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Sustained Drought, but Not Short-Term Warming, Alters the Gut Microbiomes of Wild *Anolis* Lizards

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ABSTRACT As rising temperatures threaten biodiversity across the globe, tropical ectotherms are thought to be particularly vulnerable due to their narrow thermal tolerance ranges. Nevertheless, physiology-based models highlighting the vulnerability of tropical organisms rarely consider the contributions of their gut microbiota, even though microbiomes influence numerous host traits, including thermal tolerance. We combined field and lab experiments to understand the response of the slender anole lizard (*Anolis apletophallus*) gut microbiome to climatic shifts of various magnitude and duration. First, to examine the effects of long-term climate warming in the wild, we transplanted lizards from the mainland Panama to a series of warmer islands in the Panama Canal and compared their gut microbiome compositions after three generations of divergence. Next, we mimicked the effects of a short-term "heat-wave" by using a greenhouse experiment and explored the link between gut microbiome composition and lizard thermal physiology. Finally, we examined variation in gut microbiomes in our mainland population in the years both before and after a naturally occurring drought. Our results suggest that slender anole microbiomes are surprisingly resilient to short-term warming. However, both the taxonomic and predicted functional compositions of the gut microbiome varied by sampling year across all sites, suggesting that the drought may have had a regional effect. We provide evidence that short-term heat waves may not substantially affect the gut microbiota, while more sustained climate anomalies may have effects at broad geographic scales.

IMPORTANCE As climate change progresses, it is crucial to understand how animals will respond to shifts in their local environments. One component of this response involves changes in the microbial communities living in and on host organisms. These "microbiomes" can affect many processes that contribute to host health and survival, yet few studies have measured changes in the microbiomes of wild organisms experiencing novel climatic conditions. We examined the effects of shifting climates on the gut microbiome of the slender anole lizard (*Anolis apletophallus*) by using a combination of field and laboratory studies, including transplants to warm islands in the Panama Canal. We found that slender anole microbiomes remain stable in response to short-term warming but may be sensitive to sustained climate

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anomalies, such as droughts. We discuss the significance of these findings for a species that is considered highly vulnerable to climate change.

KEYWORDS microbiome, climate change, *Anolis*, drought, tropical ectotherm, thermal physiology

As global temperatures are expected to rise through the end of the century and beyond, understanding the impacts of climate warming on animal physiology is increasingly important. Organisms living in areas experiencing the greatest magnitude of temperature change were originally expected to suffer disproportionately. However, there has been an increasing awareness over the past two decades that a susceptibility to the negative effects of climate warming depends on multiple factors, not only on the extent of climate warming but also on the fundamental thermal niches of the species affected (1–5). Because of this, tropical ectotherms living in environments which were historically thermally stable may be disproportionately affected due to their narrow ranges of thermal tolerance (2). Indeed, warming of even a degree or two is of particular concern for many species (2, 6). Although an integrative framework by which to assess organism susceptibility to climate change has emerged, taking into account behavior, physiology, and local environmental variation (1, 7), a unified understanding of susceptibility to climate change will require the inclusion of microbiome-mediated effects on host fitness.

The microbiome is a major mediator of an organism's interaction with the world. In recent years, the hologenome and metaorganism concepts have emerged as a way to describe the sum of the contribution of host and microbial genes to host phenotypic variation (8, 9). The way that an organism develops, adapts, and functions in the world depends on both its innate physiology and the contributions of symbiotic microorganisms (10–12). The microbiome is responsible for modulating many aspects of host survival, including development, metabolism, and immune function, and studies on many species have shown a link between microbial composition and physiology (13–25). Additionally, evidence is emerging to support the claim that physiological tolerance limits, such as the body temperatures at which normal organismal function is maintained, can depend on microbiome composition (26–29). In insect and lizard hosts, for example, warming-induced losses of microbiota can reduce host thermal tolerance (26–28). In salamanders, digestive performance and microbiome diversity decrease in tandem under exposure to warming (29). Thus, if an animal's microbiome is altered due to environmental change, this may result in a greater susceptibility to disease or an altered thermal physiology, which in turn leads to reduced survival or reduced reproductive success in the changing environment. These effects may be particularly pronounced in ectotherms, whose body temperatures are directly influenced by the environmental temperature (30).

Tropical forest lizards are thought to be especially at risk from rising temperatures because they often have narrow thermal tolerance ranges and live in thermally homogenous environments that preclude the behavioral buffering of stressful climatic conditions (3, 31–33). While the microbiome is poorly characterized for most lizard species (34), recent studies have begun to reveal links between the gut microbiomes of temperate-zone lizards and exposure to thermal stress. In common lizards (*Zootoca vivipara*) from Eurasia, Bestion et al. found reduced microbial richness and changes in the relative abundance of some microbial taxa under low levels of warming (35). In western fence lizards (*Sceloporous occidentalis*) from North America, Moeller et al. observed a decrease in one of the predominant bacterial phyla and a shift in community composition under a 10°C increase (27). While these recent studies provided valuable insight into the gut microbiomes of lizards in response to warming in laboratory and mesocosm settings, to our knowledge, variation in lizard microbiomes across years and in response to climatic fluctuations in wild populations has never been examined.

We studied the response of the gut microbiome to climate change in both wild and captive populations of a lowland, tropical forest lizard. First, to examine the potential

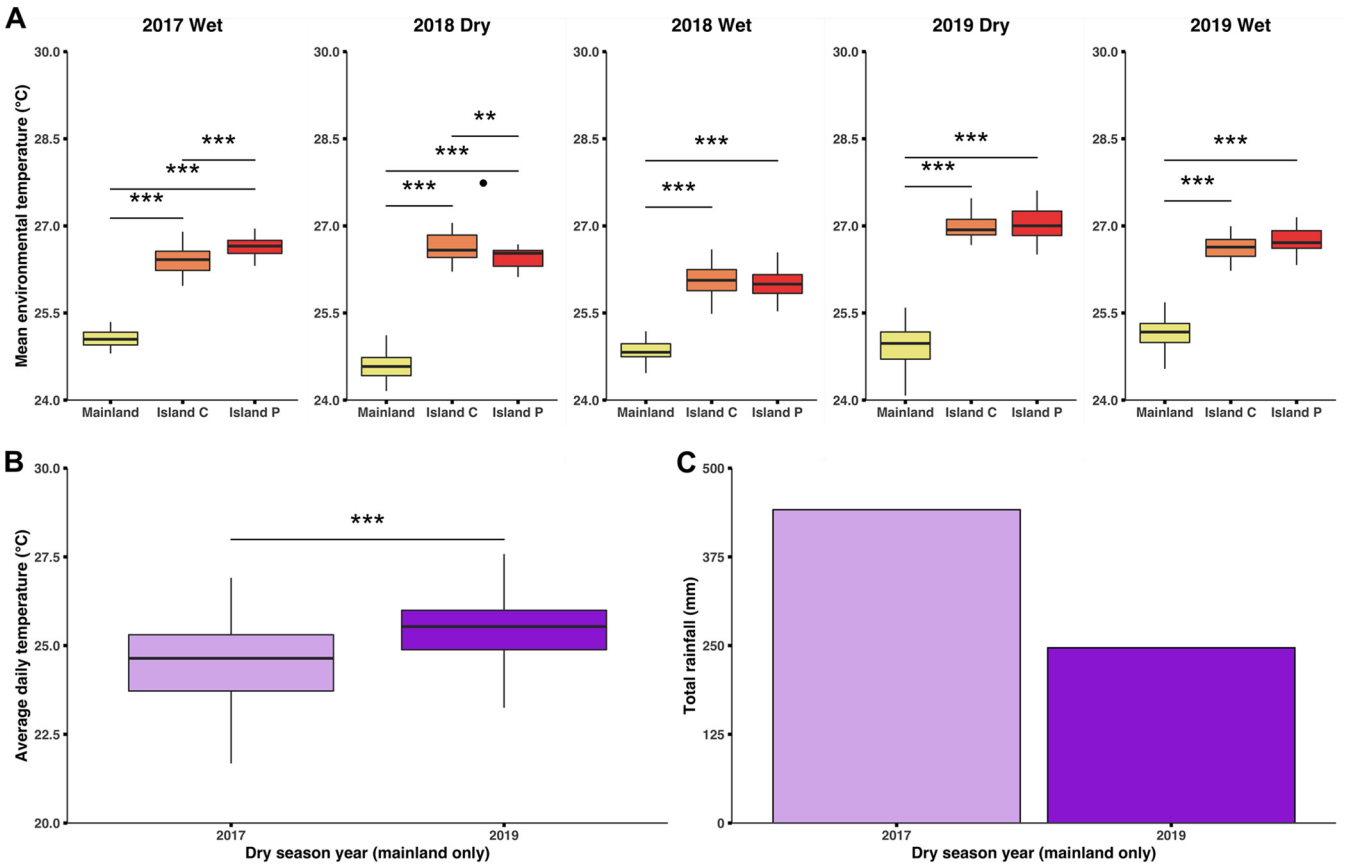

FIG 1 Climatic variation among sites, years, and seasons. (A) Mean environmental temperatures (recorded using data loggers deployed randomly in space) were warmer on experimental islands than on the mainland across all years and seasons. (B) Average daily temperatures in central Panama were higher in 2019 (during a severe drought) compared to the equivalent time period in the previous year (data from the Clearing Weather Station on Barro Colorado Island). Boxplots display the median, interquartile range, and range of the data. (C) Total dry season rainfall was substantially lower during the drought in 2019, relative to the same period in 2017. Significant differences are indicated with asterisks (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$).

of longer-term climate change to drive shifts in gut microbiome communities, we transplanted slender anoles (*Anolis apletophallus*, sensu Köhler and Sunyer, 2008) from a single source population on the mainland to several islands in the Panama Canal (36). These islands were substantially warmer than the ancestral habitat, and we compared the gut microbiomes of these populations after three generations of divergence. Next, to test whether the gut microbiomes remained stable during short-term heat wave events, we conducted a greenhouse experiment in which we maintained slender anoles in either control conditions or warm conditions for 5 weeks and then measured shifts in their gut microbiome characteristics. In these same individuals, we also tested for associations between the abundance of specific microbial taxa and variation in host cold tolerance, heat tolerance, and thermal preference. Finally, we compared microbiomes sampled from the same mainland population in both 2017 and 2019, before and after a severe drought affected central Panama. We hypothesized that gut microbiome structure and function would change after three generations on the warmer islands, through exposure to short-term warming in greenhouses, and over time on the mainland (possibly in response to the drought).

RESULTS

Climatic differences among sites and years. The mean environmental temperatures of Islands C and P were 1.34 and 1.36°C hotter than the mainland across all seasons and years (Fig. 1A). According to the weather station on Barro Colorado Island, the average daily temperature in the 2019 dry season (January through May) was 0.8°C hotter than the same period in 2017 (Fig. 1B). Data from this same weather station also

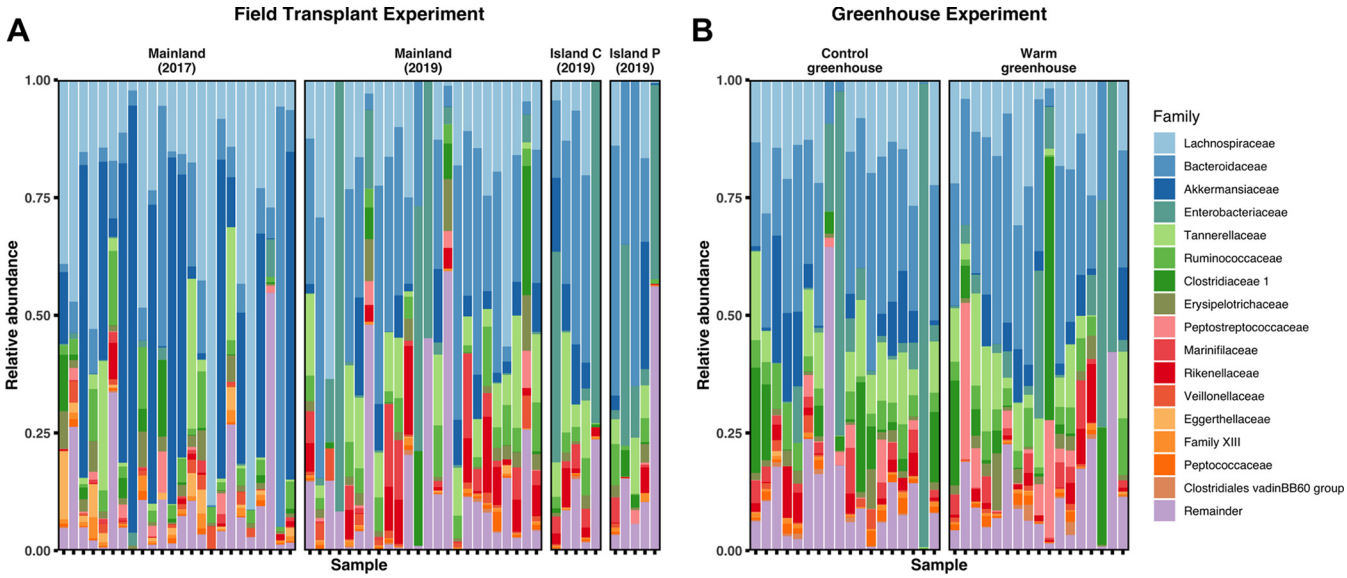

FIG 2 Microbial taxonomic composition among hosts, sites, and treatments. Microbial taxonomic composition of lizard gut samples at the family level for our field sites (A) and greenhouses (B). Each bar is a sample from an individual host and shows the relative abundance of ASVs in the top 16 most abundant microbial families. Note that while statistical analytical comparisons were done at the ASV level, here we present patterns at the family level for ease of visualization.

showed that the drought which occurred during the 2019 dry season resulted in 194.57 mm less rainfall compared to the same time period in 2017 (Fig. 1C). Further, the mean rainfall of the past 10 dry seasons was 442.62 mm, and in 2019, central Panama received just 246.89 mm of rain, or only about half of the rainfall of a typical dry season (Table S4).

Taxonomic and functional composition of the slender anole microbiome. We sequenced a total of 93 lizard intestinal tracts from all samples across all sites and treatments. Sequencing resulted in 10,981,185 reads after quality control with a mean sequence length of 244 bp and 9,364 unique ASVs. Samples had a mean sequence content of 109,285 sequences and ASVs had a mean frequency of 1,163 sequences.

When we considered our entire data set, the slender anole microbiome was dominated by the phyla Bacteroidetes, Firmicutes, Proteobacteria, and Verrucomicrobia (Fig. 2A and B). We found no significant differences in metrics of diversity between sexes (Table S1). The mean Jaccard distance between any two samples in this data set was 0.656, indicating that, on average, any two lizards shared only 34% of their ASVs. At both the ASV and the 98% identity operational taxonomic units (OTU) levels, zero features were present in 100% of lizards in this data set. Only one ASV was found in at least 50% of the lizards. Even when the ASVs were clustered into 98% OTUs, only seven OTUs were shared by at least 75% of lizards. All overlapping ASVs or OTUs were members of Bacterioidetes, Firmicutes, Proteobacteria, or Verrucomicrobia.

The relative abundance of the most common predicted cellular functions was more consistent among samples than that of the specific taxa present (Fig. 2A and B; Fig. 3 and B). The mean Jaccard distance across all samples with regard to KEGG function was 0.227 (0.429 lower than by taxonomy), meaning that on average any two lizards shared 76% of their functional orthologs. Further, 2,162 out of 7,760 predicted KEGG orthologs were present in all of the lizards that we sequenced.

Effects of long-term climatic shifts in the wild. The prevailing influence on the taxonomic structure of the slender anole gut microbiome was the year that the microbiome was sampled, irrespective of the population sampled (Fig. 4A to D). The impacts of sampling location and year were visually apparent in the overall taxonomic composition (Fig. 4A), relative abundance of high-prevalence taxa (Fig. 4B), community evenness (Kruskal-Wallis H: 12.697; *P* value 0.005) (Fig. 4C), and community dissimilarity (unweighted UniFrac PERMANOVA pseudo-F: 2.306; *P* value 0.001) (Fig. 4D). Subsequent

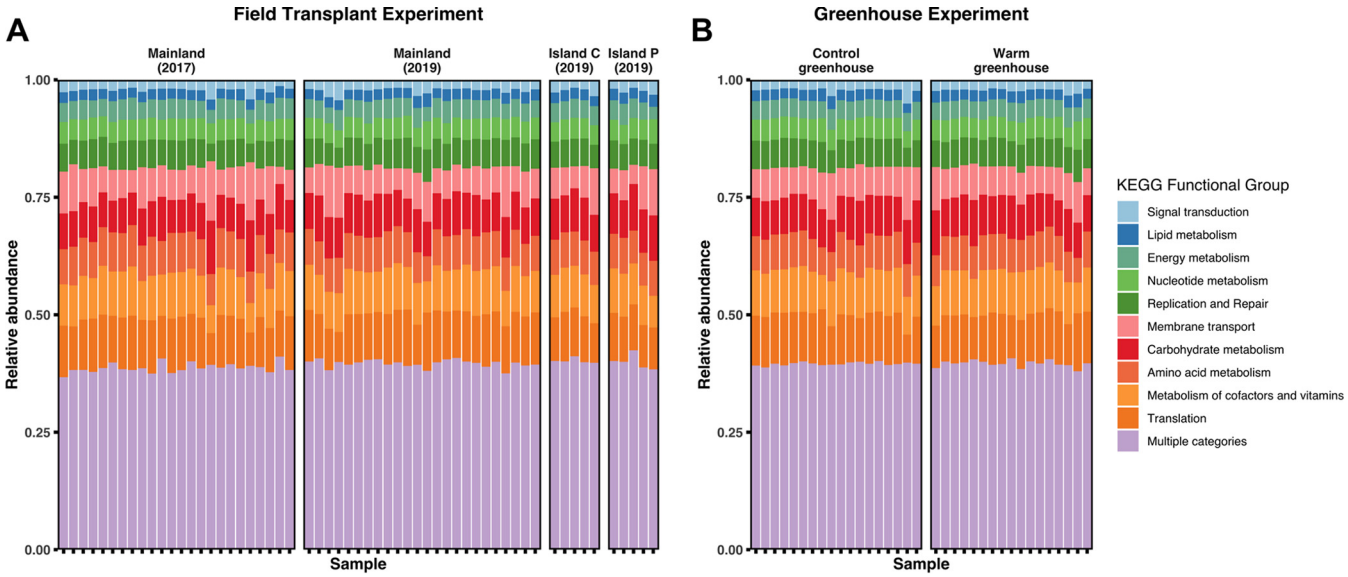

FIG 3 Functional variation in gut microbiome composition among hosts, sites, and treatments. Individual hosts varied little in the functional composition of gene pathways related to the metabolism found within their gut microbiomes, irrespective of whether the hosts were sampled from the field (A) or from the greenhouses (B). Each bar is a sample from an individual host and shows the top 10 KEGG categories by abundance, grouped at level B in the KEGG BRITE functional hierarchy. "Multiple categories" refers to functional annotations that were present in multiple level B functional groups.

pairwise PERMANOVAs revealed that differences between sampling groups were driven by year as differences in beta-diversity were only significant when comparing between each group in 2019 and the ancestral population in 2017 (BH-adjusted unweighted UniFrac pairwise PERMANOVA P values < 0.05), and there was no significant difference in the beta-diversity between the mainland and island lizards in 2019 (unweighted UniFrac PERMANOVA pseudo-F: 1.310; $P = 0.124$) (Table S1). Further, the differences in evenness were driven only by the differences between the 2017 and 2019 mainland lizards (BH-corrected pairwise Kruskal-Wallis P value = 0.011) (Fig. 4C). No significant difference in the number of ASVs was seen between years (Table S1). Between 2017 and 2019, we observed changes in the relative abundance of two of the five most abundant bacterial phyla (Fig. 4B). The relative abundance of Actinobacteria changed the most between the years, with this taxon decreasing in abundance between 2017 and 2019 across all sites (Bonferroni-adjusted pairwise Wilcoxon P values < 0.05). We also observed an increase in the relative abundance of Bacteroidetes between all sites in 2017 and 2019, though this was only significantly different between the two mainland samples (Bonferroni-adjusted pairwise Wilcoxon P value = 0.009).

With the changes in the microbial taxonomic profiles, the predicted functional diversity also shifted between years (Fig. 5A; Fig. 5C). Functional community evenness was significantly different among wild populations (Kruskal-Wallis H: 13.891, $P = 0.003$) (Fig. 5A), and *post hoc* tests showed that this significant difference was apparent only between the 2017 and 2019 mainland populations (pairwise Wilcoxon rank-sum with BH correction, H: 12.230, $P = 0.003$) and not between the mainland and island groups in 2019 (Fig. 5A). On a principal coordinates analysis (PCoA) plot of functional community dissimilarity (Bray-Curtis), the 2017 and 2019 groups clustered distinctly along PC1 (PERMANOVA pseudo-F: 2.730, $P = 0.002$) (Fig. 5C). We also observed a significant difference in the richness of functional orthologs among the wild populations (Kruskal-Wallis H: 7.806, $P = 0.05$) (Table S3). This was again driven by a significant difference between 2017 and 2019 mainland samples (Wilcoxon rank-sum with BH correction, H: 7.106, $P = 0.046$), whereas no difference was seen between islands and the mainland (all $P > 0.5$). This contrasted with the lack of significant differences that we found when simply comparing ASVs (Table S1). 100 functions were present in higher relative abundance in 2019 compared to 2017 (Table S5). These changes were predominantly related to metabolism (particularly carbohydrate metabolism), genetic information

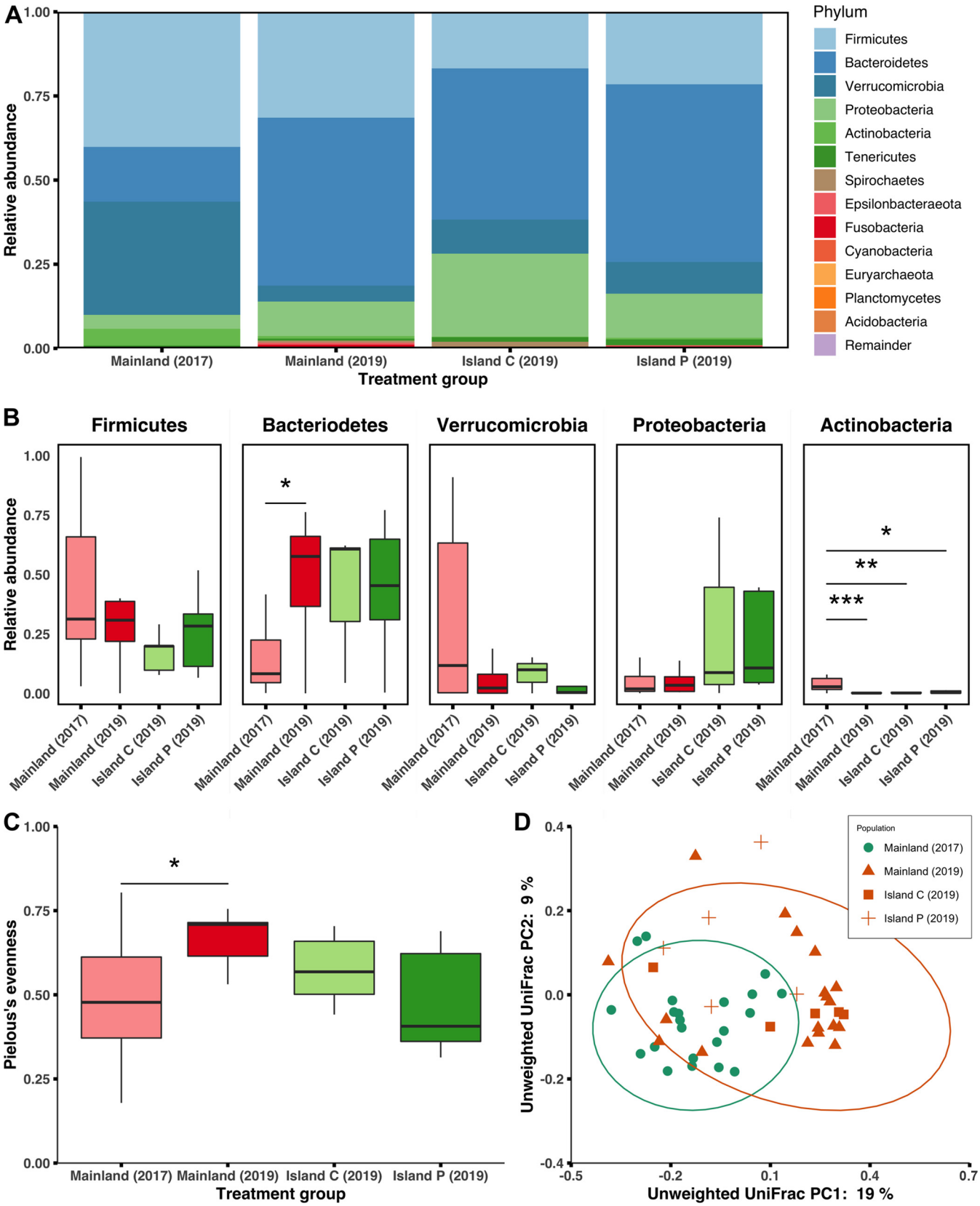

FIG 4 Community composition of microbial taxa across sites and years. (A) The relative abundance of ASVs of microbial taxa (phyla) in the gut of the slender anole was similar between the mainland and experimental islands in 2019 but differed between the mainland in 2017 and all 2019 groups. (B) The relative abundance of ASVs assigned to Actinobacteria differed between all sites and years, whereas the relative abundance of three of the four other most

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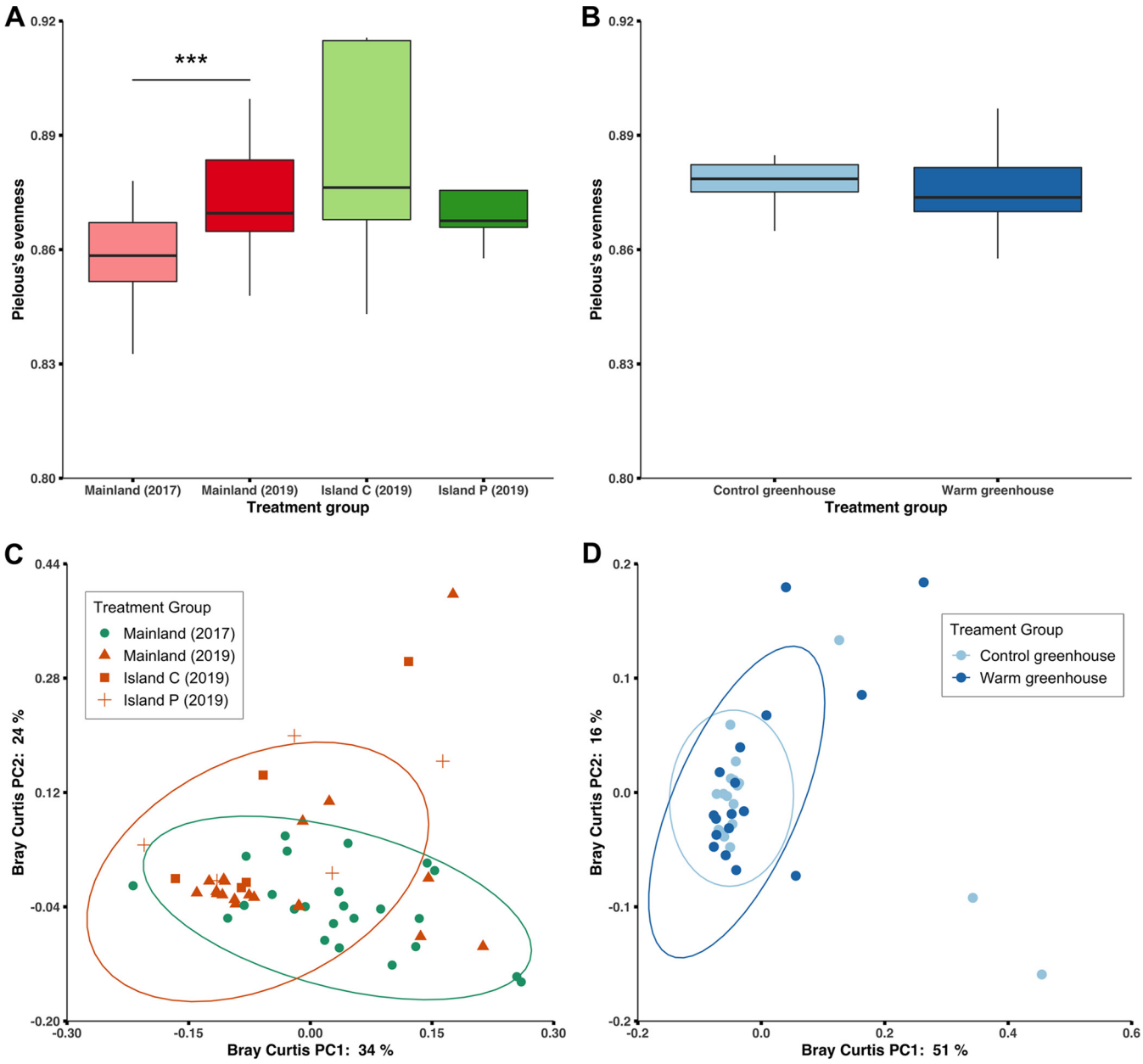

FIG 5 Diversity of functional composition among lizard hosts and sites. (A) The community evenness of functional orthologs was elevated in the mainland population in 2019. (B) The community evenness of functional orthologs was similar in control and warm greenhouses. (C) Year (green = 2017, orange = 2019) had a significant effect on the community dissimilarity of the functional compositions of the lizard gut microbiomes. (D) Short-term warming had no effect on the community dissimilarity of the functional composition of the lizard gut microbiomes. Boxplots display the median, interquartile range, and range of the data. Significant differences for the boxplots are indicated by asterisks (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$).

processing, signaling and cellular processes, and environmental information processing (Table S5).

Effects of short-term warming. During the greenhouse experiments, lizards in warm conditions experienced elevated body temperatures compared to those in control conditions. After the onset of the artificial heat wave, lizard surface body temperatures in

FIG 4 Legend (Continued)
abundant taxa did not differ among sites or years (Bacteroidetes increased in relative abundance between 2017 and 2019 on the mainland). (C) The evenness of microbial communities increased between 2017 (predrought) and 2019 (postdrought) on the mainland, whereas evenness did not differ between any site in 2019. (D) PCoA plot showing that the slender anole gut microbial community structure on the mainland in 2017 differed from all other years and sites. Boxplots display the median, interquartile range, and range of the data. Significant differences for the boxplots are indicated by asterisks (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$).

warm conditions were on average 3.0°C warmer during the day (08:00 to 18:00) than lizards kept in control conditions (control greenhouse: mean = 26.0°C, N = 187; warm greenhouse: mean = 29.0°C, N = 149). Lizards were also more likely to experience thermally stressful conditions in the warm greenhouse. 27.7% of the surface body temperatures that we measured in the warm greenhouse were above 30°C, whereas only 2.0% of surface temperatures were above 30°C in the control greenhouse. Note that these surface body temperature measurements include seven additional individuals that were not included in the final microbiome sequencing data set.

Short-term warming did not affect the taxonomic or functional composition of the slender anole gut microbiome. Average taxonomic composition (Fig. 6A), evenness (Fig. 6B), and community dissimilarity did not differ between the warming and control treatments (Fig. 6C; Table S1). Also, short-term warming did not alter any estimate of functional diversity (Fig. 5B and D; Table S3). Captivity itself, however, slightly altered the microbial community dissimilarity, and the captive and noncaptive anoles (collected simultaneously from our mainland field site) clustered distinctly along PC2 in a PCoA plot (Fig. 5C). This difference was only detected with the unweighted UniFrac metric (Fig. 5C; Table S3) (PERMANOVA pseudo-F: 1.384, *P* value 0.046). Finally, there were no significant relationships between the host thermal physiology or the thermal preference and any metric of microbiome composition, diversity, or a change in the abundance of select taxa (Table S2).

DISCUSSION

Symbiotic gut microorganisms play an important role in the health and survival of many organisms. Changing environmental temperatures, both in the short term and in the long term, can impact the structure, stability, and function of the gut microbiome, potentially resulting in negative impacts on a host (30, 37, 38). Here, we used a long-term island transplant experiment in tandem with a short-term greenhouse warming experiment to test whether and how different aspects of environmental change might impact the microbiome and physiology of a species vulnerable to climate warming. We found that an experimentally induced, artificial "heat wave" had little effect on any metric of the diversity of the microbiome in terms of either taxonomic composition or functional makeup. Instead, we found significant changes in the taxonomic and functional structures of the microbiome between 2017 and 2019 across all of the wild populations we studied. A possible explanation for these differences is that the composition of slender anole gut microbiomes was impacted by the severe drought that occurred between the end of 2018 and mid 2019, which dramatically altered the temperature and rainfall regimes in central Panama. If so, our results suggest that the gut microbiome of slender anoles is resilient to short-term warming but can be restructured by longer-term climate anomalies, such as sustained droughts. Our results have implications for the interpretation of results from short-term experiments performed in captivity as well as for longer-term changes that may occur due to global climate change.

In the wild populations we studied, we found little effect of transplantation to islands but strong effects of sampling year on both the structure and the function of the gut microbiome. The taxonomic composition of the microbiome, the community dissimilarity, and the relative abundance of the top five microbial phyla did not differ between the mainland and experimental islands in 2019, even though the islands were nearly 1.5°C warmer than the mainland (a large difference for the lowland tropics) over the three-generation duration of the study. We had hypothesized that the microbiomes of third-generation lizards on the warmer islands would have diverged as a result of plasticity or evolution. Although this may have occurred, the fact that both the mainland and island populations exhibited extremely similar microbiome communities in 2019 suggests that regional effects may have influenced the gut microbiomes of lizards at all sites in this year and swamped any prior variation that may have arisen from spatial differences between the sites. A notable occurrence that affected the entire region during our study was a drought that occurred just prior to

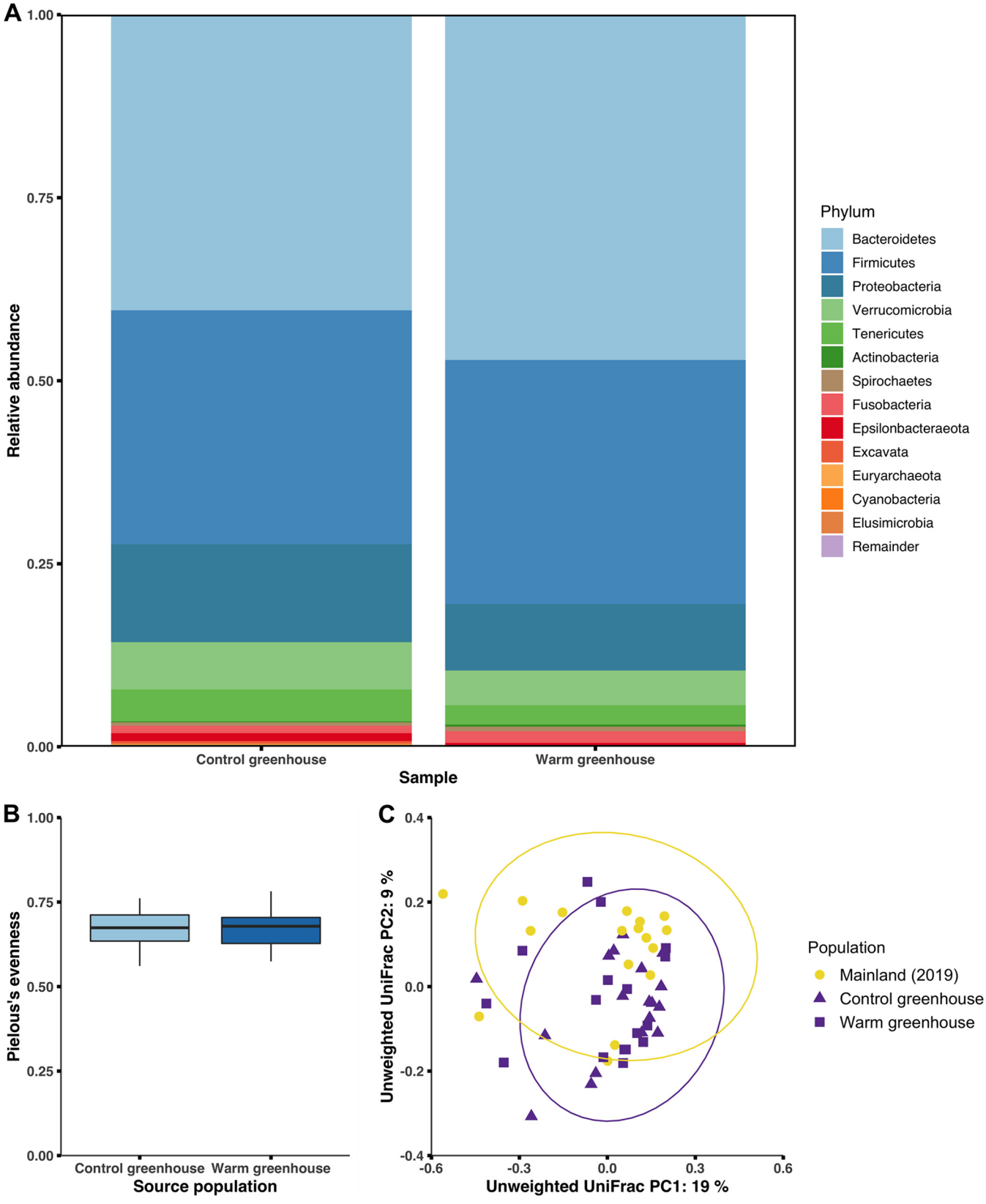

FIG 6 Community composition of microbial taxa before and after exposure to short-term warming in a greenhouse. The relative abundance of the top 13 taxa (A) and the evenness of microbial communities (B) were similar between control and warm conditions. (C) Captive lizards (control and warm greenhouses) differed in community structure compared to wild population collected from the same site in the same year (2019). Boxplots display the median, interquartile range, and range of the data.

our final sampling that year. This drought (which was one of the most severe ever recorded in Panama) is a likely suspect for a driver of the observed temporal changes in microbiome composition. Nevertheless, it is important to note that other, perhaps more subtle, environmental changes may have occurred in addition to the drought, and even if the drought was important, our data cannot determine whether its effects were direct (changes in temperature and precipitation) or indirect (e.g., changes in prey availability; see the caveat below). Regardless, compared to 2017, the microbiome samples from 2019 displayed an increased community evenness, a shift in community dissimilarity, a reduction in the relative abundance of the phylum Actinobacteria, and an increase in the relative abundance of the phylum Bacteroidetes. These effects were observed despite high intraspecific variation in microbial community membership.

Another piece of evidence that supports the hypothesis that a regional event, such as the drought, may have been the key driver of microbiome variation between years is the observation that the mainland and islands shifted in their microbial diversity in similar directions across all metrics, despite the islands and mainland likely differing in their native arthropod and plant communities. Prey communities are known to affect the gut microbiomes of higher trophic level consumers (39, 40). Thus, it is difficult to explain how gut microbiomes among experimental island populations would have remained similar after multiple generations without invoking a strong regional event that affected all populations in similar ways. Nevertheless, additional longitudinal sampling of our mainland and island populations is needed to confirm this conclusion.

In contrast to our work, previous studies in lizards using the 16S rRNA gene have almost always found that warming altered the gut microbiome, although these studies measured warming over shorter time scales and under captive conditions (27, 35, 41). In the common lizard (*Zootoca vivipara*), warmer temperatures reduced overall bacterial richness and altered the relative abundance of dominant phyla, enriching Proteobacteria and Actinobacteria and reducing Bacteroidetes and Firmicutes (35). In western fence lizards (*Sceloporus occidentalis*) exposed to temperatures at the upper ranges of their thermal tolerance, no decrease in microbial richness was observed, but the abundance of Firmicutes was reduced (27). While a similar change in the abundance of Firmicutes has been observed using both 16S sequencing (29, 37) and shotgun metagenomics (42) in other tetrapods exposed to thermal stress, including tadpoles, salamanders, and hens, we did not observe this effect in any of our comparisons, possibly indicating that the responses are idiosyncratic and are dependent on the particular host taxon being considered. Indeed, 16S studies in fish and invertebrates have not shown this decrease in Firmicutes (30, 43, 44). Finally, slender anoles are generalist, insectivorous lizards. Thus, if the observed changes between 2017 and 2019 were indeed induced by the drought, this may have affected their microbiome indirectly by altering the abundance and the composition of invertebrate (prey) communities. Additional controlled experiments in which the temperature, humidity, and prey availability are independently manipulated over longer time periods and the changes in the microbiomes are assessed could help elucidate the mechanisms at play.

Interestingly, the yearly differences in the gut microbiome were apparent not only in the taxonomic structure of the gut microbiome but also on its predicted functional profile. This was true, even though the predicted functional makeup of the lizard gut microbiomes was substantially more consistent among individuals than in the taxonomic makeup. While we found a low overlap of ASVs among the lizards in our data set, 2,162 out of 7,790 inferred KEGG orthologs were present in all of our samples. A previous study of *Anolis* microbiomes (*Anolis cristatellus* and *A. sagrei*) also highlighted high taxonomic diversity, and this result, although constrained by the limits of predictive tools like PICRUSt2, is motivation for additional work into the functional consistency of the reptile microbiota (45). Despite this consistency of predicted functional makeups, we still observed an increase in the evenness of functions and distinct PCoA clustering between 2017 and 2019. Genes which were present in a significantly higher abundance in 2019 were those predominantly encoding functions related to metabolism (particularly

carbohydrate metabolism), genetic information processing, signaling and cellular processes, and environmental information processing. A similar pattern was found in the common lizard (*Zootoca vivipara*), in which predicted genes associated with metabolism, information processing, and cellular abundances were enriched following warming in male lizards (35). As previous work has shown that warming can impact digestive efficiency in ectotherms, it is possible that changes in the microbial community and the resulting functional potential could impact host nutrient acquisition (29). This emerging pattern, in which certain functional profiles are enriched under warmer conditions, suggests that evaluating changes in microbial function after extreme climatic events is likely to be a fruitful avenue for future research on the resilience of animals to global change.

If the differences in the environmental temperature between 2017 and 2019 caused changes in the slender anole gut microbiomes in the wild, this effect was not recapitulated in our greenhouse experiment. Instead, anole microbiomes were unchanged by short-term warming. There was little effect from a simulated "heat wave", in which lizards regularly experienced stressful body temperatures. After exposure to the warm greenhouse, no metric of microbial taxonomic or functional structure changed. Interestingly, this result is abnormal, compared to previous 16S amplicon studies on lizards, which have invariably found that short-term warming changes the gut microbiome composition (27, 35, 41). All of these previous studies were on species from temperate latitudes, suggesting the possibility that there is a latitudinal gradient in microbiome resilience to heat waves, although much more work is needed to test this hypothesis. Despite short-term warming having no significant impact on our study species, captivity did alter the microbiome community composition, in that the lizards from both greenhouses (control and warm) ended with divergent microbiomes relative to the mainland lizards sampled in the same year. This effect was only apparent when measured by unweighted UniFrac, a metric which gives equal precedence to rare and abundant taxa, implying that rare taxa may be important in shaping these differences (46). Captivity-driven changes to the microbiota have previously been observed in a multitude of host taxa, using both 16S and shotgun metagenomics (22, 47–49). These studies include lizards, with effects varying among host species, and include changes to the relative abundance of dominant phyla (50), decreased alpha diversity (45), distinct clustering on PCoA plots (45), and an increased richness of and changes to the community structure (51). The changes that we observed in the captive lizard gut microbiomes could have been caused by cage conditions, contact with researchers, the artificial diet of domestic crickets that we fed to all of the individuals, or a combination of these factors.

Previous studies using both 16S amplicon sequencing and qPCR, including one on lizards, found that variations in the abundance of certain microbial taxa were associated with differences in host thermal tolerance (26–28). In contrast, we did not detect any relationship between the microbiome community structure or the abundance of particular microbial taxa on slender anole thermal physiology (cold tolerance, heat tolerance, or thermal preference in a laboratory arena). This, in combination with the high functional redundancy among gut microbiomes of individuals, raises the possibility that the physiological tolerances of some tropical species are robust to shifts in microbiomes brought on by climate change.

Several of our results should be interpreted with caution. First, the high microbial diversity among host individuals may have obscured the population-level effects that we expected to see. This issue is particularly salient, given the small number of individuals that we sampled from our experimental islands, in which we were limited due to small population sizes and logistical constraints. However, despite the small sample sizes on the islands, we revealed a number of consistent patterns between the islands and the mainland in 2019, indicating that our sample sizes were sufficient to capture broad patterns across sites. A more fundamental issue is that we were not able to sample island lizards in 2018. Therefore, we cannot determine whether environmental differences between the mainland and islands generated the divergence in the lizard microbiomes in the period between the sampling years. Finally, 16S rRNA gene sequencing is sometimes thought to have limited power to determine the predicted

functional profiles of microbial communities, particularly when using a small section of the 16S rRNA gene, which can have limited taxonomic resolution in some host species (52). Nevertheless, more advanced methods (e.g., shotgun metagenomics) often find high functional overlap, despite taxonomic diversity among samples, just as we observed here (53, 54). Despite these limitations, our analysis improves our understanding of the effect of increased temperature on host microbiomes, which is imperative in understanding host physiology and resiliency to climate change.

As the global climate shifts, the responses of animals will be influenced, at least partly, by the composition of their resident microbial communities. This is thought to be especially true for tropical ectotherms, which are thermal specialists and have body temperatures that equilibrate rapidly to changes in ambient temperature. Our results suggest that while the slender anole's gut microbiome may be resilient to shorter bouts of environmental warming, year-to-year variation in microbiome composition and function can be substantial and might be driven by regional climate anomalies, such as droughts. The specific mechanisms by which climate change alters microbiomes, or whether these changes affect population dynamics and the fitness of hosts, remain open questions worthy of future study. Regardless, it is these "meta-organisms" (hosts and their microbial symbionts) that may be the fundamental biological unit responding to environmental change.

MATERIALS AND METHODS

Study system. The slender anole is a small, insectivorous, semiarboreal Dactyloid lizard that occupies the understory of secondary and primary rainforests in central Panama (55–57). Slender anoles have greater than 90% annual mortality and, therefore, are essentially an annual species (58). They have a narrow thermal tolerance range (31, 32, 59) and are considered vulnerable to climate warming (32, 60). Indeed, field surveys over several decades have shown that the abundances of some populations have been declining in recent years due to shifts in the local climate (60).

Experimental design: field transplants and longitudinal sampling on the mainland. Lake Gatún is an artificial lake that comprises the middle 21 miles of the Panama Canal. This lake was created when the Chagres River was dammed during the canal's construction, which flooded the Chagres Valley. Within Lake Gatún, there are hundreds of small islands, which were formerly hilltops that remained above water level. These islands are warmer than the adjacent mainland environments, primarily due to the decreased canopy cover and the higher edge-to-interior ratio. Given their small size and isolation, the islands almost certainly have lower species diversity than the mainland. Nevertheless, Lake Gatún is positioned within the historic geographic range of the slender anole, and thus, the larger islands in the lake (not considered in this study) have permanent populations. In our study, we utilized smaller, anole-free islands as natural arenas in which to expose wild populations to consistently warmer environments over several years. These islands are a sufficient distance from the mainland and from other anole-occupied islands so as to prevent the movement of individuals between our study sites. Our mainland (ancestral) site was in Soberanía National Park, Panama (9°08.120′N, −79°43.388′W). We transplanted lizards from along a central trail called Pipeline Road at our mainland site to two experimental island sites, Islands C and P (9°08.025′N, −79°50.322′W and 9°11.362′N, −79°49.933′W, respectively), in 2017 (transplantation occurred between June and September; note that we conducted parallel transplants during this time to other islands that are not considered in this study). We transplanted 70 lizards to each island (equal sex ratios) after giving each individual a unique identifier using injections of visual implant elastomer (61). We conducted mark recapture each wet season (May to November) on the experimental islands, although we only sampled the microbiomes from third-generation adults captured in 2019 (see below).

To quantify the thermal differences between the mainland and islands (and between years), we deployed temperature loggers programmed to measure environmental temperatures every 100 min. Each temperature logger consisted of an iButton (calibrated at factory: Embedded Data Systems, Lawrenceburg, KY, USA) that was coated in Plasti-Dip (Plasti Dip International, Blaine, MN, USA) for waterproofing and was glued to a small piece of wooden trim. We have used these data loggers in the past to reliably estimate the environmental temperature distributions of slender anoles (32, 59, 62). On the mainland, we deployed temperature loggers each year in both the wet and dry seasons in random locations along a set of transects that radiated from Pipeline Road into the forest. We deployed two transects in the wet season of 2017 and six in both the wet and dry seasons of 2018 and 2019 (~15 data loggers per transect). The temperature loggers were fastened to branches using zip ties at random distances along each transect (in 1 m intervals), on a random side of each transect (left or right), at a random height in the vegetation (0.5 to 2 m in 0.5 m intervals), and in a random orientation on the branch (top, side, or bottom). During the same time periods, we deployed between 25 and 30 temperature data loggers on both experimental islands by haphazardly choosing locations that covered the majority of each island and then choosing a random cardinal direction, distance (0 to 3 m in 1 m intervals), height (0.5 to 2 m in 0.5 m intervals), and orientation on the branch (top, side, or bottom).

In the wet season (May to November) of 2017, we collected 15 male and 15 female adult (>38 mm SVL) slender anoles from our mainland site to sample their microbiomes as a pretransplant control. In

2019, during the same time of year, we collected an additional 15 males and 15 females from our mainland site (to serve as a longitudinal sample and a control) as well as five third-generation males from each experimental island. We only sampled males from the experimental islands because these small populations are part of an ongoing experiment, and the removal of females or large numbers of males would have a high impact on population growth rates. All lizards were clean-collected by hand, using nitrile gloves. We determined the sex of all individuals by examining the dewlap and then immediately euthanized them (via decapitation) in the field and stored them on ice in clean plastic bags. They were then transported to the Smithsonian facility in Gamboa, where we preserved them at −20°C.

Experimental design: greenhouse experiment. To study the effects of short-term warming on the slender anole gut microbiome, we conducted a controlled greenhouse experiment in 2019. We collected a total of 35 adult slender anoles from our mainland site (17 females and 18 males). The lizards were allowed to acclimate to the laboratory conditions for 48 h, after which we measured several indices of thermal tolerance and thermoregulatory behavior. First, we measured each lizard's critical thermal minimum (CT_{min}) and voluntary thermal maximum (VT_{max}), which are estimates of cold and heat tolerance, respectively. We randomized the order in which lizards were assayed for each of these variables, and all of the individuals were given a minimum of 2 h of rest between assay types. To measure CT_{min}, the lizards were placed in an incubator (myTemp Mini, Benchmark Scientific, Sayreville, NJ, USA) that was set to 2°C for 10 min to lower their body temperatures below the point at which they lose their righting ability. The lizards were then removed from the incubator and allowed to warm toward room temperature. During this period, we placed each lizard on its dorsal surface every 10 s to determine whether it had regained its righting response. When a lizard was able to right itself, we recorded its body temperature using a cloacal thermometer (Omega thermocouple with a type K temperature probe, Norwalk, CT, USA). We have validated this approach as a reliable way to measure CT_{min} in previous studies (32, 59, 62). VT_{max} is the upper body temperature at which a lizard displays clear escape behavior and is therefore an estimate of the temperature at which the lizard may begin to seek shade in nature (63–65). To measure VT_{max}, lizards were placed, one at a time, within a small plastic container, which was itself placed inside an incubator set to 50°C. We observed the lizards for signs of fleeing behavior, which occurs at a threshold body temperature and is clearly distinguishable from other behaviors (such as exploratory behavior). When a lizard displayed fleeing behavior, it was removed from the incubator, and its body temperature was measured with a cloacal thermometer.

Next, following our previously established protocol (32, 59), we evaluated thermoregulatory behavior by introducing each individual to a thermal gradient in the laboratory. Laboratory-based thermal arenas do not contain barriers to movement, and the body temperatures that lizards achieve in these gradients are therefore assumed to be thier "preferred" temperatures that optimize their physiological performance (63, 66–69). We built our gradients from plastic bins measuring 0.85 m × 0.4 m × 0.4 m (length × width × height) and had an infrared heat lamp (250 W) suspended over one end. We adjusted the height of the heat lamps to generate a temperature gradient ranging from 22°C on the cooler end (controlled by the thermostat in the room) to 38°C on the warmer end (verified with an infrared temperature gun). We gently heated a pot of water over a hot plate in the room with the thermal gradients to approximate natural relative humidity levels (approximately 60 to 80%) in slender anole habitats. Before placing lizards into the thermal gradients, we inserted a type-T thermocouple 5 mm into the cloaca and fixed it in place with medical tape. The other ends of the thermocouples were connected to a MadgeTech TCTempXLCD 8-channel data logger (MadgeTech, Warner, NH, USA). The body temperatures of all individuals were recorded automatically (no people were present in the room) for 2 h in the gradients, but we discarded the first hour of the measurements from the analyses (i.e., we treated the first hour as an acclimation period). All of the measurements of thermoregulatory behavior were made during the times of the day when slender anoles are typically active (between 07:00 and 18:00 h), and all individuals had been fasted for 48 h prior to the onset of trials. We considered the mean temperature that the lizards achieved in the gradient as their thermal preference, or T_{pref}.

After we measured the thermal tolerance and preference of all individuals, we randomly assigned them to a control or warm treatment group (equal sex ratios per treatment). The lizards were housed individually in 30 cm × 30 cm × 46 cm (width × depth × height) mesh cages with a four-inch base of leaf litter as the substrate and a vertical branch for perching. The cages were then placed inside two thermostat-controlled outdoor greenhouses at the Smithsonian Tropical Research Institution's facilities in Gamboa, Panama. The positions of males and females were positioned in an alternating fashion within the greenhouses to reduce the stress induced by male-male competition. The thermostat of the control greenhouse was originally set to 24°C for 5 days, then set to 25°C for 5 days, and raised to a final temperature of 26°C at which it remained for 21 days. The warm greenhouse was originally set to 24°C and was raised to a final temperature of 30°C over a period of 14 days to mimic the onset of a short-term heat wave. The warm greenhouse remained at 30°C for 17 days. Note that these greenhouses have glass roofs and were therefore exposed to natural shifts in solar radiation and cloud cover such that the thermostats dampened moment-to-moment shifts in temperature but did not prevent them completely. Thus, the warm greenhouse was exposed to bursts of temperature increase that went several degrees higher than 30°C. We maintained humidity in both greenhouses with a bucket of water placed in the center of each greenhouse and with daily misting of each cage. Each lizard was fed four to six crickets (*Acheta domesticus*) every 3 days. To validate that greenhouse temperatures resulted in sufficiently divergent thermal environments from the perspective of the lizards, we measured surface body temperatures over the course of the experiment by using a Fluke infrared temperature gun (Fluke, Wilmington, NC, USA). Towards the end of the exposure period, we fasted the lizards for 48 h (the lizards remained at experimental temperatures while fasting) and then remeasured all of the thermal tolerance and

thermoregulatory behavior assays exactly as described above. After this second round of physiological and behavioral assays, we released the lizards back into the greenhouses to be maintained for 7 days at experimental temperatures without further manipulation and with normal access to food and water such that the microbiome sampling would not reflect the short-term stress of the laboratory experiments. At the end of this 7 day post-experimentation period, the lizards were removed from the greenhouses, euthanized within 15 min, and preserved for later dissection at −20°C.

Lizard dissection, DNA extraction, and library preparation. We defrosted the lizards on ice until the tissues were pliable enough to be dissected. We then aseptically dissected each individual, using tools that were dipped in 100% ethanol and flame-sterilized. A vertical cut was made from the anterior portion of the chest to the vent (cloaca). We separated the cut skin in a manner to avoid contact of the skin with the internal organs. We then removed the entire GI tract from the stomach to the cloaca and detached the stomach from the intestines. The intestinal samples were stored at −20°C overnight in 100% ethanol. Only the intestinal portion of the gut was used for subsequent DNA extraction. A Qiagen DNeasy PowerSoil Kit (Cat No: 12888-100, Qiagen, Hilden, Germany) was used for whole DNA extraction with several minor modifications to optimize the procedure, as follows. We washed the gut material with two 500 μL volumes of PowerBead Solution to remove leftover ethanol. We cut the gut material into small pieces (approximately 1 mm^3) using scissors that had been dipped in 100% ethanol and flame sterilized. We then homogenized this material in the PowerBead tubes on a bead beater for 2 min (Biospec, Bartlesville, OK, USA). After the addition of solutions C2 and C3 that were provided in the Qiagen kit, we incubated samples at 4°C for 5 min. After the addition of C3, the resultant ~1,000 μL of homogenate was vortexed thoroughly and split into two aliquots. We then used 500 μL of the homogenate for the remainder of the protocol and used the resulting DNA for downstream PCR and for library preparation.

We quantified the DNA by using a NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA). Out of 108 dissected guts, 94 were of a high enough quality and concentration to be included in the final library preparation. We used a two-step, PCR-based 16S rRNA gene library preparation protocol. We amplified the V4 region of the bacterial 16S rRNA gene in triplicate using 515F and 806R IL, IL-N, and IL-2N phased primers (ordered from Integrated DNA Technologies, Coralville, IA, USA) (70, 71). For the first PCR, we used 6.1 μL H_2O, 5 μL Platinum *Taq* MasterMix (Thermo Fisher Scientific, Waltham, MA, USA), 0.2 μL of each primer, and 1 μL of sample DNA in a 12.5 μL total reaction volume. The cycling conditions were as follows: 3 min at 94°C; 25 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 1 min and 30 s; and a final hold for 10 min at 72°C. We verified amplification via gel electrophoresis (1% agarose with GelRed [Biotium, Fremont, CA, USA] run at 110V for 20 min). We then pooled 3 μL of each replicate for a final volume of 9 μL per sample, which was used for barcoding in the second polymerase chain reaction (PCR).

In a second, limited-cycle PCR, we added dual-indexed barcodes. Forward barcode primers SB501-SB508 and reverse barcode primers SB701-SB712 were used (72). In a total reaction volume of 10 μL, we combined 2 μL water, 5 μL Platinum *Taq* MasterMix, 1 μL of each respective barcoding primer, and 1 μL of pooled PCR product from the first PCR. The cycling conditions were as follows: 3 min at 94°C; 6 cycles of 94°C for 45 s, 50°C for 1 min, 72°C for 1 min and 30 s; and a final hold for 10 min at 72°C. We purified and normalized the final PCR product with a Charm Biotechnology 96-well PCR normalization and purification plate (Charm Biotech, Cape Girardeau, MO, USA), following the manufacturer's protocol. We pooled 10 μL of each barcoded sample into a microcentrifuge tube. The library was purified and concentrated using a 1× volume of SPRI beads (made in-house in the Smithsonian Ecological and Evolutionary Genomics Laboratory). We washed the beads two times with 80% ethanol. The final elution volume was 50 μL. We determined library concentrations using the Qubit fluorometer dsDNA high sensitivity assay (Thermo Fisher, Waltham, MA, USA), and quality was assessed using a high sensitivity DNA assay on a BioAnalyzer (Agilent, Santa Clara, CA, USA). We sequenced the libraries (including a DNA and a PCR negative control) on a full lane of an Illumina MiSeq (Illumina, San Diego, CA, USA) with 2 × 250 paired end reads. All of the methods and procedures described were carried out under the Institutional Animal Care and Use protocols issued by the Smithsonian Tropical Research Institute.

Statistical and bioinformatic analyses: environmental temperature data. To evaluate environmental differences among sites, we calculated the mean temperature measured from each data logger and then compiled these data by site, year, and season. We then conducted multiple regression, with temperature as the dependent variable and site, year, and season as interacting independent variables in a global model. Backwards stepwise model selection was performed in R (version 4.0.4) (73), using the Akaike information criterion (AIC) (74) to compare the models, and the best model included the three-way interaction between site, year, and season. This approach was compared to the output of the dredge function in the R package MuMIn, which provides a ranked list of all model possibilities (75). Both approaches yielded the same best model. *Post hoc* comparisons were then performed by utilizing pairwise *t* tests with the Bonferroni correction in R.

Panama experienced a severe drought during the dry season of 2019. Thus, we further compared the dry season conditions on the mainland in 2017 with those of the 2018 and 2019 dry seasons. To that end, we obtained data from the BCI Clearing Weather Station and extracted the average daily temperature and total precipitation for each time period. We then compared the differences in temperature between years using an analysis of variance (ANOVA) in R, with temperature as the dependent variable and dry season year as the independent variable. Subsequent *post hoc t* tests with Bonferroni corrections were then used to determine which years were driving these differences (76). We compared the raw values of precipitation, as these data were collected by the weather station as single values for each time period (total amount of rain per unit time).

Statistical and bioinformatic analyses: microbiome comparisons and links with host physiology and behavior. Our microbiome analyses fell into three primary sets of comparisons: (i) we compared mainland lizard microbiomes (collected in either 2017 or 2019) to those of island lizards collected in 2019 to understand the effects of multiple generations of adaptation to warm island environments; (ii) we compared mainland samples in 2017 with mainland samples from 2019 to understand te hwithin-population effect of the sampling year, which may be attributable to the drought that occurred between these sampling periods; and (iii) we compared the control and warm treatments from our greenhouse experiments to understand the role of short-term heat stress on lizard microbiomes and to identify potential links between the microbial community structure, host thermal tolerance, and host thermoregulatory behavior. For all of these comparisons, we followed identical procedures to characterize the microbiome community structure, as described below.

We imported and demultiplexed raw reads in QIIME2 version 2019.7 in the Casava 1.8 format (77). The reads were quality filtered and trimmed to remove primers and then clustered into amplicon sequence variants (ASVs), a proxy for species, in the QIIME2 DADA2 plug-in (78). We used ASVs for all analyses. When we attempted to determine the core microbiome members, we also clustered the ASVs into OTUs by using the vsearch plug-in in QIIME2 to ensure that the core members were not missed due to an artificial splitting of genomes in the ASV generation (79, 80). We constructed a phylogenetic tree using Mafft and Fasttree in QIIME2 (81–83), and this tree was rooted using QIIME2 midpoint-root. Taxonomy was assigned with the QIIME2 feature classifier (84), using a naive Bayes taxonomy pretrained classifier that was provided by QIIME2 and was trained on SILVA full-length 99% OTU 16S sequences (85, 86). We removed contaminants and controls by processing sequences in the R package *decontam* with the PCR and DNA negatives as references (87). Sequences that could not be assigned to at least a phylum and those which matched mitochondria or chloroplasts were also removed. We conducted alpha rarefaction in QIIME2 to explore sequencing depth. For all diversity metrics, we rarefied samples (subsampled without replacement) at 18,979 reads in QIIME2, as this depth enabled us to retain all of our island samples, and a rarefaction curve showed that the vast majority of samples plateaued in increasing diversity at this read depth (Fig. S1).

We calculated alpha and beta diversity metrics using QIIME2. The alpha diversity metrics were computed using the core-metrics-phylogenetic plugin in QIIME2. To determine whether lizards from different sites (mainland, Island C, Island P) and treatments (control greenhouse, warm greenhouse) differed in their number of unique microbial amplicon sequence variants (ASVs), we measured the feature count in QIIME2. To determine whether the evenness or alpha diversity of the microbial communities differed between sites and treatments, we computed the Pielou's evenness of samples and Shannon index (88, 89). Statistically significant differences for all alpha diversity and evenness metrics were calculated using Kruskal-Wallis tests that were followed by pairwise Wilcoxon rank-sum tests with the Benjamini-Hochberg (BH) false discovery rate correction if significant differences were observed (90, 91). To determine whether sites and treatments differed in microbial community structure, we measured the Jaccard distance (a qualitative measure of community dissimilarity), unweighted UniFrac (a qualitative measure incorporating phylogenetic relationships), and weighted UniFrac (a quantitative measure incorporating phylogenetic relationships) (46, 92, 93). Significant differences for all beta diversity metrics (Jaccard, weighted UniFrac, unweighted UniFrac) were calculated using a permutational multivariate analysis of variance (PERMANOVA) followed by pairwise PERMANOVAs with the BH correction when significant differences were observed. To visualize differences in the beta diversity metrics, we used a principal coordinates analysis (PCoA). To visualize and compare the overall taxonomic structure of the microbial communities, we imported ASV tables into R and grouped them by sample or treatment in the Phyloseq package (94). To test whether the five most abundant phyla (several of which have been reported to be sensitive to heat in other lizard microbial communities, e.g., [27], [35]) were significantly different in their relative abundances, we filtered the target taxa from rarefied feature tables and compared their relative abundances in each treatment via Wilcoxon rank-sum tests with Bonferroni *P* value corrections.

To determine whether the functional potential of the gut microbiome differed between sites and treatments, we used the PICRUSt2 QIIME2 plug-in to infer potential functional pathways possessed by the microbes (52). For the alpha and beta diversity analyses of the functional orthologs in QIIME2, we rarefied samples at 19,872,671 features. We calculated the alpha and beta diversity metrics in the same manner as described for the microbial ASVs; however, we used the Bray-Curtis distance in place of UniFrac to compare the composition of the functional orthologs between treatments, as the UniFrac metrics require a phylogenetic tree (95). The Kyoto Encyclopedia of Genes and Genomes (KEGG) system of IDs was used to identify functional traits, using the online KEGG mapper search pathway tool (96). The KEGG IDs were grouped at level B in the KEGG BRITE functional hierarchy, and the 10 most abundant functional groups were used to construct a composition plot. Finally, we used ALDEx2 in R to determine any KEGG orthologs which were differentially abundant (97).

Lastly, we tested for relationships between host physiological traits and gut microbiome composition by examining correlations between the gut microbiome composition at the end of the greenhouse experiment and lizard physiology (VT_{max}, CT_{min}) and behavior (mean T_{pref}). We also tested for associations between the gut microbiome composition at the end of the experiment and any changes in physiological or behavioral traits that had occurred over the course of the experiment. We explored the relationship between the relative abundance of the top five most abundant phyla as well as the variation in microbial communities described by the first two unweighted and weighted UniFrac axes and by the Bray-Curtis dissimilarity axes on these behavioral and physiological traits. We tested for relationships between each of the metrics of microbiome diversity with each of VT_{max}, CT_{min}, and T_{pref} as separate dependent variables

using linear models (ANOVA) in R. The *P* values of these ANOVAs were corrected using a Bonferroni multiple-comparison correction.

Data availability. Upon publication of the manuscript, the raw sequences and corresponding metadata will be available in the NCBI Sequencing Read Archive (BioProject PRJNA861826). All other relevant data and scripts for analysis are available at https://github.com/claireewilliams/Williamsetal2022AnolisMicrobiome.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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C.E.W., J.G.K., W.O.M., C.L.C., and M.L.L., designed the study. C.E.W., J.G.K., D.J.N., A.A.R., E.F., B.C., M.A.G.-K., L.K.N., J.D.C., C.L.C., and M.L.L. collected data. C.E.W., D.J.N., A.A.R., E.F., B.C., L.K.N., and M.L.L. built the lizard cages. C.E.W., A.A.R., E.F., B.C., M.A.G.-K., and M.L.L. conducted lizard husbandry. C.E.W. analyzed data. C.E.W. produced the first draft of the manuscript. All authors revised and approved the final version of the manuscript.

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