguanas of the Galápagos islands using 16S rRNA-based pyrosequencing. *The ISME Journal* **5**, 1461–70.

Ingala MR, Simmons NB, Wultsch C, Krampis K, Speer KA, Perkins SL (2018). Comparing microbiome sampling methods in a wild mammal: fecal and intestinal samples record different signals of host ecology. *Frontiers in Microbiology* **9**, 803.

Jiménez-Cruz E, Ramírez-Bautista A, Marshall JC, Lizana-Avia M, Nieto-Montes de Oca A (2005). Reproductive cycle of *Sceloporus grammicus* (Squamata: Phrynosomatidae) from Teotihuacan, state of Mexico. *The Southwestern Naturalist* **50**, 178–87.

Kohl KD, Brun A, Caviedes-Vidal E, Karasov WH (2019). Age-related changes in the gut microbiota of wild House Sparrow nestlings. *International Journal of Avian Science* **161**, 184–91.

Kohl KD, Brun A, Magallanes M *et al.* (2017). Gut microbial ecology of lizards: insights into diversity in the wild, effects of captivity, variation across gut regions and transmission. *Molecular Ecology* **26**, 1175–89.

Langille MGI, Zaneveld J, Caporaso JG *et al.* (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology* **31**, 814–21.

Lemos-Espinal JA, Ballinger RE (1995). Ecology of growth of the high altitude lizard *Sceloporus grammicus* on the eastern slope of Iztaccihuatl volcano, Puebla, Mexico. *Transactions of the Nebraska Academy of Sciences* **22**, 77–85.

Leyte-Manrique A, Ramírez-Bautista A (2010). Diet of two populations of *Sceloporus grammicus* (Squamata: Phrynosomatidae) from Hidalgo, Mexico. *The Southwestern Naturalist* **55**, 98–103.

Li D (2018). hillR: taxonomic, functional, and phylogenetic diversity and similarity through Hill Numbers. *Journal of Open Source Software* **3**, 1041.

Linnenbrink M, Wang J, Hardouin EA, Künzel S, Metzler D, Baines JF (2013). The role of biogeography in shaping diversity of the intestinal microbiota in house mice. *Molecular Ecology* **22**, 1904–16.

Lkhagva E, Chung HJ, Hong J *et al.* (2021). The regional diversity of gut microbiome along the GI tract of male C57BL/6 mice. *BMC Microbiology* **21**, 44.

Martin MO, Gilman FR, Weiss SL (2010). Sex-specific asymmetry within the cloacal microbiota of the striped plateau lizard, *Sceloporus virgatus*. *Symbiosis* **51**, 97–105.

McMurdie PJ, Holmes S (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **8**, e61217.

Miller BM, Liou MJ, Lee J-Y, Bäumler AJ (2021). The longitudinal and cross-sectional heterogeneity of the intestinal microbiota. *Current Opinion in Microbiology* **63**, 221–30.

Moeller AH, Ivey K, Cornwall MB *et al.* (2020). The lizard gut microbiome changes with temperature and is associated with heat tolerance. *Applied and Environmental Microbiology* **86**, e01181.

Montoya-Ciriaco N, Gómez-Acata S, Muñoz-Arena LC *et al.* (2020). Dietary effects on gut microbiota of the mesquite lizard *Sceloporus grammicus* (Wiegmann, 1828) across different altitudes. *Microbiome* **8**, 6.

Moon CD, Young W, Maclean PH, Cookson AL, Bermingham EN (2018). Metagenomic insights into the roles of Proteobacteria in the gastrointestinal microbiomes of healthy dogs and cats. *MicrobiologyOpen* **7**, e00677.

Oksanen J, Blanchet FG, Friendly M *et al.* (2020). Vegan: Community Ecology Package, V.2.5-7. https://cran.r-project.org/package=vegan.

Palarea-Albaladejo J, Martín-Fernández JA (2015). zCompositions—R package for multivariate imputation of left-censored data under a compositional approach. *Chemometrics and Intelligent Laboratory Systems* **143**, 85–96.

Parada-Vanegas D, De la Fuente MK, Landskron G *et al.* (2019). Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Frontiers in Immunology* **10**, 277.

R Core Team (2021). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Ruiz-Rodríguez M, Soler JJ, Lucas FS *et al.* (2009). Bacterial diversity at the cloaca relates to an immune response in magpie *Pica pica* and to body condition of great spotted cuckoo *Clamator glandarius* nestlings. *Journal of Avian Biology* **40**, 42–8.

Sottas C, Schmiedová L, Kreisinger J *et al.* (2021). Gut microbiota in two recently diverged passerine species: evaluating the effects of species identity, habitat use and geographic distance. *BMC Ecology and Evolution* **21**, 41.

Stanley D, Geier MS, Chen H, Hughes RJ, Moore RJ (2015). Comparison of fecal and cecal microbiotas reveals qualitative similarities but quantitative differences. *BMC Microbiology* **15**, 51.

Suzuki TA, Phifer-Rixey M, Mack KL *et al.* (2019). Host genetic determinants of the gut microbiota of wild mice. *Molecular Ecology* **28**, 3197–207.

Tang W, Zhu G, Shi Q *et al.* (2019). Characterizing the microbiota in gastrointestinal segments of *Rhabdophis subminiatus*: Dynamic changes and functional predictions. *Microbiologyopen* **8**, e789.

Trevelline BK, MacLeod KJ, Langkilde T, Kohl KD (2019). Gestation alters the gut microbiota of an oviparous lizard. *FEMS Microbiology Ecology* **95**, fiz086.

Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI (2007). The human microbiome project. *Nature* **449**, 804–10.

Videvall E, Strandh M, Engelbrecht A, Cloete S, Cornwallis CK (2017). Measuring the gut microbiome in birds: Comparison of faecal and cloacal sampling. *Molecular Ecology Resources* **18**, 424–34.

White J, Richard M, Massot M, Meylan S (2011). Cloacal bacterial diversity increases with multiple mates: evidence of sexual transmission in female common lizards. *PLoS ONE* **6**, e22339.

Zaher M, El-Ghareeb AW, Hamdi H, Essa E, Lahsik S (2012). Anatomical, histological and histochemical of the reptilian alimentary canal to their food habits: I. *Uromastyx aegyptiaca*. *Life Science Journal* **9**, 84–104.

Zarepour M, Bhullar K, Montero M *et al.* (2013). The mucin Muc2 limits pathogen burdens and epithelial barrier dysfunction during *Salmonella enterica* serovar Typhimurium colitis. *Infection and Immunity* **81**, 3672–83.

Zhang Y, Simon SE, Johnson JA, Allen MS (2017). Spatial microbial composition along the gastrointestinal tract of captive Attwater's prairie chicken. *Microbial Ecology* **73**, 966–77.

Zhou J, Nelson TM, Rodriguez Lopez C, Sarma RR, Zhou SJ, Rollins LA (2020). A comparison of nonlethal sampling methods for amphibian gut microbiome analyses. *Molecular Ecology Resources* **20**, 844–55.

Zúñiga-Vega JJ, Méndez de la Cruz FR, Cuellar O (2008). Demography of the lizard *Sceloporus grammicus* exploring temporal variation in population dynamics. *Canadian Journal of Zoology* **86**, 1397–409.

SUPPLEMENTARY MATERIALS

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supporting Information

Supporting information of method: DNA extraction protocol for gastrointestinal tract segments and fecal samples.

Table S1 Morphometric data of *Sceloporus grammicus* individuals used in this study

Table S2 Concentration of the extracted DNA from each sample and the number of reads recovered after quality checks and bioinformatic steps

Table S3 Pairwise-perMANOVA results based on Aitchison distance of the bacterial communities among gastrointestinal tract segments with the feces and cloaca of *Sceloporus grammicus*. Bold text indicates a P-value < 0.05

Table S4 Wilcoxon signed-rank test comparison of Hill numbers to measure alpha taxonomic diversity between gastrointestinal tract segments with the feces and cloaca

of *Sceloporus grammicus*. Bold text indicates a *P*-value < 0.05.

Table S5 Wilcoxon signed-rank test comparison of Hill numbers to measure alpha functional diversity between gastrointestinal tract segments with the feces and cloaca of *Sceloporus grammicus*. Bold text indicates a *P*-value < 0.05.

Figure S1 Relative abundance of the dominant (a) phyla and (b) genera among gastrointestinal tract segments, feces and cloaca of *Sceloporus grammicus*. Box and whisker plots (median, interquartile and 10–90 percentiles) of the relative abundance and raincloud plot with the distribution among individuals (avg, average; sd, standard deviation of the mean).

Cite this article as:

Hernández M, Ancona S, Hereira-Pacheco S, Díaz de la Vega-Pérez AH, Navarro-Noya YE (2023). Comparative analysis of two nonlethal methods for the study of the gut bacterial communities in wild lizards. *Integrative Zoology* **00,** 1–16. https://doi.org/10.1111/1749-4877.12711

6. Chapter II: Is habitat more important than phylogenetic relatedness for elucidating the gut bacterial composition in sister lizard species?

Mauricio Hernández, Sergio Ancona, Aníbal H. Díaz de la Vega-Pérez, Ligia C. Muñoz-Arenas, Stephanie Hereira-Pacheco and Yendi E. Navarro-Noya. *Microbes and Environments* 2022; 37(3): ME21087. DOI: 10.1264/jsme2.ME21087. [IF: 2.6]

Since fecal bacterial communities accurately resemble the bacterial communities of the GIT (Hernández and cols. 2023), in the **Second Chapter**, we characterized the fecal bacterial communities (hereafter gut microbiota) of two closely related species, *S. aeneus* and *S. bicanthalis*, inhabiting at different elevations within La Malinche volcano, 2600 and 4150 m a.s.l. respectively. These two species are morphologically and ecologically similar (Méndez de la Cruz and cols. 2018), which renders them ideal subjects to examine interspecific differences in their bacterial assemblages. Because gut microbiota variation among populations is strongly influenced by microbial local exposure (Lankau and cols. 2012), altitudinal gradient (Montoya-Ciriaco and cols. 2020) and particular evolutionary history of species (Li and cols. 2017), we hypothesized that *S. aeneus* and *S. bicanthalis* would exhibit differences in their gut microbiota diversity and composition. Furthermore, to elucidate the influence of environmental conditions and phylogenetic relatedness on bacterial communities, we compared the core gut microbiota between *S. grammicus* and *S. aeneus* that coexist at ~2600 m a.s.l., and between *S. grammicus* and *S. bicanthalis* that coexist at ~4150 m a.s.l. in the study area. There were significant differences in diversity and composition of the gut microbiota between species, and these results support the hypothesis that gut microbiota differ among them. Gut bacterial alpha diversity was higher in *S. bicanthalis* living at ~4150 m a.s.l. compared to *S. aeneus* living at ~2600 m a.s.l., which probably implies that more diverse microbiotas in *S. bicanthalis* may contribute to a better adaptation and thrive at high-altitude regions. Additionally, core microbial community varied between *S. grammicus* with *S. aeneus* and *S. bicanthalis*, but not between closely related species *S. aeneus* and *S. bicanthalis*, suggesting that habitat conditions and evolutionary history impact on gut microbiota variation. Find more information about the published research in *Microbes and Environments* (Hernández and cols. 2022).

Microbes Environ. 37(3), 2022
https://www.jstage.jst.go.jp/browse/jsme2 doi:10.1264/jsme2.ME21087

Is Habitat More Important than Phylogenetic Relatedness for Elucidating the Gut Bacterial Composition in Sister Lizard Species?

Mauricio Hernández[1], Sergio Ancona[2], Aníbal H. Díaz De La Vega-Pérez[3]*, Ligia C. Muñoz-Arenas[4], Stephanie E. Hereira-Pacheco[5], and Yendi E. Navarro-Noya[6]*

[1]*Doctorado en Ciencias Biológicas, Centro Tlaxcala de Biología de la Conducta, Universidad Autónoma de Tlaxcala, Carretera Tlaxcala-Puebla km 1.5, 90062, Tlaxcala, Mexico;* [2]*Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, C.P., 04510, Mexico City, Mexico;* [3]*Consejo Nacional de Ciencia y Tecnología-Centro Tlaxcala de Biología de la Conducta, Universidad Autónoma de Tlaxcala, Carretera Tlaxcala-Puebla km 1.5, 90062, Tlaxcala, Mexico;* [4]*Facultad de Ingeniería Ambiental, UPAEP, Puebla, Mexico;* [5]*Estación Científica La Malinche, Centro Tlaxcala de Biología de la Conducta, Universidad Autónoma de Tlaxcala, Carretera Tlaxcala-Puebla km 1.5, 90062, Tlaxcala, Mexico; and* [6]*Laboratorio de Interacciones Bióticas, Centro de Investigación en Ciencias Biológicas, Universidad Autónoma de Tlaxcala, Carretera San Martín Texmelucan km 10.5, 90120, San Felipe Ixtacuixtla, Tlaxcala, Mexico*

(Received December 7, 2021—Accepted May 14, 2022—Published online June 30, 2022)

The gut microbiota influences the phenotype and fitness of a host; however, limited information is currently available on the diversity and functions of the gut microbiota in wild animals. Therefore, we herein examined the diversity, composition, and potential functions of the gut microbiota in three *Sceloporus* lizards: *Sceloporus aeneus*, *S. bicanthalis*, and *S. grammicus*, inhabiting different habitats in a mountainous ecosystem. The gut bacterial community of *S. bicanthalis* from alpine grasslands at 4,150 m a.s.l. exhibited greater taxonomic, phylogenetic, and functional alpha diversities than its sister species *S. aeneus* from cornfields and human-induced grasslands at 2,600 m a.s.l. Bacteria of the genus *Blautia* and metabolic functions related to the degradation of aromatic compounds were more abundant in *S. bicanthalis* than in *S. aeneus*, whereas *Oscillibacter* and predicted functions related to amino acid metabolism and fermentation were more abundant in *S. aeneus*. The structure of the dominant and most prevalent bacteria, i.e., the core microbiota, was similar between the sister species from different habitats, but differed between *S. grammicus* and *S. aeneus* cohabiting at 2,600 m a.s.l. and between *S. grammicus* and *S. bicanthalis* cohabiting at 4,150 m a.s.l. These results suggest that phylogenetic relatedness defines the core microbiota, while the transient, *i.e.*, non-core, microbiota is influenced by environmental differences in the habitats. Our comparisons between phylogenetically close species provide further evidence for the specialized and complex associations between hosts and the gut microbiota as well as insights into the roles of phylogeny and ecological factors as drivers of the gut microbiota in wild vertebrates.

Key words: gut microbiota, mountain ecosystem, related species, reptile microbiome, wild lizard

The gut microbiota strongly influences the health of its vertebrate hosts via energy and nutrient acquisition (Matsuyama *et al.*, 2019) or protection against pathogens, either by competing against pathogenic microbes or by boosting the host's immune system (Belkaid and Hand, 2014). Importantly, the gut microbiota shapes the phenotype of the host, and, thus, plays a critical role in how natural populations respond to environmental conditions (Alberdi *et al.*, 2016). Nevertheless, the majority of research on the composition and functions of the vertebrate gut microbiota have focused on mammals, and predominantly on humans and captive mammalian populations (Colston and Jackson, 2016). Therefore, limited information is currently available on the composition and functions of the gut microbiota in wild vertebrate populations.

Non-avian reptiles are taxonomically very diverse (Uetz and Hošek, 2021), and are widely distributed and play important ecological functions in their habitats (Pereira de Miranda, 2017); however, research on the composition and functions of their gut microbial communities is in its infancy. Only a few studies have examined the wild and captive reptilian gut microbiota, and the findings obtained showed that several factors may influence gut microbiota variations in this group of animals, *e.g.*, climate change (Bestion *et al.*, 2017), an altitudinal gradient (Zhang *et al.*, 2018; Montoya-Ciriaco *et al.*, 2020), gestation (Trevelline *et al.*, 2019), diet and captivity (Kohl *et al.*, 2017), multiple mating (White *et al.*, 2011), and phylogeny and ecomorphism (Ren *et al.*, 2016).

Coevolution between gut microbial communities and hosts has been documented in mammals (Li *et al.*, 2017; Ingala *et al.*, 2018), reptiles (Scheelings *et al.*, 2020), and birds (Sottas *et al.*, 2021), with the general pattern being that

* Corresponding authors.
Yendi E. Navarro-Noya:
E-mail: nyendi@hotmail.com, yendiebenezer.navarro.n@uatx.mx;
Tel: +52–2484815482; Fax: +52–2484815482.
Aníbal H. Díaz de la Vega-Pérez: E-mail: anibal.helios@gmail.com;
Tel: +52–2464621557; Fax: +52–2464621557.

Citation: Hernández, M., Ancona, S., Díaz de la Vega-Pérez, A. H., Muñoz-Arenas, L. C., Hereira-Pacheco, S. E., and Navarro-Noya, Y. E. (2022) Is Habitat More Important than Phylogenetic Relatedness for Elucidating the Gut Bacterial Composition in Sister Lizard Species?. *Microbes Environ* **37**: ME21087.
https://doi.org/10.1264/jsme2.ME21087

the gut microbiota is more similar in closely related species than among distantly related species. Nevertheless, similarities in the composition of the gut microbiota of phylogenetically close species may not be unambiguously dissociated from ecological similarities between hosts. A previous study reported that the gut bacterial composition did not significantly differ between sympatric populations of closely related species of the deer mice *Peromyscus leucopus* and *P. maniculatus gracilis*, which have similar diets (Baxter *et al.*, 2015). These findings raised the question as to whether this was due to ecological similarities rather than phylogenetic relatedness between host species (see Sottas *et al.*, 2021 for a similar example in the nightingale birds *Luscinia megarhynchos* and *L. luscinia*).

Reptiles provide striking examples of the complex relationships between the composition of the gut microbiota and the ecology and phylogeny of hosts. Small dietary variations may explain differences in the gut microbiota compositions and structures of two *Liolaemus* lizard species (*Liolaemus parvus* and *L. ruibali*; Kohl *et al.*, 2017). Similarly, fine-scale exposure to different local pools of microbial species resulted in differences in the gut microbial communities of the land iguanas *Conolophus subcristatus* and *C. pallidus* cohabiting the Galápagos islands (Hong *et al.*, 2011; Lankau *et al.*, 2012). Variations in gut bacterial communities were detected between two species of anoles, *Anolis cristatellus* and *A. sagrei*, which exhibit convergent trunk-ground ecomorphs (Ren *et al.*, 2016). However, further research on other reptiles is needed to confirm the generality of these patterns.

In the present study, we used 16S rRNA gene sequencing to compare the taxonomic, phylogenetic, and functional diversities of the fecal bacterial biota (hereafter referred to as the gut microbiota) of two closely related lizard species of the genus *Sceloporus* (*Phrynosomatidae*): the oviparous lizard *Sceloporus aeneus* Wiegmann, 1828, and the viviparous lizard *S. bicanthalis* Smith, 1937 inhabiting the volcano La Malinche (4,460 m a.s.l.) in the Trans-Mexican Volcanic Belt. These sister species diverged from their common ancestor ~5.5 million years ago (Wiens *et al.*, 2013), and exhibit similar morphologies and body sizes (snout to vent length 51–59 mm). Both species are terrestrial and inhabit grasslands (Méndez de la Cruz *et al.*, 2018), their maximum average lifespan is approximately one year (Rodríguez-Moreno, 2004), and they are generalist insectivorous (Canseco-Márquez and Gutiérrez-Mayén, 2010; Cruz-Elizalde *et al.*, 2021). In La Malinche, these lizard species occupy contrasting habitats. *S. aeneus* is mainly found in cornfields, human-induced grasslands, and shrubs located at 2,600 m a.s.l., with a mean air temperature of 13.20±6.69°C and mean relative humidity of 66.68±22.09% (Domínguez-Godoy *et al.*, 2020). In contrast, *S. bicanthalis* is mainly found in alpine grasslands located at 4,150 m a.s.l., where mean air temperature is 6.02±4.7°C and mean relative humidity is 67.74±29.93% (Domínguez-Godoy *et al.*, 2020).

After the gut microbiota of *S. aeneus* was shown to differ from that of *S. bicanthalis* despite them being sister species, we compared the core gut microbiota of these species with that of another member of the genus *Sceloporus*, the mesquite lizard *S. grammicus* Wiegmann, 1828, which coexists with both species in the studied sites. *S. grammicus* is an insectivorous lizard with arboreal and saxicolous habits that lives at 2,300–4,400 m a.s.l. in La Malinche (Díaz de la Vega-Pérez *et al.*, 2019a). We speculated that if the core gut bacterial composition differs between *S. grammicus* and the two other *Sceloporus* species, then differences in the gut bacterial composition between *S. aeneus* and *S. bicanthalis* may be attributed to species identity (Sottas *et al.*, 2021) rather than differences in the ecological conditions to which these lizards are subject. Data on the core gut microbiota of *S. grammicus* were taken from Montoya-Ciriaco *et al.* (2020).

Materials and Methods

Ethics statement

The sampling and handling of lizards complied with ethical and legal regulations in Mexico to conduct research on wild organisms, as stipulated in the Norma Oficial Mexicana (NOM-126-ECOL-2000). Permission for the sampling and handling of lizards was granted by the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT, Mexico) under the collecting permits SGPA/DGVS/15396/15 and SGPA/DGVS/007736/20.

Study area and sampling

La Malinche is an eroded stratovolcano situated in the Mexican states of Tlaxcala and Puebla (N 19°, 14′ W 98°02′). This volcano is mainly covered by cornfields, shrubs, and herbaceous plants (low-zone at 2,600 m a.s.l.), coniferous (*Pinus* spp. and *Abies* spp.) and oak (*Quercus* spp.) forests (medium-zone at 3,200 m a.s.l.), and rocky alpine grassland and shrubs of *Juniperus monticola* (high-zone at 4,150 m a.s.l.) (Domínguez-Godoy *et al.*, 2020). Lizards were sampled in February 2020 at different elevations: 9 individuals of *S. aeneus* were collected at 2,600 m a.s.l. (19°12′ N, 97°55′ W) and 9 of *S. bicanthalis* at 4,150 m a.s.l. (19°14′ N, 98°01′ W). Lizards were captured by hand between 0900 and 1600 h. Each captured specimen was transported individually to the La Malinche Scientific Station, located at 3,100 m a.s.l. (19°14′ N, 97°59′ W), for fecal sampling. Lizards were housed individually in sanitized cages and maintained at 20–25°C until they naturally defecated. The base of each cage was covered with a sterile paper sheet and fecal samples were collected with sterile forceps. Fecal samples were placed in separate 1.5-mL sterile polypropylene tubes, stored and transported into a cooler with ice (<4°C), and then kept at –20°C until DNA extraction. All lizards were released alive in good physical condition at the site at which they were captured.

DNA isolation and library preparation

DNA was extracted from feces using two different methods of cell lysis and pooled as previously described by Montoya-Ciriaco *et al.* (2020). DNA quality was verified by electrophoresis through 1% agarose gels. Amplification of the V3–V4 region of the 16S rRNA gene was performed using the 341F (5′-CCTACGGGNGGC WGCAG-3′) and 805R (5′-ACHVGGGTATCTAATCC-3′) primers (Herlemann *et al.*, 2011) modified with adapters for the Illumina sequencing platform. The thermal cycling conditions of PCR were as follows: denaturation at 95°C for 2 min, followed by 28 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s, and a final extension at 72°C for 5 min. A negative control was included in each PCR to detect reagent contamination. PCR was performed in triplicate, pooled, purified using the FastGene Gel/PCR Extraction Kit (Nippon Genetics), quantified using a NanoDrop 3300 fluorospectrometer (Thermo Fisher Scientific) with the PicoGreen dsDNA assay (Invitrogen),

and combined at equal molar concentrations. Sequencing was conducted by Macrogen with 300-bp PE MiSeq runs (Illumina). Raw sequence databases are available at the Sequence Read Archive (SRA) from the NCBI under the project number PRJNA816478.

Bioinformatic analysis

A sequencing data analysis was performed using the open-source software QIIME. Demultiplexing was conducted with QIIME v1.9.1 (Caporaso *et al.*, 2010). Sequences were imported into QIIME2 v2021.4.0 (Bolyen *et al.*, 2019). Denoising, quality filtering, trimming, paired-end sequence merging, dereplication in Amplicon Sequence Variants (ASVs), and chimera filtering were performed with the DADA2 plugin (Callahan *et al.*, 2016). Standard filtering parameters (maxEE=2, truncQ=2, p-pooling-method=pseudo) were applied to forward and reverse reads, and forward reads were trimmed to 260 nt and reverse to 200 nt. Query sequences (rep-set) were assigned taxonomically with classify-sklearn with a Naive Bayes supervised learning algorithm using the trained SILVA 16S rRNA gene database version v.138.1. Organellar 16S rRNA sequences, *i.e.*, from mitochondria and chloroplasts, were eliminated. After these filtering steps, samples with fewer than 1,000 reads were eliminated from the dataset. To construct the phylogeny for the calculation of phylogenetic diversity, the rep-set was aligned with MAFFT (Katoh and Standley, 2013) and a rooted maximum likelihood tree was built using IQ-TREE multicore version 2.0.3 (Minh *et al.*, 2019) with the best substitution model for our dataset as selected with the ModelFinder algorithm, *i.e.*, the GTR+F+R10 model. The potential functions of the microbiome were investigated with PICRUSt2. The rep_set and a reference database of genomes from the Integrated Microbial Genomes database were aligned with hidden Markov models to insert ASVs into a reference tree. Genome predictions were performed with a hidden-state algorithm. Pathway abundance based on Enzyme Classification number (EC number) abundance was inferred with MetaCyc (Caspi *et al.*, 2016).

Statistical analysis

Downstream statistical analyses were performed within the R environment (R Core Team, 2020). We used Hill numbers to measure true taxonomic, phylogenetic, and functional alpha diversities at different q diversity orders: q=0 corresponds to the total number of ASVs or species richness, q=1 corresponds to frequent ASVs and is equivalent to the exponential of the Shannon entropy, and q=2 corresponds to dominant ASVs and is equivalent to the reciprocal of the Simpson index (Chao *et al.*, 2014; Alberdi and Gilbert, 2019). Taxonomic, functional, and phylogenetic alpha diversities were obtained using the *hillR* R package (Ma and Li, 2018). Taxonomic alpha diversity was calculated with the frequency table of ASVs. Functional diversity was assessed as the mean functional diversity per species (MD_q), which calculates the effective sum of pairwise distances between a fixed species and all other species using the frequency table of ASVs (community) and that of EC numbers (functional traits). Hill numbers for phylogenetic diversity incorporate the tree's branching pattern, the relative branch lengths, and the relative abundance of each node/branch, and the unit of measurement is the effective total branch length (Chao *et al.*, 2010). A non-parametric Mann-Whitney-Wilcoxon test was implemented to detect significant differences in alpha diversities between *S. aeneus* and *S. bicanthalis*. Adjusted P-values were considered to be significant at P<0.05.

Due to the compositional nature of the microbiome data, we applied a centered-log-ratio transformation "*clr*" to the frequency table of ASVs, which makes the data symmetric and linearly related, with the *ALDEx2* R package (Gloor *et al.*, 2017). A Robust Aitchison Principal Component Analysis (RPCA), which is a proper distance metric for compositional data (Aitchison *et al.*, 2000), was obtained to examine variations in bacterial community assemblages, and these differences were assessed using a permutational multivariate analysis of variance (perMANOVA) with 999 permutations using the *vegan* R package (Oksanen *et al.*, 2017). An ANOVA-like Differential Expression (ALDEx) analysis was used to examine differences in the abundance of taxonomic groups and EC numbers among *S. aeneus* and *S. bicanthalis* with the *ALDEx2* R package. Raw counts were used as an input and Monte Carlo Dirichlet instances of *clr* transformation values were generated with the function "*aldex.clr*". To test for differences in abundance between bacterial taxa, a Mann-Whitney-Wilcoxon test was conducted using the function "*aldex.ttest*". A Benjamini-Hochberg sequential correction was applied to the resulting P-value. Heatmaps of differentially abundant taxa and functions were constructed with the *ComplexHeatmap* R package (Gu *et al.*, 2016).

We compared the gut bacterial community structure of the two populations of *S. grammicus* sampled by Montoya-Ciriaco *et al.* (2020) with *S. aeneus* and *S. bicanthalis*: one population coexisting with *S. aeneus* at 2,600 m a.s.l., and another population coexisting with *S. bicanthalis* at 4,150 m a.s.l. Sequences from the gut bacterial communities of both populations of *S. grammicus* were obtained from the NCBI (https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA544140). To reduce the bias of comparing two different datasets, the two fasta files of the representative sequences were clustered with a closed-reference clustering method at a similarity threshold of 97% using VSEARCH within QIIME2 and against the Greengenes 16S rRNA gene database version 13_8 (http://greengenes.lbl.gov/Download/). The resulting OTUs were taxonomically assigned with classify-sklearn and frequency tables of taxonomic compositions at the genus level were used for further analyses. It was necessary to use the core gut bacterial communities instead of the whole bacterial communities of the gut, which include both the core microbiota and non-core microbiota, because samples were collected in different years (*S. grammicus* was sampled in 2015; *S. aeneus* and *S. bicanthalis* were sampled in 2020). The core gut microbiota is more stable over time than the non-core gut microbiota (Huse *et al.*, 2012) and, thus, comparisons of the core microbiota allowed us to reduce the potential confounding effect of interannual variations in the composition of gut bacterial communities. Core gut bacterial communities were defined as bacterial genera with a prevalence >55% in the samples of each species. The resulting frequency table that contained the samples from *S. aeneus*, *S. bicanthalis*, and *S. grammicus* at the genus level was *clr* transformed, and perMANOVA and PCA were performed as described above for the comparisons of interest. A Venn diagram was constructed with the *VennDiagram* R package (Chen and Boutros, 2011). A network was built to show the co-occurrence patterns of the core bacterial genus between the three lizard species using the *NetCoMi* R package (Peschel *et al.*, 2021). Zeros from the observation matrix were replaced with pseudocounts with a predefined value of 0.5 and data was *clr* transformed. Correlations (edges) between nodes (core bacterial genera) were obtained with the *SparCC* function (≥0.3) (Friedman and Alm, 2012). The adjacency matrix was obtained with the function "*graph_from_adjacency_matrix*" from the *igraph* R package (Csardi and Nepusz, 2006). Clusters, components, and hubs were identified based on a fast greedy modularity optimization algorithm. Components with unconnected nodes were removed from the network for visualization. The R scripts for the statistical analysis may be found at GitHub (https://github.com/Steph0522/Sceloporus_species).

Results

Eighteen fecal samples were used to characterize the gut bacterial communities of *S. aeneus* (n=9) and *S. bicanthalis* (n=9), and resulted in 107,919 good quality sequences (min frequency=1300, max frequency=14503; Table S1). Sixty-one fecal samples from *S. grammicus* (n=38 collected at 2,600 m a.s.l. and n=23 collected at 4,150 m a.s.l.) were

used to compare the core gut bacterial communities between *S. grammicus* and *S. aeneus* and between *S. grammicus* and *S. bicanthalis*.

Alpha diversity of gut bacterial communities

Across all gut bacterial communities, 886 ASVs were identified with an average of 136 ASVs per sample. Except for phylogenetic diversity at q=2, the taxonomic, phylogenetic, and functional diversities of gut bacterial communities at q=1 and 2 were higher in *S. bicanthalis* than in *S. aeneus* (P<0.05; Fig. 1B–I and Table S2). Taxonomic, phylogenetic, and functional richness were similar in both species.

Taxonomic compositions and structures of gut bacterial communities

The gut bacterial communities of *S. aeneus* and *S. bicanthalis* contained 11 bacterial phyla with *Bacteroidota* (42.55±14.50%) being the most abundant, followed by *Firmicutes* (40.71±13.04%), *Proteobacteria* (11.75±15.09%), *Desulfobacterota* (2.16±1.67%), and *Verrucomicrobiota* (2.06±2.32%). The remaining six bacterial phyla had a relative abundance <1% (Fig. 2A). The most abundant genera across all samples were, in decreasing order, *Bacteroides* (19.18±8.48), *Odoribacter* (11.81±5.23), *Parabacteroides* (8.67±6.04), *Hafnia-Obesumbacterium* (7.45±16.47), *Alistipes* (6.77±5.81), [Eubacterium] (3.47±2.70), *Roseburia* (2.95±6.72), and *Akkermansia* (2.62±2.87) (Supplementary Fig. S1). The most abundant genera were also the most prevalent. The core gut bacterial microbiota of *Sceloporus* spp. comprised *Bacteroides*, *Parabacteroides*, *Odoribacter*, *Hafnia-Obesumbacterium*, and *Alistipes*, but also included *Lachnospiraceae*, *Oscillibacter*, *Blautia*, *Akkermansia*, and *Desulfovibrio* (Supplementary Fig. S1). A differential abundance analysis with Aldex revealed that *Oscillibacter* and *Blautia* were differentially abundant genera (considering an effect size >|0.8|) between *S. aeneus* and *S. bicanthalis* (effect size of –0.86 and 0.91, respectively), with *Oscillibacter* being more abundant in *S. aeneus* and *Blautia* in *S. bicanthalis* (Fig. 2B). An ordination analysis separated the gut bacterial communities of *S. aeneus* from those of *S. bicanthalis* (Fig. 2C). Accordingly, perMANOVA showed a significant difference in the gut bacterial community structure of *S. aeneus* and *S. bicanthalis* (F=1.869, df=1, P<0.001, adjusted R^2=0.103; Table S3).

Prediction of metabolic functions

A total of 1,851 functional genes were predicted and annotated. The most abundant predicted functions across all samples were related to nucleic acid processing. Fifteen functions were different (considering an effect size >|0.8|) between *S. aeneus* and *S. bicanthalis* (Fig. 3A). Functions related to amino acid synthesis and fermentation were more abundant in *S. aeneus* than in *S. bicanthalis*, whereas metabolic functions associated with the degradation of aromatic compounds were more abundant in *S. bicanthalis*. However, none of these predicted functions were significantly different. The ASVs identified as *Hafnia-Obesumbacterium*

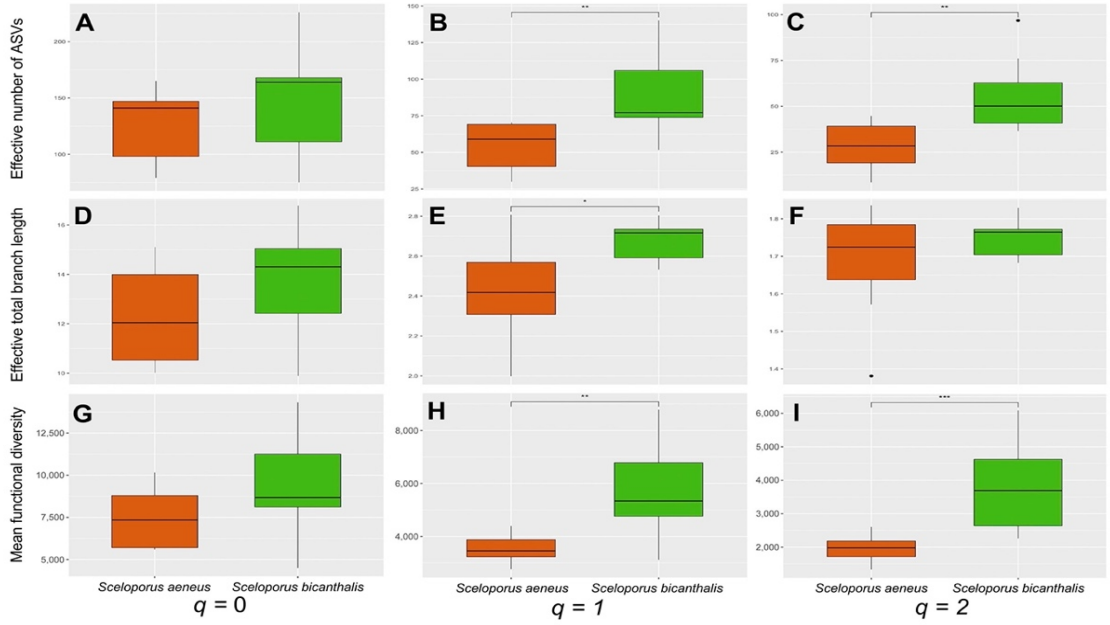

Fig. 1. Box and whisker plots (medians, interquartiles, 10–90% percentiles) of the true alpha diversity estimated as Hill numbers of gut bacterial communities of two *Sceloporus* lizard species inhabiting a high-mountain ecosystem. Taxonomic alpha diversity (A, B, C), phylogenetic alpha diversity (D, E, F), and functional alpha diversity (G, H, I) were calculated at diversity orders q=0 (A, D, G), q=1 (B, E, H), and q=2 (C, F, I). Significant differences among lizard species were tested by the Mann-Whitney-Wilcoxon test.

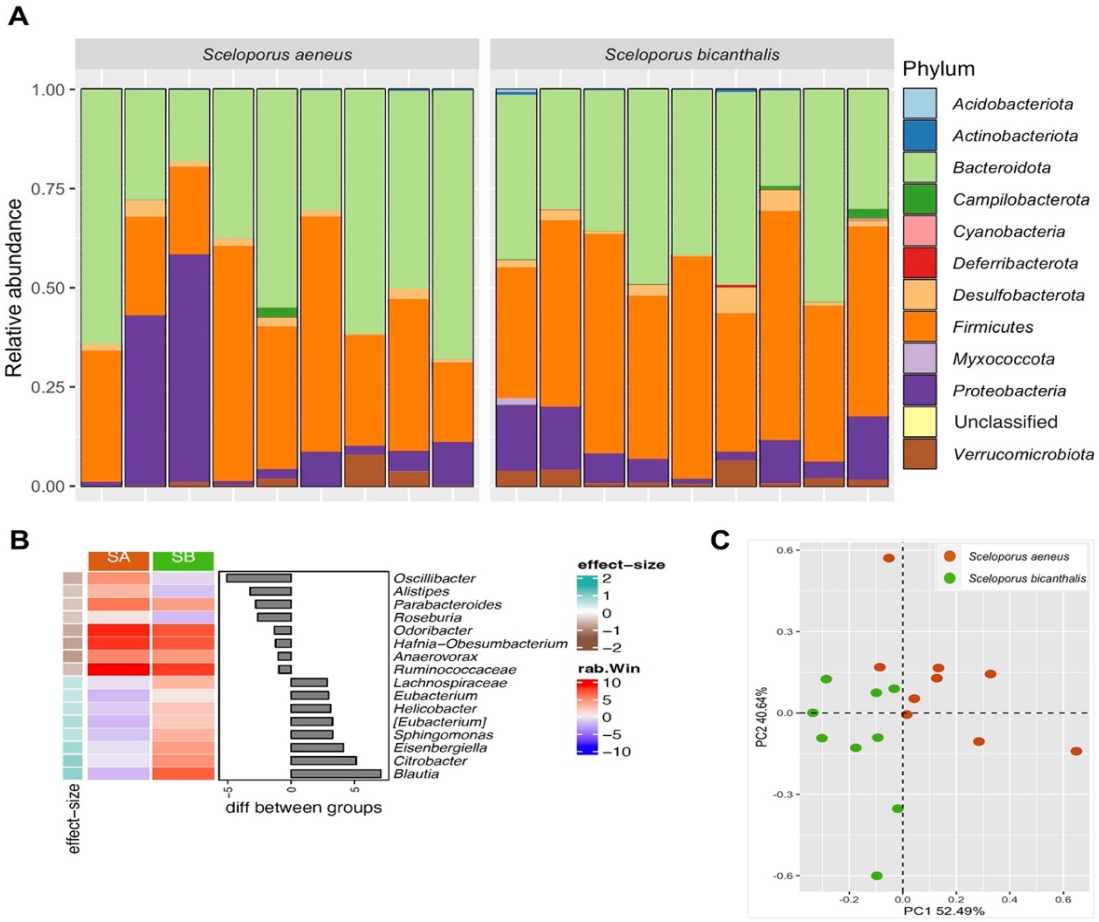

Fig. 2. Taxonomic compositions of gut bacterial communities of two *Sceloporus* lizard species inhabiting a high-mountain ecosystem. (A) Bar plot of individual relative abundance at the phylum level, (B) heatmaps with comparisons between *Sceloporus aeneus* (SA) and *Sceloporus bicanthalis* (SB) of the median *clr* value of the 15 most abundant genera, as assessed by an ANOVA-like differential expression tool for compositional data. Bar plots represent the median difference between species, and (C) comparisons of the gut bacterial communities of the *Sceloporus* species from this study by a Robust Principal Component analysis (RPCA).

and *Serratia* equally contributed to amino acid biosynthesis pathways (Fig. 3B). Meanwhile, ASVs belonging to 11 different genera contributed to the degradation of aromatic compounds.

Comparison of core gut bacterial communities between Sceloporus *species*

Based on the result showing that the gut microbiota of *S. aeneus* was different from that of *S. bicanthalis*, we compared the core gut bacterial biota of these species with that of *S. grammicus*, which coexists with both species at the studied sites (Fig. 4A). The core gut bacterial communities of *Sceloporus* members were separated by species in the ordination analysis (Fig. 4B). Similarly, the population of *S. grammicus* coexisting with *S. aeneus* at 2,600 m a.s.l. (Fig. 4C) and *S. grammicus* and *S. bicanthalis* at 4,150 m a.s.l. (Fig. 4D) were separated by species in the ordination analysis. The core gut bacterial communities of *S. grammicus* sampled at two different elevations did not significantly differ from each other ($P>0.05$; Table S3), and neither did the core bacterial genera of *S. aeneus* and *S. bicanthalis* ($P>0.05$; Table S3). Core gut bacterial communities were significantly different between *S. grammicus* and *S. aeneus* at 2,600 m a.s.l. and between *S. grammicus* and *S. bicanthalis* at 4,150 m a.s.l. ($P<0.05$; Table S3). Nine core bacterial genera were shared between the three lizard species, *S. bicanthalis* and *S. aeneus* shared 11 genera, while *S. bicanthalis* had four unique genera and *S. aeneus* had none. *S. grammicus* shared six bacterial genera with both sister species and had six unique genera (Fig. 5A). A co-occurrence network analysis clustered core genera into two components. One of them positively connected

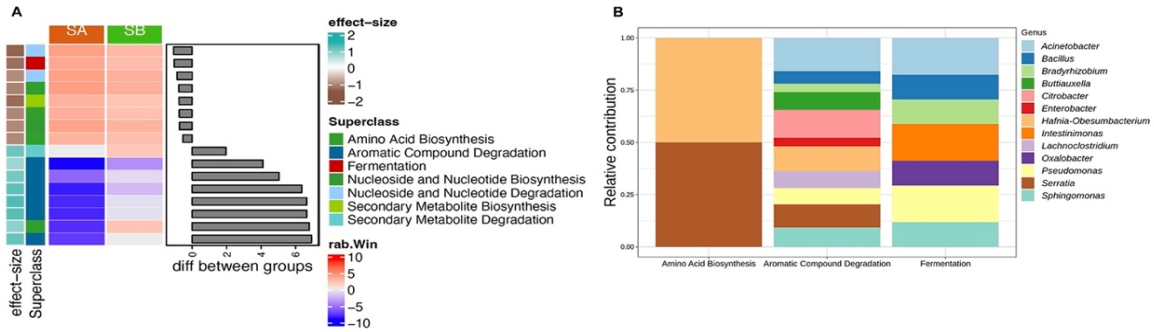

Fig. 3. Predicted functions of *Sceloporus aeneus* (SA) and *Sceloporus bicanthalis* (SB). Functional predictions were examined with the ancestral reconstruction algorithm in PICRUSt2. (A) Differentially abundant functions with an effect size >|0.8| as selected by an ANOVA-like differential expression tool for compositional data and Benjamini-Hochberg sequential correction. Median *clr* values (rab.Win) and the effect size were plotted as heatmaps and the median difference of *clr* values between species as bar plots. (B) Bar plots with the relative contribution of bacterial genera to the differential predicted functional pathways.

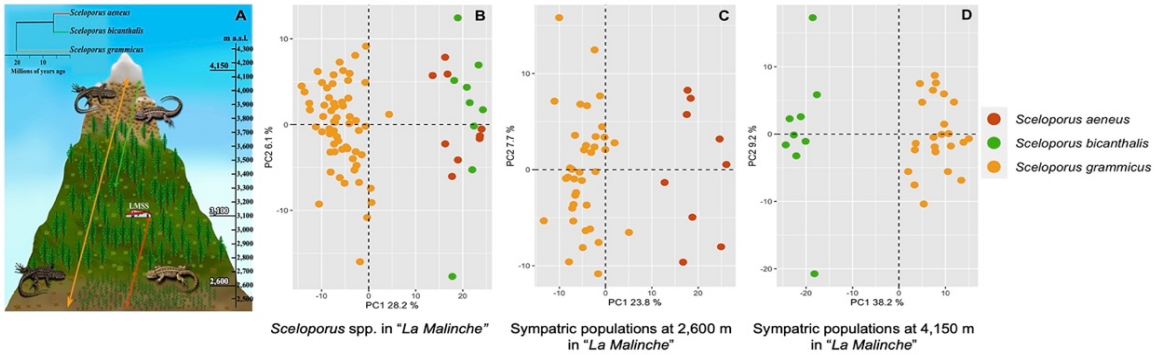

Fig. 4. Comparisons of gut bacterial communities of *Sceloporus* species inhabiting a high-mountain ecosystem. (A) Schematic representation of the distribution within La Malinche and phylogenetic relatedness: the lines depict the altitudinal distribution of *Sceloporus grammicus* (orange), *S. aeneus* (red), and *S. bicanthalis* (green). The phylogenetic tree of the three lizard species was represented based on Wiens *et al.* (2013). (B) Principal Component Analysis (PCA) plots using Aitchison dissimilarities of the gut bacterial communities of the three *Sceloporus* lizard species inhabiting the study area, (C) sympatric populations of the *Sceloporus* species at 2,600 m a.s.l., and (D) sympatric populations of the *Sceloporus* species at 4,150 m a.s.l. in La Malinche. The La Malinche Scientific Station (LMSS) is located at 3,100 m a.s.l.

Eubacterium, *Holdemania*, *Bacteroides*, *Parabacteroides*, *Coprococcus*, and *Dorea* among others, which co-occur in the three lizard species, and the other connected those co-occurring mostly in *S. grammicus* (*Akkermansia*, *Serratia*, *Oscillospira*, *Clostridium*, and *Roseburia* were positively connected and *Ruminococcus*, *Blautia*, and *Sphingomonas* were negatively connected to *Oscillospira*) (Fig. 5B). Both components included members of *Ruminococcaceae* and *Lachnospiraceae*. *Lachnospiraceae* and *Odoribacter* nodes mostly connected the components in the network; therefore, they were identified as hubs. The whole network had a clustering coefficient of 0.53, positive edge percentage of 69.4, and modularity of 0.32.

Discussion

The present study revealed differences in the composition of the gut microbiota between two closely related lizard species of the genus *Sceloporus* that feed on insects and exhibit similar body sizes and terrestrial habits, but inhabit grasslands with contrasting temperatures and vegetation compositions located at different elevations in La Malinche. *S. bicanthalis*, living in alpine grasslands located at 4,150 m a.s.l. with an average temperature of 6.0°C, exhibited greater taxonomic, phylogenetic, and functional alpha diversities in its gut bacterial community than *S. aeneus*, which inhabits cornfields, human-induced grasslands, and shrubs located at 2,600 m a.s.l. with an average temperature of 13.2°C. We infer that these differences are mainly driven by non-core bacterial communities and are likely due to differences in food resources.

Habitats impose different environmental conditions on host species and affect gut bacterial diversity

Specimens of *S. bicanthalis* living at 4,150 m a.s.l. must cope with low atmospheric oxygen concentrations, high levels of ultraviolet radiation, and low temperature and humidity levels (Díaz de la Vega-Pérez *et al.*, 2019a; Domínguez-Godoy *et al.*, 2020). These limiting conditions are associated with increased metabolic rates in lizards

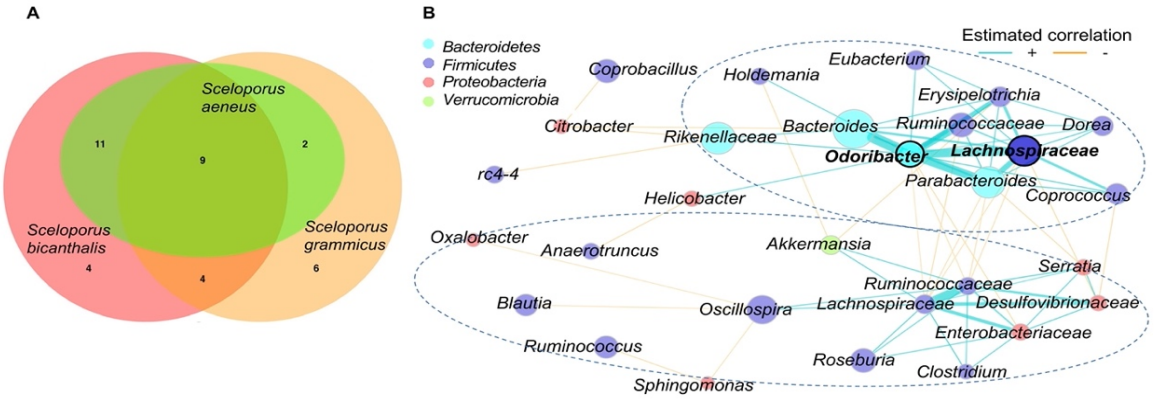

Fig. 5. Core gut bacterial communities of *Sceloporus aeneus*, *S. bicanthalis*, and *S. grammicus*. (A) Venn diagram showing the shared and unique core genera of the lizard species and (B) the co-occurrence network of the core-bacterial genera. Nodes represent the bacterial genera and edges show the degree of correlations as obtained with SparCC (≥0.3). Edges in blue are positive correlations and those in orange are negative. Sub-communities, *i.e.*, components, were identified based on a fast greedy modularity optimization algorithm (only components with more than two connected nodes are shown). Nodes with thick black borders were hubs within clusters, *i.e.*, nodes with a connection degree larger than the third quantile within a cluster. Ellipses delimit the components.

(Yuni *et al.*, 2015; Plasman *et al.*, 2020), which are required in order to maintain an optimal energetic balance under these conditions (Yuni *et al.*, 2015). Diverse gut microbial communities, particularly short-chain fatty acid-producing bacteria (*e.g.*, *Blautia*, Eubacterium, and *Lachnospiraceae*) that predominate in the gut of *S. bicanthalis*, allow them to satisfy their physiological or energy demands in the challenging environments they occupy at 4,150 m a.s.l. (Zhang *et al.*, 2016; Wu *et al.*, 2020). In marked contrast to *S. bicanthalis*, specimens of *S. aeneus* were captured at 2,600 m a.s.l., where temperatures are warmer and oxygen availability is higher, and, thus, energy requirements may be lower and diverse gut bacterial communities less important.

Higher diversity in the gut microbiota in *S. bicanthalis* than in *S. aeneus* may also be attributed to parallel differences in diet breadth because high diversity in gut bacterial communities is related to broad diets in reptiles (Hong *et al.*, 2011). However, differences in diet breadth are unlikely to explain the present results. *S. bicanthalis* living at 4,150 m a.s.l. may be exposed to a lower diversity of insect prey than *S. aeneus* living at 2,600 m a.s.l because insect diversity has been reported to slightly decrease at high elevations elsewhere (McCoy, 1990) and also in La Malinche, as suggested by the number of *Arthropoda* families found in the feces of *S. grammicus* at 4,150 m a.s.l. being 10-fold lower than in those living at 2,600 m a.s.l. (Montoya-Ciriaco *et al.*, 2020). Nevertheless, diet breadth needs to be estimated for *S. aeneus* and *S. bicanthalis* living at different elevations in order to assess its role as a driver of differences in gut microbiota compositions between these lizard species. The availability of bacterial inoculums acquired from insect prey may be a plausible explanation for the differences observed in gut microbiota compositions between *S. aeneus* and *S. bicanthalis*. Grasslands inhabited by *S. bicanthalis* at 4,150 m a.s.l. in La Malinche are less accessible and, thus, less perturbed by human activities (Díaz de la Vega-Pérez *et al.*, 2019b), whereas *S. aeneus* living in cornfields and human-induced grasslands and shrubs at 2,600 m a.s.l. is exposed to agrochemicals, including pesticides and chemical fertilizers, which are frequently used to promote growth and protect crops from insects and competitor weeds (García-Juárez *et al.*, 2019). Habitat alterations and exposure to agrochemicals may reduce the diversity of gut bacterial communities in insects (Syromyatnikov *et al.*, 2020), plants (Perazzolli *et al.*, 2014), and animals at higher trophic levels (Amato *et al.*, 2013), and, thus, insects, arachnids, and plant material occasionally eaten by *S. aeneus* (Cruz-Elizalde *et al.*, 2021) may provide less diverse bacterial inoculums than prey eaten by *S. bicanthalis* at less perturbed areas, which, in turn, may translate into differences in the diversity of the gut microbiota.

Are differences due to different species or habitats?

We compared the core gut microbiota between *S. aeneus* and *S. grammicus* at 2,600 m a.s.l. and between *S. bicanthalis* and *S. grammicus* at 4,150 m a.s.l. to establish whether differences in the gut bacterial beta diversity between *S. aeneus* and *S. bicanthalis* are due to differences in their environments rather than to differences in species-specific characteristics, such as host genetics, life history, and behavior (Sottas *et al.*, 2021). If environmental conditions are the major driver of the composition of the gut microbiota, no differences in the core gut microbiota were expected between *S. grammicus* and *S. aeneus* coexisting at 2,600 m a.s.l. or between *S. grammicus* and *S. bicanthalis* coexisting at 4,150 m a.s.l. (we are not aware of areas at which *S. bicanthalis* and *S. aeneus* coexist in La Malinche, and comparisons with *S. grammicus* were the best control we had). In addition, we compared the core microbiota between *S. aeneus* and *S. bicanthalis* to investigate whether more stable gut bacterial communities also differ between these closely related species living in different environments. The core microbiota differed between *S. grammicus* and *S. aeneus* and between *S. grammicus* and *S.*

bicanthalis, but not between *S. aeneus* and *S. bicanthalis*. These results are perplexing and imply that dissimilarities in the gut bacterial communities of *S. aeneus* and *S. bicanthalis* are mainly due to differences in non-core bacterial taxa, which are highly influenced by environmental conditions (Grieneisen *et al.*, 2017). Moreover, these results add to evidence for the core microbiota being highly conserved in sister taxa (Baxter *et al.*, 2015; Li *et al.*, 2017), and suggest that differences in overall gut bacterial communities between *S. aeneus* and *S. bicanthalis* may be partially driven by species identity (Baxter *et al.*, 2015; Sottas *et al.*, 2021) because core bacterial communities in the gut differed from those observed in coexisting specimens of *S. grammicus*. Nevertheless, species identity explained only a small part of the variance in gut microbiota compositions between *S. aeneus* and *S. bicanthalis* (R^2: 0.10), and may account for a small portion of the variance in other iguanian lizards, such as *L. parvus* and *L. ruibali* (R^2: 0.05) (Kohl *et al.*, 2017). Comparisons of the overall and core gut microbiota between sympatric populations of *S. aeneus* and *S. bicanthalis* will provide insights into the role of ecological factors and species-specific characteristics in the composition of the gut microbiota.

Differences in taxonomic and functional compositions between lizard hosts

Differences in the composition of the overall gut microbiota between *S. aeneus* and *S. bicanthalis* may be due to genetic differences that these sister species have accumulated since they diverged from their common ancestor ~5.5 million years ago (Wiens *et al.*, 2013). Similarities in their core gut microbiota may be related to a high degree of genetic similarity (Wiens *et al.*, 2010), historically convergent diets (Canseco-Márquez and Gutiérrez-Mayén, 2010), and habitat use (Méndez de la Cruz *et al.*, 2018). The gut bacterial communities of *S. aeneus* and *S. bicanthalis* were dominated by three phyla: *Bacteroidota*, *Firmicutes*, and *Proteobacteria*. These phyla are representative of the bacterial communities of many vertebrates, *e.g.*, birds (Hird *et al.*, 2015) and mammals (Ingala *et al.*, 2018), and, thus, the present results add to evidence for these bacterial phyla maintaining a close and ancient relationship with their vertebrate hosts (Colston and Jackson, 2016). Regarding bacterial genera, the abundance of *Oscillibacter* (*S. aeneus*) and *Blautia* (*S. bicanthalis*) differed between these sister lizards. This pattern is consistent with the higher prevalence of *Blautia* in humans living at high elevations (Han *et al.*, 2021), a bacterial genus associated with short-chain fatty acid production (Liu *et al.*, 2021). Furthermore, *Oscillibacter* was isolated from the gut of the Hawaiian turtle (McDermid *et al.*, 2020) and this genus has been associated with the maintenance of gut barrier integrity (Lam *et al.*, 2012).

Predicted genes involved in the degradation of aromatic compounds were more abundant in *S. bicanthalis* than in *S. aeneus*. Previous studies indicated that under extreme environmental conditions, *Sceloporus* spp. may feed on plant material. Serrano-Cardozo *et al.* (2008) detected plant material in the gastrointestinal tract of *Sceloporus* spp. in a semiarid region of Mexico, while Montoya-Ciriaco *et al.* (2020) identified considerable amounts of the genetic material of plants in the feces of *S. grammicus* in alpine-grasslands. If *S. bicanthalis* feeds on plant material, this may explain the high abundance of functions, such as the degradation of aromatic compounds, but also the large taxonomic, phylogenetic, and functional diversities of the bacterial communities in its digestive tract. In contrast, functions related to amino acid biosynthesis were more frequent in *S. aeneus* than in *S. bicanthalis*. Further research on the metabolism and diet of hosts and the actual functions of bacterial groups is needed to elucidate the underlying causes of this difference; however, one plausible explanation is that bacterial genes associated with carbohydrate and amino acid metabolism may help specimens of *S. aeneus* to process a diet richer in proteins than that of *S. bicanthalis*.

Conclusion

The present study showed that the taxonomic, phylogenetic, and functional alpha diversities of the gut microbiota were greater in *S. bicanthalis* living at 4,150 m a.s.l. than in *S. aeneus* living at 2,600 m a.s.l., which may be because more diverse gut bacterial communities allow *Sceloporus* lizards to cope with the limiting conditions that they are exposed to at high elevations (*e.g.*, low temperatures and humidity levels, low atmospheric oxygen concentrations, and high levels of ultraviolet radiation) (Zhang *et al.*, 2016). Differences in the gut microbiota between *S. aeneus* and *S. bicanthalis* appear to mainly be driven by environmentally induced changes in non-core gut bacterial communities; core gut bacterial communities are shared and well conserved in these sister taxa. Further research on the diet and metabolic requirements of *Sceloporus* lizard hosts living at different elevations, and the diversity of bacterial inoculums available in different habitats is warranted to obtain a more detailed understanding of the role of ecological factors as drivers of gut microbiota compositions in wild animals.

Funding

Funding was provided by Consejo Nacional de Ciencia y Tecnología (CONACyT): Infraestructura project number: 205945, Ciencia de Frontera project number: 137748 and Cátedras CONACyT project number: 883. MH received Ph.D. scholarship number: 967648 and S H-P postdoctoral grant number: 929602 by CONACyT. This article is a requirement for obtaining a Ph.D. degree for the first author.

Acknowledgements

The authors thank M. Martínez-Gómez, the La Malinche Scientific Station, and Centro Tlaxcala de Biología de la Conducta for access and logistic support. We thank Miguel Domínguez and Erick Gómez for their support during the fieldwork.

References

Aitchison, J., Barceló-Vidal, C., Martín-Fernández, J.A., and Pawlowsky-Glahn, V. (2000) Log-ratio analysis and compositional distance. *Math Geol* **32**: 271–275.

Alberdi, A., Aizpurua, O., Bohmann, K., Zepeda-Mendoza, M.L., and Gilbert, M.T.P. (2016) Do vertebrate gut metagenomes confer rapid ecological adaptation? *Trends Ecol Evol* **31**: 689–699.

Alberdi, A., and Gilbert, M.T. (2019) A guide to the application of Hill numbers to DNA-based diversity analyses. *Mol Ecol Resour* **19**: 804–817.

Amato, K.R., Yeoman, C.J., Kent, A., Righini, N., Carbonero, F., Estrada, A., *et al.* (2013) Habitat degradation impacts black howler monkey (*Alouatta pigra*) gastrointestinal microbiomes. *ISME J* **7**: 1344–1353.

Baxter, N.T., Wan, J.J., Schubert, A.M., Jenior, M.L., Myers, P., and Schloss, P.D. (2015) Intra- and interindividual variations mask interspecies variation in the microbiota of sympatric *Peromyscus* populations. *Appl Environ Microbiol* **81**: 396–404.

Belkaid, Y., and Hand, T.W. (2014) Role of the microbiota in immunity and inflammation. *Cell* **157**: 121–141.

Bestion, E., Jacob, S., Zinger, L., Di Gesu, L., Richard, M., White, J., *et al.* (2017) Climate warming reduces gut microbiota diversity in a vertebrate ectotherm. *Nat Ecol Evol* **1**: 0161.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G., *et al.* (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* **37**: 852–857.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., and Holmes, S.P. (2016) DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* **13**: 581–583.

Canseco-Márquez, L., and Gutiérrez-Mayén, M.G. (2010) Anfibios y reptiles del valle de Tehuacán-Cuicatlán. Primera edición, Página (in Spanish).

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**: 335–336.

Caspi, R., Billington, R., Ferrer, L., Foerster, H., Fulcher, C.A., Keseler, I.M., *et al.* (2016) The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Res* **44**: 471–480.

Chao, A., Chiu, C.H., and Jost, L. (2010) Phylogenetic diversity measures based on Hill numbers. *Philos Trans R Soc B* **365**: 3599–3609.

Chao, A., Chiu, C.H., and Jost, L. (2014) Unifying species diversity, phylogenetic diversity, functional diversity, and related similarity and differentiation measures through hill numbers. *Annu Rev Ecol Evol Syst* **45**: 297–324.

Chen, H., and Boutros, P.C. (2011) VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinf* **12**: 35.

Colston, T.J., and Jackson, C.R. (2016) Microbiome evolution along divergent branches of the vertebrate tree of life: What is known and unknown. *Mol Ecol* **25**: 3776–3800.

Cruz-Elizalde, R., Ramírez-Bautista, A., and Núñez de Cáceres-González, F.F. (2021) Sexual dimorphism and feeding ecology of the black-bellied bunchgrass lizard *Sceloporus aeneus* (Squamata: Phrynosomatidae) in Central Mexico. *South Am J Herpetol* **18**: 46–55.

Csárdi, G., and Nepusz, T. (2006) The igraph software package for complex network research. *Int J Complex Syst* **1695**: 1–9. URL https://igraph.org

Díaz de la Vega-Pérez, A.H., Barrios-Montiel, R., Jiménez-Arcos, V.H., Bautista, A., and Bastiaans, E. (2019a) High-mountain altitudinal gradient influences thermal ecology of the mesquite lizard (*Sceloporus grammicus*). *Can J Zool* **97**: 659–668.

Díaz de la Vega-Pérez, A.H., Jiménez-Arcos, V.H., Centenero-Alcalá, E., Méndez de la Cruz, F.R., and Ngo, A. (2019b) Diversity and conservation of amphibians and reptiles of a protected and heavily disturbed forest of central Mexico. *Zookeys* **830**: 111–125.

Domínguez-Godoy, M.A., Hudson, R., Pérez-Mendoza, H.A., Ancona, S., and Díaz de la Vega-Pérez, A.H. (2020) Living on the edge: lower thermal quality but greater survival probability at a high altitude mountain for the mesquite lizard (*Sceloporus grammicus*). *J Therm Biol* **94**: 102757.

Friedman, J., and Alm, E.J. (2012) Inferring correlation networks from genomic survey data. *PLoS Comput Biol* **8**: e1002687.

García-Juárez, G., Hernández-Vázquez, M., and Orozco-Bolaños, G. (2019) Aflatoxins presence and agrochemicals in stored corn: security food's risks in Tlaxcala state, Mexico. *CIBA Revista Iberoamericana De Las Ciencias Biológicas Y Agropecuarias* **8**: 106–130.

Gloor, G.B., Macklaim, J.M., Pawlowsky-Glahn, V., and Egozcue, J.J. (2017) Microbiome datasets are compositional: and this is not optional. *Front Microbiol* **8**: 2224.

Grieneisen, L.E., Livermore, J., Alberts, S., Tung, J., and Archie, E.A. (2017) Group living and male dispersal predict the core gut microbiome in wild baboons. *Integr Comp Biol* **57**: 770–785.

Gu, Z., Eils, R., and Schlesner, M. (2016) Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* **32**: 2847–2849.

Han, N., Pan, Z., Liu, G., Yang, R., and Yujing, B. (2021) Hypoxya: the "invisible pusher" of gut microbiota. *Front Microbiol* **12**: 690600.

Herlemann, D.P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J.J., and Andersson, A.F. (2011) Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J* **5**: 1571–1579.

Hird, S.M., Sánchez, C., Carstens, B.C., and Brumfield, R.T. (2015) Comparative gut microbiota of 59 neotropical bird species. *Front Microbiol* **6**: 1403.

Hong, P-Y., Wheeler, E., Cann, I.K., and Mackie, R.I. (2011) Phylogenetic analysis of the fecal microbial community in herbivorous land and marine iguanas of the Galápagos islands using 16S rRNA-based pyrosequencing. *ISME J* **5**: 1461–1470.

Huse, S.M., Ye, Y., Zhou, Y., and Fodor, A.A. (2012) A core human microbiome as viewed through 16S rRNA sequence clusters. *PLoS One* **7**: e34242.

Ingala, M.R., Simmons, N.B., Wultsch, C., Krampis, K., Speer, K.A., and Perkins, S.L. (2018) Comparing microbiome sampling methods in a wild mammal: fecal and intestinal samples record different signals of host ecology. *Front Microbiol* **9**: 803.

Katoh, K., and Standley, D.M. (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* **30**: 772–780.

Kohl, K.D., Brun, A., Magallanes, M., Brinkerhoff, J., Laspiur, A., Acosta, J.C., *et al.* (2017) Gut microbial ecology of lizards: insights into diversity in the wild, effects of captivity, variation across gut regions and transmission. *Mol Ecol* **26**: 1175–1189.

Lam, Y.Y., Ha, C.W., Campbell, C.R., Mitchell, A.J., Dinudom, A., Oscarsson, J., *et al.* (2012) Increased gut permeability and microbiota change associate with mesenteric fat inflammation and metabolic dysfunction in diet-induced obese mice. *PLoS One* **7**: e34233.

Lankau, E.W., Hong, P.Y., and Mackie, R.I. (2012) Ecological drift and local exposures drive enteric bacterial community differences within species of Galápagos iguanas. *Mol Ecol* **21**: 1779–1788.

Li, H., Qu, J., Li, T., Yao, M., Li, J., and Li, X. (2017) Gut microbiota may predict host divergence time during Glires evolution. *FEMS Microbiol Ecol* **93**: fix009.

Liu, X., Mao, B., Gu, J., Wu, J., Cui, S., Wang, G., *et al.* (2021) *Blautia*—a new functional genus with potential probiotic properties? *Gut Microbes* **13**: 1875796.

Ma, Z., and Li, L. (2018) Measuring metagenome diversity and diversity with Hill numbers. *Mol Ecol Resour* **18**: 1339–1355.

Matsuyama, M., Morrison, M., Lê Cao, K-M., Pruilh, S., Davies, P.S.W., Wall, C., *et al.* (2019) Dietary intake influences gut microbiota development of healthy Australian children from the age of one to two years. *Sci Rep* **9**: 12476.

McCoy, E.D. (1990) The distribution of insects along elevational gradients. *Oikos* **58**: 313–322.

McDermid, K.J., Kittle-Ill, R.P., Veillet, A., Plouviez, S., Muehlstein, L., and Balazs, G.H. (2020) Identification of gastrointestinal microbiota in Hawaiian green turtles (*Chelonia mydas*). *Evol Bioinf Online* **16**: 1–18.

Méndez de la Cruz, F.R., Díaz de la Vega-Pérez, A.H., Centenero-Alcalá, H., and Jiménez-Arcos, V.H. (2018) *Anfibios y Reptiles del Parque Nacional La Malinche*. San Pablo eds Monte, Mexico: Universidad Autónoma de Tlaxcala (in Spanish).

Minh, B.Q., Trifinopoulos, J., Schrempf, D., and Schmidt, H.A. (2019) IQTREE version 2.0: tutorials and manual phylogenomic software by maximum likelihood. URL http://www.iqtree.org

Montoya-Ciriaco, N., Gómez-Acata, S., Muñoz-Arenas, L.C., Dendooven, L., Estrada-Torres, A., Díaz de la Vega-Pérez, A.H., *et al.* (2020) Dietary effects on gut microbiota of the mesquite lizard *Sceloporus grammicus* (Wiegmann, 1828) across different altitudes. *Microbiome* **8**: 6.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., *et al.* (2017) Vegan: community ecology package, v. 2.4–5. URL https://cran.r-project.org/package=vegan

Perazzolli, M., Antonielli, L., Storari, M., Puopolo, G., Pancher, M., Giovannini, O., *et al.* (2014) Resilience of the natural phyllosphere microbiota of the grapevine to chemical and biological pesticides. *Appl Environ Microbiol* **80**: 3585–3596.

Pereira de Miranda, E.B. (2017) The plight of reptiles as ecological actors in the tropics. *Front Ecol Evol* **5**: 159.

Peschel, S., Müller, C.L., von Mutius, E., Boulesteix, A.L., and Depner, M. (2021) NetCoMi: network construction and comparison for microbiome data in R. *Briefings Bioinf* **22**: bbaa290.

Plasman, M., Bautista, A., McCue, M.D., and Díaz de la Vega-Pérez, A.H., (2020) Resting metabolic rates increase with elevation in a mountain-dwelling lizard. *Integr Zool* **15**: 363–374.

R Core Team (2020) R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. URL https://www.R-project.org/

Ren, T., Kahrl, A.F., Wu, M., and Cox, R.M. (2016) Does adaptive radiation of a host lineage promote ecological diversity of its bacterial communities? A test using gut microbiota of *Anolis* lizards. *Mol Ecol* **25**: 4793–4804.

Rodríguez-Moreno, F. (2004) Demografía comparada de dos especies de lacertilios emparentados del género *Sceloporus* (Sauria: Phrynosomatidae) con diferente modo reproductor. PhD Thesis, The National Autonomous University of Mexico.

Scheelings, T.F., Moore, R.J., Van, T.T.H., Klaassen, M., and Reina, R.D. (2020) Microbial symbiosis and coevolution of an entire clade of ancient vertebrates: the gut microbiota of sea turtles and its relationship to their phylogenetic history. *Anim Microbiome* **2**: 17.

Serrano-Cardozo, V.H., Lemos-Espinal, J.A., and Smith, G.R. (2008) Comparative diet of three sympatric *Sceloporus* in the semiarid Zapotitlán Valley, Mexico. *Rev Mex Biodivers* **79**: 427–434.

Sottas, C., Schmiedová, L., Kreisinger, J., Albrecht, T., Reif, J., Osiejuk, T.S., *et al.* (2021) Gut microbiota in two recently diverged passerine species: evaluating the effects of species identity, habitat use and geographic distance. *BMC Ecol Evol* **21**: 41.

Syromyatnikov, M.Y., Usuwa, M.M., Savinkova, O.V., Derevshchikova, M.I., and Popov, V.N. (2020) The effect of pesticides on the microbiome of animals. *Agriculture (Basel, Switz)* **10**: 79.

Trevelline, B.K., MacLeod, K.J., Langkilde, T., and Kohl, K.D. (2019) Gestation alters the gut microbiota of an oviparous lizard. *FEMS Microbiol Ecol* **95**: fiz086.

Uetz, P., and Hošek, J. (2021) The Reptile Database. URL http://www.reptile-database.org/

White, J., Richard, M., Massot, M., and Meylan, S. (2011) Cloacal bacterial diversity increases with multiple mates: evidence of sexual transmission in female common lizards. *PLoS One* **6**: e22339.

Wiens, J.J., Kuczynski, C.A., Arif, S., and Reeder, T.W. (2010) Phylogenetic relationships of phrynosomatid lizards based on nuclear and mitochondrial data, and a revised phylogeny for *Sceloporus*. *Mol Phylogenet Evol* **54**: 150–161.

Wiens, J.J., Kozak, K.H., and Silva, N. (2013) Diversity and niche evolution along aridity gradients in North American lizards (Phrynosomatidae). *Evolution* **67**: 1715–1728.

Wu, Y., Yao, Y., Dong, M., Xia, T., Li, D., Xie, M., *et al.* (2020) Characterisation of the gut microbial community of rhesus macaques in high-altitude environments. *BMC Microbiol* **20**: 68.

Yuni, L.P.E.K., Jones, S.M., and Wapstra, E. (2015) Energy expenditure of the spotted snow skink, *Niveoscincus ocellatus*, at two climate extremes of its distribution range. *J Therm Biol* **52**: 208–216.

Zhang, W., Li, N., Tang, X., Liu, N., and Zhao, W. (2018) Changes in intestinal microbiota across an altitudinal gradient in the lizard *Phrynocephalus vlangalii*. *Ecol Evol* **8**: 4695–4703.

Zhang, Z., Xu, D., Wang, L., Hao, J., Wang, J., Zhou, X., *et al.* (2016) Convergent evolution of rumen microbiomes in high-altitude mammals. *Curr Biol* **25**: 1873–1879.

7. Chapter III: DNA metabarcoding reveals seasonal changes in diet composition across four arthropod-eating lizard species (Phrynosomatidae: *Sceloporus*)

Mauricio Hernández, Stephanie Hereira-Pacheco, Antton Alberdi, Aníbal H. Díaz de la Vega-Pérez, Arturo Estrada-Torres, Sergio Ancona and Yendi E. Navarro-Noya. *Integrative Zoology* 2023; 00: 1-16. DOI: 10.1111/1749-4877.12755. [IF: 3.3]

It has been proven that the diet greatly influences gut microbial communities. Recent studies indicate that species with different feeding habits exhibit a distinctive gut microbial composition (Phillips and cols. 2012; Ingala and cols. 2018). Likewise, seasonal shifts in gut microbiota composition are correlated with seasonal dietary variation in wild mammal populations (Guo and cols. 2021; Fan and cols. 2022). Since previous surveys have revealed dietary seasonal variation in *Sceloporus* lizard species (Leyte-Manrique and Ramírez-Bautista 2010; Cruz-Elizalde and cols. 2020), we expected that seasonal dietary changes lead to seasonal fluctuations in their gut microbiota. It is worth noting that, to date, no study has evaluated the diet composition in *Sceloporus* species using DNA metabarcoding approach, considered as an accurate method to identify prey items at different taxonomic levels (Alberdi and cols. 2017).

Therefore, in the **Third Chapter**, using DNA metabarcoding approach, we first investigated the seasonal variation in diet composition and diversity across four *Sceloporus* lizard species (*S. aeneus*, *S. bicanthalis*, *S. grammicus* and *S. spinosus*) during the dry and rainy seasons. We predicted that dietary richness is greater during the dry season than the rainy season, since previous studies on *Sceloporus* species have reported a higher dietary diversity in the dry season compared to the rainy season (Castro-Franco et al. 2017; García-Rosales et al. 2019). Overall, our results revealed that both dietary (genus level) and phylogenetic (lineage level) richness was higher during the dry than the rainy season, which is in line with our prediction. A possible explanation for this finding is that during the dry season lizard individuals find reduced prey availability and consume alternative prey to meet their energy requirements when they emerge from their winter shelters. Turnover of seasonal diet varied among species, being significantly greater in *S. spinosus* than *S. bicanthalis*, two species occupying contrasting habitats in the study area. A broader dietary breadth was observed during the dry season in *S. bicanthalis* living at

~4150 m a.s.l. in the La Malinche volcano, and the three other species did not show seasonal differences. Detailed information is provided in the following published paper (Hernández and cols. 2023).

Integrative Zoology 2023; 0: 1–16 doi: 10.1111/1749-4877.12755

ORIGINAL ARTICLE

INTEGRATIVE ZOOLOGY

DNA metabarcoding reveals seasonal changes in diet composition across four arthropod-eating lizard species (Phrynosomatidae: *Sceloporus*)

Mauricio HERNÁNDEZ,[1] Stephanie HEREIRA-PACHECO,[2] Antton ALBERDI,[3] Aníbal H. DÍAZ DE LA VEGA-PÉREZ,[4] Arturo ESTRADA-TORRES,[2] Sergio ANCONA[5] and Yendi E. NAVARRO-NOYA[6]

[1]Doctorado en Ciencias Biológicas, Centro Tlaxcala de Biología de la Conducta, Universidad Autónoma de Tlaxcala, Tlaxcala, Mexico, [2]Estación Científica La Malinche, Centro Tlaxcala de Biología de la Conducta, Universidad Autónoma de Tlaxcala, Tlaxcala, Mexico, [3]Center for Evolutionary Hologenomics, The Globe Institute, University of Copenhagen, Copenhagen, Denmark, [4]Consejo Nacional de Ciencia y Tecnología-Centro Tlaxcala de Biología de la Conducta, Universidad Autónoma de Tlaxcala, Tlaxcala, Mexico, [5]Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, Mexico City, Mexico and [6]Laboratorio de Interacciones Bióticas, Centro de Investigación en Ciencias Biológicas, Universidad Autónoma de Tlaxcala, San Felipe Ixtacuixtla, Tlaxcala, Mexico

Abstract

Diet composition and its ecological drivers are rarely investigated in coexisting closely related species. We used a molecular approach to characterize the seasonal variation in diet composition in four spiny lizard species inhabiting a mountainous ecosystem. DNA metabarcoding revealed that the lizards *Sceloporus aeneus*, *S. bicanthalis*, *S. grammicus*, and *S. spinosus* mostly consumed arthropods of the orders Hemiptera, Araneae, Hymenoptera, and Coleoptera. The terrestrial lizards *S. aeneus* and *S. bicanthalis* mostly predated ants and spiders, whereas the arboreal–saxicolous *S. grammicus* and saxicolous *S. spinosus* largely consumed grasshoppers and leafhoppers. The taxonomic and phylogenetic diversity of the prey was higher during the dry season than the rainy season, likely because reduced prey availability in the dry season forced lizards to diversify their diets to meet their nutritional demands. Dietary and phylogenetic composition varied seasonally depending on the species, but only dietary composition varied with altitude. Seasonal dietary turnover was greater in *S. spinosus* than in *S. bicanthalis*, suggesting site-specific seasonal variability in prey availability; no other differences among species were observed. *S. bicanthalis*, which lives at the highest altitude in our study site, displayed interseasonal variation in diet breadth. Dietary differences were correlated with the species' feeding strategies and elevational distribution, which likely contributed to the coexistence of these lizard species in the studied geographic area and beyond.

Key words: arthropod-eating lizards, diet breadth, environmental barcoding, mountainous ecosystem, seasonal dietary shifts

Correspondence: Sergio Ancona, Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, 04510, Mexico City, Mexico.
Email: sergio.ancona@iecologia.unam.mx
Yendi E. Navarro-Noya, Laboratorio de Interacciones Bióticas, Centro de Investigación en Ciencias Biológicas, Universidad Autónoma de Tlaxcala, Carretera San Martín Texmelucan km 10.5, 90120, San Felipe Ixtacuixtla, Tlaxcala, Mexico.
Email: nyendi@hotmail.com

INTRODUCTION

Dietary analyses provide key information on species interactions, trophic webs, and ecosystem functioning (Nielsen *et al.* 2017). Vertebrate diets are dynamic and can be influenced by several factors that affect prey availability, including seasonal and distributional changes in rainfall, humidity, and ambient temperature (Rubolini *et al.* 2003; Goodyear & Pianka 2011). These factors, alone or in combination, may lead to dietary changes at the individual or population level, which can be evident at different spatial and temporal scales (Goodyear & Pianka 2011).

Arthropod-eating lizards are widely distributed, occupy a diversity of habitats, and are exposed to broad environmental variation (Wiens *et al.* 2013). This makes them ideal subjects to investigate dietary changes driven by ecological factors. For instance, the number and relative frequency of prey eaten by the rough lizard *Sceloporus horridus* Wiegmann, 1834 (Castro-Franco *et al.* 2017) and the red minor lizard *S. minor* Cope, 1885 (García-Rosales *et al.* 2019) vary by season and habitat. Interestingly, closely related species that coexist locally may exhibit different feeding habits or foraging strategies, which allow them to exploit different food sources and reduce interspecific competition. For example, sympatric populations of the congeneric teiid lizards *Cnemidophorus abaetensis* Dias, Rocha & Vrcibradic, 2002 and *C. ocellifer* (Spix, 1825) showed marked differences in their main prey, despite having minor differences in their daily activity patterns and microhabitat use (Dias & Rocha 2007). However, this may not be widely generalizable; sympatric populations of other arthropod-eating lizards, such as *Psammodromus algirus* (Linnaeus, 1758) and *Podarcis vaucheri* (Boulenger, 1905) ingested similar prey (Mamou *et al.* 2016). Specialization in habitat use plays an important role in prey selection and, consequently, in dietary differences among cohabiting species. For example, microhabitat segregation between species may reduce diet overlap. This is the case among lizards of the genera *Agama* and *Acanthocercus*, in which ground-dwelling lizards mostly feed on ants, whereas rock-dwelling lizards primarily feed on flying insects (Tan *et al.* 2021).

The richness and abundance of insects in lizard diets often exhibit temporal and spatial variability. For instance, the dietary composition of four lizard species of the genus *Ctenotus* that coexist in the Great Victoria Desert varied considerably over time and among sampling locations (Goodyear & Pianka 2011). Similar spatial and temporal variation in dietary composition has been documented in *Sceloporus* lizards. For example, *S. horridus* (Castro-Franco *et al.* 2017) and *S. minor* (García-Rosales *et al.* 2019) consumed a higher total number of prey items during the dry season than during the rainy season, and diet composition differed between lizards from pine-oak forests and those from xerophilous scrub. Furthermore, it has been documented that the taxonomic richness of insect communities often decreases with altitude (McCoy 1990; Joseph *et al.* 2019 but see Widhiono *et al.* 2017), and this association can be reflected by the diet of lizards. Recent analyses revealed differences in dietary composition among lizards of *S. grammicus* sampled at 2600, 3100, and 4150 m above sea level (m a.s.l.) on the La Malinche Volcano, where the number of Arthropoda families at the lowest sampling site was roughly 10-fold higher than at the highest sampling site (Montoya-Ciriaco *et al.* 2020). However, the availability and richness of arthropods consumed by lizards were found to be positively associated with elevation in other areas. For example, in *P. algirus* in the Sierra Nevada massif (Spain), prey availability and diet breadth increased with elevation between 300 and 2500 m a.s.l. (Moreno-Rueda *et al.* 2018). Hence, the influence of ecological factors such as elevation, habitat type, or seasonality on diet composition can differ among lizard species, among regions, or across altitudinal gradients.

High-throughput DNA sequencing offers a powerful tool for dietary studies in wild populations (Pompanon *et al.* 2012; Nielsen *et al.* 2017) because it offers greater taxonomic resolution than microscopic analyses of stomach contents and has the potential to analyze multiple samples in parallel (Gil *et al.* 2020). Despite the great potential of this methodology to improve our knowledge of diet and its major determinants, only a few studies have used DNA metabarcoding to estimate diet composition in reptiles (e.g. Kartzinel & Pringle 2015; Pereira *et al.* 2019; Gil *et al.* 2020).

In this study, we analyzed the diet of four arthropod-eating lizard species belonging to the genus *Sceloporus* (*S. aeneus* Wiegmann, 1828, *S. bicanthalis* Smith, 1937, *S. grammicus* Wiegmann, 1828, and *S. spinosus* Wiegmann, 1828) inhabiting a mountainous ecosystem in central Mexico. Using DNA metabarcoding of the mitochondrial gene cytochrome C oxidase subunit I (COI), we compared the diet composition of these species between the dry and rainy seasons of the year 2020. Additionally, we evaluated the suitability of DNA metabarcoding versus visual inspection of stomach contents for diet characterization by comparing the results of our molecular analysis to visual inspection data previously published by Cruz-Elizalde *et al.* (2020) in *S. aeneus*. The

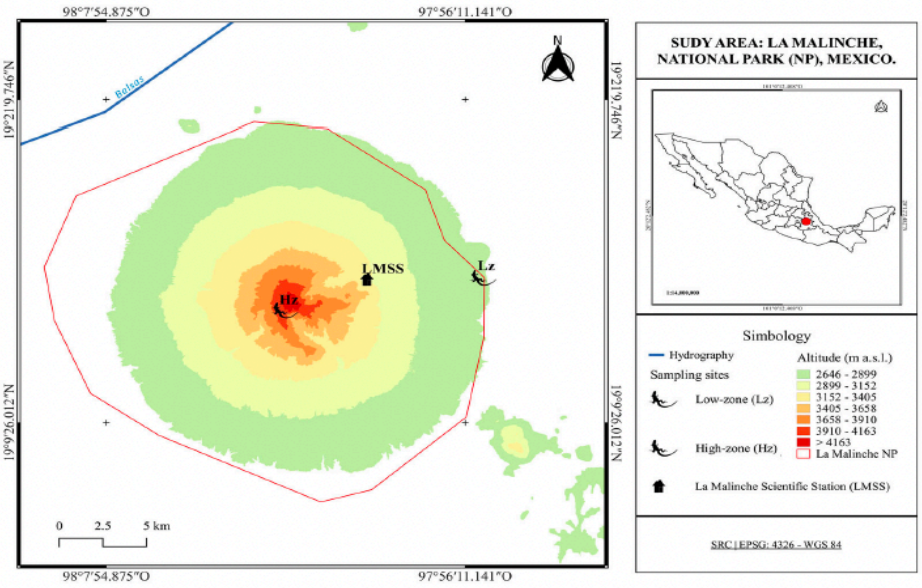

Figure 1 Map of the study area. Topographic slopes are shaded from pale green to pale red. Individuals were captured in two different sites in La Malinche National Park: 2600 m a.s.l. (Lz: low-zone) and 4150 m a.s.l. (Hz: high-zone). Lizards were transported to La Malinche Scientific Station located at 3100 m a.s.l. (Mz: medium-zone) to collect fecal samples.

four focal lizard species differ in their ecology and life histories, which may result in differences in their diets. *Sceloporus aeneus* and *S. bicanthalis* are two closely related terrestrial species, which occupy grasslands (Méndez de la Cruz *et al.* 2018) and have an average lifespan of 1 year (Rodríguez-Romero *et al.* 2002). *Sceloporus grammicus* is arboreal and saxicolous (Domínguez-Godoy *et al.* 2020); inhabits various habitats, including low and montane shrublands, forests, and deserts (Lemos-Espinal *et al.* 1998); and has a lifespan of 4 years (Ortega-Rubio *et al.* 1999). *Sceloporus spinosus* is predominantly saxicolous and occurs in arboreal, rocky, and xeric landscapes (Torres Barragán *et al.* 2020) and can live up to 5 years (Méndez de la Cruz *et al.* 2018). Previous studies have shown that *S. grammicus* and *S. aeneus* mainly feed on Formicidae, Coleoptera, Hymenoptera, Orthoptera, and Hemiptera (Leyte-Manrique & Ramírez-Bautista 2010; Cruz-Elizalde *et al.* 2020), but there is no published information on the diet of *S. bicanthalis* and *S. spinosus*. Our study aims to fill this knowledge gap and provide insights into the diet composition of related and/or cohabiting lizard species using molecular methods.

MATERIALS AND METHODS

Ethical statement

This study was approved by the Mexican "Secretaría de Recursos Naturales y Medio Ambiente" (SEMARNAT) under animal capture and biological sample collection permit number SGPA/DGVS/007736/20. All field and laboratory procedures were performed in accordance with the ethical guidelines of the Official Mexican Norm NOM-126-ECOL-2000.

Study area and sample collection

Fieldwork was conducted on the La Malinche Volcano (Fig. 1), a protected area located in the states of Tlaxcala and Puebla. This area has a temperate subhumid climate with an annual average rainfall of 800 mm and an annual average temperature of 15°C (Montoya *et al.* 2004). The dry season occurs between November and April, and the rainy season spans from May to October (Gay-García *et al.* 2004). La Malinche belongs to

Table 1 Number of samples collected from the four *Sceloporus* lizard species during the dry and rainy seasons

	Samples collected					Samples amplified and sequenced				
	Dry season		Rainy season			Dry season		Rainy season		
Species	♂	♀	♂	♀	Total samples	♂	♀	♂	♀	Total samples
Sceloporus aeneus	5	4	6	7	22	2	3	3	5	13
S. bicanthalis	5	4	8	7	24	3	2	6	5	16
S. grammicus	7	8	9	4	28	6	6	8	3	23
S. spinosus	8	3	6	4	21	5	3	6	2	16
Total	25	19	29	22	95	16	14	23	15	68

Symbols (♂) and (♀) represent male and female, respectively.

the Mexican Transvolcanic Belt and is the sixth-highest mountain in Mexico, with an elevation of 4461 m (Díaz de la Vega-Pérez *et al.* 2019). The vegetation varies and the temperature decreases with increasing altitude. At ~2600 m (the lowest elevation sampled in this study), the predominant vegetation types are cornfields, human-induced grasslands, and herbaceous plants. At ~3100 m (mid-elevation, near the La Malinche Scientific Station), the volcano is mainly covered by coniferous (*Pinus* spp. and *Abies* spp.) and oak (*Quercus* spp.) forests. Near the summit of the volcano at ~4150 m, alpine rocky grasslands and shrubs of *Juniperus monticola* predominate (Domínguez-Godoy *et al.* 2020).

We collected fecal samples from *Sceloporus* lizards for DNA extraction during the dry season (February) and rainy season (October) of 2020. Lizards were caught by hand or noosing during their daily activity period, between 0900 and 1600 hours (Méndez de la Cruz *et al.* 2018) at two sampling sites. Individuals of *S. aeneus*, *S. grammicus*, and *S. spinosus* were captured at ~2600 m a.s.l. (19°12′N, 97°55′W), whereas specimens of *S. bicanthalis* were captured at ~4150 m a.s.l. (19°14′N, 98°01′W). The number of specimens collected per lizard species, sex, and season are shown in Table 1. Lizards were categorized as adults according to their snout–vent length (SVL) measurements: *S. aeneus* > 45 mm (Manríquez-Morán *et al.* 2013), *S. bicanthalis* > 46 mm (Gribbins *et al.* 2011), *S. grammicus* > 44 mm (Jiménez-Cruz *et al.* 2005), and *S. spinosus* > 70 mm (Méndez de la Cruz *et al.* 2013). Morphometric data are presented in Table S1, Supporting Information. Lizards were transported in cloth bags to La Malinche Scientific Station, located at 3100 m a.s.l., where they were individually housed in plastic containers (30 cm length × 20 cm width × 15 cm height) that were previously disinfected with 70% alcohol for fecal sample collection. No food was provided at any time to avoid biasing our results. We collected a single fecal sample per individual. Immediately upon defecation, fresh fecal samples were collected using sterile tweezers and transferred into sterile 1.5-mL tubes. Samples were transported in a cooled icebox at 4°C to the laboratory in Tlaxcala city and stored at −20°C until DNA extraction. All lizards were released unharmed at their capture sites 12–24 h after fecal sampling.

DNA extraction and library preparation

The procedures used to isolate DNA from the fecal samples are detailed in Hernández *et al.* (2023). We used the primers mlCOIintF (5′-GGWACWGGWT GAACWGTWTAYCCYCC-3′) (Leray *et al.* 2013) and jgHCO2198 (5′-TAIACYTCI GGRTGICCRAARA AYCA-3′) (Geller *et al.* 2013) to amplify the mitochondrial gene COI, which is considered a suitable molecular marker for animal species identification (Alberdi *et al.* 2018). The PCR was performed under the following conditions: initial denaturation at 95°C for 2 min, followed by 28 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s, and a final extension of 5 min at 72°C. In parallel, a PCR blank was included as a negative control to monitor for laboratory contamination, and no contamination was detected. The resulting PCR amplicons were purified using the QIAquick PCR kit (QIAGEN, Germany) and quantified using a NanoDrop® 3300 fluorospectrometer (Thermo Fisher Scientific, MA, USA) with PicoGreen dsDNA assay (Invitrogen, CA, USA). Finally, PCR products were pooled in equimolar concentrations and sequenced by Macrogen Inc. (Seoul, South Korea) on an Illumina MiSeq platform (Illumina, CA, USA) using

300-bp PE. Raw sequences are available at the NCBI Sequence Read Archive (accession number PRJNA942030). Although a total of 95 individuals were captured, we were able to amplify 68 out of 95 samples.

Bioinformatic analysis

The raw sequence data were processed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline. First, the barcode reads were extracted using "*extract_barcode.py*" script in QIIME v1.9.1 (Caporaso *et al.* 2010), and the separated sequences and barcodes were imported to QIIME2 v2021.4.0 (Bolyen *et al.* 2019). Raw sequences were demultiplexed using the "*qiime demux*" plugin, and both forward and reverse Illumina adapters were removed using the "*cutadapt*" plugin (Martin 2011). Reads were merged using the "*join-pairs*" method within the VSEARCH plugin (Rognes *et al.* 2016), then filtered based on quality scores with a minimum parameter of 20 using the "*q-score-joined*" method of the "*quality-filter*" plugin. Quality filtered sequences were dereplicated using VSEARCH and then clustered into operational taxonomic units (OTUs) with a similarity threshold of 97% using the "*cluster-features-de-novo*" method. Finally, the taxonomy of each OTU was assigned using BLAST against the BOLD COI sequence database (Ratnasingham & Hebert 2007 "https://www.boldsystems.org") and then filtered within QIIME2 using the "*RESCRIPt*" plugin (O'Rourke *et al.* 2020; Robeson *et al.* 2021).

All sequences assigned to genus *Sceloporus*, phylum Chordata, and kingdom Fungi were removed from the representative OTU sequences and OTU table. Only OTUs identified as class Insecta, and orders Araneae, Stylommatophora, Isopoda, Haplotaxida (genus *Octolasion*), and Chordeumatida were considered to be deliberately consumed prey. The selection of these taxa as potential prey was based on previous reports of their consumption by *Sceloporus* lizards (Feria-Ortiz *et al.* 2001; Leyte-Manrique & Ramírez-Bautista 2010; García-Rosales *et al.* 2019). We also detected some OTUs belonging to the microscopic invertebrate orders Sarcoptiformes (percent frequency of occurrence 6.42 ± 9.30), Rhabditida (3.03 ± 7.96), Trombidiformes (2.33 ± 6.06), Haplotaxida (genus *Chaetogaster* 0.24 ± 1.15), Adinetida (2.95 ± 5.25), Ploima (2.05 ± 7.02), Anomopoda (1.86 ± 5.87), Calanoida (0.45 ± 2.05), and Symphypleona (0.18 ± 1.52). However, microscopic organisms of the first four orders are likely commensals, ectoparasites, or endoparasites of the prey ingested by lizards (Potapov *et al.* 2022) rather than prey themselves, and microscopic organisms of the remaining orders may live in temporary ponds or mosses and can be ingested by lizards through water or plant consumption, since plant material is often consumed by *Sceloporus* lizards (Serrano-Cardozo *et al.* 2008; Cruz-Elizalde *et al.* 2020; Montoya-Ciriaco *et al.* 2020). Therefore, we considered these records to constitute incidental ingestion and excluded them from the statistical analyses. The frequency table of OTUs was collapsed to the genus level for further analysis. To construct the phylogeny used to calculate phylogenetic richness, the OTU sequences were aligned with MAFFT (Katoh & Standley 2013) and a rooted maximum likelihood tree was built using IQTREE multicore v.2.0.3 (Minh *et al.* 2019) with the best nucleotide substitution model (UNREST + FO + I + G4) as selected by ModelFinder (Kalyaanamoorthy *et al.* 2017).

Statistical analysis

All further data processing and statistical analyses were carried out using the software R v.4.1.2 (R Core Team 2021). Diet composition and richness assessments were based on occurrence (presence/absence) of a given taxon. Dietary phylogenetic richness was the number of invertebrate lineages calculated using *hillR* v.0.5.1 (Li 2018). Shapiro–Wilk tests were used to test for normal distributions of dietary data. The sampling coverage was estimated with *iNEXT* v.2.0.20 (Hsieh *et al.* 2016).

We investigated the influence of seasonality (dry and rainy seasons) on dietary and phylogenetic richness by fitting separate generalized linear models (GLMs) with a quasi-Poisson and a Poisson error distribution, respectively, and log link function using the "*glm*" function of *stats* v.4.0.3 (Crawley 2007). Quasi-Poisson distribution accounted for overdispersion in the number of invertebrate genera. We included the interactive effect of lizard species (four-level factor) and seasonality in these models, since changes in diet may differ among lizard species in response to temporal variation in prey availability. We also included the SVL (mm) of each individual and the elevation (m) of the sampling site as covariates, since larger lizards may feed on larger prey (Costa *et al.* 2008), and prey abundance and diversity often vary with altitude (Moreno-Rueda *et al.* 2018; Montoya-Ciriaco *et al.* 2020). To simplify the models, we confirmed that diet does not differ between the sexes in *Sceloporus* lizards (Leyte-Manrique & Ramírez-Bautista 2010; Cruz-Elizalde *et al.* 2020) by comparing dietary and phylogenetic richness between the sexes within each species using Wilcoxon rank sum tests before fitting linear models. Since sex did not influence diet (all P values > 0.05), this variable was not included in the GLMs. We used deletion tests to compare the simplified minimal

adequate model with the model including a non-significant term or with the model excluding a significant term to assess the statistical significance of the increase in deviance for each model (Crawley 2005).

We calculated dietary niche breadth for each lizard species using Levin's standardized index as follows: $BA = ((1/\Sigma pi^2) - 1)/n - 1$, where *BA* refers to the standardized index of diet breadth, *pi* represents the proportion of individuals of a given prey taxon found in the diet, and *n* is the total number of prey categories recorded per lizard species (Hurlbert 1978). Levin's index ranges from 0 to 1; values closer to 0 indicate a specialist diet, while values closer to 1 indicate a generalist diet. This ecological metric was computed using the number of invertebrate genera with *MicroNiche* v.1.0.0 (Finn *et al.* 2020). Statistical differences were assessed by Kruskal–Wallis tests followed by Bonferroni correction for multiple comparisons. Within each lizard species, niche breadth was compared between the dry and the rainy seasons using a Wilcoxon rank sum test.

To assess beta diversity (i.e. differences in prey items among lizard species), we calculated two measures: the Jaccard dissimilarity index to compare the diet composition at the genus level, and unweighted Unifrac distances to assess dissimilarities in phylogenetic composition among lizard species (i.e. invertebrate lineages). Data were visualized using a principal coordinate analysis (PCoA) ordination plot. A permutational multivariate analysis of variance (perMANOVA) with 999 permutations was computed to test differences in community composition using both distance matrices, fitting the following model: richness ~ species × season + elevation + SVL, using the "*adonis2*" function in *vegan* v.2.6-2 (Oksanen *et al.* 2022). Additionally, a permutational multivariate analysis of dispersion (PERMDISP) was done to test the homogeneity of dispersion between groups using "*betadisper*" function in *vegan*. After finding that elevation influenced lizards' diet composition, we repeated the analysis excluding *S. bicanthalis*—the only species living at ~4150 m a.s.l.—to determine whether this elevational effect remained when including only the three lizard species living at ~2600 m a.s.l. Seasonal dietary turnover was estimated as pairwise dissimilarities based on the Jaccard index for each lizard species between the dry and rainy seasons, using the function "*pair_dis*" in *hilldiv* v.1.5.3 (Alberdi & Gilbert 2019). We compared the seasonal dietary turnover among lizard species using a two-tailed Kruskal–Wallis test followed by Dunn's post hoc multiple comparisons. To visualize the relationship between each lizard species and its prey items, a bipartite network graph was constructed with *bipartite* v.2.16 (Dormann *et al.* 2009), using the relative frequencies of prey at the order, family, and genus levels. The relative occurrence of a given prey taxon was calculated as the percentage of total samples per lizard species in which it was detected. In all statistical analyses, a P value < 0.05 was considered statistically significant.

Last, we compared the diet inferred from visual inspections of stomach contents in *S. aeneus* (Cruz-Elizalde *et al.* 2020) versus the diet inferred using DNA metabarcoding analysis to assess the consistency between methods and suitability of metabarcoding analysis to unveil dietary diversity of arthropod-eating lizards. Since visual inspection is often unable to identify prey at fine taxonomic levels, we evaluated the frequency of occurrence of prey items at the order level using both methods to ensure that they were comparable in terms of taxonomic precision. We expected that metabarcoding analysis would reveal a greater diversity of prey items than visual analysis.

RESULTS

Considering both deliberately and incidentally consumed prey items, the sequencing data analysis yielded 35 030 high-quality sequences with a mean of 515 sequences per sample. DNA metabarcoding of the COI gene detected 5 phyla, 12 classes, 21 orders, 56 families, and 70 genera of invertebrates. We detected 48 OTUs in *S. aeneus*, 52 in *S. bicanthalis*, 47 in *S. grammicus*, and 59 in *S. spinosus*. The estimated sampling coverage was 0.78 for *S. aeneus* and *S. grammicus* and 0.77 for *S. bicanthalis* and *S. spinosus*.

Relative frequency of prey taxa in lizard feces (deliberate consumption)

Across all individual samples, the most frequent invertebrate classes were Insecta (100%) and Arachnida (82%). Other classes were recorded in less than 10% of samples. At the order level, the bipartite network revealed that Hemiptera and Araneae contributed similarly to the diets of the four lizard species, whereas Hymenoptera and Coleoptera were mostly ingested by *S. aeneus*, *S. bicanthalis*, and *S. grammicus*, and Orthoptera by *S. spinosus* (Fig. 2a). The families Formicidae, Anthocoridae, and Linyphiidae were similarly consumed by *S. aeneus*, *S. bicanthalis*, and *S. grammicus*; Thomisidae was frequently ingested by *S. bicanthalis*; whereas Pyrgomorphidae was more commonly consumed by *S. grammicus* and *S. spinosus* (Fig. 2b). At the genus level, *Formica*, *Linepithema*, and *Myrmarachne* were

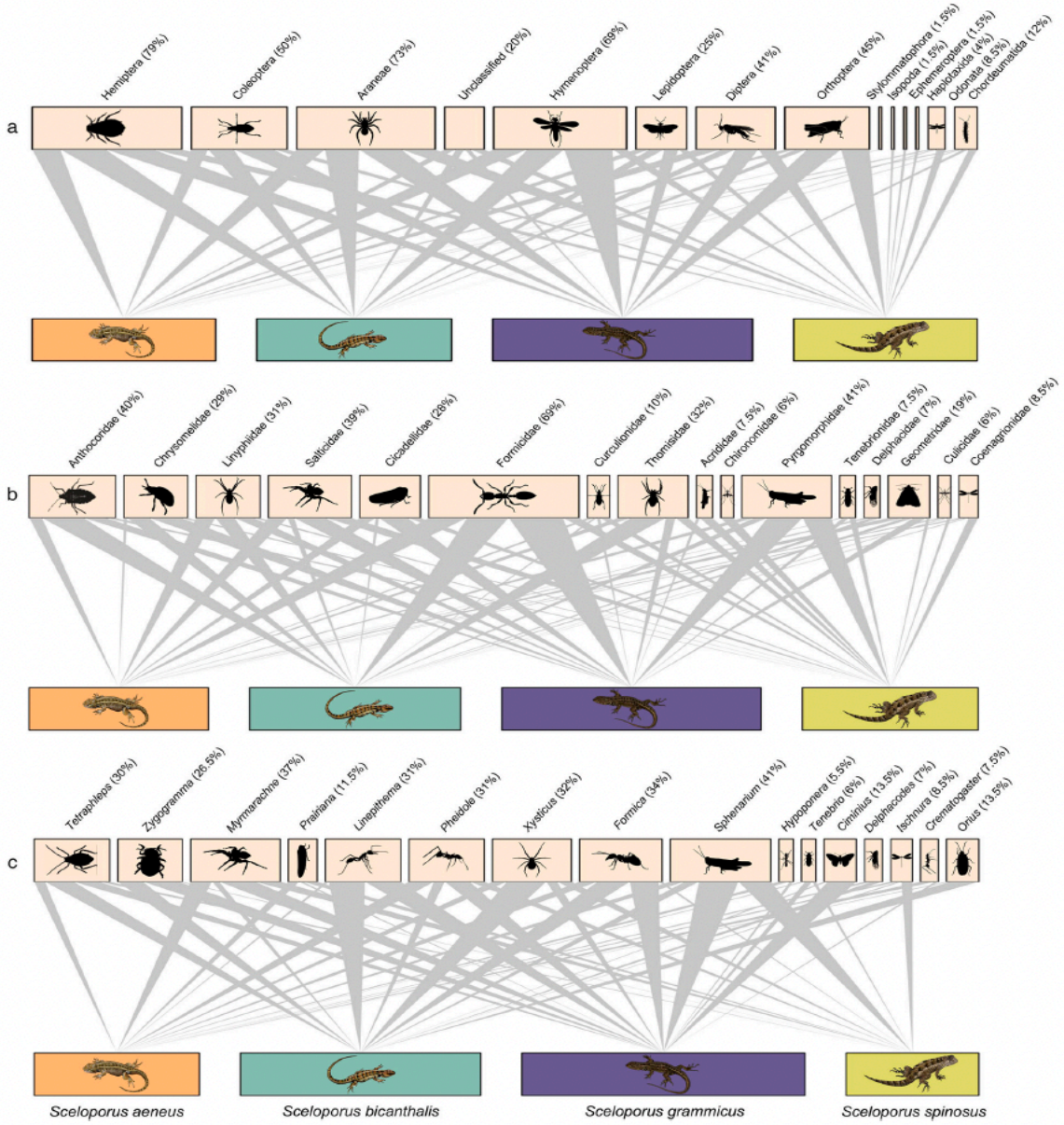

Figure 2 Bipartite networks showing connections between prey and their predatory lizard species. The lower boxes represent the predators (i.e. the four lizard species), and the upper boxes represent the food items at order level (a), family level (b) and genus level (c). Only taxa contributing ≥6% (family level) and ≥ 5% (genus level) were plotted in the bipartite networks. The width of the gray lines depicts the frequency of occurrence of prey categories consumed by the four *Sceloporus* lizard species.

widely consumed by *S. grammicus*, *S. bicanthalis*, and *S. aeneus*; *Sphenarium* by *S. grammicus* and *S. spinosus*; and *Xysticus* by *S. bicanthalis* (Fig. 2c). The diet compositions at different taxonomic levels for each lizard species are given in Table S2, Supporting Information, and the information about incidental consumption can be found in Table S3, Supporting Information.

Influence of season on diet composition across lizard species

Insecta and Arachnida were similarly ingested in both seasons by all lizard species. Diplopoda was consumed by *S. grammicus* during the rainy season and by *S. spinosus* during the dry season. The orders Hemiptera, Araneae, Hymenoptera, Coleoptera, and Orthoptera were similarly consumed by all lizard species in both seasons, except Orthoptera which was not detected during the rainy season in *S. bicanthalis*. The families Formicidae and Linyphiidae were commonly ingested in the rainy season by the four lizard species. In addition, Pyrgomorphidae was mostly consumed by *S. grammicus* and *S. spinosus* in both seasons. Anthocoridae was consumed more by *S. bicanthalis* during the rainy season. *Formica*, *Linepithema*, and *Xysticus* genera were frequent in the diet of the four lizard species during the rainy season, whereas *Sphenarium* and *Myrmarachne* were commonly detected in *S. grammicus* and *S. spinosus* during the dry season. The frequency of occurrence and percentages of both deliberate and incidental consumption by season at different taxonomic levels are shown in Tables S4 and S5, Supporting Information, respectively.

Influence of seasonality on taxonomic and phylogenetic richness across lizard species

The taxonomic richness of prey (excluding incidental consumption) did not differ among lizard species ($F_{62,65} = 1.930$, $P = 0.134$), was unrelated to lizards' SVL ($F_{61,62} = 0.007$, $P = 0.933$) and did not vary across sampling site elevations ($F_{65,66} = 0.552$, $P = 0.460$). However, it was higher during the dry season than during the rainy season ($\beta = 1.941 \pm 0.103$, 95% CI: 1.730 ± 2.137, $F_{66,67} = 0.746$, $P = 0.001$), independent of the lizard species (species × season: $F_{58,61} = 2.043$, $P = 0.118$). Similarly, phylogenetic richness of deliberate prey did not differ among lizard species ($F_{62,65} = 0.853$, $P = 0.465$) and was unrelated to lizards' SVL ($F_{61,62} = 0.923$, $P = 0.337$) and elevation of the capture sites ($F_{65,66} = 0.139$, $P = 0.709$). Lizards showed a higher phylogenetic diversity during the dry season compared to the rainy season ($\beta = 1.191 \pm 0.478$, 95% CI: 1.382 ± 0.181, $F_{65,66} = 9.432$, $P = 0.002$), and this seasonal effect did not differ among lizard species (species × season: $F_{58,61} = 1.305$, $P = 0.271$).

Differences in diet composition among and within lizard species

Dietary composition at the genus level

The PCoA plot based on Jaccard distances revealed that samples from the same season were clustered together for each lizard species, except for *S. spinosus*, which did not show a clear pattern (Fig. 3a). Overall, diet composition showed lower variation during the dry season than during the rainy season. According to the perMANOVA test, the taxonomic composition of the diet varied considerably between seasons, and this seasonal effect depended on the species (species × season: $F_3 = 2.304$, adjusted $R^2 = 0.085$, $P = 0.001$). Dietary composition was also influenced by the elevation of sampling sites ($F_1 = 2.059$, adjusted $R^2 = 0.025$, $P = 0.001$), but it was unrelated to lizards' SVL ($F_1 = 1.347$, adjusted $R^2 = 0.017$, $P = 0.087$). The PERMDISP test did not show significant differences in the dispersion of dietary composition among species ($F_3 = 2.343$, $P = 0.081$) and between seasons in each species (*S. aeneus*: $F_1 = 0.427$, $P = 0.527$; *S. bicanthalis*: $F_1 = 0.615$, $P = 0.446$; *S. grammicus*: $F_1 = 1.689$, $P = 0.208$; *S. spinosus*: $F_1 = 0.816$, $P = 0.381$). This suggested that the differences between groups were due to separation of the centroid of the groups. In addition, the influence of elevation on dietary composition remained statistically significant even after removing data from *S. bicanthalis* ($F_1 = 2.987$, adjusted $R^2 = 0.034$, $P = 0.003$), the only lizard species living at 4150 m a.s.l. in this study.

Dietary composition at the lineage level

The unweighted Unifrac distance PCoA analysis did not reveal a clear separation between the dry and rainy seasons for lizard species, although *S. aeneus* displayed subtle variation in phylogenetic composition between seasons (Fig. 3b). The perMANOVA test indicated that the phylogenetic composition of the diet was influenced by the species × season interaction ($F_3 = 2.163$, adjusted $R^2 = 0.088$, $P = 0.007$), but it was unrelated to elevation ($F_1 = 1.605$, adjusted $R^2 = 0.022$, $P = 0.104$) and lizards' SVL ($F_1 = 0.715$, adjusted $R^2 = 0.010$, $P = 0.661$). The PERMDISP test showed no significant

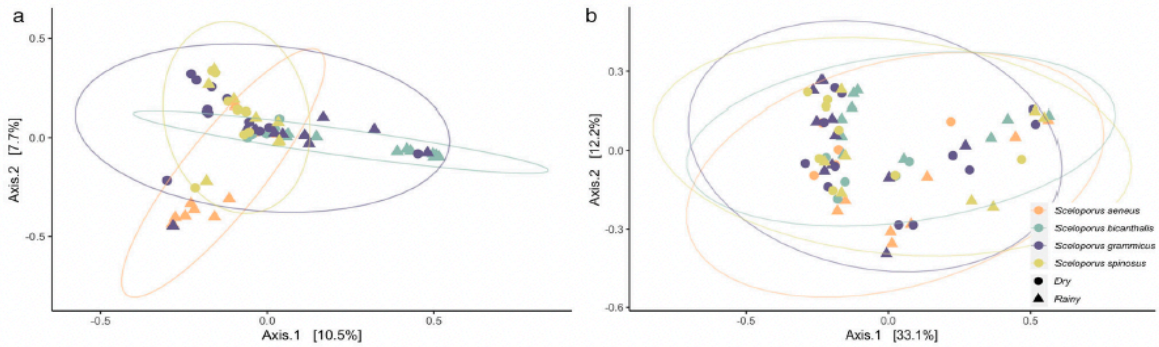

Figure 3 Principal coordinate analysis (PCoA) of the dietary composition at the genus level based on Jaccard distance matrix (a) and phylogenetic composition based on unweighted UniFrac distance matrix (b). Lizard species are represented by different colors and seasonal variation by circles (dry) and triangles (rainy). Colored ellipses represent 95% confidence intervals.

dispersion in phylogenetic composition among species ($F_3 = 0.6578$, $P = 0.581$), as well as intra-species (*S. aeneus*: $F_1 = 0.452$, $P = 0.990$; *S. bicanthalis*: $F_1 = 0.384$, $P = 0.703$; *S. grammicus*: $F_1 = 0.447$, $P = 0.109$; *S. spinosus*: $F_1 = 0.453$, $P = 0.667$).

Pairwise dissimilarities

Seasonal dietary turnover was statistically different among lizard species ($H = 21.932$, df = 3, $P = 0.001$). The multiple comparison Dunn's test showed that *S. bicanthalis* had lower seasonal dietary turnover (median = 0.68) than *S. spinosus* (median = 0.83) ($P = 0.001$, Fig. 4). The remaining comparisons among species were not statistically significant.

Dietary niche breadth (BA)

The Kruskal–Wallis test showed no significant differences in diet breadth among lizard species ($H = 4.261$, df = 3, $P = 0.235$; Fig. 5a). At the intra-species level, there were no seasonal differences in diet breadth in *S. aeneus*, *S. grammicus*, or *S. spinosus*, but the diet of *S. bicanthalis* was broader during the dry season ($BA = 0.58$) than during the rainy season ($BA = 0.30$) (Wilcoxon rank sum test, $P = 0.013$; Fig. 5b).

Comparison between metabarcoding analysis and visual inspection in *Sceloporus aeneus*

Visual analysis yielded nine arthropod prey categories at the order level, as well as plant material. The most frequently recorded arthropod orders were Hymenoptera,

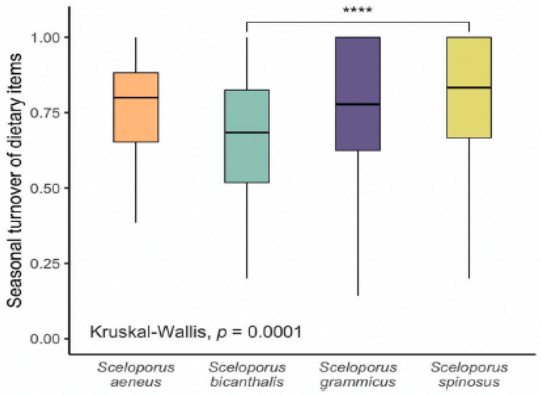

Figure 4 Seasonal dietary turnover within lizard species calculated as pairwise Jaccard dissimilarities between the dry and rainy seasons. Seasonal dietary turnover was statistically different between *Sceloporus bicanthalis* and *S. spinosus*.

Hemiptera, Araneae, and adults of Coleoptera. The incidentally consumed taxa were not detected by visual inspection. When including only the taxa considered to constitute deliberately consumed prey, our metabarcoding analysis identified a total of 10 orders; Hymenoptera, Hemiptera, Araneae, Diptera, and Coleoptera were the most frequently detected in *S. aeneus*. Since we used arthropod-specific primers, our COI metabarcoding analysis did not allow us to detect plant material. Five orders of incidentally consumed taxa were detected: Sarcoptiformes, Adinetida, Rhabditida, Ploima, and Anomopoda (Fig. S1, Supporting Information).

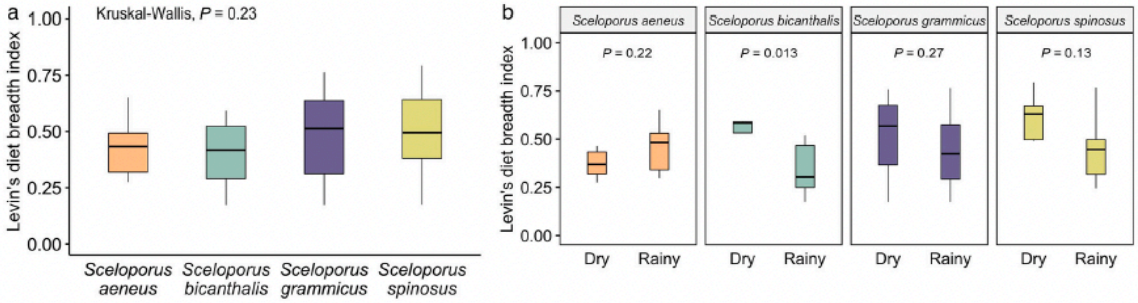

Figure 5 (a) Levin's standardized index calculated for four *Sceloporus* species. (b) Dietary niche breadth during the dry and the rainy season was compared within species. Values closer to 0 indicate a narrow diet breadth (dietary specialists), while values closer to 1 indicate a wide diet breadth (dietary generalists).

DISCUSSION

Our fecal DNA metabarcoding analysis of *Sceloporus* species provided four main results. First, both the taxonomic and phylogenetic richness of prey consumed by these lizards were greater during the dry season than during the rainy season. Second, diet composition of genera and lineages differed between the dry and rainy seasons among *Sceloporus* species, except for *S. spinosus*, while elevation only influenced diet composition at the genus level. Third, seasonal dietary turnover was lower for *S. bicanthalis* than for *S. spinosus* (which showed the highest value) but did not differ among the remaining lizard species. Fourth, only *S. bicanthalis* exhibited seasonal differences in diet breadth, which was broader during the dry season than during the rainy season.

The higher prey diversity at the taxonomic level during the dry season compared to the rainy season in the four species studied is consistent with the pattern observed in other members of the genus *Sceloporus*, such as *S. horridus* (Castro-Franco *et al.* 2017) and *S. minor* (García-Rosales *et al.* 2019) from other localities in central Mexico. Our analysis also revealed that this pattern also applies to phylogenetic diversity. These results probably imply that during the dry season, when emergence and abundance of insects and other arthropods decrease (Richards & Windsor 2007), lizards may be forced to exploit additional food resources to satisfy their nutritional demands, resulting in a more diversified diet.

It is also possible that a more diversified diet in our dry season sampling (February) could be attributable to increased foraging activity of lizards after emerging from their shelters at the end of winter in the study area. Some lizards from central Mexico are not active during part of the winter, and they can recover from the resulting decrease in energy intake by increasing their post-winter foraging times (Anderson *et al.* 2022), potentially consuming a wider array of prey during the recovery period (García-Rosales *et al.* 2019). Under this hypothesis, we would have expected to find lower body condition (i.e. SVL–mass residuals) during the dry season; however, this was not supported by our data, since we found no differences in body condition between seasons in any lizard species (Wilcoxon tests, P values > 0.05; see Table S6, Supporting Information). Body condition of *Sceloporus* lizards may differ between seasons in other areas of central Mexico, though (Rivera-Rea *et al.* 2023).

Conversely, the higher availability of arthropods during the rainy season may allow lizards to exploit a limited variety of valuable prey, leading to a less diversified diet during this period. Seasonal dietary shifts in lizards and other vertebrates are common (Feria-Ortiz *et al.* 2001; Cruz-Elizalde *et al.* 2020) and are often correlated to temporal variation in precipitation, landscape features, temperature, and humidity (Rubolini *et al.* 2003; Goodyear & Pianka 2011). These factors may be influencing the diet of the lizard species studied here, and further analyses are required to elucidate their individual or combined influence on the main prey detected by our DNA metabarcoding analysis.

Overall, diet overlap among focal lizard species was high but not absolute, suggesting that these species could compete for similar food sources to some degree on the La Malinche Volcano. Competition for prey may be particularly strong among *S. aeneus*, *S. grammicus*, and *S. spinosus*, which coexist in the sites where we sampled

them at ~2600 m a.s.l., an area mainly covered by farmlands, shrubs, and herbaceous plants. The coexistence of these three species despite competition could be facilitated by seasonal dietary niche partitioning, for example, if each overlapping species specializes or consumes prey taxa in different proportions between seasons, as has been documented in sympatric populations of mice (Reid *et al.* 2013). This partitioning could be relaxed to some extent when the availability of arthropod prey increases during the rainy season.

Differences in microhabitat use and feeding habits may also facilitate the coexistence of arthropod-eating lizards that exploit similar prey sources (Tan *et al.* 2021). The bipartite network revealed variation in dietary niche partitioning among lizard species when diet was analyzed at the genus level. The terrestrial lizards *S. aeneus* and *S. bicanthalis* consumed a greater proportion of ants belonging to genera *Pheidole*, *Formica*, and *Linepithema* than the saxicolous *S. spinosus*. Meanwhile, the arboreal–saxicolous *S. grammicus* and saxicolous *S. spinosus* consumed a greater quantity of flying insects such as grasshoppers and leafhoppers (genera *Sphenarium*, *Ciminius*, and *Ischnura*) than terrestrial lizards. Dominance of ants in terrestrial lizards compared to saxicolous lizards, or flying insects in arboreal and saxicolous lizards compared to terrestrial lizards has been documented elsewhere, and this pattern probably promotes their coexistence in a variety of ecosystems (Tan *et al.* 2021). Differences in foraging behavior (e.g. active foraging vs. sit-and-wait foraging) could also facilitate the coexistence of arthropod-eating lizards. However, in the case of our study species, this explanation seems unlikely because most species of the genus *Sceloporus* use the sit-and-wait foraging strategy (Vitt *et al.* 1981), and our focal species spend much of their time perched on rocks and fallen trees. Nonetheless, further studies would be needed to formally test this hypothesis.

Like other members of the genus *Sceloporus* (Gadsden *et al.* 2011; Castro-Franco *et al.* 2017; García-Rosales *et al.* 2019), the four lizard species studied here mostly consumed specimens of Hemiptera, Araneae, Hymenoptera, and Coleoptera. Hence, our findings based on molecular analyses support the proposition that members of the genus *Sceloporus* can be considered generalist insectivores (Serrano-Cardozo *et al.* 2008), and more broadly, arthropod-eating lizards. *Sceloporus* species may exhibit a more generalist diet though since they often consume other invertebrates such as mollusks and annelids (Feria-Ortiz *et al.* 2001; Leyte-Manrique & Ramírez-Bautista 2010). Notably, patterns of seasonal variation in diet composition are in line with results provided by our diversity analyses as well as data on the diet composition of other members of the genus *Sceloporus*. For instance, phytophagous insects such as Coleoptera, Hymenoptera, and Orthoptera were more frequently ingested during the rainy season than the dry season, which is consistent with patterns observed in *S. jalapae* Günther, 1890, *S. horridus*, and *S. aeneus* from central Mexico (Serrano-Cardozo *et al.* 2008; Cruz-Elizalde *et al.* 2020). Seasonal variation in diet composition was also revealed by dietary turnover, which was high (>0.6) across all lizard species and greater in *S. spinosus* than in *S. bicanthalis*. Species turnover can vary along temporal or spatial axes (Goodyear & Pianka 2011). Thus, the difference in seasonal dietary turnover between *S. spinosus* and *S. bicanthalis* could be explained by both the distance and the elevation differences between their sampling sites, which may lead to site-specific seasonal variability in prey abundance (Moreno-Rueda *et al.* 2018). Intriguingly, dietary seasonal turnover did not differ between *S. bicanthalis*, *S. aeneus*, and *S. grammicus*, despite the elevation differences in their sampling sites. We speculate that the lack of differences in dietary seasonal turnover among these species could be due to similarities in their body size or foraging strategies, and in the case of *S. bicanthalis* and *S. aeneus*, to their phylogenetic closeness (Grummer *et al.* 2014).

Spatial segregation related to differences in the elevation of sampling sites could have led to differences in dietary composition among lizard species. For instance, *S. bicanthalis* was the only species that displayed a broader diet during the dry season compared to the rainy season. Individuals of *S. bicanthalis* living at ~4150 m a.s.l. on La Malinche are exposed to low temperatures and humidity, low atmospheric pressure, and reduced gas concentrations, as well as high ultraviolet radiation, as it has been previously described in La Malinche by demographic and physiological studies in *S. grammicus* (Domínguez-Godoy *et al.* 2020; González-Morales *et al.* 2023), and other mountainous ecosystems inhabited by lizards (e.g. *Phrynocephalus vlangalii* Strauch, 1876; Wu *et al.* 2018). These conditions can limit prey availability and foraging activity of lizards, as suggested by an earlier analysis on La Malinche, which showed that the number of Arthropoda families consumed by *S. grammicus* decreases at 4150 m a.s.l. (Montoya-Ciriaco *et al.* 2020). Therefore, *S. bicanthalis* is thought to access a limited variety of prey items in its montane habitat compared to the three other lizard species. Nevertheless, individuals of *S. bicanthalis* may consume alternative prey during the dry season, when the abundance of insects decreases at high elevations (Wardhaugh *et al.* 2018). In addition,

as has been previously reported in other lacertids living at high elevations (Moreno-Rueda *et al.* 2018), spiders contributed strongly to the diet of *S. bicanthalis* during the rainy season. Spiders are considered to be profitable prey for lizards (Herrel *et al.* 2001), so reduced dietary richness during the rainy season could be linked with a high preference for spiders by *S. bicanthalis* during this season.

Dietary composition of *S. aeneus* retrieved from DNA metabarcoding differed from what was documented for this species by Cruz-Elizalde *et al.* (2020) using visual inspection. Whereas visual analysis identified Coleoptera, Formicidae, and Hemiptera as the most common prey, our molecular approach detected a much broader range of prey, including Hymenoptera, Hemiptera, Araneae, Diptera, and Coleoptera, as well as five other orders with lower frequencies of occurrence. Furthermore, we detected (likely incidental consumption) Sarcoptiformes, Adinetida, Rhabditida, Ploima, and Anomopoda. It seems likely that the differences between our results using DNA metabarcoding versus visual analysis are largely because visual inspection detects insect prey that have chitinous exoskeletons and thus harder bodies, but it fails to detect soft-bodied prey that are more easily digested by lizards (Kok *et al.* 2021), while DNA metabarcoding is not subject to this particular bias. Nonetheless, the detection of incidentally consumed taxa by DNA metabarcoding analysis could lead to overestimation and biases in dietary diversity and composition. Furthermore, in contrast to visual methods, DNA metabarcoding does not allow the distinction of different life stages of prey (Bessey *et al.* 2019), and in our case, additional molecular markers would have to be included to detect plant material. Therefore, results obtained by visual and molecular methods should be interpreted cautiously. Ideally, dietary analysis should implement both methodological approaches, with larger sample sizes, to ensure more robust inferences on dietary composition and its major ecological drivers.

Our sample sizes were limited by the occurrence/encounter of lizards in the study area and DNA amplification success rates. Small sample sizes are a common limitation among dietary studies of reptiles (Dalhuijsen *et al.* 2014; Cruz-Elizalde *et al.* 2020; Guarino 2001), which can potentially lead to biased estimates of dietary niche breadth. Nevertheless, sample sizes did not affect the statistical power of our analyses, and sample completeness estimators indicated even sample coverage across contrasted lizards (Hsieh *et al.* 2016). Thus, it is unlikely that our inferences were biased by limited sample sizes (Cross *et al.* 2020).

In conclusion, our findings indicate that arthropod-eating *Sceloporus* lizards exhibit dynamic dietary changes, suggesting that during periods of low prey abundance and after emerging from shelters, they incorporate a great variety of diet items, resulting in more diverse diets. Differences in dietary composition among species that coexist locally may be associated with differences in microhabitat use and potentially reflect distinct energetic demands faced by each species in their respective habitats.

ACKNOWLEDGMENTS

The authors thank La Malinche Scientific Station and Centro Tlaxcala de Biología de la Conducta for access and logistic support. We thank Dr. Lynna Kiere for her revision of the English language. Funding was provided by the Consejo Nacional de Ciencia y Tecnología (CONACyT) through the Infraestructura project (205945), the Ciencia de Frontera project (137748), and the Cátedras CONACyT project (883), and by the Universidad Nacional Autónoma de México (UNAM). M.H. received PhD scholarship (967648) and S. H.-P., postdoctoral grant (929602) from CONACyT. This article is a requirement for obtaining a PhD degree for the first author.

CONFLICT OF INTEREST

The authors declare that there are no competing interests.

REFERENCES

Alberdi A, Aizpurua O, Gilbert MTP, Bohmann K (2018). Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution* **9**, 134–47.

Alberdi A, Gilbert MTP (2019). Hilldiv: An R package for the integral analysis of diversity based on Hill numbers. *BioRxiv*, https://doi.org/10.1101/545665

Anderson RO, Alton LA, White CR, Chapple DG (2022). Ecophysiology of a small ectotherm tracks environmental variation along an elevational cline. *Journal of Biogeography* **49**, 405–15.

Bessey C, Jarman SN, Stat M *et al.* (2019). DNA metabarcoding assays reveal a diverse prey assemblage for *Mobula* rays in the Bohol Sea, Philippines. *Ecology and Evolution* **9**, 2459–74.

Bolyen E, Rideout JR, Dillon MR *et al.* (2019). Reproducible, interactive, scalable and extensible

microbiome data science using QIIME 2. *Nature Biotechnology* **37**, 852–7.

Caporaso JG, Kuczynski J, Stombaugh J *et al.* (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**, 335–6.

Castro-Franco R, Bustos-Zagal MG, Trujillo-Jiménez P (2017). Diet composition of *Sceloporus horridus horridus* (Sauria: Phrynosomatidae) in tropical deciduous forest, Mexico. *Acta Zoológica Mexicana* **33**, 443–9.

Costa GC, Vitt LJ, Pianka ER, Mesquita DO, Colli GR (2008). Optimal foraging constrains macroecological patterns: Body size and dietary niche breadth in lizards. *Global Ecology and Biogeography* **17**, 670–7.

Crawley MJ (2005). *Statistics: An Introduction Using R*. John Wiley and Sons Ltd., Chichester, West Sussex, England, UK, pp. 103–24.

Crawley MJ (2007). *The R Book*. John Wiley and Sons Ltd., Chichester, West Sussex, England, UK, 942 p.

Cross SL, Craig MD, Tomlinson S, Bateman PW (2020). I don't like crickets, I love them: Invertebrates are an important prey source for varanid lizards. *Journal of Zoology* **310**, 323–33.

Cruz-Elizalde R, Ramírez-Bautista A, Núñez de Cáceres-González FF (2020). Sexual dimorphism and feeding ecology of the black-billied bunchgrass lizard *Sceloporus aeneus* (Squamata: Phrynosomatidae) in central Mexico. *South American Journal of Herpetology* **18**, 46–55.

Dalhuijsen K, Branch WR, Alexander GJ (2014). A comparative analysis of the diets of *Varanus albigularis* and *Varanus niloticus* in South Africa. *African Zoology* **49**, 83–93.

Dias EJR, Rocha CFD (2007). Niche differences between two sympatric whiptail lizards (*Cnemidophorus abaetensis* and *C. ocellifer*, Teiidae) in the restinga habitat of northeastern Brazil. *Brazilian Journal of Biology* **67**, 41–6.

Díaz de la Vega-Pérez AH, Jiménez-Arcos VH, Centenero-Alcalá E, Méndez-de la Cruz FR, Ngo A (2019). Diversity and conservation of amphibians and reptiles of a protected and heavily disturbed forest. *ZooKeys* **830**, 111–25.

Domínguez-Godoy MA, Hudson R, Pérez-Mendoza HA, Ancona S, Díaz de la Vega-Pérez AH (2020). Living on the edge: Lower thermal quality but greater survival probability at a high altitude mountain for the mesquite lizard (*Sceloporus grammicus*). *Journal of Thermal Biology* **94**, 102757.

Dormann CF, Fründ J, Blüthgen N, Gruber B (2009). Indices, graphs and null models: Analyzing bipartite ecological networks. *The Open Ecology Journal* **2**, 7–24.

Feria-Ortiz M, Nieto-Montes de Oca A, Salgado Ugarte IH (2001). Diet and reproductive biology of the viviparous lizard *Sceloporus torquatus torquatus* (Squamata: Phrynosomatidae). *Journal of Herpetology* **35**, 104–12.

Finn DR, Yu J, Ilhan ZE *et al.* (2020). MicroNiche: An R package for assessing microbial miche breadth and overlap from amplicon sequencing data. *FEMS Microbiology Ecology* **96**, fiaa131.

Gadsden H, Estrada-Rodríguez JL, Quezada-Rivera DA, Leyva-Pacheco SV (2011). Diet of the yarrow's spiny lizard *Sceloporus jarrovii* in the Central Chihuahuan Desert. *The Southwestern Naturalist* **56**, 89–94.

García-Rosales A, Cruz-Elizalde R, Ramírez-Bautista A, Mata-Silva V (2019). Feeding ecology of two populations of *Sceloporus minor* (Squamata: Phrynosomatidae) inhabiting contrasting environments in central Mexico. *Salamandra* **55**, 103–14.

Gay-García C, Hernández-Vázquez M, Jiménez-López J *et al.* (2004). Evaluation of climatic forecasts of rainfall for the Tlaxcala State (México): 1998–2002. *Atmosfera* **17**, 127–50.

Geller J, Meyer C, Parker M, Hawk H (2013). Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Molecular Ecology Resources* **13**, 851–61.

Gil V, Pinho CJ, Aguiar CAS, Jardim C, Rubelo R, Vasconcelos R (2020). Questioning the proverb "more haste, less speed": Classic versus metabarcoding approaches for the diet study of a remote island endemic gecko. *PeerJ* **8**, e8084.

González-Morales JC, Fajardo V, Díaz de la Vega-Pérez AH *et al.* (2023). Elevation and blood traits in the mesquite lizard: Are patterns repeatable between mountains? *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **276**, 111338.

Goodyear SE, Pianka ER (2011). Spatial and temporal variation in diets of sympatric lizards (genus *Ctenotus*) in the Great Victoria Desert, Western Australia. *Journal of Herpetology* **45**, 265–71.

Gribbins K, Anzalone M, Collier M, Granados-González G, Villagrán-Santa Cruz M, Hernández-Gallegos O (2011). Temporal germ cell development strategy during continuous spermatogenesis within the montane

lizard, *Sceloporus bicanthalis* (Squamata; Phrynosomatidae). *Theriogenology* **76**, 1090–9.

Grummer JA, Bryson RW, Reeder TW (2014). Species delimitation using bayes factors: Simulations and application to the *Sceloporus scalaris* species group (Squamata: Phrynosomatidae). *Systematic Biology* **63**, 119–33.

Guarino F (2001). Diet of a large carnivorous lizard, *Varanus varius*. *Wildlife Research* **28**, 627–30.

Hernández M, Ancona S, Hereira-Pacheco S, Díaz de la Vega-Pérez AH, Navarro-Noya YE (2023). Comparative analysis of two nonlethal methods for the study of the gut bacterial communities in wild lizards. *Integrative Zoology*, 1–16. https://doi.org/10.1111/1749-4877.12711

Herrel A, Van Damme R, Vanhooydonck B, De Vree F (2001). The implications of bite performance for diet in two species of lacertid lizards. *Canadian Journal of Zoology* **79**, 662–70.

Hsieh TC, Ma KM, Chao A (2016). iNEXT: an R package for rarefaction and extrapolation of species diversity (Hill numbers). *Methods in Ecology and Evolution* **7**, 1451–6.

Hurlbert SH (1978). The measurement of niche overlap and some relatives. *Ecology* **59**, 67–77.

Jiménez-Cruz E, Ramírez-Bautista A, Marshall JC, Lizana-Avia M, Nieto-Montes de Oca A (2005). Reproductive cycle of *Sceloporus grammicus* (Squamata: Phrynosomatidae) from Teotihuacan, state of Mexico. *The Southwestern Naturalist* **50**, 178–87.

Joseph GS, Muluvhahothe MM, Seymour CL, Munyai TC, Bishop TR, Foord SH (2019). Stability of Afromontane ant diversity decreases across an elevation gradient. *Global Ecology and Conservation* **17**, e005962.

Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS (2017). ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nature Methods* **14**, 587–9.

Kartzinel TR, Pringle RM (2015). Molecular detection of invertebrate prey in vertebrate diets: Trophic ecology of Caribbean island lizards. *Molecular Ecology Resources* **15**, 903–14.

Katoh K, Standley DM (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution* **30**, 772–80.

Kok PJR, Broholm TL, Mebs D (2021). Thriving in a hostile world: Insights from the dietary strategy of two allopatric, closely related tepui summit endemic amphibians. *Ecology and Evolution* **11**, 8730–42.

Lemos-Espinal JA, Ballinger RE, Smith G (1998). Comparative demography of the high-altitude lizard, *Sceloporus grammicus* (Phrynosomatidae), on the Iztaccihuatl volcano, Puebla, Mexico. *The Great Basin Naturalist* **58**, 375–9.

Leray M, Yang JY, Meyer CP *et al.* (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Frontiers in Zoology* **10**, 34.

Leyte-Manrique A, Ramírez-Bautista A (2010). Diet of two populations of *Sceloporus grammicus* (Squamata: Phrynosomatidae) from Hidalgo, Mexico. *The Southwestern Naturalist* **55**, 98–103.

Li D (2018). hillR: taxonomic, functional, and phylogenetic diversity and similarity through Hill Numbers. *Journal of Open Source Software* **3**, 1041.

Mamou R, Marniche F, Amroun M, Herrel A (2016). Trophic ecology of two sympatric lizard species: The Algerian sand lizard and the wall lizard in Djurdjura, northern Algeria. *Zoology and Ecology* **26**, 1–9.

Manríquez-Morán NL, Villagrán-Santa Cruz M, Méndez de la Cruz FR (2013). Reproductive activity in females of the oviparous lizard *Sceloporus aeneus*. *The Southwestern Naturalist* **58**, 325–9.

Martin M (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet Journal* **17**, 10.

McCoy ED (1990). The distribution of insects along elevational gradients. *Oikos* **58**, 313–22.

Méndez de la Cruz FR, Díaz de la Vega-Pérez AH, Centenero-Alcalá H, Jiménez-Arcos VH (2018). *Anfibios y Reptiles del Parque Nacional La Malinche*. Universidad Autónoma de Tlaxcala, San Pablo del Monte, Mexico.

Méndez de la Cruz FR, Villagrán-Santa Cruz M, López-Ortíz ML, Hernández-Gallegos O (2013). Reproductive cycle of a high-elevation, oviparous lizard (*Sceloporus spinosus*: Reptilia: Phrynosomatidae). *The Southwestern Naturalist* **58**, 54–63.

Minh BQ, Trifinopoulos J, Schrempf D, Schmidt HA (2019). IQTREE version 2.0: Tutorials and Manual Phylogenomic Software by Maximum Likelihood. Available from URL: http://www.iqtree.org

Montoya A, Kong A, Estrada-Torres A, Cifuentes J, Caballero J (2004). Useful wild fungi of La Malinche Nacional Park, Mexico. *Fungal Diversity* **17**, 115–43.

Montoya-Ciriaco N, Gómez-Acata S, Muñoz-Arenas LC *et al.* (2020). Dietary effects on gut microbiota of the mesquite lizard *Sceloporus grammicus* (Wiegmann, 1828) across different altitudes. *Microbiome* **8**, 6.

Moreno-Rueda G, Melero E, Reguera S, Zamora-Camacho FJ, Álvarez-Benito I (2018). Prey availability, prey selection, and trophic niche width in the lizard *Psammodromus algirus* along an elevational gradient. *Current Zoology* **64**, 603–13.

Nielsen JM, Clare E, Hayden B, Brett MT, Kratina P (2017). Diet tracing in ecology: Method comparison and selection. *Methods in Ecology and Evolution* **9**, 278–91.

Oksanen J, Blanchet FG, Friendly M *et al.* (2022). *Vegan: Community Ecology Package. R Package Version 2.6-2*. Available from URL: https://CRAN.R-project.org/package=vegan

O'Rourke DR, Bokulich NA, Jusino MA, MacManes MD, Foster JT (2020). A total crapshoot? Evaluating bioinformatic decisions in animal diet metabarcoding analyses. *Ecology and Evolution* **10**, 9721–39.

Ortega-Rubio A, Barrault R, Halffter G (1999). Population dynamics of *Sceloporus grammicus* (Sauria: Phrynosomatidae) at Durango, Mexico. *The Southwestern Naturalist* **44**, 64–72.

Pereira A, Xavier R, Perera A, Salvi D, Harris DJ (2019). DNA metabarcoding to assess diet partitioning and feeding strategies in generalist vertebrate predators: A case study on three syntopic lacertid lizards from Morocco. *Biological Journal of the Linnean Society* **127**, 800–9.

Pompanon F, Deagle BE, Symondson WOC, Brown DS, Jarman SN, Taberlet P (2012). Who is eating what: Diet assessment using next generation sequencing. *Molecular Ecology* **21**, 1931–50.

Potapov AM, Beaulieu F, Birkhofer K *et al.* (2022). Feeding habits and multifunctional classification of soil-associated consumers from protists to vertebrates. *Biological Reviews* **97**, 1057–117.

R Core Team (2021). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Ratnasingham S, Hebert PDN (2007). BOLD: The barcode of life data system (www.barcodinglife.org). *Molecular Ecology Notes* **7**, 355–64.

Reid REB, Greenwald EN, Wang Y, Wilmers CC (2013). Dietary niche partitioning by sympatric *Peromyscus boylii* and *P. californicus* in a mixed evergreen forest. *Journal of Mammalogy* **94**, 1248–57.

Richards LA, Windsor DM (2007). Seasonal variation of arthropod abundance in gaps and the understorey of a lowland moist forest in Panama. *Journal of Tropical Ecology* **23**, 169–76.

Rivera-Rea J, Macotela L, Moreno-Rueda G *et al.* (2023). Thermoregulatory behavior varies with altitude and season in the sceloporine mesquite lizard. *Journal of Thermal Biology* **114**, 103539.

Robeson MS, O'Rourke DR, Kaehler BD *et al.* (2021). RESCRIPt: Reproducible sequence taxonomy reference database management. *PLoS Computational Biology* **17**, e1009581.

Rodríguez-Romero F, Méndez FR, García-Collazo R, Villagrán-Santa Cruz M (2002). Comparación del esfuerzo reproductor en dos especies hermanas del género *Sceloporus* (Sauria: Phrynosomatidae) con diferente modo reproductor. *Acta Zoológica Mexicana* **85**, 181–8.

Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016). VSEARCH: A versatile open source tool for metagenomics. *PeerJ* **4**, e2584.

Rubolini D, Pirovano A, Borghi S (2003). Influence of seasonality, temperature and rainfall on the winter diet of the long-eared owl, *Asio otus*. *Folia Zoologica* **52**, 67–76.

Serrano-Cardozo VH, Lemos–Espinal JA, Smith GR (2008). Comparative diet of three sympatric *Sceloporus* in the semiarid Zapotitlán Valley, Mexico. *Revista Mexicana Biodiversidad* **79**, 427–34.

Tan WC, Measey J, Vanhooydonck B, Herrel A (2021). The relationship between bite force, morphology, and diet in southern African agamids. *BMC Ecology and Evolution* **21**, 126.

Torres Barragán CA, Hernández Salinas U, Ramírez-Bautista A (2020). Do growth rate and survival differ between undisturbed and disturbed environments for *Sceloporus spinosus* Wiegmann, 1828 (Squamata: Phrynosomatidae) from Oaxaca, Mexico? *Amphibian and Reptile Conservation* **14**, 43–54.

Vitt LJ, van Loben Sels RC, Ohmart RD (1981). Ecological relationships among arboreal desert lizards. *Ecology* **62**, 398–410.

Wardhaugh CW, Stone MJ, Stork NE (2018). Seasonal variation in a diverse beetle assemblage along two elevational gradients in the Australian Wet Tropics. *Scientific Reports* **8**, 8559.

Widhiono I, Sudiana E, Darsono D (2017). Diversity of wild bees along elevational gradient in an agricultural area in Central Java, Indonesia. *Psyche: A Journal of Entomology* **2017**, 2968414.

Wiens JJ, Kozak KH, Silva N (2013). Diversity and niche evolution along aridity gradients in North American lizards (Phrynosomatidae). *Evolution* **67**, 1715–28.

Wu Q, Dang W, Hu Y-C, Lu H-L (2018). Altitude influences thermal ecology and thermal sensitivity of locomotor performance in a toad-headed lizard. *Journal of Thermal Biology* **71**, 136–41.

SUPPLEMENTARY MATERIALS

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Morphometric data of *Sceloporus* individuals used in this study.

Table S2 Diet composition in terms of frequency of occurrence (FO = frequency of occurrence) at class, order, family and genus levels.

Table S3 Frequency of occurrence (FO = frequency of occurrence) of incidental dietary items at class, order, family and genus levels found in the four *Sceloporus* lizard species.

Table S4 Diet composition in terms of frequency of occurrence (FO = frequency of occurrence) at class, order, family and genus levels during dry and rainy seasons.

Table S5 Frequency of occurrence (FO = frequency of occurrence) of incidental dietary items at class, order, family and genus levels found in the four *Sceloporus* lizard species during the dry and rainy seasons.

Table S6 Residual values of the simple linear regression model (snout-vent length-mass residuals) in the four *Sceloporus* lizard species.

Figure S1 Methodological comparison of dietary analysis of *Sceloporus aeneus* between visual inspection of stomach contents (Cruz-Elizalde *et al.* 2020) and DNA metabarcoding approach. Comparisons were performed at the order level to make the two methods comparable. A = adult, L = larvae.

Cite this article as:

Hernández M, Hereira-Pacheco S, Alberdi A *et al.* (2023). DNA metabarcoding reveals seasonal changes in diet composition across four arthropod-eating lizard species (Phrynosomatidae: *Sceloporus*). *Integrative Zoology* **00**, 1–16. https://doi.org/10.1111/1749-4877.12755

8. Chapter IV: Seasonal variation in diet composition shapes gut microbial communities in arthropod-eating lizards

In natural conditions, vertebrate taxa are exposed to a wide variety of microbial inoculums (Hong and cols. 2011; Lankau and cols. 2012) which in turn influence their gut microbiota. Recently, diet has been identified as a key factor that dynamically influences gut microbial communities of vertebrates (Kartzinel and cols. 2019). However, few studies have examined in parallel the association between dietary seasonal patterns and temporal variability in gut microbial community composition. In the **Fourth Chapter,** we tested whether gut microbial communities exhibit seasonal variation and this variation relates to dietary seasonal changes (Guo and cols. 2021; Fan and cols. 2022) we previously documented in the studied species (Hernández and cols. 2023). The results of the fourth chapter revealed that Firmicutes, Bacteroidota and Proteobacteria were highly abundant between seasons, and *S. aeneus* and *S. bicanthalis* shared the major number of ASVs than any other species. There was a significant effect of the interaction species*season on gut bacterial communities, in which bacterial alpha diversity was higher during the dry season for *S. bicanthalis*, whereas *S. aeneus* showed opposite patterns, and no seasonal differences were detected in *S. grammicus* and *S. spinosus*. In addition, dietary seasonal composition showed a significant effect on gut microbiota composition, but did not influence bacterial alpha diversity. In absence of an effect of the diet, this result suggests that lizard species may experiment seasonal shifts in microbial inoculums, temperature, humidity and physiological conditions, which in turn may affect their gut bacterial diversity. Since lizards consume a similar variety of arthropod genera between seasons (Hernández and cols. 2023), gut microbiota composition varies seasonally, without notable changes in microbiota diversity between seasons. However, future studies will be needed to elucidate the influence of other extrinsic and intrinsic factors on lizard gut microbiota.

9. Discussion

There is growing evidence that the gut microbial communities largely contribute to ecological adaptation of animal hosts (Gilbert and cols. 2015; Alberdi and cols. 2016). Furthermore, bacterial community assemblages often respond to environmental conditions in which animals live. For instance, less diverse bacterial communities were observed in wild primates inhabiting suboptimal habitats compared to their counterparts living in pristine environments (Amato and cols. 2013), and these differences were associated with a low short-chain fatty acids production by microbial communities in those individuals occupying disturbed habitats. Therefore, gut microbial communities play an essential role in the health and fitness of free-ranging animals.

Fecal samples and cloacal swabs are two non-invasive sampling methods used for investigating intestinal microbiota of captive and wild populations. However, it is well-known that these methods do not reflect the entire GIT microbiota (Videvall and cols. 2017; Zhou and cols. 2020), and the microbial community composition often differs among host species. Actually, it is imperative to assess the suitability of fecal or cloacal samples to study spatial heterogeneity of lizard gut microbiota and avoid lethal procedures in wild populations. We explored the intestinal microbiota profiles and compared them with fecal and cloacal microbiota profiles in *S. grammicus*. As we expected, gut bacterial communities exhibited spatial patterns along the GIT, in which the rectum harbored the most diverse taxonomic and functional microbial community. Moreover, our results highlighted that in terms of community membership, fecal samples provide a more accurate non-lethal approach to evaluate lizard gut microbiota. Our findings are consistent with previous studies in birds (Videvall and cols. 2017), mice (Suzuki and Nachman 2016) and primates (Yasuda and cols. 2015), showing that fecal samples are useful to evaluate the GIT microbiota. Therefore, fecal samples comprise an ideal method due to easy collection, non-destructive approach and its potential to be implemented when repeated sampling is needed in longitudinal studies in natural populations (Suzuki and Nachman 2016).

A large body of evidence suggests that the gut microbiota vary among different taxa, but related species often share more microbial taxa than unrelated species (Li and cols. 2017), and this

pattern could be associated with the coevolutionary trajectory between hosts and their gut microbiota (Youngblut and cols. 2019). However, phylogenetically distant species often differ in their genetic composition, feeding habits and microhabitat use, making difficult to separate ecological factors and evolutionary history (Ingala and cols. 2018). Therefore, knowledge gaps still exist concerning to differences of gut microbiota between related species with similar ecological features, as occur between *S. aeneus* and *S. bicanthalis*, two closely related species living in the La Malinche volcano. Our comparative study between these two recently diverged species revealed differences in their gut microbiota diversity and composition. *S. bicanthalis* living in the high-zone at ~4150 m a.s.l. showed a greater bacterial diversity compared to *S. aeneus* living at ~2600 m a.s.l. We speculate that a higher gut bacterial diversity in *S. bicanthalis* may assist lizards to adapt and survive in high-altitude environments. Furthermore, clear differences in core microbial community composition were found between coexisting species, i.e. *S. aeneus* and *S. grammicus* (at 2600 m) and *S. bicanthalis* and *S. grammicus* (at 4150 m), indicating that differences in their life-history traits and evolutionary history (Wiens and cols. 2013) lead to variation in gut bacterial communities. In contrast, core microbiota convergence between *S. aeneus* and *S. bicanthalis* largely reflects similarities in habitat use and genetic composition (Grummer and cols. 2014) and recent divergence time (Wiens and cols. 2013), despite occupying contrasting environments in the study area. Hence, these results suggest that the core microbiota remains stable over evolutionary time in closely related species (Li and cols. 2017). In line with this finding, but including whole microbiota (transient and core microbial taxa), Sottas and cols. (2020) revealed that the gut microbiota composition of two closely related passerine species did not differ between either sympatric or allopatric populations.

In wildlife populations, particularly in insectivorous species, previous studies have demonstrated significant dietary changes across seasons as a result of temporal variation in insect richness and abundance (Grimbacher and Stork 2009; Sánchez-Reyes and cols. 2019). For instance, analysis of stomach contents and fecal DNA metabarcoding have revealed temporal shifts in diet composition among different lizard species (Gadsden and cols. 2011; Alemany and cols. 2022). Such changes have been attributed to temporal variation in food availability and ecological factors. Here, we were interested in describing how diet of four

Sceloporus species varies by season at intra- and inter-species level. We expected a higher dietary diversity during the rainy season because of great resource abundance, however, our results showed that the taxonomic and phylogenetic diversity of prey was greater during the dry season than the rainy season. One possible explanation would be that during the dry season lizard species experience food shortages and potentially exploit alternative prey to satisfy their nutritional demands or increase their foraging time when emerge from winter shelters (Anderson and cols. 2022), leading to more generalist food regime. This finding is consistent with earlier reports based on macroscopic analyses (Castro-Franco et al. 2017; García-Rosales et al. 2019), in which dietary diversity increased during the dry season compared to rainy season. Using DNA metabarcoding approach, we obtained a detailed quantitative dietary analysis of four *Sceloporus* species whose diet is still very limited. However, further studies would be needed to unravel the influence of other environmental factors on seasonal diet variation. Our study also contributes to expand our knowledge about the factors influencing gut microbiota composition in these lizard species, since diet has been recognized as a key driver of gut microbiota composition.

While the core microbiota often shows less variation over time in vertebrate taxa (Li and cols. 2017; Hernández and cols. 2022), the whole microbial community—transient and core taxa—appear to be highly diverse and dynamic over time (Risely 2020). Additionally, more pronounced shifts in gut microbiota composition have been observed across seasons (Baniel and cols. 2021; Guo and cols. 2021), but few studies have investigated whether such changes occur in parallel with temporal variation in dietary consumption. Given the current gaps in knowledge, we focus on the role of seasonality (*per se*) and temporal dietary changes on the gut microbiota variation among four *Sceloporus* species considered as generalist predators (Hernández and cols. 2023). Our results indicated an increase in gut bacterial alpha diversity during the dry season than the rainy season for *S. bicanthalis*, whereas *S. aeneus* exhibited an opposite pattern and no seasonal differences were observed in the two other species. *Sceloporus aeneus* and *S. bicanthalis* are two species with short life and rapid growth rate that show seasonal and continuous reproductive activity, respectively (Hernández-Gallegos and cols. 2002; Manríquez-Morán and cols. 2013). These features may influence their seasonal activity patterns (e.g. energetic costs) and correspondingly could promote seasonal shifts in their gut microbiota

composition. In addition, it has been reported that *Sceloporus* species tend to use a major variety of microhabitats during the dry season than the rainy season (Siliceo-Cantero and cols. 2016), exposing them to a more variety of environmental sources (soil, plant material, water, fallen trees, etc.) which may influence the gut bacterial richness of *S. bicanthalis* (Hong and cols. 2011; Lankau and cols. 2012). Similar to our results, wild primates showed a greater alpha diversity in the dry season (Orkin and cols. 2019; Rudolph and cols. 2022), whereas in small mammals gut bacterial diversity was higher during the rainy season compared to dry season (Fan and cols. 2022). Furthermore, as previously reported in other vertebrate taxa (Kartzinel and cols. 2019; Murillo and cols. 2022), we also observed that changes in gut microbiota composition were associated with seasonal dietary consumption. However, richness of the consumed prey items (arthropod genera) did not influence the gut bacterial alpha diversity, suggesting that other factors not included here (e.g. microbial inoculum sources, temperature, humidity, microhabitat use, reproduction activity, behavioral and physiological shifts between seasons) may impact the bacterial diversity of these *Sceloporus* species. A similar phenomenon has been documented in mammals (Guo and cols. 2021) and birds (Bodawatta and cols. 2022), where microbial community assembly was correlated with dietary composition, but bacterial diversity did not linearly increase with dietary richness. We speculate that the lack of an effect of dietary richness on bacterial diversity may be a result of the small sample sizes, since our analysis was limited to only 68 out of 95 samples that amplified for COI gene (Hernández and cols. 2023). Bodawatta and cols. (2022) also emphasize that small sample sizes often obscured the association between dietary richness and bacterial diversity, or simply exist a little influence of diet-associated microbes on animal gut microbiota.

10. General Conclusion

To summarize, we demonstrated that our focal lizard species comprise remarkable examples to assess simultaneously the gut bacterial communities and dietary composition. Our results suggest that fecal samples comprise a reliable non-lethal method for investigating GIT microbial communities in wild lizard populations, highlighting its suitability when repeated sampling is needed. We also documented similarities in core bacterial communities between closely related

species *S. aeneu*s and *S. bicanthalis*, which probably relate to their phylogenetic closeness, but significant differences were observed in whole bacterial community between these species, suggesting that local environmental conditions (e.g. temperature, humidity, food availability, ultraviolet radiation, oxygen concentrations, atmospheric pressure) may play an essential role in the dynamics of gut microbial communities. In addition, diet is a prominent factor in structuring the gut microbiota composition of wild vertebrates. We found that the diet composition of the four *Sceloporus* lizard species was influenced by seasonal fluctuations of the study area. Furthermore, the gut bacterial communities were highly dynamics between seasons in these lizard species, and this temporal variation was associated in part by seasonal shifts in diet composition. However, future studies will be required to fully elucidate the influence of other ecological factors, as well as reproductive status, seasonal physiological changes, and mating behaviour (monandrous and polyandrous) on the dynamics of lizard gut microbial communities. Additionally, genome-resolved metagenomics (a robust method to assess taxonomic diversity and functional attributes of microbial communities) will provide a better understanding of the successful adaptation of *Sceloporus* species to a wide variety of habitats.

11. References

Alberdi A, Aizpurua O, Bohmann K, Zepeda-Mendoza ML, Gilbert MTP. 2016. Do vertebrate gut metagenomes confer rapid ecological adaptation? Trends Ecol Evol 31: 689-699.

Alberdi A, Aizpurua O, Gilbert MTP, Bohmann K. 2017. Scrutinizing key steps for reliable metabarcoding of environmental samples. Methods Ecol Evol 9: 134-147.

Alemany I, Pérez-Cembranos A, Pérez-Mellado V, Castro JA, Picornell A and cols. 2022. DNA metabarcoding the diet of *Podarcis* lizards endemic to the Balearic Islands. Curr Zool, zoac073.

Amato KR, Yeoman CJ, Kent A, Righini N, Carbonero F and cols. 2013. Habitat degradation impacts black howler monkey (*Alouatta pigra*) gastrointestinal microbiomes. ISME J 7: 1344-1353.

Anderson RO, Alton LA, White CR, Chapple DG. 2022. Ecophysiology of a small ectotherm tracks environmental variation along an elevational cline. J Biogeogr 49: 405-415.

Andrews RM. 1998. Geographic variation in field body temperature of *Sceloporus* lizards. J Therm Biol 23: 329-334.

Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. 2005. Host-bacterial mutualism in the human intestine. Science 307: 1915-1920.

Baniel A, Amato KR, Beehner JC, Bergman TJ, Mercer A and cols. 2021. Seasonal shifts in the gut microbiome indicate plastic responses to diet in wild geladas. Microbiome 9: 26.

Bodawatta KH, Klečková I, Klečka J, Pužejová K, Koane B and cols. 2022. Specific gut bacterial responses to natural diets of tropical birds. Sci Rep 12: 713.

Carding S, Verbeke K, Vipond DT, Corfe BM, Owen LJ. 2015. Dysbiosis of the gut microbiota in disease. Microb Ecol Health Dis 26: 26191.

Castro-Franco R, Bustos-Zagal MG, Trujillo-Jiménez P. 2017. Diet composition of *Sceloporus horridus horridus* (Sauria: Phrynosomatidae) in tropical deciduous forest, Mexico. Acta Zool Mex 33: 443-449.

Colston TJ, Jackson CR. 2016. Microbiome evolution along divergent branches of the vertebrate tree of life: What is known and unknown. Mol Ecol 25: 3776-3800.

Cruz-Elizalde R, Ramírez-Bautista A, Núñez de Cáceres-Gonzáles FF. 2020. Sexual dimorphism and feeding ecology of the black-bellied bunchgrass lizard *Sceloporus aeneus* (Squamata: Phrynosomatidae) in Central Mexico. S Am J Herpetol 18: 46-55.

Díaz de la Vega-Pérez AH, Jiménez-Arcos VH, Centenero-Alcalá E, Méndez de la Cruz FR, Ngo A. 2019. Diversity and conservation of amphibians and reptiles of a protected and heavily disturbed forest of centra Mexico. Zookeys 830: 111-125.

Fan C, Zhang L, Jia S, Tang X, Fu H and cols. 2022. Seasonal variations in the composition and functional profiles of gut microbiota reflect dietary changes in plateau pikas. Integr Zool 17: 379-395.

Gadsden H, Estrada-Rodríguez JL, Quezada-Rivera DA, Leyva-Pacheco SV. 2011. Diet of the yarrow's spiny lizard *Sceloporus jarrovii* in the central Chihuahuan Desert. Southwest Nat 56: 89-94.

García-Rosales A, Cruz-Elizalde R, Ramírez-Bautista A, Mata-Silva V. 2019. Feeding ecology of two populations of *Sceloporus minor* (Squamata: Phrynosomatidae) inhabiting contrasting environments in central Mexico. Salamandra 55: 103-114.

Gilbert SF, Bosch TCG, Ledón-Rettig C. 2015. Eco-Evo-Devo: developmental symbiosis and developmental plasticity as evolutionary agents. Nat Rev Genet 16: 611-622.

Grimbacher PS, Stork NE. 2009. Seasonality of a diverse beetle assemblage inhabiting lowland tropical rain forest in Australia. Biotropica 41: 328-337.

Grond K, Sandercock BK, Jumpponen A, Zeglin LH. 2018. The avian gut microbiota: community, physiology and function in wild birds. J Avian Biol 49: e01788.

Grummer JA, Bryson RW, Reeder TW. 2014. Species delimitation using bayes factors: simulations and application to the *Sceloporus scalaris* species group (Squamata: Phrynosomatidae). Syst Biol 63: 119-133.

Guo N, Wu Q, Shi F, Niu J, Zhang T and cols. 2021. Seasonal dynamics of diet-gut microbiota interaction in adaptation of yaks to life at high altitude. NPJ Biofilms Microbiomes 7: 38.

Hernández M, Ancona S, Díaz de la Vega-Pérez AH, Muñoz-Arenas LC, Hereira-Pacheco SE and cols. 2022. Is habitat more important than phylogenetic relatedness for elucidating the gut bacterial composition in sister lizard species? Microbes Environ 37: ME21087.

Hernández M, Ancona S, Hereira-Pacheco S, Díaz de la Vega-Pérez AH, Navarro-Noya YE. 2023. Comparative analysis of two nonlethal methods for the study of the gut bacterial communities in wild lizards. Integr Zool 00: 1-16.

Hernández M, Hereira-Pacheco S, Alberdi A, Díaz de la Vega-Pérez AH, Estrada-Torres A and cols. 2023. DNA metabarcoding reveals seasonal changes in diet composition across four arthropod-eating lizard species (Phrynosomatidae: *Sceloporus*). Integr Zool 00: 1-16.

Hernández-Gallegos O, Méndez-de la Cruz FR, Villagrán-Santa Cruz M, Andrews RM. 2002. Continuous spermatogenesis in the lizard *Sceloporus bicanthalis* (Sauria: Phrynosomatidae) from high elevation habitat of central Mexico. Herpetologica 58: 415-421.

Hong P-Y, Wheeler E, Cann IKO, Mackie RI. 2011. Phylogenetic analysis of the fecal microbial community in herbivorous land and marine iguanas of the Galapagos Islands using 16S rRNA-based pyrosequencing. ISME J 5: 1461-1470.

Ingala MR, Simmons NB, Wultsch C, Krampis K, Speer KA and cols. 2018. Comparing microbiome sampling methods in a wild mammal: fecal and intestinal samples record different signals of host ecology, evolution. Front Microbiol 9: 803.

Jacobs L, McMahon BH, Berendzen J, Longmire J, Gleasner C and cols. 2019. California condor microbiomes: Bacterial variety and functional properties in captive-bred individuals. PLoS One 15: e0230738.

Kartzinel TR, Hsing JC, Musili PM, Brown BRP, Pringle RM. 2019. Covariation of diet and gut microbiome in African megafauna. PNAS 116: 23588-23593.

Khan I, Bai Y, Zha L, Ullah N, Ullah H and cols. 2021. Mechanism of the gut microbiota colonization resistance and enteric pathogen infection. Front Cell Infect Microbiol 23: 716299.

Kohl KD, Brun A, Magallanes M, Brinkerhoff J, Laspiur A and cols. 2017. Gut microbial ecology of lizards: insights into diversity in the wild, effects of captivity, variation across gut regions and transmission. Mol Ecol 26: 1175-1189.

Lankau EW, Hong P-Y, Mackie RI. 2012. Ecological drift and local exposures drive enteric bacterial community differences within species of Galapagos iguanas. Mol Ecol 21: 1779-1788.

Leaché AD. 2010. Species trees for spiny lizards (genus *Sceloporus*): identifying points of concordance and conflict between nuclear and mitochondrial data. Mol Phylogenet Evol 54: 162-171.

Lemos-Espinal JA, Ballinger RE. 1995. Comparative thermal ecology of the high-altitude lizard *Sceloporus grammicus*. Can J Zool 73: 2184-2191.

Leyte-Manrique A, Ramírez-Bautista A. 2010. Diet of two populations of *Sceloporus grammicus* (Squamata: Phrynosomatidae) from Hidalgo, Mexico. Southwest Nat 55: 98-103.

Li H, Qu J, Li T, Yao M, Li J, and cols. 2017. Gut microbiota may predict host divergence time during Glires evolution. FEMS Microbiol Ecol 93: fix009.

Manríquez-Morán NL, Villagrán-Santa Cruz M, Méndez-de la Cruz FR. 2013. Reproductive activity in females of the oviparous lizard *Sceloporus aeneus*. Southwest Nat 58: 325-329.

Marchesi JR, Ravel J. 2015. The vocabulary of microbiome research: a proposal. Microbiome 3: 31.

McKenzie VJ, Song SJ, Delsuc F, Prest TL, Oliveiro AM and cols. 2017. The effects of captivity on the mammalian gut microbiome. Integr Comp Biol 57: 690-704.

Méndez de la Cruz FR, Díaz de la Vega-Pérez AH, Centenero-Alcalá H, Jiménez-Arcos VH. 2018. Anfibios y reptiles del Parque Nacional La Malinche. San Pablo Eds Monte, Mexico. Universidad Autónoma de Tlaxcala.

Méndez-Salazar E, Ortiz-López M, Granados-Silvestre M, Palacios-Gonzáles B, Menjivar M. 2018. Altered gut microbiota and compositional changes in Firmicutes and Proteobacteria in Mexican undernourished and obese children. Front Microbiol 9: 2494.

Montoya-Ciriaco N, Gómez-Acata S, Muñoz-Arenas LC, Dendooven L, Estrada-Torres A and cols. 2020. Dietary effects on gut microbiota of the mesquite lizard *Sceloporus grammicus* (Wiegmann, 1828) across different altitudes. Microbiome 8: 6.

Murillo T, Schneider D, Fichtel C, Daniel R. 2022. Dietary shifts and social interactions drive temporal fluctuations of the gut microbiome from wild redfronted lemurs. ISME Commun 2: 3.

Orkin JD, Campos FA, Myers MS, Cheves Hernandez SE, Guadamuz A and cols. 2019. Seasonality of the gut microbiota of free-ranging white-faced capuchins in a tropical dry forest. ISME J 13: 183-196.

Phillips CD, Phelan G, Dowd SE, McDonough MM, Ferguson AW and cols. 2012. Microbiome analysis among bats describes influences of host phylogeny, life history, physiology and geography. Mol Ecol 21: 2617-2627.

Pincheira-Donoso D, Bauer AM, Meiri S, Uetz P. 2013. Global taxonomic diversity of living reptiles. PloS One 8: e59741.

Prabhu VR, Wasimuddin, Kamalakkannan R, Arjun MS, Nagarajan M. 2020. Consequences of domestication on gut microbiome: a comparative study between wild gaur and domestic mithun. Front Microbiol 11: 133.

Pyron RA, Burbrink FT, Wiens JJ. 2013. A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. BMC Evol Biol 13: 93.

Risely A. 2020. Applying the core microbiome to understand host-microbe systems. J Anim Ecol 89: 1549-1558.

Rodríguez-Romero F, Méndez FR, García-Collazo R, Villagrán-Santa Cruz M. 2002. Comparación del esfuerzo reproductor en dos especies hermanas del género *Sceloporus* (Sauria: Phrynosomatidae) con diferente modo reproductor. Acta Zool Mex 85: 181-188.

Rudolph K, Schneider D, Fichtel C, Daniel R, Heistermann M and cols. 2022. Drivers of gut microbiome variation within and between groups of a wild Malagasy primate. Microbiome 10:28.

Sánchez-Reyes UJ, Niño-Maldonado S, Clark SM, Barrientos-Lozano L, Almaguer-Sierra P. 2019. Successional and seasonal changes of leaf beetles and their indicator value in a fragmented low thorn forest of northeastern Mexico (Coleoptera, Chrysomelidae). ZooKeys 825: 71-103.

Siliceo-Cantero HH, García A, Gao Y. 2016. Abundance and habitat use of the lizard *Sceloporus utiformis* (Squamata: Phrynosomatidae) during the seasonal transition in a tropical environment. Rev Mex Biodivers 87: 1301-1307.

Sottas C, Schmiedová L, Kreisinger J, Albrecht T, Reif J and cols. 2020. Gut microbiota in two recently diverged passerine species: evaluating the effects of species identity, habitat use and geographic distance. BMC Ecol Evo 21: 41.

Suzuki TA, Nachman MW. 2016. Spatial heterogeneity of gut microbial composition along the gastrointestinal tract in natural populations of house mice. PLoS One 11: e0163720.

Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R and cols. 2007. The human microbiome project. Nature 449: 804-810.

Uetz P, Freed P, Aguilar R, Hošek J. 2022. The Reptile Database, http://www.reptile-database.org. Assessed on 19 November 2022.

Videvall E, Strandh M, Engelbrecht A, Cloete S, Cornwallis CK. 2017. Measuring the gut microbiome in birds: comparison of faecal and cloacal sampling. Mol Ecol Resour 18: 424-434.

Weiss SL. 2001. The effect of reproduction on food intake of a sit-and-wait foraging lizard, *Sceloporus virgatus*. Herpetologica 57: 138-146.

Wiens JJ, Reeder TW. 1997. Phylogeny of the spiny lizards (*Sceloporus*) based on molecular and morphological evidence. Herpetol Monogr 11: 1-101.

Wiens JJ, Kuczynski CA, Arif S, Reeder TW. 2010. Phylogenetic relationships of phrynosomatid lizards based on nuclear and mitochondrial data, and a revised phylogeny for *Sceloporus*. Mol Phylogenet Evol 54: 150-161.

Wiens JJ, Kozak KH, Silva N. 2013. Diversity and niche evolution along aridity gradients in North American lizards (Phrynosomatidae). Evolution 67: 1715-1728.

Wu H, Wu E. 2012. The role of gut microbiota in immune homeostasis and autoimmunity. Gut Microbies 3: 4-14.

Yasuda K, Oh K, Ren B, Tickle TL, Franzosa EA and cols. 2015. Biogeography of the intestinal mucosal and lumenal microbiome in the rhesus macaque. Cell Host Microbe. 17: 385-391.

Youngblut ND, Reischer GH, Walters W, Schuster N, Walzer C and cols. 2019. Host diet and evolutionary history explain different aspects of gut microbiome diversity among vertebrate clades. Nat Commun 10: 2200.

Zhang Z, Li D, Tang R. 2019. Changes in mouse gut microbial community in response to the different types of commonly consumed meat. Microorganisms 7: 76.

Zhou J, Nelson MN, Rodriguez Lopez C, Sarma RR, Zhou SJ and cols. 2020. A comparison of nonlethal sampling methods for amphibian gut microbiome analyses. Mol Ecol Resour 20: 844-855.