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Invasive Amphibian Gut Microbiota and Functions Shift Differentially in an Expanding Population but Remain Conserved Across Established Populations

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Abstract
Studies of laboratory animals demonstrate extensive variation of host gut microbiomes and their functional capabilities across populations, but how does anthropogenic change impact the microbiomes of non-model species? The anthropogenic movement of species to novel environments can drastically alter animals' microbiomes; however, factors that shape invasive species gut microbiota during introduction remain relatively unexplored. Through 16S amplicon sequencing on guttural toad (*Sclerophrys gutturalis*) faecal samples, we determine that residence time does not impact microbiome variation between source and introduced populations. The youngest population (~ 20 years in Cape Town) has the most distinct microbiome and associated functional capabilities, whereas longer residence times (~ 100 years in Réunion and Mauritius) produce less divergent microbial compositional, phylogenetic, and predicted functional diversity and differential abundance from source populations (Durban). Additionally, we show extensive variation of microbial and functional diversity, as well as differential abundance patterns in an expanding introduced population (Cape Town) between core and periphery sites. Contrasting previous studies, we suggest that introduction pathways might be an important factor impacting host microbial divergence. These findings also imply that the microbiome can diverge in accordance with host population dynamics.

Keywords Biological invasions · Residence time · Central-marginal hypothesis · Gut microbiome · Intestinal microbiota

Introduction

Vertebrates are host to diverse gut bacterial communities that profoundly influence host health and physiology through the metabolic and functional components they express [1, 2]. Changes in environmental conditions have been shown to produce extensive microbial compositional and functional variation in human, laboratory, and commercial animal populations [3]. The susceptibility of gut microbial communities to extrinsic environmental conditions indicates that anthropogenic disturbance, which rapidly reshapes numerous environmental factors, can alter wildlife gut microbiomes [3, 4]. However, how anthropogenic disturbance impacts non-model wildlife species' microbiomes remains relatively unexplored [3–5].

Anthropogenic movement of species to novel environments can drastically alter animals' gut microbiome [5]. It has been shown that the loss and/or gain of single microbial symbionts, due to introduction of species into new environments, can have strong evolutionary effects on invasive host performance, reproduction, and dispersal [6–10]. Biological invasions, therefore, provide us with a valuable opportunity as natural experiments to investigate population and symbiotic microbial responses to novel conditions. Introduction of species can occur through various pathways, but are often the result of deliberate introductions, with significant efforts dedicated to detecting and monitoring these populations [11]. Invasion histories provide valuable co-variates, such as propagule pressure, life stage of individuals introduced, and time of introduction, that could impact host microbial variation within an introduced population. Variation in residence time (i.e. time since population introduction), for instance, can produce divergent microbial communities between

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source and introduced populations. Increased residence time facilitates the accumulation of novel microbes from the new environment and, furthermore, microbial genetic differentiation due to selection pressures imposed by new abiotic and biotic pressures can also potentially increase with residence time [12]. Additionally, longer residence time at the invasion core results in higher population densities, presumably increasing transfer of microbial symbionts, while the opposite is true for individuals at the population's periphery [13, 14]. Biological invasions, therefore, allow us to examine the response of population change to novel environmental conditions across multiple spatial and temporal scales.

How invasion dynamics might influence vertebrate microbiome divergence has received almost no attention, compared to other environmental change factors (e.g. habitat fragmentation and captivity) [3–5, 15] and invasive plant species [12]. Here, we use the guttural toad (*Sclerophrys gutturalis*) invasion as a unique natural experiment to better investigate how invasion impacts the hosts' gut microbiome. We examined gut bacterial differentiation of guttural toads in Cape Town, the result of a recent, accidental introduction approximately 20 years ago, and on the islands of Mauritius and Réunion, after almost 100 years of colonization, and compare these invasive toad gut microbiomes to their known source population in Durban, South Africa [16]. Our aim was to test the hypotheses (1) that older invasive populations of the guttural toad with longer residence times (~ 100 years and ~ 47 generations: Réunion & Mauritius) will result in significant divergence of microbial compositional, phylogenetic, and predicted functional diversity and differential abundance patterns from the source population (Durban), while younger populations with shorter residence times (~ 20 years and ~ 9 generations: Cape Town) will show no or limited shifts in these diversity and abundance metrics from the source population and (2) that residence time will, furthermore, produce distinct compositional, phylogenetic, and predicted functional microbial diversity and differential abundance patterns between the core and periphery of the expanding invasive population in Cape Town [17].

Materials and Methods

Species and Study Site Description

The guttural toad (*Sclerophrys gutturalis*) is distributed among various natural and peri-urban habitats in sub-Saharan Africa commonly characterized by tropical and subtropical climates (i.e. summer rainfall) [18]. Guttural toad diets consist of insects, gastropods, and other invertebrates [18, 19]. Guttural toads are widely established across the Mascarene Islands, where the oldest introductions of adult toads were to Mauritius in 1922 and a subsequent introduction of adults took place in 1927 to Réunion (Fig. 1) [20]. Both introductions were an intentional attempt of insect biocontrol [20]. These islands are characterized by tropical climates similar to guttural toads' native range. A younger accidental introduction of eggs or tadpoles likely occurred through a consignment of aquatic plants to peri-urban Constantia, Cape Town, where animals were first heard calling in 2000 (Fig. 1) [21]. The Cape Town area is characterized by a Mediterranean (i.e. winter-rainfall) climate. Due to its recent introduction, this population consists of a core (site of introduction) and continuously expanding range edge (naturally dispersed sites) [17]. Concerns regarding the guttural toad invasions' impact on the endemic, Endangered *Sclerophrys pantherina* (western leopard toad), have led to considerable control efforts by the City of Cape Town since 2010 [22]. All guttural toad invasive populations have been genetically determined to be from the same population in Durban, South Africa [16].

Sample Collection

A total of 33 adult toads were collected in peri-urban residential gardens in Durban, Mauritius, and Réunion (11 per population), from February to July 2019. In Cape Town, a collection of 22 individuals was made, 11 each from the core and periphery in February 2019. The core was defined as sites where toads were routinely caught as part of the eradication programme, while sites where toads have never been previously recorded were defined as the periphery. Adult toads were captured by hand after sunset (19:00 h). Toad sex was confirmed through visual inspection for white colouration of the gular region and a greater than 40 mm snout-to-vent length (SVL) measurement [23].

Immediately after capture, toads were weighed, and their SVL was measured. Toads were placed individually in plastic containers (195 × 195 × 180 mm). Plastic containers were sterilized with a 10% bleach solution and a 70% ethanol solution before use with an individual toad. Faecal samples were collected from toads within the first 8 h of captivity. After sample collection, invasive toads were euthanized by immersion in a 1 gl^{-1} solution of tricaine ethane sulfonate (MS-222) for 20 min and native toads were released. At least 0.4 g faecal matter was obtained from each individual with ethanol-sterile forceps. Samples were subsequently submersed in 1.0 ml *RNAlater*™ (Ambion, Austin, TX) within 2-ml polypropylene tubes and stored at – 20 °C. After 6 weeks, faecal samples were centrifuged (2 min at 10 000 × g), the supernatant was removed, and the pellet stored at – 80 °C. Empty tubes and tubes containing *RNAlater*™ were kept as negative controls for DNA processing.

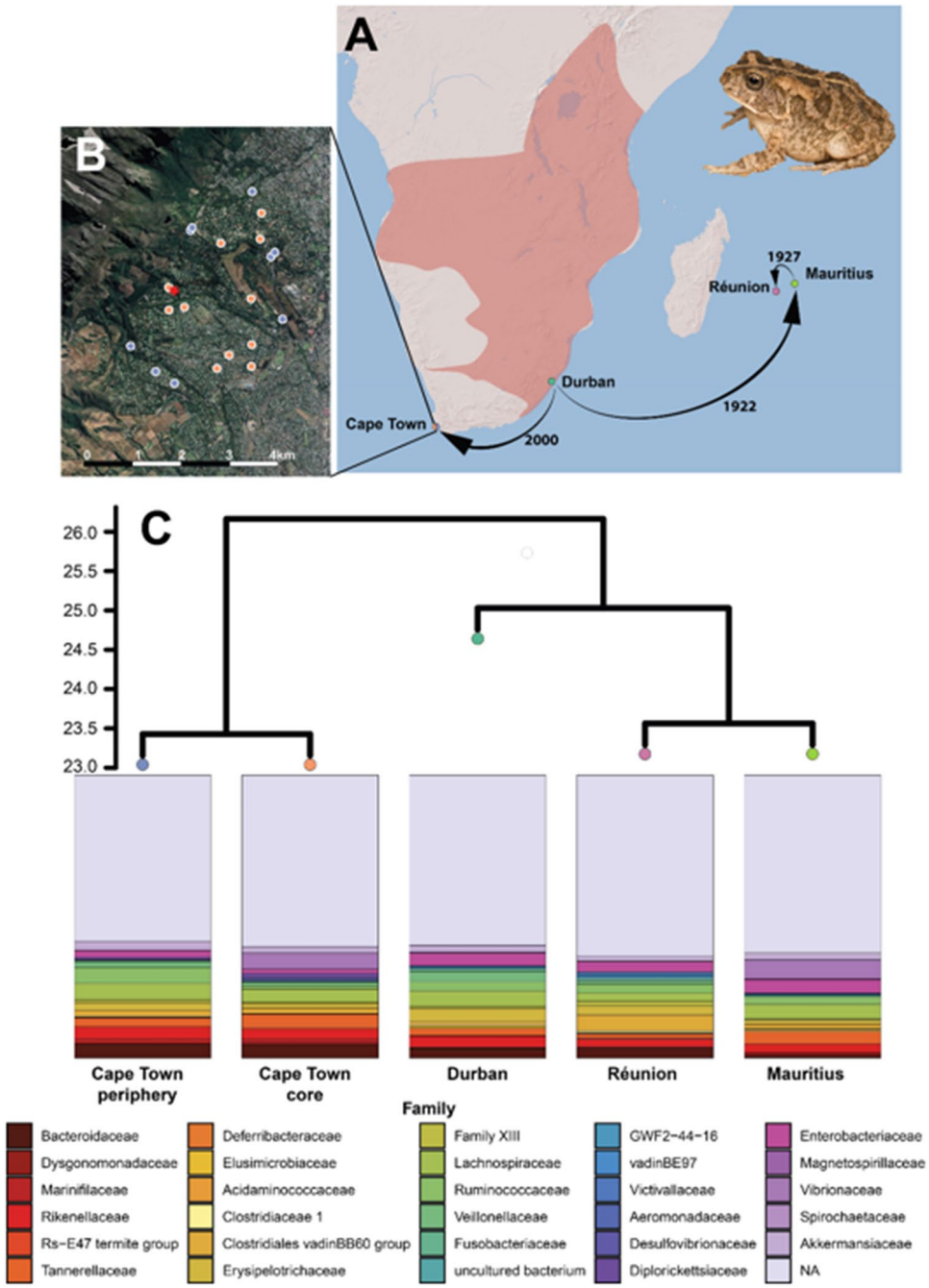

Fig. 1 Map and UPGMA cluster dendrogram of guttural toad (*Sclerophrys gutturalis*) invasive and native populations. **A** Location of sampling areas for guttural toad adults from invasive population; Mauritius (green), Réunion (pink), and Cape Town (red) and the source population; Durban (blue) ($n = 44$). The natural distribution of toads is indicated by the red polygon. **B** Location of sampling areas for guttural toads from the core and periphery from Cape Town ($n = 22$). **C** UPGMA cluster dendrogram of CLR-distances between gut microbial communities of the different populations and relative proportions of the taxa that are present in gut microbiome samples. ASVs were assigned taxonomy up to the family level

DNA Extraction and Purification

The DNeasy® PowerSoil® kit (QIAGEN, Hilden, Germany) was used, according to the manufacturer's protocol, to extract genomic DNA from 0.25 g of each faecal sample. Tubes containing blank templates (nuclease-free PCR water) and no templates were included as negative controls throughout the entire process from DNA extraction to PCR (polymerase chain reactions) amplification.

DNA samples were quantified using the Qubit 4.0 Fluorometer (ThermoFisher Scientific) and the Qubit 1 × dsDNA HS assay kit (ThermoFisher Scientific) according to the manufacturer's protocol. To determine the purity of the genomic DNA samples, spectrophotometry was performed on the NanoDrop® ND-1000 (ThermoFisher Scientific). Genomic quality scores were determined on the LabChip GXII Touch using the DNA Extended Range LabChip and Genomic DNA Reagent Kit (PerkinElmer, Waltham, MA, USA), according to the manufacturer's protocol.

PCR Amplification

The V3 and V4 hypervariable rRNA regions were targeted during sequencing. Target 16S rRNA sequences were amplified using the universal bacterial primer set, 314F 5′ – CCTACGGGNGGCWGCAG – 3′ and 785R 5′ – GAC TACHVGGGTATCTAATCC – 3′ [24]. Fragments were amplified from 5 ng genomic DNA in a reaction volume of 20 µl (0.5 µM of each primer, 200 µM dNTPs, 0.4 U Phusion® hot-start II high-fidelity (HF) DNA polymerase and 1 × Phusion® HF buffer with a final concentration of 1.5 mM $MgCl_2$). Amplification of each sample was performed in duplicate. PCRs were performed on the SimpliAmp™ Thermal Cycler (ThermoFisher Scientific). Initial template DNA denaturation at 98 °C for 30 s was followed by 25 cycles consisting of 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 30 s, with a final product extension at 72 °C for 10 min.

Presence of amplified products was verified on the PerkinElmer LabChip® GXII Touch (PerkinElmer, Waltham, MA, USA), using the X-mark chip and HT DNA NGS 3 K reagent kit, according to the manufacturer's protocol. PCR products were then purified with 1.8 × volume Agencourt™ AMPure™ XP reagent (Beckman Coulter, Brea, CA, USA) and eluted in 25 µl nuclease-free water. Purified amplicons were quantified on the Qubit 4.0 Fluorometer using the Qubit 1 × dsDNA HS assay kit (ThermoFisher Scientific), according to the manufacturer's protocol.

Library Preparation

Library preparation from 100 ng PCR product per sample was performed using the NEXTflex™ DNA Sequencing Kit (Bio Scientific Corporation) according to the manufacturer's protocol. From each purified PCR product, 40 µl was end-repaired at 22 °C for 30 min using 3 µl End-repair enzyme mix and 7 µl End-repair buffer in a final volume of 50 µl. The end-repaired products were purified with 1.8 × volume Agencourt™ AMPure™ XP reagent (Beckman Coulter). From the purified, end-repaired products, 9 µl was ligated to 4 µl IonCode™ Barcode Adapter (ThermoFisher Scientific) with the addition of 31.5 µl Ligation mix at 22 °C for 15 min. The adapted-ligated, barcoded libraries were then purified with 1.8 × Agencourt™ AMPure™ XP reagent (Beckman Coulter) and quantified using the Ion TaqMan Library Quantitation Kit (ThermoFisher Scientific). Using the StepOnePlus™ Real-time PCR system (ThermoFisher Scientific), qPCR amplification was performed. Library fragment size distributions were assessed on the LabChip® GXII Touch (PerkinElmer, Waltham, MA, USA), using the X-mark chip and HT DNA NGS 3L reagent kit according to the manufacturer's protocol.

Sequencing

Massive parallel sequencing was performed on the Ion GeneStudio™ S5 Prime System using the Ion S5™ Sequencing solutions and reagents according to the manufacturer's protocol.

Sequencing Data Pre-processing

Resulting sequences were stored in FASTQ formatted files generated for each sample. Single-end raw reads (12 452 682) were imported into QIIME2 (version 2020.2) for preprocessing [25]. The divisive amplicon denoising algorithm (DADA2) plugin was used to de-noise sequencing reads [26]. Briefly, low-quality sequences (sequences < 400 bp in length and < 20 quality score, sequences containing ambiguous characters, unreadable barcodes, or without primer sequences), chimeric sequences, and singletons were removed. The resulting sequences were then used to generate amplicon sequence variants (ASVs) for downstream analyses. This resulted in 7 240 389 sequences ranging from 55 358 to 141 380 sequences per sample representing a total of 16 602 unique ASVs. ASV sequences were aligned with mafft (q2-alignment plugin) [27], high entropy positions were filtered from the resulting alignment [28], an unrooted tree was constructed with FastTree 2 (q2-phylogeny plugin) [29], and the tree was rooted using midpoint rooting. Taxonomy was assigned to ASVs with a classify-sklearn classifier trained against the most recent SILVA 16S rRNA gene reference database (release 138) (q2-feature-classifier plugin) [30]. The ASV table, phylogenetic tree, and assigned taxonomy table were used in all downstream analyses.

The ASV table and its corresponding phylogenetic tree were additionally used to predict functional profiles of samples through the PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2, NSTI cutoff = 2) pipeline in QIIME2 (q2-picrust2 plugin) [31] and the KO Database of Molecular Functions by ortholog annotation (KEGG orthologues, KO, https://www.genome.jp/kegg/ko.html).

All negative controls were removed due to low sequence number (< 100) and sequence quality score (< 20).

Statistical Analysis

Preliminary analyses showed that body mass was positively correlated with SVL. Therefore, toad body condition (or scaled mass index) [32] was used as a covariate in all downstream analyses.

All statistical analyses were performed in R version 3.6.2 [33]. Metadata, ASV table, taxonomy, and phylogenetic tree were imported using the qiime2R package [34]. A phyloseq object was built from these datasets using the

phyloseq package [35]. Prior to all downstream analyses, alpha rarefaction curves were inspected to assess sequencing depth. Visual inspection confirmed that sequencing depth was adequate for each sample with regard to number of ASVs detected (Fig. S1). ASV counts of each sample were then filtered, removing ASVs present in less than 5% of the samples, and normalized according to the read depth of each sample using the phyloseq and microbiomeutilities packages [36].

Diversity metrics inverse Shannon diversity, Evenness, Chao1 species richness, and Faith's phylogenetic diversity were calculated using the vegan package in R [37]. Linear mixed-effects models (LMM) were used to determine whether alpha diversity metrics (response variables) vary across populations. Prior to analyses, model assumptions (normality, homogeneity, and independence) were assessed following Zuur et al. [38]. Phylogenetic diversity estimates did not meet assumptions of normality. Data were square-root transformed and subsequently met model assumptions. A full model included two fixed factors, population, and body condition. All models were fitted with the random intercept collection site. Relative variable importance of competing models was evaluated using Akaike information criterion (AIC). To evaluate the variance of data explained by each model, marginal (fixed effects) and conditional (fixed and random effects) R^2 were calculated using the 'r.squaredGLMM' function in the package MuMIn [39, 40]. Chi-square statistic and associated *p*-values were investigated to examine the effect of fixed effects on the dependent variables.

Variation of bacterial alpha diversity among gut communities of toads at core and periphery sites of the Cape Town invasion was similarly investigated as described above. LMM was used to determine the effect of site (core or periphery) on alpha diversity metrics. None of the diversity metrics met model assumptions of normality. Inverse Shannon data was log-transformed, Chao1 and Phylogenetic diversity datasets were square-root transformed, and Evenness estimates were reciprocally (1/x) transformed in order to meet model assumptions. Full models included two fixed factors, site (core or periphery) and body condition. All models were fitted with the random intercept collection site.

Beta diversity of populations were examined by PERMANOVA analyses using CLR- and PHILR-distance matrices. Euclidean distance matrices with CLR- and PHILR-transformations produce metrics that are equivalent to the Bray–Curtis and Unifrac beta diversity metrics, respectively, but account for the compositional nature of the data [41, 42]. Feature tables containing read counts were first subjected to centre log-ratio (CLR)- and PHILR-transformation, using phyloseq and philr packages [42], respectively. Euclidean distance matrices were constructed from the transformed ASV count tables with the adonis function [vegan package, 37]. Distance matrices were subjected to PERMANOVA analyses to evaluate the effect of population, body condition, and collection site on toad gut microbial composition. Additionally, post hoc pairwise comparisons were completed to examine which groups significantly varied from each other. PERMANOVA analyses were also similarly conducted to examine the effect of site (core and periphery) on gut microbial composition of toads from the Cape Town invasive population.

As PERMANOVA is sensitive to differences in dispersion of data within groups (assumes a homogenous within-group dispersion), we inspected this assumption with the betadisper and permutest functions of vegan. Clustering analysis using PCoA and UPGMA methods on CLR- and PHILR-metrics were used to visualize similarity of population and site gut microbiomes.

To investigate differential abundance of ASVs across populations, likelihood ratio tests (LRT) were employed through the DeSeq2 package [43]. This test was implemented using a full model with population and body condition against a reduced model with body condition as the only predictive variable. Prior to analyses, read counts were normalized using a regularized logarithm. The Benjamini–Hochberg method for reducing the false discovery rate (FDR) was employed with a cutoff of <0.05 for identifying differentially abundant microbes. Corresponding log-fold change, *p*-values, and FDR-adjusted *p*-values were estimated. To investigate differences in abundances of ASVs across populations, pairwise comparisons of populations were performed using DeSeq2. LRT and pairwise comparisons were also similarly implemented with DeSeq2 to investigate the effect of site (core or periphery) on gut microbial abundances in the Cape Town invasive population.

Lastly, functional components of bacterial communities were assessed. Prior to all analyses, pathway abundances derived from the PICRUSt2 pipeline were filtered to exclude pathways present in less than 5% of the samples. Data was then subjected to beta diversity and differential abundance (DeSeq2) analyses similar to those described above.

Results

Gut Bacterial Communities Vary Across Native and Invasive Populations

Alpha diversity of guttural toad gut microbiomes did not vary between populations (Fig. S2, Table S1). However, compositional and phylogenetic beta diversity varied significantly across populations (Table 1). While guttural toads from all populations varied significantly in their compositional diversity (Fig. 1, Fig. 2A, Table S2), only the youngest populations' (Cape Town) phylogenetic diversity was

Table 1 Summary of PERMANOVA results analyzing the effect of population, body condition, and collection site on *Sclerophrys gutturalis* (guttural toad) gut microbial communities as measured by compositional CLR- and phylogenetic PHILR-Euclidean metrics. For each comparison, dependent and explanatory variables, degrees of freedom (d.f.), sum of squares (SS), pseudo-*F*-statistic, *r*-squared values (R^2), and *p*-values are reported

Dependent variable	Explanatory variable	d.f	SS	Pseudo-*F*	R^2	*p*-value
CLR-Euclidean	**Population**	**3**	**3599.8**	**2.56**	**0.16**	**0.001**
	Body condition	1	665.0	1.42	0.03	0.051
	Collection site	7	3797.1	1.16	0.16	0.069
	Residuals	32	15,002.2		0.65	
	Total	43	23,064.1		1.00	
PHILR-Euclidean	**Population**	**3**	**517.6**	**2.71**	**0.16**	**0.001**
	Body condition	1	103.5	1.62	0.03	0.088
	Collection site	7	565.7	1.27	0.18	0.060
	Residuals	32	2039.1		0.63	
	Total	43	3225.7		1.00	

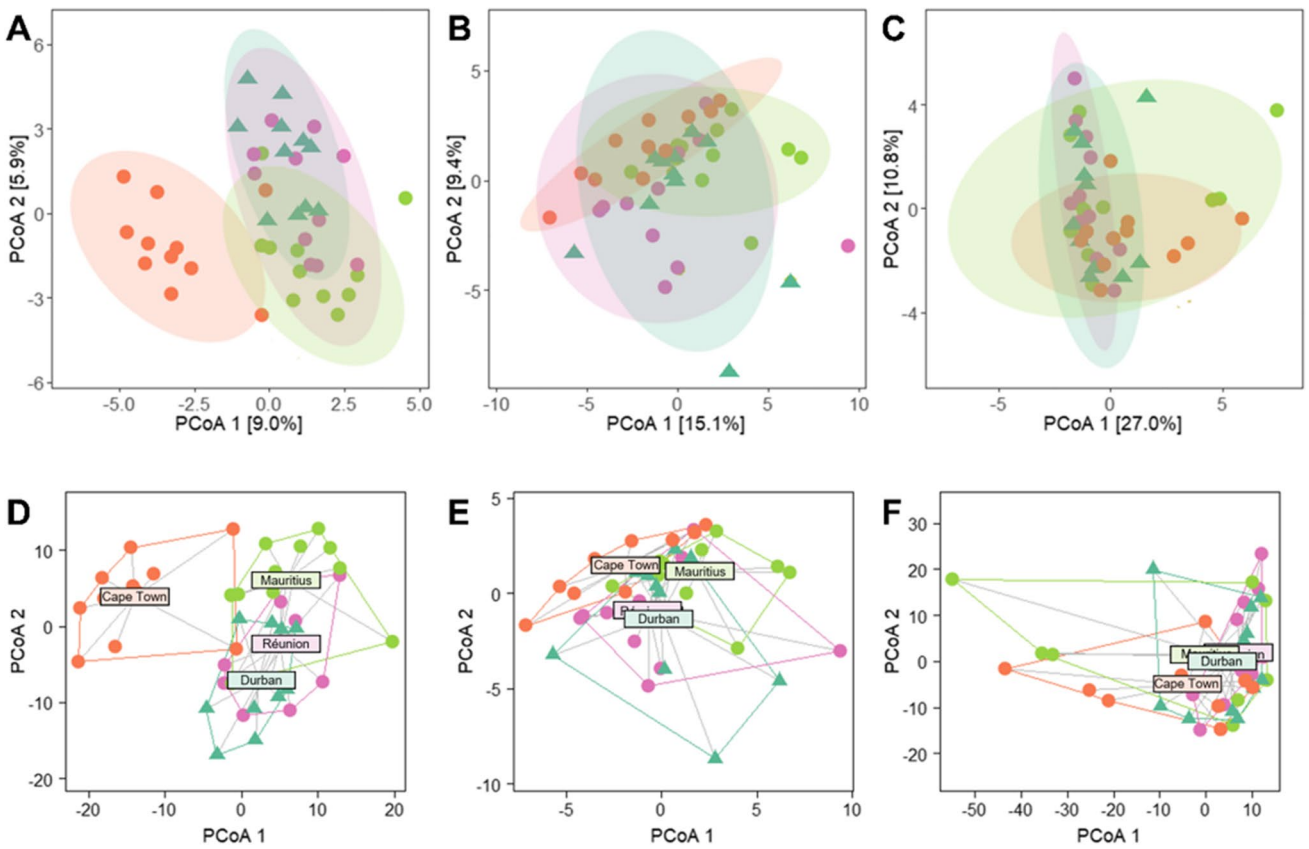

Fig. 2 Principle Coordinates Analysis (PCoA) of gut microbiomes of invasive and native guttural toad (*Sclerophrys gutturalis*) populations. PCoA of **A** CLR-Euclidean compositional beta diversity, **B** PHILR-Euclidean phylogenetic beta diversity, and **C** CLR-Euclidean functional beta diversity. Gut microbial communities significantly differed among guttural toad native (triangles) and invasive (circles) populations. PCoA dispersion plots of **D** CLR-Euclidean compositional beta diversity, **E** PHILR-Euclidean phylogenetic beta diversity, and **F** CLR-Euclidean functional beta diversity. Permutational test of dispersions (PERDISP) showed no differences in dispersion between populations' gut microbiomes. Guttural toads were collected from invasive populations in Mauritius (green), Réunion (pink), Cape Town (red), and native populations in Durban (blue)

significantly different from all other populations gut microbial communities (Fig. S3, Fig. 2B, Table S2). Betadisper analysis indicated that variation between population microbiomes was not due to different dispersion levels (Fig. 2D, E). Additionally, body condition and collection site had no effect on toad gut microbiomes (Table 1).

A total of 5205 ASVs were differentially abundant across populations (Table S3). The youngest population had the most differentially abundant ASVs, with 1809 ASVs differentially abundant between Durban and Cape Town, 1015 between Durban and Réunion (Fig. 3). The gut bacterial communities of the different populations were mainly composed of the same phyla (largely Bacteroidetes, Firmicutes, and Proteobacteria), with the exception of greater Tenericutes spp. abundances in Durban and Mauritius and enrichment of Cyanobacteria in Cape Town bacterial communities. Several differences of abundance in lower taxonomic levels were found: toads from Cape Town had higher levels of Vibrionaceae (*Salinivibrio* sp.) and Clostridiales sp. and lower abundances of Rikenellaceae, Erysipelotrichaceae, and Prevotellaceae present in their gut bacterial communities (Table S4).

Only Functional Profiles of the Youngest Invasive Population Are Distinct from the Source Population

Analysis of predicted functions revealed that population had an effect on the functional profiles of guttural toad gut microbial communities (Fig. 2C, Table 2). However, post hoc analyses showed that only the functional profiles of the youngest populations' gut microbial communities are significantly distinct from the other populations (Table S5). Betadispr analysis indicated that variation between population-predicted functional pathways was not due to different dispersion levels (Fig. 2F). Body condition and collection site also had no significant effect on the predicted functionality of toad gut microbial communities (Table 2).

Only 86 of 397 predicted metabolism-associated functional features of the gut microbial communities were differentially abundant across toad populations (Table S6). Among populations, the youngest population had the most differentially abundant functional pathways, with significant enrichment of vitamin biosynthesis, carbohydrate and sugar degradation, fatty acid and lipid degradation,

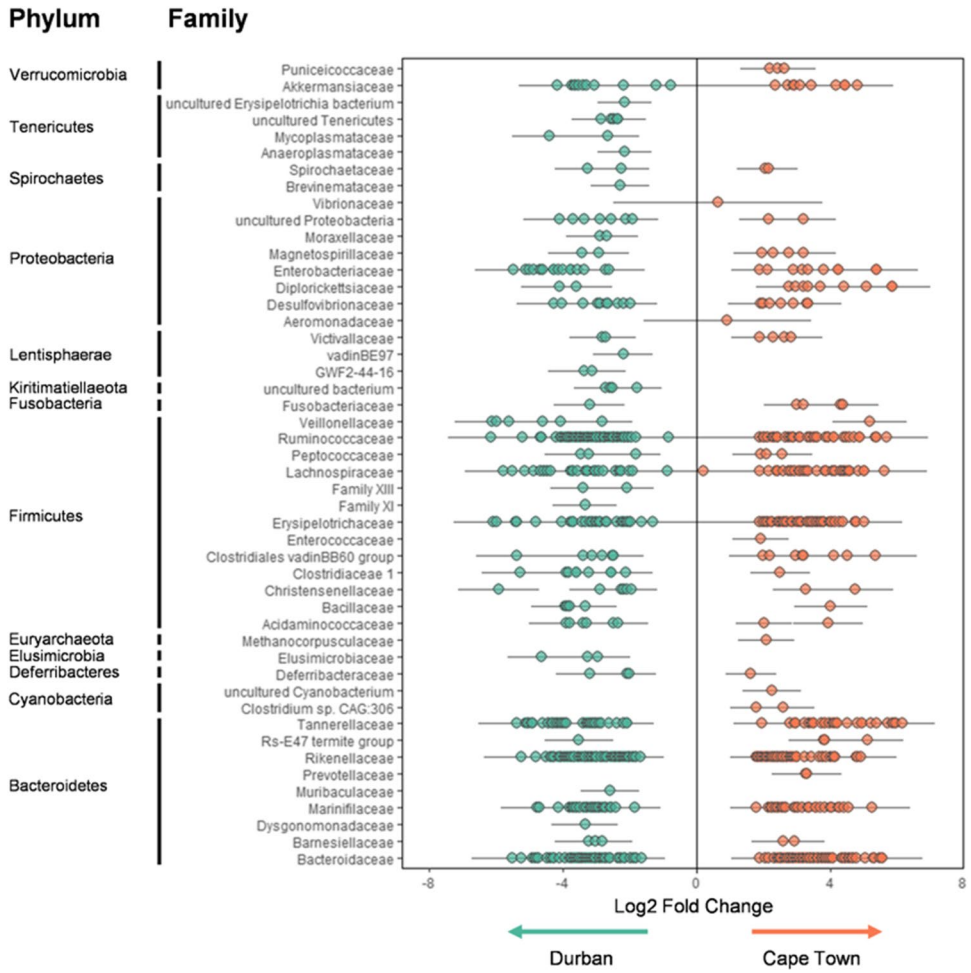

Fig. 3 Differential abundance of gut bacterial ASVs between native and invasive guttural toad (*Sclerophrys gutturalis*) populations. Differences in the relative abundances of individual ASVs are depicted by log2 fold change. A total of 1809 ASVs significantly differed in abundance between native (Durban, blue) and invasive (Cape Town, red) toad populations (DESeq2: adj-$P < 0.05$). Of these, 957 ASVs were not assigned taxonomy to the family level and were removed for plotting purposes (see table S4 for a complete list of differentially abundant ASVs). Family and Phylum classification is provided where available

Table 2 Summary of PERMANOVA results analyzing the effect of population, body condition, and collection site on *Sclerophrys gutturalis* (guttural toad) predicted gut microbial functional capabilities as measured by CLR-Euclidean metrics. For each comparison, dependent and explanatory variables, degrees of freedom (d.f.), sum of squares (SS), pseudo-*F*-statistic, *r*-squared values (R^2), and *p*-values are reported

Dependent variable	Explanatory variable	d.f	SS	Pseudo-*F*	R^2	*p*-value
CLR-Euclidean	**Population**	**3**	**4375**	**1.61**	**0.11**	**0.029**
	Body condition	1	1205	1.33	0.03	0.167
	Collection site	6	4781	0.88	0.12	0.706
	Residuals	33	29,919		0.74	
	Total	43	40,280		1.00	

and glycan and starch biosynthesis functional pathways compared to those from Durban, Mauritius, and Réunion (Fig. 4, Table S7).

Expanding Populations Have Divergent Compositional and Phylogenetic Bacterial Communities Across Its Core and Periphery

In Cape Town, alpha diversity did not vary between core and periphery sites (Fig. S4, Table S8). However, beta diversity of gut microbial communities varied both compositionally and phylogenetically between core and periphery sites (Table 3, Fig. 5). Betadispr analysis indicated that this variation was not the result of different dispersion levels (Fig. 5D, E). Body condition and collection site also had no significant effect on the beta diversity of toad gut microbial communities (Table 3). Gut bacterial communities differed greatly in abundance at phylum level and lower taxonomic levels (1360 ASVs significantly differed, Fig. 6, Table S9). Toads at the periphery had lower abundances of Bacteroidetes, Firmicutes, Lentisphaerae, and Tenericutes species and significant enrichment of Proteobacteria (Table S9).

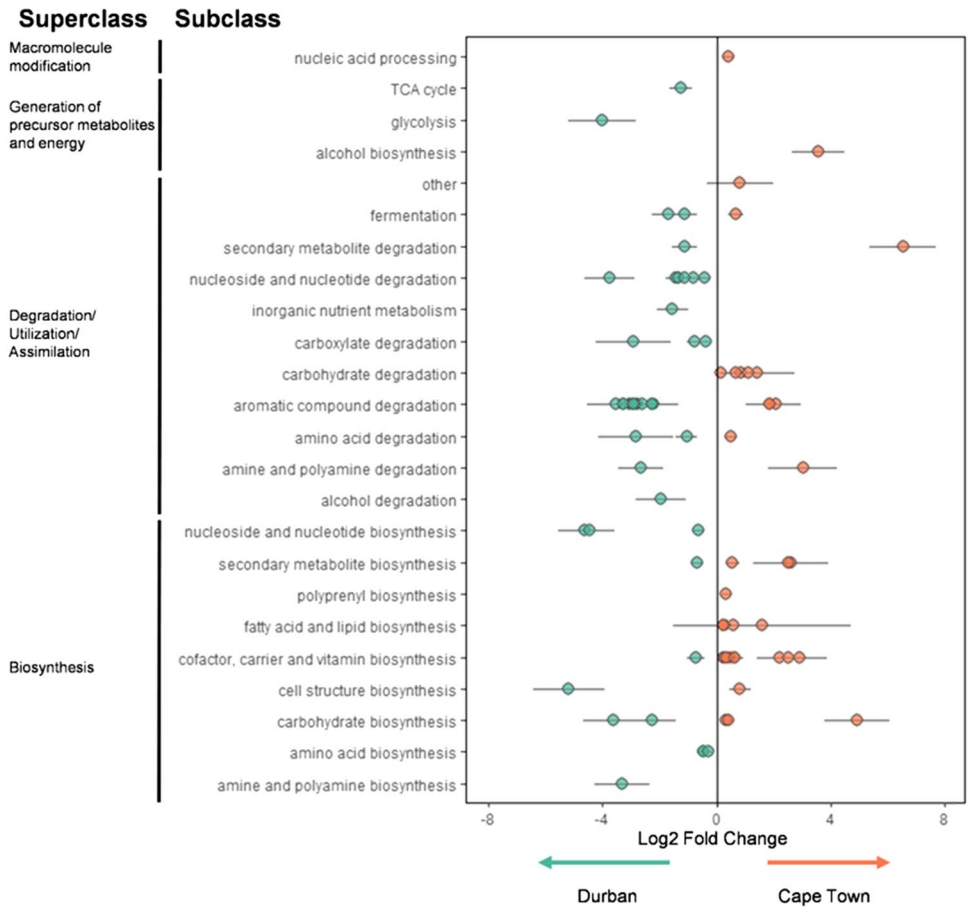

Fig. 4 Differential abundance of gut bacterial predicted functional pathways between native and invasive guttural toad (*Sclerophrys gutturalis*) populations. Differences in the relative abundances of individual functional pathways are depicted by log2 fold change. A total of 37 functional pathways significantly differed in abundance between native (Durban, blue) and invasive (Cape Town, red) toad populations (DESeq2: adj-$P < 0.05$; see table S7 for a complete list of differentially abundant functional pathways)

Table 3 PERMANOVA results analyzing the effect of site (core and periphery), body condition, and collection site on *Sclerophrys gutturalis* (guttural toad) gut microbial communities as measured by compositional CLR- and phylogenetic PHILR-Euclidean metrics. For each comparison, dependent and explanatory variables, degrees of freedom (d.f.), sum of squares (SS), pseudo-*F*-statistic, *r*-squared values (R^2), and *p*-values are reported

Dependent variable	Explanatory variable	d.f	SS	Pseudo-*F*	R^2	*p*-value
CLR-Euclidean	**Population**	**1**	**1053.4**	**1.49**	**0.07**	**0.007**
	Body condition	1	787.6	1.11	0.05	0.204
	Collection site	3	2456.1	1.16	0.16	0.052
	Residuals	16	11,326.9		0.72	
	Total	21	15,624.0		1.00	
PHILR-Euclidean	**Population**	**1**	**271.6**	**1.55**	**0.07**	**0.049**
	Body condition	1	171.0	0.98	0.05	0.482
	Collection site	3	491.7	0.94	0.13	0.634
	Residuals	16	2800.3		0.75	
	Total	21	3734.6		1.00	

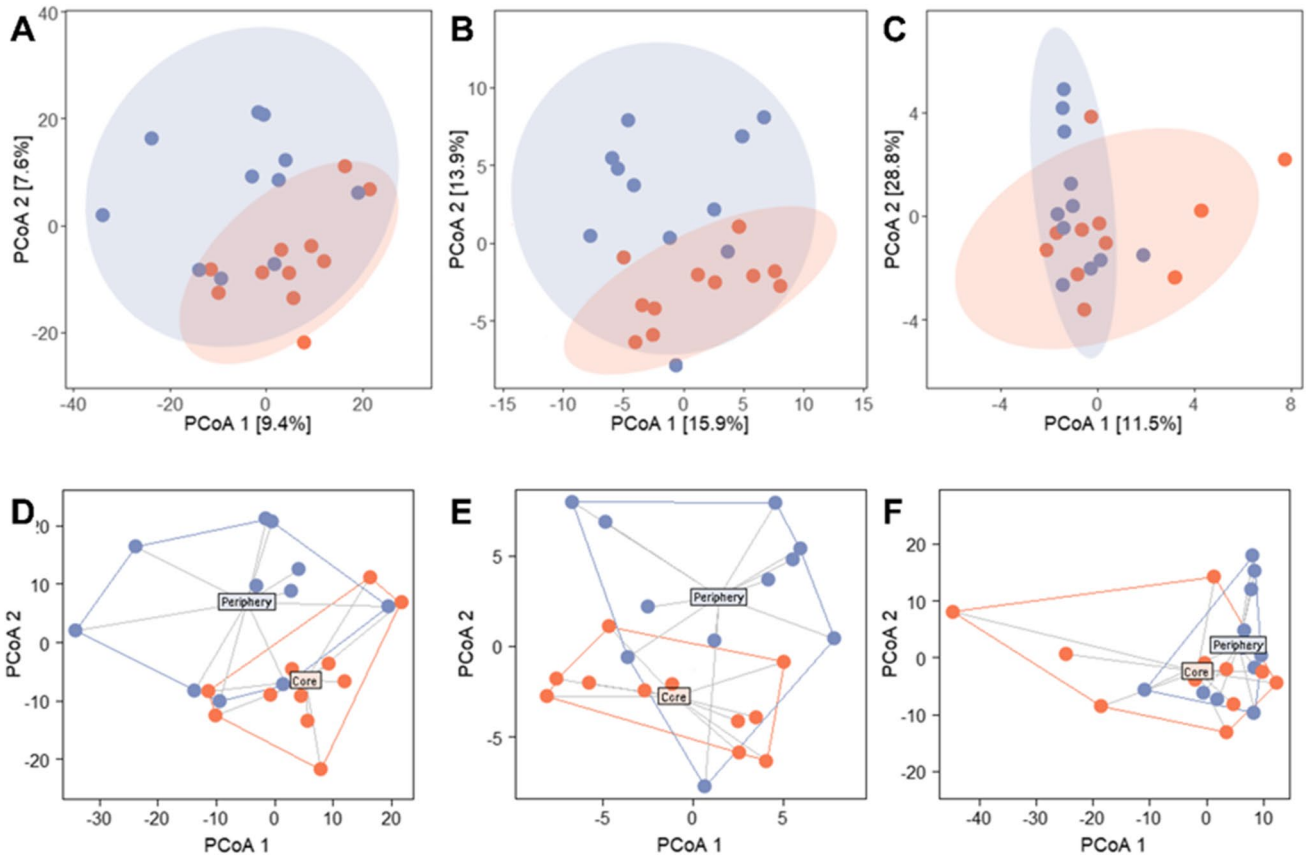

Fig. 5 Principle Coordinates Analysis (PCoA) of gut microbiomes between the core and periphery of an expanding invasive guttural toad (*Sclerophrys gutturalis*) population. PCoA of **A** CLR-Euclidean compositional beta diversity, **B** PHILR-Euclidean phylogenetic beta diversity, and **C** CLR-Euclidean functional beta diversity of guttural toad gut bacterial communities. Gut microbial communities significantly differed among core (purple) and periphery (red) sites in the guttural toad invasive Cape Town population. PCoA dispersion plots of **D** CLR-Euclidean compositional beta diversity, **E** PHILR-Euclidean phylogenetic beta diversity, and **F** CLR-Euclidean functional beta diversity. Permutational test of dispersions (PERDISP) showed no differences in dispersion between sites' gut microbiomes

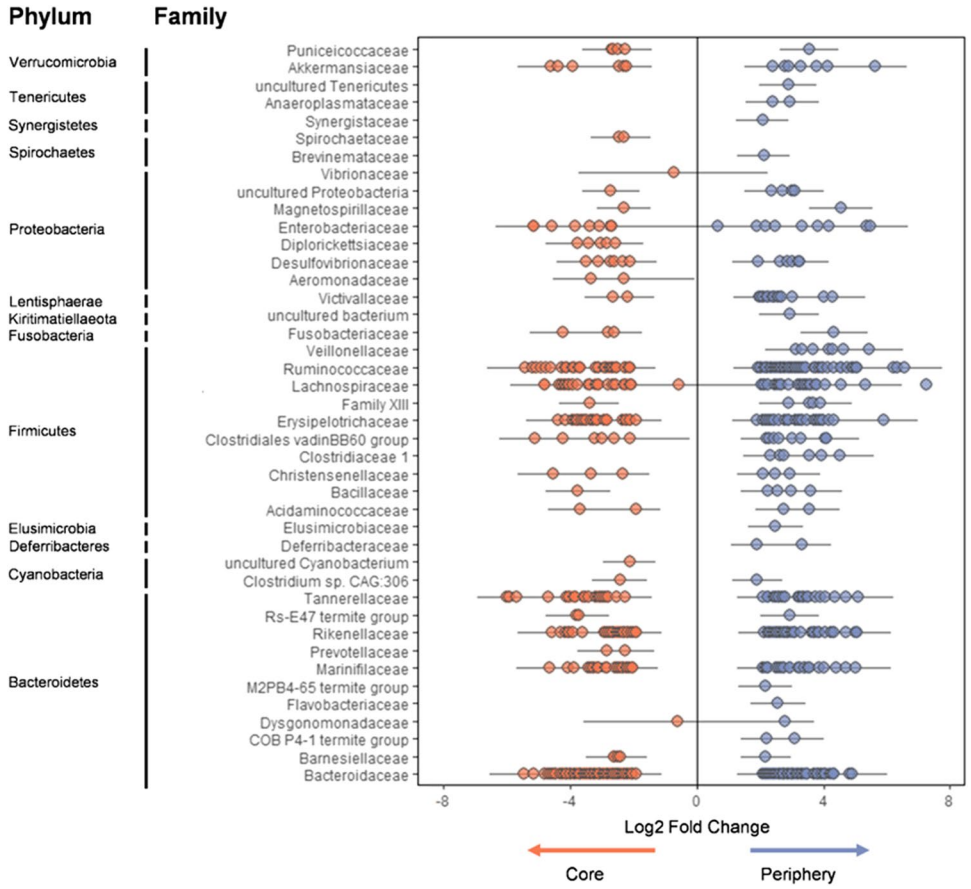

Fig. 6 Differential abundance of gut bacterial ASVs between the core and periphery of an expanding invasive guttural toad (*Sclerophrys gutturalis*) population. Differences in the relative abundances of individual ASVs are depicted by log2 fold change. A total of 1361 ASVs significantly differed in abundance between core (red) and periphery (purple) sites (DESeq2: adj-$P < 0.05$). Of these, 710 ASVs were not assigned taxonomy to the family level and were removed for plotting purposes (see table S9 for a complete list of differentially abundant ASVs). Family and Phylum classification is provided where available

Functional Profiles Vary Between Core and Periphery Sites in an Expanding Invasive Population

Functional profiles varied significantly between core and periphery sites in the Cape Town invasive population (Fig. 5C, Table 4). Additionally, eight of 397 predicted metabolism-associated functional features of the gut bacterial communities were differentially abundant between core and periphery guttural toads (Fig. 7, Table S10). Toads at the periphery were enriched in glycogen, starch and enzyme biosynthesis, and polysaccharide degradation functional groups, while core toad bacterial communities had enrichment of glycan and glycogen degradation, secondary metabolite biosynthesis, and cell wall biosynthesis functional pathways.

Discussion

Guttural toad gut bacterial communities have diverged from their source population across all invasive populations. Overall, the few studies that have explored gut microbiome differentiation of invasive species support our results in

Table 4 PERMANOVA results analyzing the effect of site (core and periphery), body condition, and collection site on *Sclerophrys gutturalis* (guttural toad) gut microbial functional capabilities as measured by CLR-Euclidean metrics. For each comparison, dependent and explanatory variables, degrees of freedom (d.f.), sum of squares (SS), pseudo-*F*-statistic, *r*-squared values (R^2), and *p*-values are reported

Dependent variable	Explanatory variable	d.f	SS	Pseudo-*F*	R^2	*p*-value
CLR-Euclidean	**Population**	**1**	**1150.1**	**1.95**	**0.09**	**0.025**
	Body condition	1	634.7	1.07	0.05	0.350
	Collection site	3	2236.9	1.26	0.17	0.134
	Residuals	16	9460.9		0.70	
	Total	21	13,482.6		1.00	

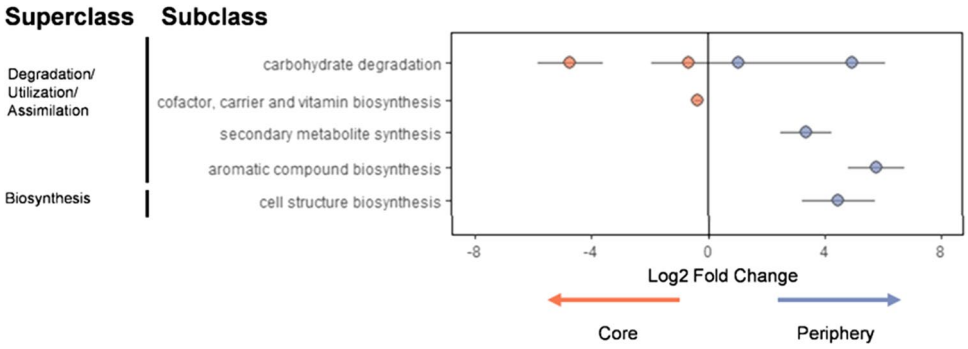

Fig. 7 Differential abundance of gut bacterial predicted functional pathways between the core and periphery of an expanding invasive guttural toad (*Sclerophrys gutturalis*) population. Differences in the relative abundances of individual functional pathways are depicted by log2 fold change. In total, 8 functional pathways significantly differed in abundance between core (red) and periphery (purple) sites (DESeq2: adj-$P < 0.05$; see table S10 for a complete list of differentially abundant functional pathways

that widespread introduction of a species produces diverse microbiomes [5, 43]. Longer residence time did not produce more phylogenetically distinct gut bacterial communities and predicted functional profiles. Furthermore, within the youngest population, extensive bacterial divergence was evident across core and periphery sites suggesting that rapid alteration of gut microbiomes can occur during the expansion of a population.

Introductory pathways are an interesting factor that could produce the observed divergent gut bacterial communities between source and introduced guttural toad populations. Adult toads deliberately introduced to Mauritius and Réunion could have been more extensively inoculated with bacterial symbionts from their adult source population, compared to the youngest population which was thought to be accidentally introduced as tadpoles [21]. Previous studies indicate that microbial loss through sampling effects (i.e. introduced hosts were by chance not inoculated) as a result of varying introduction pathways is rare [7]. However, in our case, ontogeny at/during initial introduction could have increased the chances of sampling effects impacting bacterial divergence since microbiome structure is known to vary across amphibian life stages [45]. Absence of adults during the initial introduction to Cape Town could have prevented the colonization of adult microbiomes present in the source population. However, other factors known to impact the gut microbiome could have contributed to this microbiome divergence. Cape Town is characterized by a Mediterranean climate drier and colder than that of the native, source, and older invasive populations [17]. In order to tease apart these dynamics, future studies should, thus, investigate both the variation of other habitat and host features, as well as the gut bacterial variation of tadpoles across these populations.

This study is the first demonstrating that a population can undergo rapid alteration of gut microbial composition during population expansion. Microbial genetic differentiation between population core and peripheral sites can be the result of numerous factors such as effective population size [9, 14]. Additionally, recent studies have shown that spatial proximity of hosts can play an important role in microbial shifts since it mediates host exposure to similar microbial sources and allows indirect transfer of microbes between individuals [46, 47]. Decrease of effective population size as individuals move towards the periphery can possibly minimize the amount of intraspecific interactions resulting in divergent microbiomes. Guttural toads likely experience varying population dynamics at the population edge producing divergent microbial communities in an otherwise physically homogenous habitat.

Since gut microbiota modulates the availability of ingested nutrients and the efficiency of energy harvesting, its functional potential is an important aspect to consider. In this study, although all populations have distinct gut microbial communities (in terms of composition), only the youngest population has distinct functional capabilities. At the functional level, divergent taxa could exhibit functional redundancy [48], i.e. different bacterial species exhibit similar functional capabilities across communities. Lack of correlation between bacterial composition and function could, therefore, indicate that functional pathways have minor impacts on organismal performance. On the other hand, similar responses between these variables can be interpreted as composition and functional pathways having a significant influence on organismal performance [48]. Variation of predicted functions between the Cape Town and source population could possibly be associated with digestion of different food substrates. It is well known that diet has an immense impact on species' gut microbiomes because of their functional capabilities to degrade complex dietary substrates [49, 50]. Functional pathways that increased in abundance in the Cape Town gut microbial communities were associated with the carbohydrate

metabolism, energy metabolism, amino acid metabolism and biosynthesis, secondary metabolite metabolism and biosynthesis, glycolysis, and fermentation. It is possible that dietary changes between populations could have produced changes in functional composition, but this remains to be tested as it is also likely that the predicted functional potential of populations in this study is not reflective of the true functional potential of these toads.

Despite the potential for microbiome research to improve our understanding of wild host responses to environmental change, especially as it applies to climate change and invasion biology, few efforts have been made to integrate these fields [3–5, 15]. In this study, we characterized the gut microbial composition of an invasive toad species' native source population and three introduced populations. We show that residence time does not impact the gut bacterial variability or functional pathway variation of guttural toad populations. Instead, we suggest that introduction pathways might be a more important factor determining gut microbiome differentiation between populations, especially when organisms have complex life histories such as amphibians. Furthermore, this study is one of first demonstrating that population dynamics likely influence the gut microbial composition and functional capabilities of an expanding population.

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Author Contribution CW and JM designed the study. CW and JM collected the samples. CW and MDP performed the bioinformatics. CW performed the formal data analyses. CW wrote the manuscript and JM contributed to revisions. All authors have seen and approved the manuscript.

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Availability of Data and Material Raw sequence data will be available in the NCBI Sequence Read Archive (accession number: PRJNA774346).

Declarations

Ethics Approval Ethical clearance for research was obtained from Stellenbosch University Animal Ethics Committee (Protocol Number ACU-2019–9533). Collections in Cape Town occurred as part of an ongoing eradication programme of *Sclerophrys gutturalis* to mitigate impacts on the threatened endemic, the western leopard toad *Sclerophrys pantherina* (Davies et al. 2020), and in Durban and Mauritius under the permission from KZN wildlife (OP 4353/2018) and the Mauritian National Parks and Conservation Services (NP 46/3 V3), respectively.

Conflict of Interest The authors declare no competing interests.

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