Spill your guts: The invasive amphibian gut microbiome

by

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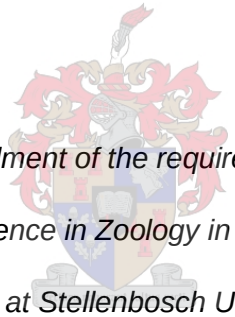

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Co-supervisor: Dr. Morne du Plessis

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Declaration

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Abstract

Invasive vertebrate species threaten global biodiversity, and significantly impact economic, agricultural and ecosystem services. Alarmingly, the number of introductions of species into non-native areas are increasing. Invasive species are introduced along with non-native microorganisms living in or on their hosts. In plant and insect invasions it has been shown that symbiotic relationships with microbial communities (collectively known as the microbiome) can enhance invasive species performance and facilitate establishment of non-native species in new environments. However, no studies have determined which factors impact the invasive vertebrate microbiome and how different microbial communities might facilitate vertebrate invasions. Therefore, in the aim of this thesis was to study how introduction of a vertebrate species into novel environments alters it's gut microbial communities and it's predicted functional capabilities, as well as whether the gut microbiome and it's functional profiles can respond adaptively to environmental change and how these responses (or lack thereof) affect host physiology and, ultimately, fitness.

To address the first aim outlined above, I made use of next-generation DNA sequencing (NGS) techniques to characterise the gut bacterial communities of guttural toad (*Sclerophrys gutturalis*) invasive populations in Mauritius, Réunion and Cape Town with varying residence times and compared these to their native source population in Durban. This allowed me to test whether residence time impacts the gut microbial compositional, phylogenetic and functional divergence across guttural toad populations. Additionally, I characterised the gut microbiome of an expanding invasive population (Cape Town) to determine how residence time impacts gut microbial divergence across the core and periphery. To address my second aim, I conducted reciprocal faecal microbial transplant experiments on native and invasive guttural toads in Durban (native area) and Cape Town (invasive area). Thereafter I exposed toads to one of two diets: natural or a dietary challenge and subsequently collected faecal microbial material in order to determine compositional, phylogenetic and functional microbial responses of toads to a novel dietary challenge. Additionally, I measured physiological performance and organ mass of toads.

I found that gut microbial communities are compositionally distinct across all invasive populations. However, only the youngest population had a phylogenetic and functionally distinct microbiome. Therefore, I found that residence time does not impact the gut microbiomes of invasive guttural toads and instead I suggest that invasion pathways (i.e. the life history of toads at introduction) might be a more important factor determining gut microbiome divergence. I found that the invasive Cape Town microbiome has adaptively diverged to become compositionally, phylogenetically and functionally flexible in response to

a novel dietary challenge compared to the native Durban microbiome. Functional pathways known to increase digestive efficiency, additionally increased in abundance in the invasive microbiome. I also found that this microbial flexibility facilitates flexibility in energy investments in hosts. Although physiological performance did not vary across diets, performance was significantly higher in toads with invasive gut microbiomes. Thus, I show for the first time that the gut microbiome facilitates ecological adaptation in an invasive amphibian population.

Opsomming

Indringer werwel spesies bedreig natuurlike biodiversiteit regoor die wêreld en het ‘n beduidende impak op ekonomiese, lanbou- en ekosisteem dienste. Die aantal spesies wat van hulle natuurlike habitat beweeg word na uitheemse omgewings is aan die toeneem. Indringer spesies bring saam met hulle mikro-organismes wat in of op hulle gashere lewe. Altesaam word alle mikro-organismes word verwys na die mikrobioom. Vir plant en insek indringer spesies is voordelige verhoudings met die mikrobioom uiters belangrik vir die gasheer se fisiologie en kan suksesvolle vesting van indringer spesies in uithemese omgewings fasiliteer. Geen studies het al ooit ondersoek of die werweldier mikrobioom die gasheer se indringer potensiaal kan verbeter nie. Dus was die doel van hierdie proefskrif om te bestudeer watse faktore die derm mikrobioom en sy funksionele vermoëns impak wanneer ‘n indringer werweldier, die gorrelskurwe padda (*Sclerophrys gutturalis*), beweeg word van ‘n natuurlike omgewing na ‘n uithemese omgewing. Boonop het hierdie proefskrif ondersoek of die indringer derm mikrobioom kan aanpas na ‘n dieetuitdaging in vergelyking met die natuurlike, inheemse derm mikrobioom en of enige verandering in die derm mikroboom veranderinge sal aanbring in die fisiologie van die paddas.

Om die bogenoemde doelwitte aan te spreek, het ek gebruik gemaak van volgende generasie DNA-basis volgorde bepalings tegnieke om die derm bakteriële gemeenskappe van paddas in Mauritius, Réunion en Kaapstad met wisselende verblyf tye te kenmerk en vergelyk aan hulle inheemse bronbevolking in Durban, Suid Afrika. Dit het my in staat gestel om te toets of verblyftyd die mikrobiese samestelling, filogenetiese en funksionele diversiteit beïnvloed van hierdie padda bevolkings. Verder het ek die derm mikrobioom van ‘n uitbreidende bevolking gekarakteriseer om vas te stel of verblyftyd die mikrobioom beïnvloed van ‘n groeiende bevolking by sy kern en periferie. Om my tweede doelwit te bereik het ek wederkerige mikrobiese fekale oorplantings op inheemse en uitheemse paddas in Durban (inheemse omgewing) en Kaapstad (uitheemse omgewing) voltooi. Daarna het ek paddas bloodgestel aan een van twee diete; ‘n natuurlike dieet of ‘n dieetuitdaging. Aan die einde van die eksperimente het ek die fekale mikrobiese materiaal van paddas versamel om die samestelling, filogenetiese en funksionele mikrobioom reaksies op die dieetuitdaging te bepaal. Verder het ek die uithouvermoë en energie berging van paddas bepaal.

Die derm mikrobioom samestelling is uniek in alle beveolkings van die gorrelskurwe padda. Alhoewel, net die jongste populasie het ‘n filogenetiese en funksioneel unieke derm mikrobioom. Dus, het ek gevind dat verblyftyd nie die derm mikrobioom van hierdie paddas beïnvloed nie. Ek stel voor dat indringsweë (dit wil sê die lewens siklus gedurende beweging van indringer spesies) ‘n kern faktor is wat die derm mikrobioom van hierdie padda beïnvloed.

Verder vind ek dat die Kaapstadse mikrobioom aangepas is om te reageer op omgewings verandering terwyl die inheemse, Durban, mikrobioom nie reageer wanneer paddas na ‘n dieetsuitdaging blootgestel word nie. Ek vind dat die mikrobioom fasiliteer die vermoë van paddas om energie te stoor en langer afstande en ‘n hoër spoed bereik wanner hulle spring. Ek wys dus vir die eerste keer dat die werweldier mikrobioom nie net kan aanpas tot ‘n nuwe ompegewing nie maar dat die mikrobioom die fisiologie van die gasheer reguleer.

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Chapter 1: General Introduction

What is the microbiome and why is it important?

Vertebrates are hosts to diverse and complex microbial communities that are adapted to live in or on their hosts (McFall-Ngai et al. 2013; Fischbach and Segre 2016). Of these, bacterial microorganisms outnumber their hosts' cells by more than an order of magnitude, with the majority present in the hosts' gastrointestinal tract (Savage, 1977; Sender et al., 2016). Studies on symbiotic bacterial communities have traditionally focused on single mutualisms, commensalisms, and parasitism (Bäckhed et al., 2005; Parniske, 2008). However, ecologists working on macroscale systems often found that microbes confound the ecological and evolutionary patterns observed in these systems. Thus, symbiosis research has expanded to study the role of microbial symbiotic relationships in other themes of biology such as speciation, evolution and coadaptation (Turnbaugh et al., 2007; Klepzig et al., 2009; Carrapiço, 2010; Lankau, 2012).

Initial efforts to determine the numbers of microbes in a community and their phylogenetic relationships (i.e. the microbiome) consisted of analysing the relatively well-conserved 16S rRNA genes in mixtures of cultured organisms (Woese & Fox, 1977; Stahl et al., 1984; Giovannoni et al., 1990; Schmidt et al., 1991; Dymock et al., 1996; Lederberg & McCray, 2001). However, up to 60% human-bacterial symbionts remains uncultivated, with a much greater percentage of uncultured bacterial species in other animal species (Pei et al., 2004; Verhelst et al. 2004; Zhou et al. 2004; Aas et al. 2005; Bik et al., 2006). With the advent of high-throughput next generation sequencing (NGS), it became possible to characterise the whole microbial composition in a single sample of interest (Eckburg et al., 2005; Caporaso et al., 2010; Douglas et al., 2020). These advancements allow for easy, comprehensive, and accessible profiling of microbial communities that are normally 'unculturable' using standard laboratory methods. Although relatively little sequencing is required to characterise the microbiome of a sample (Kuczynski et al., 2010; Parks & Beiko, 2013), deep and costly metagenomic sequencing is required to determine the functional genes expressed by these microbes (Knight et al., 2012). Development of advanced computational tools, such as PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), allows the prediction of the functional composition of a metagenome using 16S marker gene data and a database of reference genomes (Langille et al., 2013; Douglas et al., 2020). Thus, 16S marker gene profiling combined with computational tools like PICRUSt allows us to easily characterise the microbiome and its predicted function.

Recent NGS-based research emphasises the great diversity of gut bacterial communities and their functional capabilities across human, domestic- and laboratory animals (Turnbaugh et

al., 2007; Yatsunenko et al., 2012; Xiao et al., 2015; Falony et al., 2016; Laukens et al., 2016). More than 10-100 trillion distinct microorganisms live in the gut (Turnbaugh et al., 2007). While there is great variation of bacterial diversity along the gastrointestinal tract, faecal material samples are generally used to characterise interindividual variation of gut microbial communities (Rawls et al., 2006). For example, studies of faecal 16S rRNA gene sequences collected from monozygotic human twins show that fewer than 50% are shared between individuals (Turnbaugh et al., 2010). Moreover, faeces collected from unrelated human adults show extensive variation in gut bacterial community structure (Ley et al., 2006). Such high diversity within and across populations is thought to be sustained by the functional redundance (i.e. different microbes have the same functional potential) of many of these microorganisms (Hubbell, 2006; Turnbaugh et al., 2007). However, the factors producing and maintaining this microbial taxonomic and functional diversity within and across populations remains somewhat unknown.

Microbiome assembly is governed by many ecological and evolutionary processes similar to those shaping macro-ecological systems (Costello et al., 2012; Campbell et al., 2018; Trevelline et al., 2019). Numerous extrinsic environmental factors, such as diet, climate and species interactions, can rapidly produce extensive gut microbiome variation between individuals and populations (reviewed in Costello et al., 2012 and Dickey et al., 2020). The close physical contact between humans and their companion animals, for example, facilitates the acquisition and exchange of microbes. As a result, skin, oral and gut microbial communities of cohabiting humans, share more microbial taxa with their own dogs than they do with other dogs (Song et al., 2013). Conversely, the dynamics of the microbiome can also impact the host. A classic example in humans is disturbance in microbiome community composition leading to *Clostridium difficile* colonization and pathogenicity (Samarkos et al., 2018). Gut communities associated with *C. difficile* experience acute loss of microbial function which in turn results in increased growth and abundance of pathogens, decreased immunomodulation, increased inflammation, and disruption of resource accumulation in the host (Samarkos et al., 2018). Any environmentally induced alterations of the gut microbiome and its functional capabilities can, therefore, lead to the disruption of host physiological processes (Turnbaugh et al. 2007; McKenna et al. 2008; Sommer & Bäckhed 2013; Lee & Hase 2014). For example, seasonal flexibility of the gut microbiome in the brown bear, *Ursus arctos*, is related to alterations of hosts' fat accumulation and metabolism (Sommer et al., 2016). The microbiome, thus, acts as a critical mediator for how changing environmental conditions impact host function and health.

This field of research has now expanded to include wildlife species, either captive or living in their natural habitats (Jiménez & Sommer, 2017; Trevelline et al., 2019; Hauffe & Barelli,

2019). Increasingly, studies have recognised that bacteria inhabiting wild animals' guts may also be important to maintaining individual health and physiology, as has been shown extensively for humans, laboratory models and livestock (Cho & Blaser, 2012; Pascoe et al., 2017; Huaffe & Barelli, 2019). While an increasing body of research has attempted to integrate ecological and microbiome sciences (Redford et al. 2012; Amato et al., 2013; Bletz et al. 2013; Roggenbuck et al. 2014; Bahrndorff et al. 2016; Trevelline et al., 2019), fewer than 100 studies have investigated host-microbiome relationships in non-model animal species (Hauffe & Barelli, 2019). In addition, evaluation of this literature on wildlife microbiota reveals that most articles are dedicated to simply cataloguing the composition of these bacterial communities (Pascoe et al., 2017; Trevelline et al., 2019). To increase our scientific understanding and advancements in wildlife conservation, the ideas and techniques of microbiome and wildlife science need to be integrated properly.

Invaders and their microbes

Human activities continue to transform natural environments by moving species beyond the limits of their native geographic regions into areas where they do not naturally occur (Mack et al., 2000; Trakhtenbrot et al., 2005; Blackburn et al., 2011). These species must move through a series of stages while overcoming barriers to dispersal, survival, reproduction and spread in order to become invasive (Blackburn et al., 2011). Many animal and plant species that are introduced into novel environments, however, fail to cross these barriers and, thus, result in invasion failure. For example, leguminous plants may fail to naturalize if their mutualistic root-nodule bacteria (rhizobia) are not co-introduced or if the rhizobia density is low (Parker, 2001; Rodríguez-Echeverría et al., 2012). However, invasive species that do establish populations in non-native ranges can have devastating environmental and socio-economic impacts (Mack et al., 2000; Blackburn et al., 2011). Alarmingly, the rate of introductions shows no signs of decreasing and, thus, the likelihood of more species establishing in non-native ranges are increasing (Hulme, 2009; van Kleunen et al., 2015; Seebens et al., 2017). Consequently, understanding why some species become established and spread in novel environments is of great interest.

Attempts to explain why some species become successful invaders tend to focus on population ecology (Sakai et al., 2001; Liebhold & Tobin, 2008), propagule pressure (MacArthur & Wilson, 1967; Simberloff, 2009), life-history (Sæther & Bakke, 2000; Phillips et al., 2010; Allen et al., 2017), dispersal abilities and the evolutionary history of the invaders as well as the invaded ecosystems (Mack et al., 2000; Lee, 2002; Shea & Chesson, 2002). For example, dietary plasticity, or the ability to adjust one's diet, has been linked to the ability of some species to establish in novel environments (Courchamp et al., 2003; Caut et al., 2008;

Jones et al., 2008; Tonella et al., 2018). Additionally, several studies highlight cases of plant and insect invasions that were facilitated by their symbiotic microbes (Himler et al. 2011; Frago et al. 2012; Redford et al., 2012; Vilcinskas et al. 2013; Coats et al., 2014; Lu et al. 2016). For example, the rhizosphere microbiome is a primary mediator of plant establishment and success through its impact on plant immunity, pathogen abundance and stress tolerance (reviewed in Coats & Rumpho, 2014). Single strains of bacteria, their relationships and the entire microbiome have been shown to play essential roles in the performance of invasive plant and insect populations (Coats & Rumpho, 2014: Lu et al., 2016; Kamutando et al., 2019; Ramirez et al., 2019). The link between microbial communities and invasive plant and insect invasion success has been studied for many years (van der Putten et al., 2007; Pringle et al., 2009; Berendsen et al., 2012; Bakker et al., 2013; Coat & Rumpho, 2014). However, only a handful of studies have recently considered that gut microbial communities can potentially affect vertebrate invasion success (Kowalski et al., 2015; Eichmiller et al., 2016; Bahrndorff et al., 2016; Shanmuganandam et al., 2020). Given the importance of bacterial communities in regulating host health and physiology, it is imperative to understand the community assemblage of invasive hosts and the subsequent impacts this has on host health, physiology and fitness.

Besides the need to understand invasive species biology, invasive systems can be used as natural experiments to gain insights into the ecology and evolution of host-microbe relationships (Grinnell, 1919; Sax et al., 2007; Sexton et al., 2009). Biological invasions provide us with unplanned experiments across spatial and temporal scales with unique documented information as if it were planned manipulative experiments (Sax et al., 2007). Furthermore, these systems allow us to observe evolutionary and ecological processes as they are occurring in real time (Sax et al., 2007). Since host-microbe relationships have mostly been investigated in laboratory settings until now, biological invasions give us a unique opportunity to increase our comprehension of what drives these relationships and how they impact their hosts under natural conditions.

Gut, gutter, gutturalis…

The guttural toad (*Sclerophrys gutturalis*) is a highly adaptable African bufonid naturally distributed across central and southern Africa (Channing, 2001; du Preez et al., 2004) (Figure 2.1). However, it is absent from more arid regions in southern Africa, such as southern Namibia and most of southern South Africa (Channing, 2001; du Preez et al., 2004). Guttural toads inhabit a wide variety of vegetation types like Savannah, Grassland and Thicket biomes (du Preez et al., 2004). Due to its highly synanthropic behaviour, it is not uncommon to find these toads in peri-urban areas. In Mauritius, Réunion and Cape Town, South Africa, the

guttural toad has successfully established populations with all populations genetically determined to be from the same source population in Durban, South Africa (Telford et al., 2019) (Figure 2.1). Adult toads were first introduced into Mauritius in 1922 and subsequently adults were introduced in 1927 to Réunion (Cheke & Hume, 2008; Telford et al., 2019; Baxter-Gilbert et al., 2020). Both introductions were intentional attempts at insect biocontrol (Cheke & Hume, 2008; Telford et al., 2019). More recently, an accidental introduction of eggs or tadpoles occurred through a consignment of aquatic plants from Durban to peri-urban Constantia, Cape Town, where toads were first heard calling in 2000 (de Villiers, 2006; Telford et al., 2019; Davies et al., 2020a). The Cape Town population consists of a core (site of introduction) and a continuously expanding range edge (naturally dispersed sites) (Vimercati et al., 2018). Since its first detection in Constantia, the occurrence of this species raised concerns about its possible impact on the endemic and threatened western leopard toad (*Sclerophrys pantherina*), whose range overlaps partially with the introduced area of the guttural toad (de Villiers, 2006). Guttural toads could impact this endemic species through competition for resources (Vimercati, 2017) and by acting as a vector and host for both native and introduced parasites (Vimercati, 2017; Kruger, 2017). Following the detection of guttural toads in Cape Town, the City of Cape Town implemented an ongoing extirpation program since 2010 for the removal of adults, tadpoles, and eggs (Vimercati, 2017; Davies et al., 2020a; Davies et al., 2020b). However, the population is still in expansion (Vimercati, 2017).

Thesis structure and aims

The variation of host-microbe relationships and their importance in facilitating host establishment and adaptive physiology is of fundamental interest to both microbial and invasion ecology. The first aim of this thesis is to characterize the compositional variation of gut microbiomes in the source and invasive populations of the guttural toad. Specifically, the first chapter addresses whether residence time impacts the gut microbial compositional, phylogenetic and predicted functional diversity and microbial abundance of invasive populations with reference to the source populations' gut microbiome. Furthermore, whether residence time produces any variability in an expanding population is explored by determining the gut microbial composition, phylogenetic and predicted functional diversity and microbial abundance of the expanding invasive population in Cape Town. This thesis aims to further explore to what extent the invasive gut microbiome contributes to the adaptive physiology and, consequently, fitness of its host. This aim is addressed in the second chapter by examining results of reciprocal faecal microbial transplants (FMT) on invasive (of Cape Town origin) and native (of Durban origin) hosts and subsequent exposure of a dietary challenge to hosts. Compositional, phylogenetic, and predicted functional diversity changes of gut microbiomes

to dietary challenges will be examined, as well as the resource investment and physiological performance of hosts.

Chapter 2: Invasion dynamics shape the gut microbiome of a widely introduced toad (*Sclerophrys gutturalis*).

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? Increasing studies demonstrate significant shifts of gut bacterial communities when their hosts' environments are altered, whereas their microbiomes' functional capabilities remain unchanged. Here I ask whether invasive species adjust their microbial composition and predicted functionality when introduced to new environments. Through 16S amplicon sequencing on guttural toad (*Sclerophrys gutturalis*) faecal samples, I aim to determine whether longer residence times (~ 100 years) in introduced populations (Réunion and Mauritius) produces significant divergence of microbial compositional, phylogenetic and predicted functional diversity and differential abundance from source populations (Durban), compared to younger populations with shorter residence times (~ 20 years: Cape Town). Additionally, I determine whether microbial compositional, phylogenetic, and predicted functional variation exists in an expanding introduced population at its core and periphery. Residence time does not impact the microbial diversity between the source and introduced populations. The youngest population (Cape Town) has the most distinct microbiome and predicted functional microbial capabilities. Furthermore, within this expanding population there is extensive variation of microbial and functional diversity, as well as differential abundance patterns between core and periphery sites. Contrasting previous studies' findings, I suggest that introduction pathways might be a more important factor impacting the divergence of microbial compositional and functional diversity than residence time. These findings also imply that microbiome composition and predicted functional capabilities can diverge in accordance with host population dynamics.

INTRODUCTION

Vertebrates are host to diverse gut bacterial communities that profoundly influence host health and physiology through the metabolic and functional components they express (Cho & Blaser, 2012; Langille et al., 2013; Sommer & Bäckhed, 2013; Kohl & Carey, 2016; Fischbach & Segre, 2016; McKenney et al., 2017). These host-associated microbial communities are governed by the same ecological principles shaping macro-ecological systems: intrinsic and extrinsic factors (reviewed in Costello et al., 2012 and Dickey et al., 2020). Changes in environmental conditions have been shown to produce extensive microbial compositional and functional variation in human, laboratory and commercial animal populations (Trevelline et al.,

2019). The susceptibility of gut microbial communities to extrinsic environmental conditions indicates that anthropogenic disturbance, which rapidly reshapes numerous abiotic and biotic factors, can drastically alter wildlife gut microbiomes (Barelli et al., 2015; Cheng et al., 2015; Carrillo-Araujo et al., 2015; Trevelline et al., 2019; Murray et al., 2020; Teyssier et al., 2020). For example, the movement of swan geese (*Anser cygnoides*) to urbanized areas results in significant shifts in their gut microbiome as well as increases functional xenobiotic biodegradation pathways in urban geese (Wu et al., 2018). Despite the considerable compositional and functional variability of gut microbiota, how anthropogenic environmental change impacts non-model wildlife species' microbiomes remains relatively unexplored (Hauffe & Barelli, 2019; Trevelline et al., 2019).

Anthropogenic movement of species to novel environments can drastically alter animals' gut microbiome. Introduction of species into new environments are known to result in the loss and/or gain of single microbial symbionts due to changes in abiotic and biotic conditions (Colautti et al., 2004; MacLeod et al., 2010; Blackburn et al., 2015; Blackburn & Ewen, 2016; Amsellem et al., 2016). The loss and/or gain of microbial species and their associated functionality can have strong evolutionary effects on invasive host performance, reproduction, and dispersal (Colautti et al., 2004; Prior et al., 2015; Amsellem et al., 2016). Invasive plants and their co-invasive mycobionts, for example, often develop synergistic effects, where the interacting patterns promote fitness (i.e. growth rate, nutrient acquisition and stress endurance) of the host and the fungal partner, thereby exacerbating their invasive potential and ecological harm (Simberloff & Von Holle, 1999; Coats & Rumpho, 2014; Liu et al., 2020). Given the major implications of the loss and/or gain microbes to host function, it is surprising that only a handful of studies have investigated the impact of species introduction into novel environments on gut microbial communities and their functional capabilities.

Biological invasions provide a valuable opportunity as natural experiments to investigate population and symbiotic microbial responses to novel conditions. Biological invasions are often caused by deliberate introduction of species, with significant efforts dedicated to detecting and monitoring these invasions (Yoshida et al., 2007). 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 source and introduced populations. Increased residence time facilitates the accumulation of novel microbes from the new environment, producing divergent gut microbial communities (Lau & Suwa, 2016). Furthermore, genetic differentiation due to selection pressures imposed by new abiotic and biotic pressures can also potentially increase with residence time (Monzón-Argüello et al., 2014). Additionally,

accumulated residence time at the invasion core results in higher population densities, increasing transfer of microbial symbionts, while the opposite is true for individuals at the population's periphery (Brown 1984; Sagarin & Gaines, 2002; Eckert et al., 2008; Sexton et al., 2009; Couch et al., 2020). Biological invasions, therefore, allow us to examine the response of population change to novel environmental conditions across multiple spatial and temporal scales.

My aim was to test the hypotheses (1) that older invasive populations of the guttural toad with longer residence times (~ 100 years: 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: Cape Town) will show no significant 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 an expanding invasive population (Vimercati et al., 2018). This study provides important new insights into the variation of invasive amphibian gut microbiomes, an understudied group in microbiome science (Jiménez & Sommer, 2017). I highlight potential factors producing variation in gut microbiomes and their predicted functionality, as well as how population dynamics might shape the gut microbiome of non-model species under natural conditions.

MATERIALS AND METHODS

Species and study site description

The guttural toad (*Sclerophrys gutturalis*) is a common bufonid distributed among various habitats in sub-Saharan Africa (Channing, 2001; du Preez et al., 2004; Vimercati et al., 2017). It is a habitat generalist that occurs naturally in areas characterized by tropical and subtropical climates (i.e. summer rainfall) (Channing, 2001; du Preez et al., 2004). The oldest introductions of adult guttural toads were to Mauritius in 1922 and a subsequent introduction of adults took place in 1927 to Réunion (Figure 2.1; Cheke & Hume, 2008; Telford et al. 2019; Baxter-Gilbert et al., 2020). Both introductions were the result of an intentional attempt of insect biocontrol (Cheke & Hume, 2008; Telford et al., 2019). Since introduction, guttural toads have become widespread across the Mascarene Islands, particularly in Réunion and Mauritius (Cheke & Hume, 2008; Sanchez & Probst, 2016; Baxter-Gilbert et al., 2020). These islands are characterized by tropical climates similar to the native area of guttural toads. A younger accidental introduction of eggs or tadpoles through a consignment of aquatic plants from Durban to peri-urban Constantia, Cape Town, took place in 2000 (Figure 2.1; de Villiers, 2006; Telford et al., 2019; Measey et al., 2020; Davies et al., 2020a). The Cape Town area is

characterized by a mediterranean (i.e. winter-rainfall) climate. Due to its recent introduction, the Cape Town population consists of a core (site of introduction) and continuously expanding range edge (naturally dispersed sites) (Vimercati et al., 2018). All invasive populations have been genetically determined to be from the same or nearby populations in the northeast of South Africa (most likely Durban) (Telford et al., 2019). Guttural toad diets consist mostly of insects, gastropods and other invertebrates (Channing, 2001; Du Preez et al., 2004).

Sample collection

Eleven adult toads were collected within Durban, Mauritius and Réunion, from Februrary to July 2019 in peri-urban areas (residential gardens). In Cape Town, a collection of 11 individuals was made in the core of the expanding population, and an additional 11 individuals were collected from sites at the periphery in Febuary 2019. The core was defined as sites where toads were routinely caught as part of the eradication programme for the last five years, while sites where toads have never been previously recorded present were defined as the periphery. In total, 55 faecal samples were collected. Within each sampling area, adult female toads were captured by hand after sunset (19:00 h). I chose to capture animals of a single sex as intersex microbiome differences may confound results. 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 (Baxter-Gilbert et al., 2020).

Immediately after capture, toads were weighed, and their SVL was measured. Toads were placed individually in plastic containers (195 x 195 x 180 mm). Plastic containers were sterilized with a 10% bleach solution followed by further sterilization with 70% ethanol before use. Faecal samples were collected from toads within the first 8 hours of captivity. 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 sample collection, invasive toads were euthanized by immersion in a 1 gl^{-1} solution of tricaine ethane sulfonate (MS-222) for 20 minutes and native toads (in Durban, South Africa) were released.

After approximately 6 weeks, following storage at -20 °C, faecal samples were centrifuged (2 min at 10 000 x 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.

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 in an attempt to mitigate possible impacts on a threatened endemic, the western leopard toad *Sclerophrys pantherina* (Measey et al., 2020; Davies et al.,

2020a; Davies et al., 2020b) 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.

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. DNA extracts were stored at -80 °C until further processing. No template and template from blank filters were included as negative controls throughout the entire process from DNA extraction to PCR amplification.

DNA samples were quantified using the Qubit 4.0 Fluorometer (ThermoFisher Scientific) and the Qubit 1x 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 (GQS) 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' – GACTACHVGGGTATCTAATCC – 3' (Klindworth et al., 2012). 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 x Phusion® HF buffer) with a final concentration of 1.5 mM $MgCl_2$. Polymerase chain reactions (PCRs) were performed on the SimpliAmp™ Thermal Cycler (ThermoFisher Scientific). Initial template DNA denaturation at 98 °C for 30 sec was followed by 25 cycles consisting of 98 °C for 10 sec, 58 °C for 30 sec and 72 °C for 30 sec; with a final product extension at 72 °C for 10 min.

Presence of amplified products were verified on the PerkinElmer LabChip® GXII Touch (PerkinElmer, Waltham, MA, USA), using the X-mark chip and HT DNA NGS 3K reagent kit, according to the manufacturer's protocol. PCR products were then purified with 1.8x 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 1x 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 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 1.8x 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 1.8x 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 pre-processing (Bolyen et al., 2019). The divisive amplicon denoising algorithm (DADA2) plugin was used to de-noise sequencing reads (Callahan et al., 2016). 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 using default DADA2 parameters. 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 (Katoh & Standley, 2013; q2-alignment plugin), high entropy positions were filtered from the resulting alignment (Lane et al., 1991), an unrooted tree was constructed with FastTree 2 (Price et al., 2010; q2-phylogeny

plugin) 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) (Quast et al., 2013; q2-feature-classifier plugin). The ASV table, phylogenetic tree and assigned taxonomy table was used in all downstream analyses.

The ASV table and its corresponding phylogenetic tree was additionally used to predict functional profiles of samples through the PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2, NSTI cut-off = 2) pipeline in QIIME2 (Douglas et al., 2020; q2-picrust2 plugin) 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). Removal of contaminant sequences were, therefore, not required.

Statistical analysis

Preliminary analyses showed that body mass was positively correlated with SVL (snout-to-vent length). Therefore, the body condition (or scaled mass index) was calculated following Peig and Green (2009) and Vimercati et al., (2019). Body condition of toads was used as a covariate in all downstream analyses.

All statistical analyses were performed in R version 3.6.2 (R Core Team, 2019). Metadata, ASV table, taxonomy and phylogenetic tree was imported using the qiime2R package (v0.99.13, Bisanz, 2018). A phyloseq object was built from these datasets using the phyloseq package (v1.30.0, McMurdie & Holmes, 2014). Prior to all downstream analyses alpha rarefaction curves were inspected to assess sequencing depth (Figure S2.1). Visual inspection confirmed that sequencing depth was adequate for each sample with regards to number of ASVs detected. 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 (v0.99.02, Shetty et al., 2018).

Diversity metrics inverse Shannon diversity, Evenness, Chao1 species richness and Faith's phylogenetic diversity was calculated using the vegan package in R (v2.5.6, Oksanen et al., 2007). The inverse Shannon diversity metric incorporates both measures of species richness and abundance. Evenness measures how similar in abundance species in a sample are, while Chao1 measures the asymptote on a species accumulation curve to determine species richness. Faiths' phylogenetic diversity measures the cumulative branch lengths from randomly sampled species from each sample. Linear mixed-effects models (GLMM) were used to determine whether alpha diversity metrics (response variables) vary across populations. Prior to analyses, model assumptions (e.g. normality, homogeneity and

independence) were assessed. Phylogenetic diversity estimates did not meet assumptions of normality. Data was 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) R2 was calculated according to Nakagawa & Schielzeth (2013) using the 'r.squaredGLMM' function in the package MuMIn (v1.43.15, Barton, 2009). 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. GLMM 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. CLR- and PHILR-metrics are equivalent to the Bray-Curtis and Unifrac beta diversity metrics, but account for the compositional nature of the data (Gloor et al., 2017). Feature tables containing read counts were first subjected to centre log-ratio (CLR)- and PHILR-transformation, using phyloseq and philr packages (v1.12.0, Silverman et al., 2017), respectively. Euclidean distance matrices were constructed from the transformed ASV count tables with the adonis function (vegan package). Distance matrices were subjected to PERMANOVA analyses (999 permutations) 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), I inspected this assumption with the betadisper and permutest functions of vegan. Clustering analysis using PCoA and UPGMA methods on CLR- and PHILR metrics was used to visualize similarity of population and site gut microbiomes.

To investigate differential abundance of ASVs across populations, likelihood ratio tests (LTR) were employed through the DeSeq2 package (v1.26.0, Love et al., 2014). 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. LTR-tests 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 PICRUSt 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 does not vary between populations (GLMM: $p > 0.05$, Figure S2.2, Table S2.1). However, compositional and phylogenetic beta diversity varies significantly across populations (PERMANOVA on CLR-distances: $F_{(3, 43)} = 2.56$, $p < 0.05$ and PERMANOVA on PHILR-distances: $F_{(3, 43)} = 2.71$, $p < 0.05$, table 2.1). While guttural toads from all populations vary significantly in their compositional diversity ($p < 0.05$ for all pairwise comparisons, Figure 2.1, Figure 2.2A, Table S2.2), only the youngest populations' (Cape Town) phylogenetic diversity is significantly different from all other populations gut microbial communities ($p < 0.05$ for pairwise comparisons, Figure S2.3, Figure 2.2B, Table S2.2). Betadisper analysis for all comparisons indicate variation between population microbiomes are not due to different dispersion levels ($p > 0.05$, Figure 2.2D, Figure 2.2E). Additionally, body condition and collection site has no effect toad gut microbiomes (PERMANOVA: $p > 0.05$, Table 2.1).

A total of 5205 ASVs are differentially abundant across populations (DESeq2: adj-$P < 0.05$, Table S2.3). The youngest population (Cape Town) has the most differentially abundant ASVs, with 1809 ASVs differentially abundant between Durban and Cape Town (Figure 2.3), 1015 between Durban and Réunion, 1014 between Durban and Mauritius, 1341 between Mauritius and Réunion, 1430 between Mauritius and Cape Town, 1502 between Réunion and Cape Town (DESeq2: adj-$P < 0.05$ for all pairwise comparisons, Table S2.4).

Only functional profiles of the youngest invasive population is distinct from the source population

Analysis of predicted functions revealed that population has an effect on the functional profiles of guttural toad gut microbial communities (PERMANOVA: $F_{(3, 43)} = 1.61$, $p < 0.05$, Figure 2.2C, Table 2.2). However, post-hoc analyses showed that only the functional profiles of the youngest populations' gut microbial communities are significantly distinct from the other populations (PERMNOVA: $p < 0.05$ for all pairwise comparisons, Table S2.5). Betadispr analysis for all comparisons indicate that the variation between population predicted functional pathways are not due to different dispersion levels ($p > 0.05$, Figure 2.2F). Body condition and collection site also has no significant effect on the predicted functionality of toad gut microbial communities (PERMANOVA: $p > 0.05$, Table 2.2).

Only 86 predicted metabolism-associated functional features of the gut microbial communities are differentially abundant across toad populations (DESeq2: *P-adj* < 0.05, Table S2.6). Among populations, the youngest population (Cape Town) has the most differentially abundant functional pathways, with 37 differentially abundant functional pathways between Durban and Cape Town (Figure 2.4), 29 between Durban and Réunion, 5 between Durban and Mauritius, 3 between Mauritius and Réunion, 21 between Mauritius and Cape Town, 52 between Réunion and Cape Town (DESeq2: adj-*P* < 0.05 for all pairwise comparisons, Table S2.7).

Expanding populations have divergent compositional and phylogenetic bacterial communities across its core and periphery

In Cape Town, alpha diversity does not vary across core and periphery sites (GLMM: $p > 0.05$, Figure S2.4, Table S2.8). In the Cape Town invasive population, beta diversity of gut microbial communities vary both compositionally and phylogenetically between core and periphery sites (PERMANOVA on CLR-distances: $F_{(1,21)} = 1.49$, $p < 0.01$ and PERMANOVA on PHILR-distances: $F_{(1,21)} = 1.55$, $p < 0.05$, Table 2.3). Visualization of both distance matrices show clear separation of gut microbial communities between the two sites (Figure 2.5A, Figure 2.5B). Betadispr analysis indicate that this variation is not the result of different dispersion levels (PERMANOVA: $p > 0.05$, Figure 2.5D, Figure 2.5E). Body condition and collection site also has no significant effect on the beta diversity of toad gut microbial communities (PERMANOVA: $p > 0.05$, Table 2.3). Abundance of 1361 ASVs significantly differs between core and periphery gut microbiomes of Cape Town guttural toads (DESeq2: *adj-P* < 0.05, Figure 2.6, Table S2.9).

Functional profiles vary between core and periphery sites in an expanding invasive population

Functional profiles vary significantly between core and periphery sites in the Cape Town invasive population (PERMANOVA: $F_{(1,21)} = 1.95$, $p < 0.05$, Figure 2.5C, Table 2.4).

Additionally, eight predicted metabolism-associated functional features of the gut bacterial communities were differentially abundant between core and periphery guttural toads (DESeq2: *P-adj* < 0.05, Figure 2.7, Table S2.10).

DISCUSSION

Guttural toad gut microbial communities have diverged from their source population across all invasive populations. Only a few studies have explored gut microbiome differentiation across native and introduced populations (Bansal et al., 2014; Cardoso et al., 2012; Bahrndorff et al., 2016). Overall, these studies support my results in that widespread introduction of a species produces diverse microbiomes (Cardoso et al., 2012; Eichmiller et al., 2016; Rosso et al., 2018). Contrary to my expectations, longer residence time did not produce more phylogenetically distinct gut microbial communities and predicted functional profiles. This result is unexpected as residence time has been identified as a key attribute impacting pathogenic microbial richness and divergence across invasive populations (Perkins et al., 2006; Diez et al., 2010; Mitchell et al., 2010; Lau & Suwa, 2016). Furthermore, within the youngest population extensive microbial divergence was evident across core and periphery sites, suggesting for the first time that rapid alteration of gut microbiomes can occur during the expansion of a population.

Introductory pathways is an interesting factor that could produce divergent gut microbial communities between source and introduced guttural toad populations. Adult toads deliberately introduced to Mauritius and Réunion could have been more extensively infected with microbial symbionts from their source population, compared to the youngest population which was thought to be accidentally introduced into Cape Town as larvae. Previous studies indicate that microbial loss through sampling effects (i.e. introduced hosts were by chance not infected) as a result of varying introduction pathways is a rare phenomenon (MacLeod et al., 2010). However, in our case ontogeny at/during initial introduction could have increased the chances of sampling effects impacting gut microbial divergence since microbiome structure is known to vary significantly across amphibian life stages (Kohl et al., 2013; Vences et al., 2016). Introduction of tadpoles to Cape Town could have significantly reduced similarity between the adult gut microbiomes of source and invasive populations. In other words, absence of adults during the initial introduction to Cape Town could have prevented the colonization of adult microbiomes present in other populations. However, other factors known to impact the gut microbiome, such as diet and climate, could have contributed to the divergence between these populations. For example, Cape Town is characterized by a Mediterranean climate significantly drier and colder than that of the native source area and two older invasive populations (Vimercati, 2017; Vimercati et al., 2018). In order to tease apart

the impact of different factors on invasive hosts' gut microbiomes, future studies should, thus, systematically investigate both the variation of other habitat and host features, as well as the gut microbial variation of tadpoles across these populations.

This study is the first demonstrating that an expanding population can undergo rapid alteration of gut microbial composition during population expansion (but see Amor & Dal Bello, 2019 and Couch et al., 2020). Genetic differentiation between the core and peripheral sites of a population range can be the result of numerous factors including effective population size, successive founder effects and variation in extinction events (Hallatschek et al., 2007; Eckert et al., 2008). Additionally, recent studies have shown that spatial proximity of hosts can play an important role in microbial shifts (Phillips et al., 2012; Moeller et al., 2016; Ren et al., 2017; Amor & Dal Bello, 2019; Couch et al., 2020). Spatial proximity of hosts mediates exposure to similar microbial sources and allows indirect transfer of microbes between inter- and intraspecific individuals (Phillips et al., 2012; Moeller et al., 2016; Ren et al., 2017). Decrease of effective population size as individuals move towards the periphery can, therefore, 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, such as fibre, and the efficiency of energy harvesting, the functional and metabolic potential of the gut microbiome 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 (Comte et al., 2013), i.e. different bacterial species can exhibit similar functional capabilities across communities. Lack of correlation between bacterial function and composition could indicate that functional pathways have minor impacts on organismal performance (Comte et al., 2013; Bansal et al., 2014). On the other hand, similar responses between these variables can be interpreted as composition and functional pathways having a significant influence on organismal performance (Comte et al., 2013). Variation of predicted functions between the Cape Town and source population could possibly be associated with intake and digestion of different food substrates. It is well known that diet has an immense impact on species microbial composition and associated functional profiles (Turnbaugh et al., 2006; Muegge et al., 2011; David et al., 2014). Hosts rely on gut-associated microbiota to degrade complex substrates into nutrients (Bäckhed et al., 2005; Turnbaugh et al., 2006). 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.

Contrasting previous studies, alpha diversity did not vary across any comparisons. This is likely the result of the pre-processing techniques. Alpha diversity is dependent on the number of singletons/doubletons in a dataset. Singletons/doubletons present in NGS-datasets are often not the result of true sequences but sequencing errors, chimeras, artefacts, or contaminants (Chiu & Chao, 2016). This has resulted in overestimation of alpha diversity variation between populations/groups/treatments (Willis, 2019). The bioinformatics pipeline DADA2 attempts to solve this problem by treating all singletons as sequencing errors (Callahan et al., 2016). However, this results in the removal of true sequences and, consequently, underestimation of alpha diversity metrics. Until a tool is developed that can distinguish between true and false sequences, we cannot accurately estimate the alpha diversity of guttural toad populations.

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 (Jiménez & Sommer, 2017; Hauffe & Barelli, 2019; Trevelline et al., 2019; Banerjee et al., 2020). Invasive species provide us with a unique opportunity to investigate replicated natural experiments with divergent novel conditions. In this study, I characterized the gut microbial composition of an invasive toad species' native source population and three introduced populations. I show that residence time does not impact the gut microbial variability or functional pathway variation of *Sclerophrys gutturalis* populations. Instead, I suggest that introduction pathways of invasive species might be a more important factor determining microbial and functional differentiation between populations. Furthermore, this study is one of the first demonstrating that population dynamics likely influence the gut microbial composition and functional capabilities of an expanding population. Future studies, perhaps through control experiments, will be important to tease apart the potential benefits of host microbiota in facilitating host adaptation to novel environments.

FIGURES AND TABLES

Figure 2.1. (A) Location of sampling areas for *Sclerophrys gutturalis* (guttural toad) adults from invasive population; Mauritius (green), Réunion (pink) and Cape Town (red) and the source population; Durban (blue) (n = 44). (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.

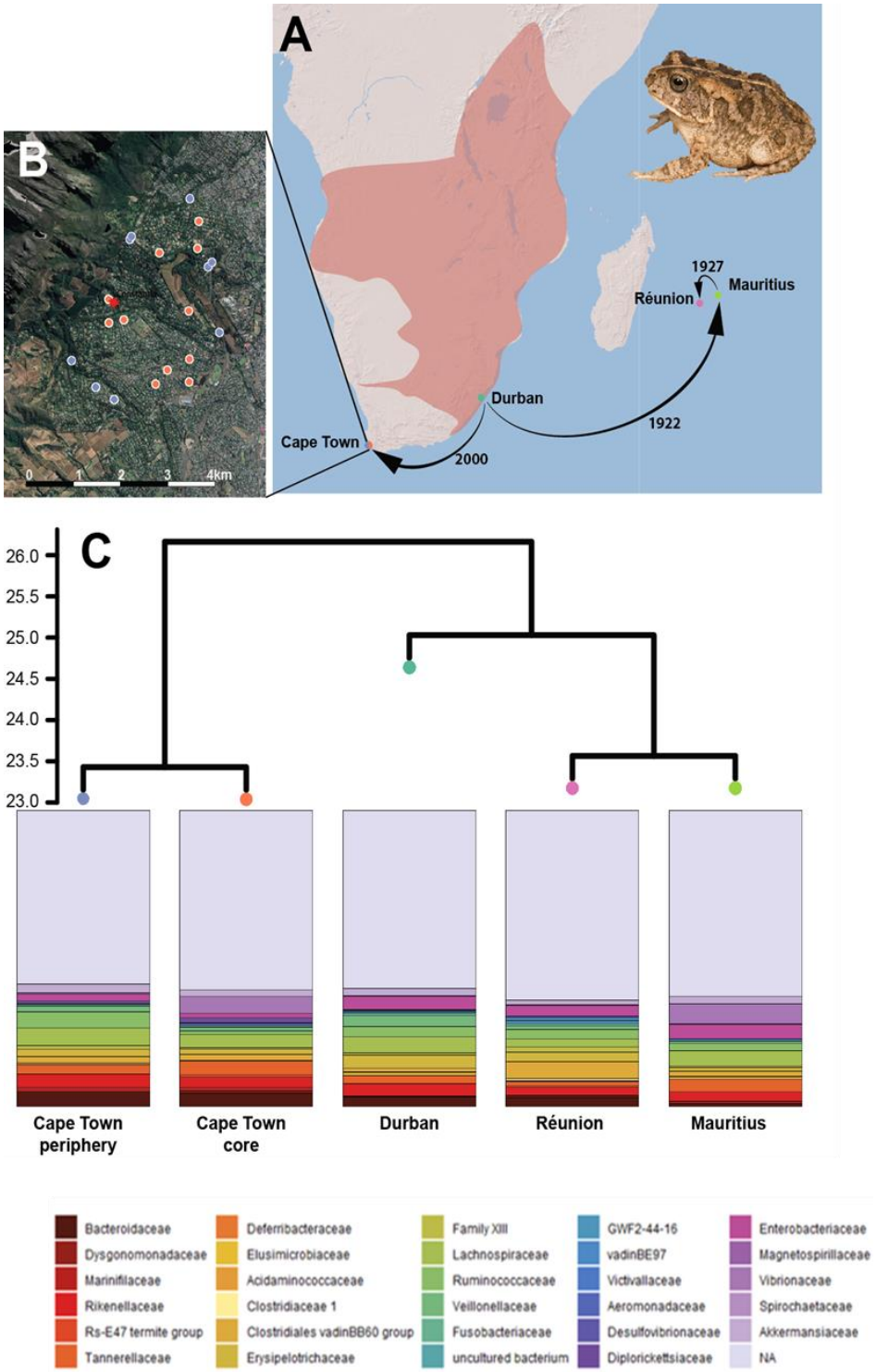

Figure 2.2. Principle Coordinates Analysis (PCoA) of (A) CLR-Euclidean compositional beta diversity, (B) PHILR-Euclidean phylogenetic beta diversity and (C) CLR-Euclidean functional beta diversity. Gut microbial communities significantly differed among *Sclerophrys gutturalis* (guttural toad) native (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).

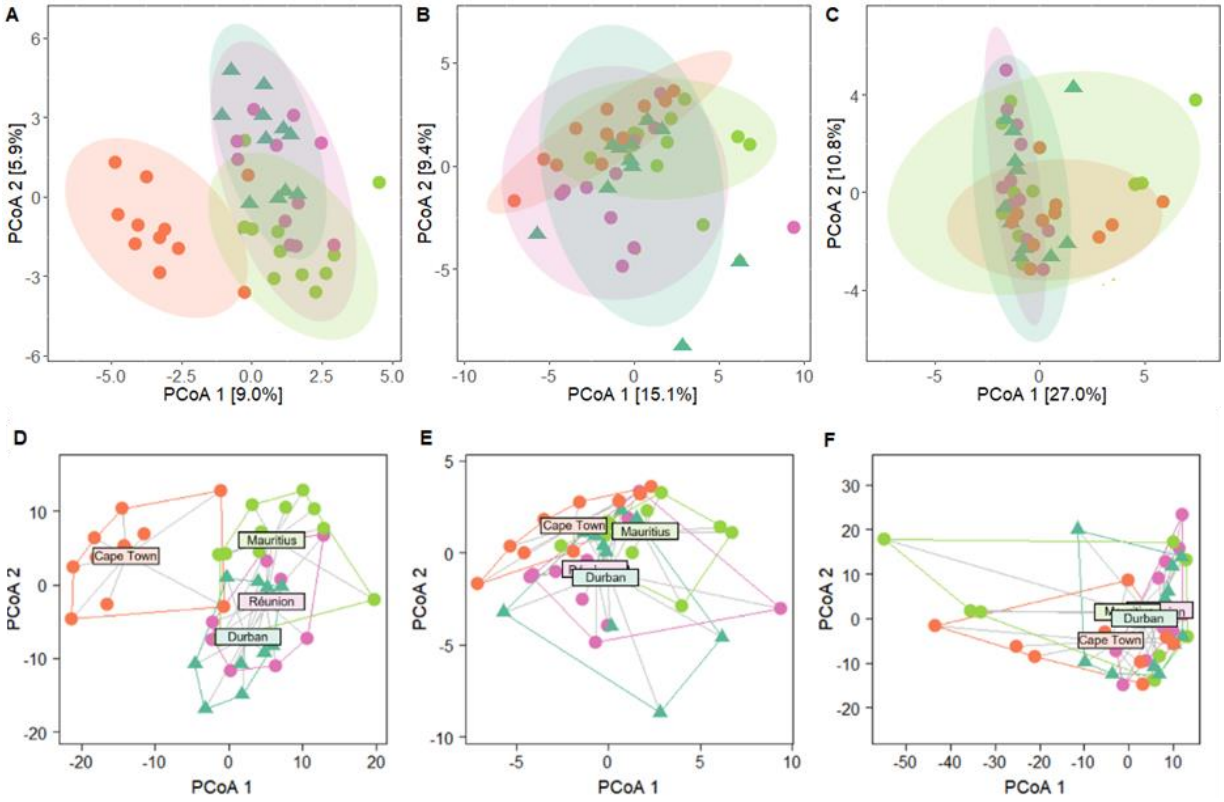

Figure 2.3. Differences in the relative abundance (depicted by log2 fold change) of ASVs between native, Durban (blue), and invasive, Cape Town (red) *Sclerophrys gutturalis* (guttural toad) populations. A total of 1809 ASVs significantly differed in abundance between native and invasive toad populations (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 S2.4 for a complete list of differentially abundant ASVs). Family and Phylum classification is provided where available.

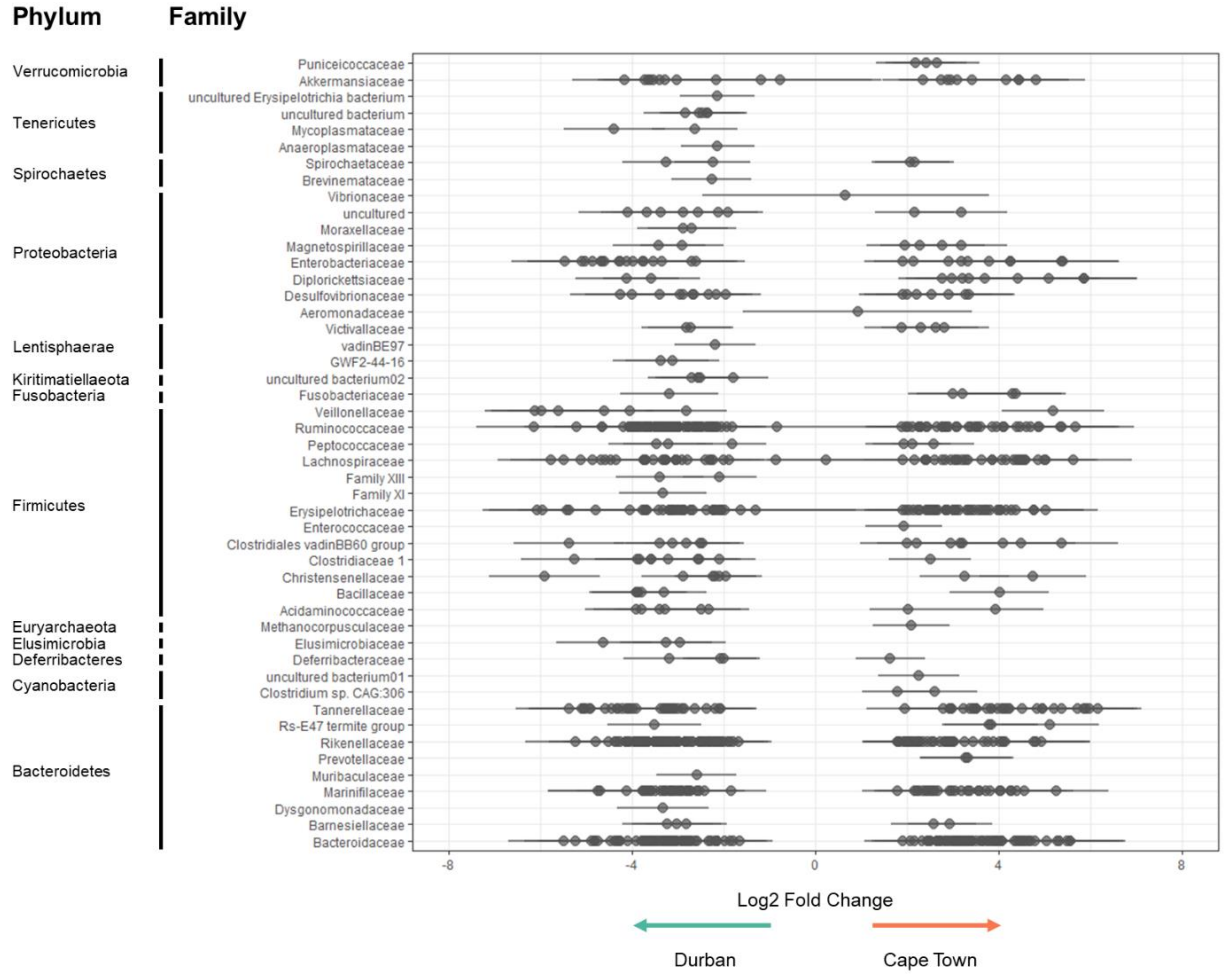

Figure 2.4. Differences in the relative abundance (as depicted by log2 fold change and relative abundance) of functional pathways between between native, Durban (blue), and invasive, Cape Town (red) *Sclerophrys gutturalis* (guttural toad) populations. A total of 37 functional pathways significantly differed in abundance between native and invasive toad populations (DESeq2: *adj-P* < 0.05; see Table S2.7 for a complete list of differentially abundant functional pathways).

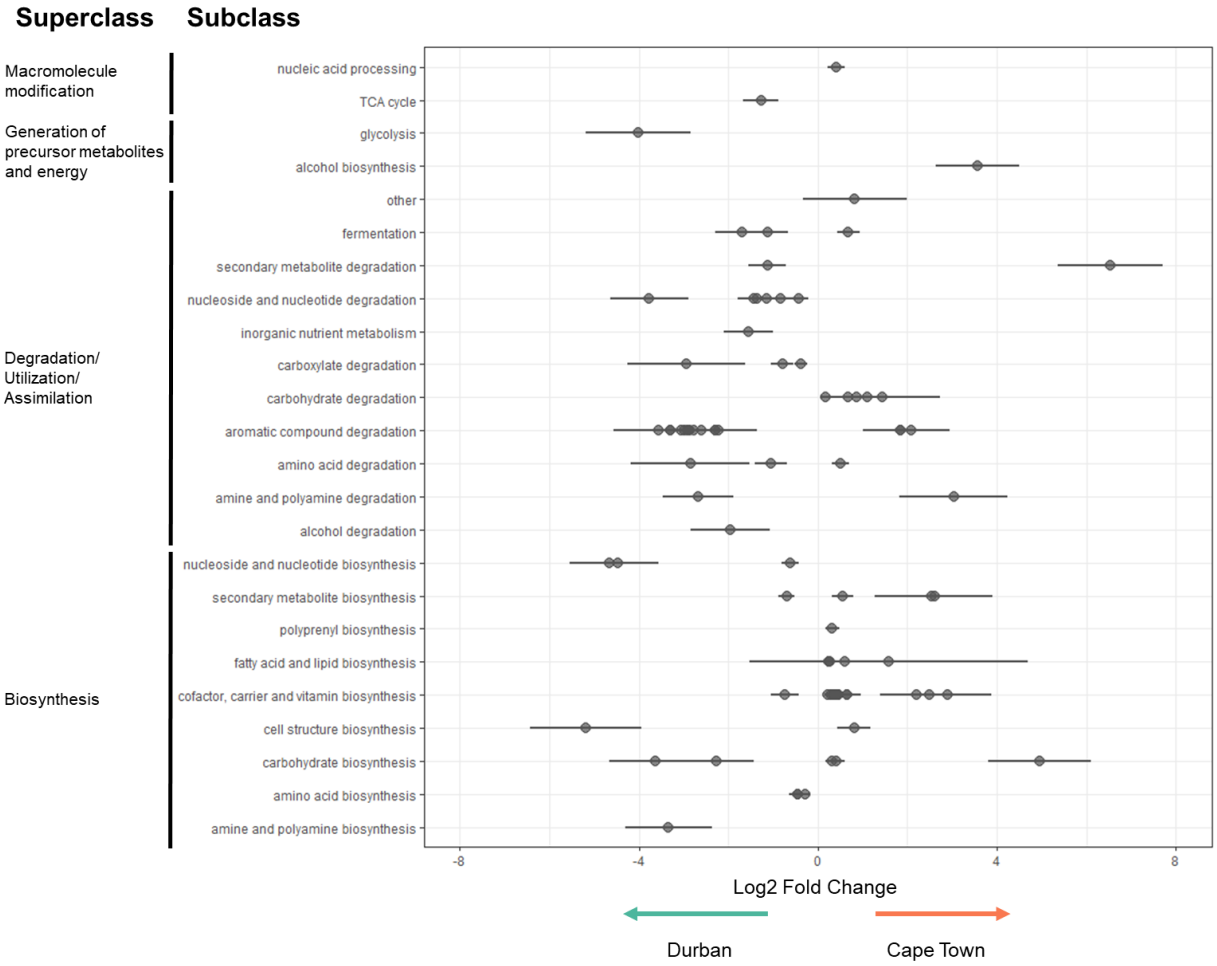

Figure 2.5. Principle Coordinates Analysis (PCoA) of (A) CLR-Euclidean compositional beta diversity, (B) PHILR-Euclidean phylogenetic beta diversity and (C) CLR-Euclidean functional beta diversity of guttural toad (*Sclerophrys gutturalis*) gut bacterial communities. Gut microbial communities significantly 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.

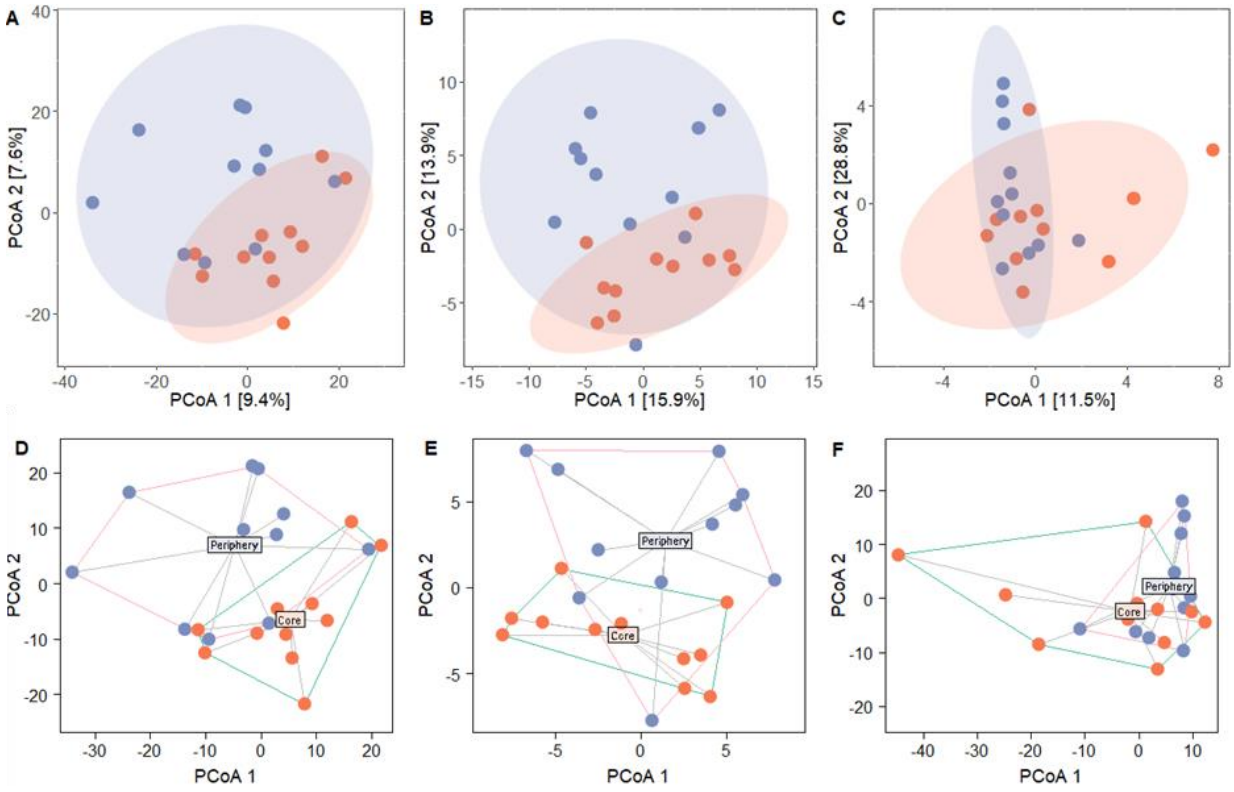

Figure 2.6. Differences in the relative abundance (depicted by log2 fold change) of ASVs between core (red) and periphery (purple) guttural toads (*Sclerophrys gutturalis*) from their invasive range in Cape Town, South Africa. A total of 1361 ASVs significantly differed in abundance between core and periphery 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 S2.9 for a complete list of differentially abundant ASVs). Family and Phylum classification is provided where available.

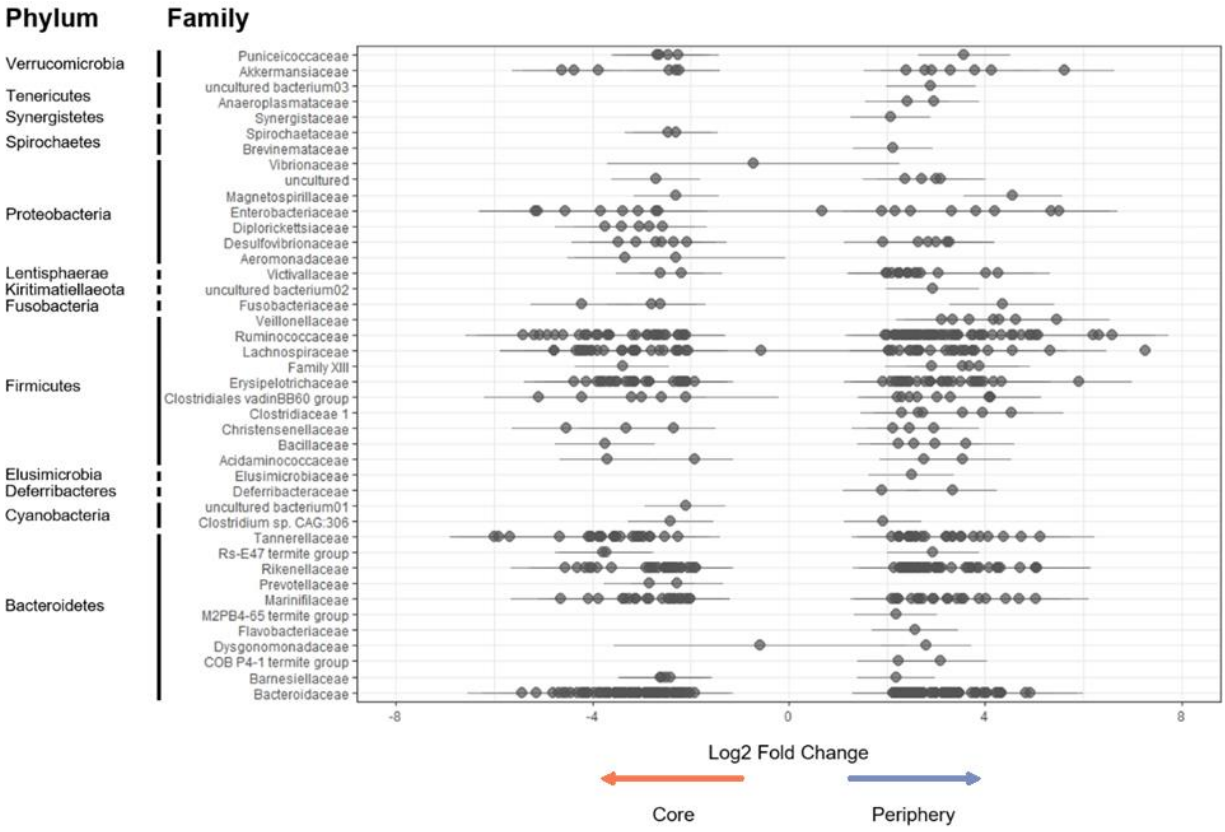

Figure 2.7. Differences in the relative abundance (as depicted by log2 fold change) of functional pathways between core (red) and periphery (purple) guttural toads (*Sclerophrys gutturalis*) from their invasive range in Cape Town, South Africa. In total 8 functional pathways significantly differed in abundance between native and invasive toad populations (DESeq2: *adj-P* < 0.05; see Table S2.10 for a complete list of differentially abundant functional pathways.

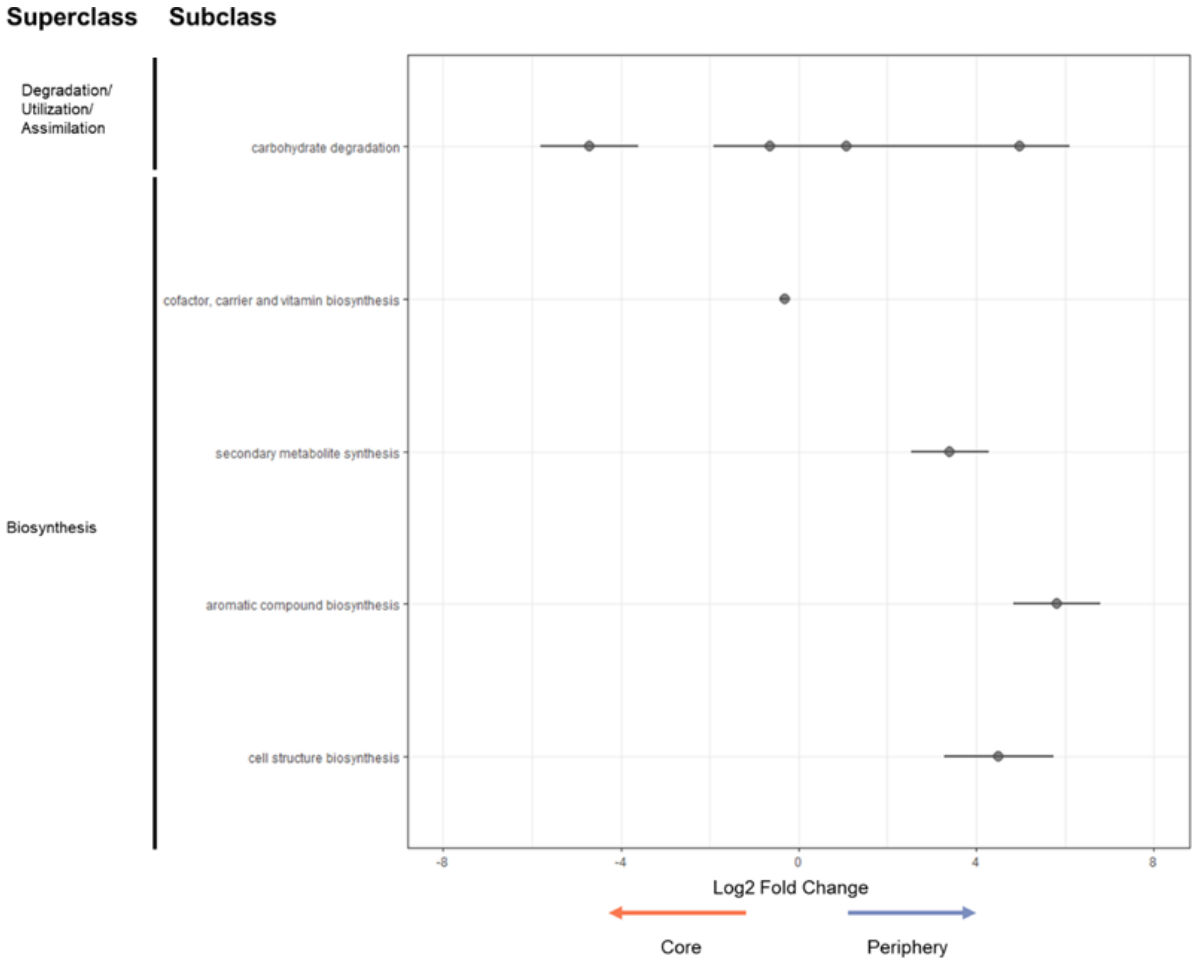

Table 2.1. Summary of PERMANOVA results analysing the effect of population, body condition and collection site on *Sclerophrys gutturalis* (guttural toad) gut microbial communities as measured by compositional CLR- and phylogenetic PHILR-Euclidean metrics. For each comparison, dependent and explanatory 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	> 0.05
	Collection site	7	3797.1	1.16	0.16	> 0.05
	Residuals	32	15002.2		0.65	
	Total	43	23064.1		1.00	
PHILR-Euclidean	Population	3	517.6	2.71	0.16	< 0.001
	Body condition	1	103.5	1.62	0.03	> 0.05
	Collection site	7	565.7	1.27	0.18	> 0.05
	Residuals	32	2039.1		0.63	
	Total	43	3225.7		1.00	

Table 2.2. Summary of PERMANOVA results analysing 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	< 0.05
	Body condition	1	1205	1.33	0.03	> 0.05
	Collection site	6	4781	0.88	0.12	> 0.05
	Residuals	33	29919		0.74	
	Total	43	40280		1.00	

Table 2.3. PERMANOVA results analysing 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	< 0.01
	Body condition	1	787.6	1.11	0.05	> 0.05
	Collection site	3	2456.1	1.16	0.16	> 0.05
	Residuals	16	11326.9		0.72	
	Total	21	15624.0		1.00	
PHILR-Euclidean	Population	1	271.6	1.55	0.07	< 0.05
	Body condition	1	171.0	0.98	0.05	> 0.05
	Collection site	3	491.7	0.94	0.13	> 0.05
	Residuals	16	2800.3		0.75	
	Total	21	3734.6		1.00	

Table 2.4. PERMANOVA results analysing 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	< 0.05
	Body condition	1	634.7	1.07	0.05	> 0.05
	Collection site	3	2236.9	1.26	0.17	> 0.05
	Residuals	16	9460.9		0.70	
	Total	21	13482.6		1.00	

SUPPLEMENTARY INFORMATION

Figure S2.1. Diversity index "observed species" of species rarefaction curve. Curves are labelled by sample number.

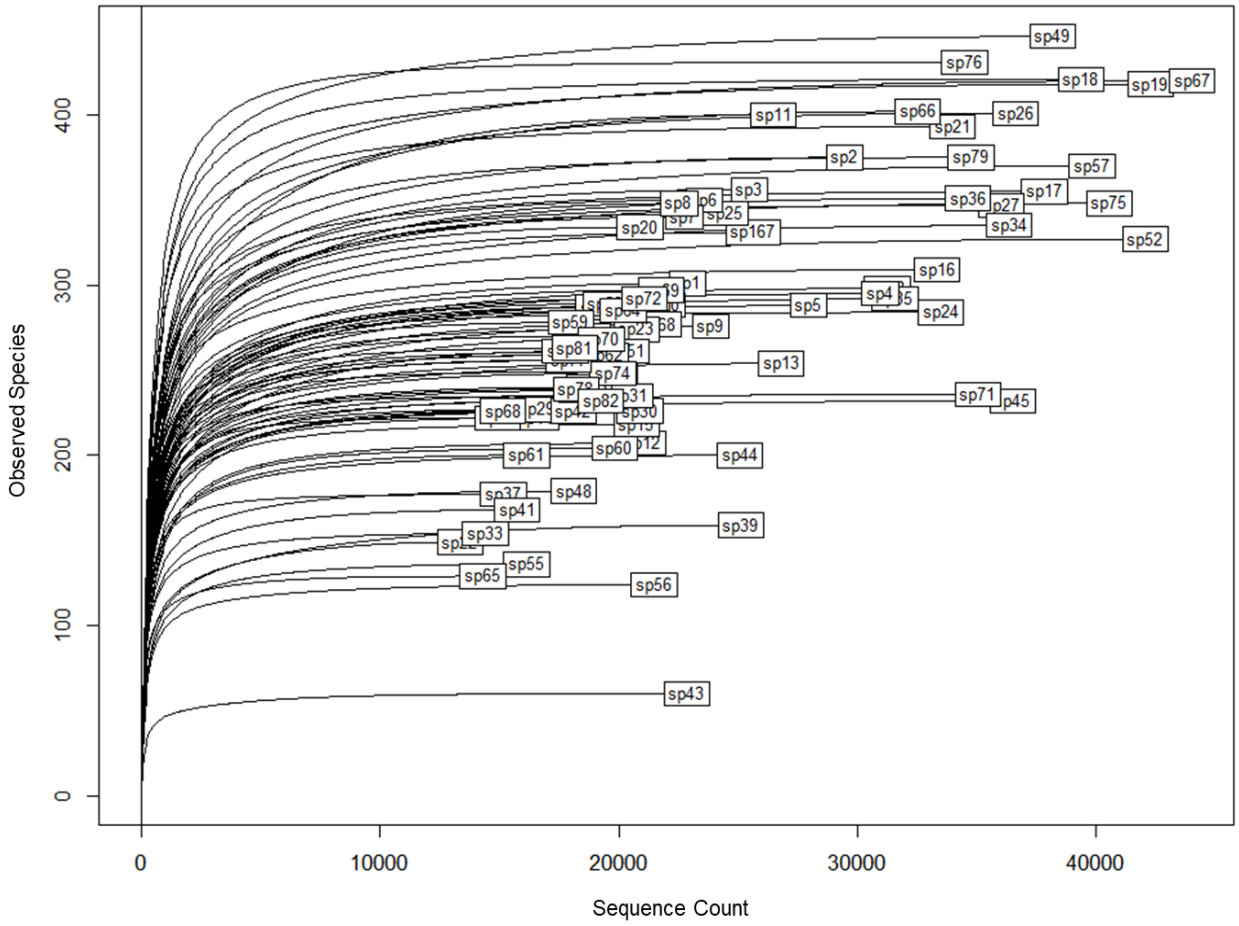

Figure S2.2. Gut bacterial alpha diversity of *Sclerophrys gutturalis* (guttural toad) native (triangles) and invasive (circles) populations. Native *S. gutturalis* were sampled in Durban, South Africa (blue) and invasive toads were sampled in Mauritius (green), Réunion (pink) and Cape Town, South Africa (red). Alpha diversity metrics (A) Shannon inverse, (B) Pielou evenness, (C) Chao1 and (D) were not significantly different between populations (GLMM, $p > 0.05$). The black line and whiskers in the box plots represent the medians and range of the lower quartile (25th percentile) and upper quartile (75th percentile).

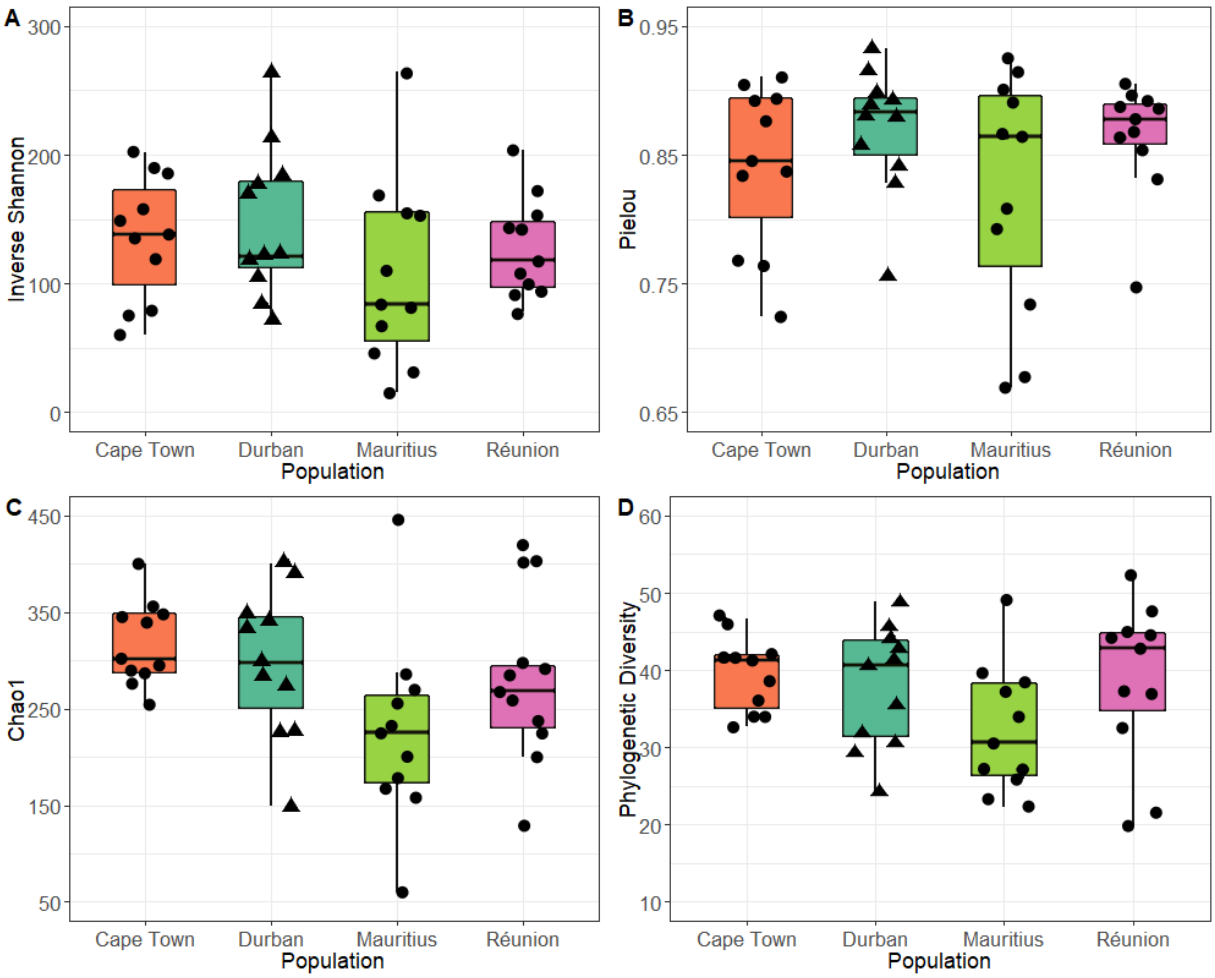

Figure S2.3. UPGMA cluster dendrogram of PHILR-distances between gut microbial communities of the different guttural toad (*Sclerophrys gutturalis*) invasive (Mauritius, Réunion and Cape Town) and native (Durban) populations.

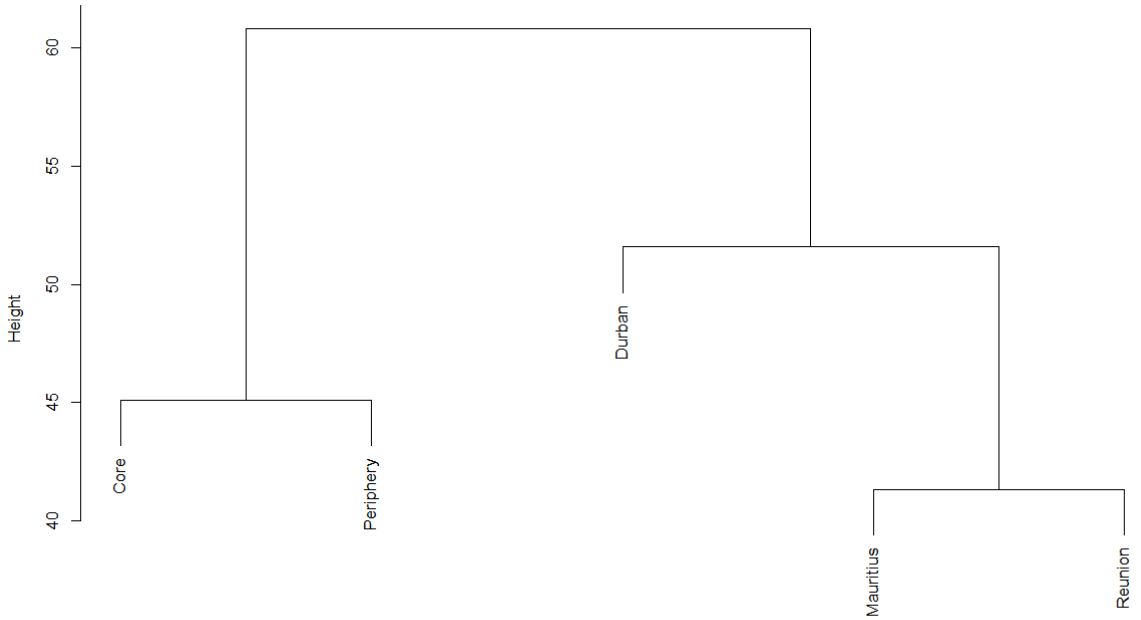

Figure S2.4. Gut bacterial alpha diversity comparisons between core (dark blue) and periphery (light blue) sites in the invasive *Sclerophrys gutturalis* (guttural toad) population, Cape Town, South Africa. Alpha diversity metrics (A) Shannon inverse, (B) Pielou evenness, (C) Chao1 and (D) were not significantly different between populations (GLMM, $p > 0.05$). The black line and whiskers in the box plots represent the medians and range of the lower quartile (25th percentile) and upper quartile (75th percentile).

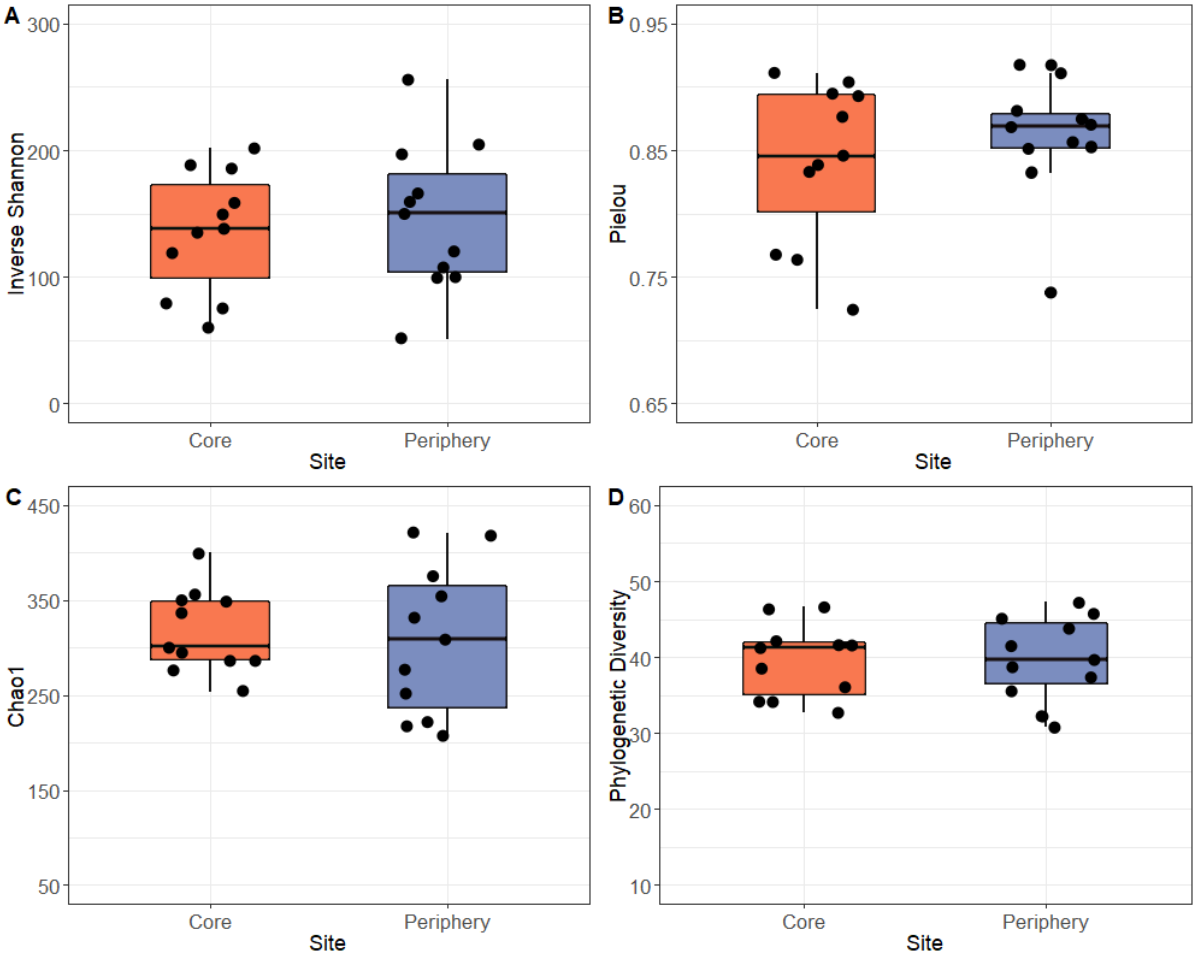

Table S2.1. Summary of best-fit mixed models analysing the differences between *Sclerophrys gutturalis* (guttural toad) population gut microbial alpha diversity estimates (inverse Shannon, Evenness, Chao1 and Faith's phylogenetic diversity metric). For each model, fixed and random explanatory variables, degrees of freedom (d.f.), *chi*-square, Akaike's information criterion (AIC), ΔAIC, marginal (R^2_m) and conditional (R^2_c) r-squared values and *p*-values are detailed.

	Explanatory variable								
Dependent variable	Fixed	Random	d.f.	*chi*-square	AIC	ΔAIC	R^2_m	R^2_c	p-value
Inverse Shannon	Population + body condition	Collection site	5	0.85	576.67	2.18	0.03	0.17	> 0.05
	Population	Collection site	4	0.61	575.68	1.20	0.02	0.19	> 0.05
	Body condition	Collection site	4	1.21	574.83	0.35	0.03	0.16	< 0.001
	Null	Collection site	3		574.48	0.00	0.00	0.20	
Evenness	Population + body condition	Collection site	5	2.82	612.69	1.93	0.07	0.18	> 0.05
	Population	Collection site	4	0.28	613.88	3.11	0.01	0.20	> 0.05
	Body condition	Collection site	4	3.09	610.76	0.00	0.07	0.18	< 0.001
	Null	Collection site	3		612.23	1.47	0.00	0.20	
Chao1	Population + body condition	Collection site	5	0.71	-130.25	2.46	0.03	0.11	> 0.05

	Population	Collection site	4	0.96	-131.93	0.79	0.02	0.11	> 0.05
	Body condition	Collection site	4	0.81	-131.72	1.00	0.02	0.10	> 0.05
	Null	Collection site	3		-132.71	0.00	0.00	0.10	
Phylogenetic diversity	Population + body condition	Collection site	5	4.24	167.64	1.34	0.08	0.18	> 0.05
	Population	Collection site	4	0.01	169.79	3.48	0.00	0.18	> 0.05
	Body condition	Collection site	4	3.13	166.30	0.00	0.07	0.18	< 0.001
	Null	Collection site	3		167.81	1.50	0.00	0.18	

Table S2.2. Summary of PERMANOVA pairwise comparisons of *Sclerophrys gutturalis* (guttural toad) gut microbial compositional and phylogenetic diversity as measured by CLR- and PHILR-Euclidean metrics, respectively. For each comparison, dependent variable, degrees of freedom (d.f.), sum of squares (SS), pseudo-*F*-statistic, r-squared values(R^2) and *p*-values are reported.

Pairwise-Comparison	Dependent variable	d.f.	SS	Pseudo-F	R^2	p-value
Cape Town and Durban	CLR-Euclidean	21	2667.5	2.57	0.11	< 0.001
	PHILR-Euclidean	21	313.3	2.87	0.13	< 0.001
Cape Town and Mauritius	CLR-Euclidean	21	2701.4	2.77	0.12	< 0.001
	PHILR-Euclidean	21	292.7	3.02	0.13	< 0.001
Cape Town and Réunion	CLR-Euclidean	21	2716.1	2.64	0.12	< 0.001
	PHILR-Euclidean	21	303.2	2.90	0.13	< 0.001
Durban and Mauritius	CLR-Euclidean	21	1662.4	1.71	0.08	< 0.01
	PHILR-Euclidean	21	180.0	1.75	0.08	> 0.05
Durban and Réunion	CLR-Euclidean	21	1541.6	1.51	0.07	< 0.01
	PHILR-Euclidean	21	145.0	1.21	0.06	> 0.05
Mauritius and Réunion	CLR-Euclidean	21	1259.1	1.31	0.06	< 0.05
	PHILR-Euclidean	21	193.4	1.83	0.08	> 0.05

Table S2.3. Differences in differential abundance of faecal bacterial ASVs across guttural toad (*Sclerophrys gutturalis*) invasive populations; Mauritius, Réunion and Cape Town, and native populations in Durban. See http://doi.org/10.5281/zenodo.4164856 for table.

Table S2.4. Pairwise comparisons of differential abundance of faecal bacterial ASVs between guttural toad (*Sclerophrys gutturalis*) invasive populations; Mauritius, Réunion and Cape Town, and native populations in Durban. See http://doi.org/10.5281/zenodo.4164856 for table.

Table S2.5. Summary of PERMANOVA pairwise comparisons of *Sclerophrys gutturalis* (guttural toad) gut microbial predicted functional diversity as measured by CLR-Euclidean metrics. For each comparison, dependent variable, degrees of freedom (d.f.), sum of squares (SS), pseudo-*F*-statistic, r-squared values(R^2) and *p*-values are reported.

Pairwise-Comparison	Dependent variable	d.f.	SS	Pseudo-F	R^2	*p*-value
Cape Town and Durban	CLR-Euclidean	21	1770.4	2.23	0.10	< 0.05
Cape Town and Mauritius	CLR-Euclidean	21	1529.9	1.36	0.06	> 0.05
Cape Town and Réunion	CLR-Euclidean	21	2604.9	3.49	0.15	< 0.001
Durban and Mauritius	CLR-Euclidean	21	1170.5	1.14	0.05	> 0.05
Durban and Réunion	CLR-Euclidean	21	798.9	1.19	0.06	> 0.05
Mauritius and Réunion	CLR-Euclidean	21	1272.5	1.30	0.06	> 0.05

Table S2.6. Differences in abundance of predicted faecal bacterial functional pathways across guttural toad (*Sclerophrys gutturalis*) invasive populations; Mauritius, Réunion and Cape Town, and native populations in Durban. See http://doi.org/10.5281/zenodo.4164856 for table.

Table S2.7. Pairwise comparisons of differential abundance of faecal bacterial predicted functional pathways between guttural toad (*Sclerophrys gutturalis*) invasive populations; Mauritius, Réunion and Cape Town, and native populations in Durban. See http://doi.org/10.5281/zenodo.4164856 for table.

Table S2.8. Summary of best-fit mixed models analysing the differences between *Sclerophrys gutturalis* (guttural toad) site (core and periphery) gut microbial alpha diversity estimates (inverse Shannon, Evenness, Chao1 and Faith's phylogenetic diversity metric). For each model, fixed and random explanatory variables, degrees of freedom (d.f.), *chi*-square, Akaike's information criterion (AIC), ΔAIC, marginal (R^2_m) and conditional (R^2_c) r-squared values and *p*-values are detailed.

	Explanatory variable								
Dependent variable	Fixed	Random	d.f.	*chi*-square	AIC	ΔAIC	R^2_m	R^2_c	*p*-value
Inverse Shannon	Site + body condition	Collection site	5	1.63	-3.11	2.86	0.06	0.24	> 0.05
	Site	Collection site	4	0.20	-4.08	1.90	0.01	0.19	> 0.05
	Body condition	Collection site	4	1.05	-4.86	1.12	0.04	0.22	< 0.001
	Null	Collection site	3		-5.97	0.00	0.00	0.17	
Evenness	Site + body condition	Collection site	5	0.97	-62.73	3.06	0.04	0.22	> 0.05
	Site	Collection site	4	0.18	-64.18	1.62	0.01	0.19	> 0.05
	Body condition	Collection site	4	0.59	-64.21	1.59	0.02	0.24	< 0.001
	Null	Collection site	3		-65.80	0.00	0.00	0.20	
Chao1	Site + body condition	Collection site	5	4.41	96.10	2.75	0.12	0.65	> 0.05

	Site	Collection site	4	1.47	95.75	2.41	0.07	0.58	> 0.05
	Body condition	Collection site	4	1.43	93.95	0.61	0.04	0.37	< 0.001
	Null	Collection site	3		93.34	0.00	0.00	0.34	
Phylogenetic diversity	Site + body condition	Collection site	5	0.59	71.08	4.27	0.03	0.21	> 0.05
	Site	Collection site	4	0.51	69.11	2.30	0.03	0.21	> 0.05
	Body condition	Collection site	4	0.01	68.79	1.98	0.00	0.12	< 0.001
	Null	Collection site	3		66.81	0.00	0.00	0.12	

Table S2.9. Differences in abundance of faecal bacterial ASVs in an expanding guttural toad (*Sclerophrys gutturalis*) population at its core and periphery. See http://doi.org/10.5281/zenodo.4164856 for table.

Table S2.10. Differences in abundance of predicted faecal bacterial functional pathways in an expanding guttural toad (*Sclerophrys gutturalis*) population at its core and periphery. See http://doi.org/10.5281/zenodo.4164856 for table.

Chapter 3: Gut microbial flexibility mediates ecological adaptation in an invasive amphibian.

ABSTRACT

Gut microbial communities regulate host physiology and health of humans and laboratory animals. The functional significance of these collective bacterial genomes (i.e. the microbiome) to the adaptive potential of wildlife hosts is still unknown. Studies demonstrating convincing examples of microbial flexibility to environmental change so far lack the experimental approaches to demonstrate the effect on host physiology. Invasive species provide natural experiments to tease apart these host-microbe relationships. However, no studies have investigated how microbial symbionts might mediate responses of invasive hosts' physiology to environmental change. In this study, I examine whether invasive gut microbiomes have significantly diverged in their ability to respond to novel environmental change (i.e. a dietary challenge) compared to native gut microbiomes by performing reciprocal faecal microbial transplant (FMT) experiments in native and invasive guttural toad (*Sclerophrys gutturalis*) populations. Subsequently, I determine how the microbiome regulates host physiological changes in response to a dietary challenge. I show that invasive gut microbiomes exhibit higher microbial compositional and predicted functional flexibility to novel dietary change, compared to native gut microbiomes. This increased microbial flexibility is coupled with significant flexibility in energy harvesting. Furthermore, my results indicate that overall invasive gut microbiomes significantly upregulate energy harvesting and physiological performance of hosts, compared to native microbiomes. This study is the first identifying gut microbiota as the sole factor contributing to the adaptive physiology of a vertebrate using a unique study design. These findings provide novel insights into the key role of gut microbial symbionts in increasing the invasive potential of its vertebrate host.

INTRODUCTION

The vertebrate digestive system is host to diverse and complex microbial communities that play a fundamental role in the development, physiology and wellbeing of their hosts (O'Hara & Shanahan, 2006; Robinson et al., 2010; Cho & Blaser, 2012; Sommer & Bäckhed, 2013; Kohl & Carey, 2016; McKenney et al., 2018). Of all the abiotic and biotic factors that affect the composition of symbiotic microbial communities (reviewed in Costello et al., 2012 and Dickey et al., 2020), diet has the largest known impact on individual gut microbiomes (Turnbaugh et al., 2009; Faith et al., 2011; David et al., 2014; Kohl et al., 2014b; Wilson et al., 2020). This is due to strong selection on gut microbial communities for their ability to degrade specific dietary molecules (Turnbaugh et al., 2009; David et al., 2014; Kohl et al., 2014b). Dietary changes

can, therefore, produce alterations in the host microbiota's ability to mediate host processes such as digestion and energy acquisition (Bäckhed et al., 2005; Turnbaugh et al., 2006; De Angelis et al., 2020), vitamin synthesis (Zmora et al., 2019), immunomodulation (Longman & Littman, 2015; Leigh & Morris, 2020; De Angelis et al., 2020), pathogen defence (Dethlefsen et al., 2007; Longman & Littman, 2015) and possibly host physiology (Alberdi et al., 2016; Fontaine & Kohl, 2020). Given the dynamic association between diet and the gut microbiome, inferring how microbiota respond to dietary changes and subsequently, shifts in hosts' physiology presents a meaningful challenge.

By using an ecological approach, the gut can be viewed as a distinct microbial habitat where gut microbial communities are affected by similar processes as those explained by island biogeography theory (i.e. immigration, colonisation, and extinction) and community ecology theory (i.e. deterministic, neutral, and historic processes of community assembly) (Walter & Ley, 2011; Costello et al., 2012; Delong, 2014; Bletz et al., 2016). Within this framework, entering a new environment by an alien host is likely to involve dietary changes, and thus, a host's gut microbial community can be predicted to adjust in response to these changing ecological conditions (i.e. microbial flexibility). However, microbial communities, like other ecological communities, can also display "resistance" to dietary (or environmental) change, i.e. communities remain essentially unchanged despite disturbance (Moya & Ferrer, 2016). Microbial resistance to diet alterations has been demonstrated in human and laboratory animal populations and is usually coupled with an inability to maintain microbial function, and host health and physiology (Smith et al., 2013; Jandhyala et al., 2015; Sonnenburg & Bäckhed, 2016). In contrast, a few studies concerning wildlife populations show that while animals display microbial flexibility in response to disturbance, this flexibility is coupled by functional maintenance or redundancy (Bletz et al., 2016; Voolstra & Ziegler, 2020; Webster et al., 2020; Fontaine & Kohl, 2020). Studying wildlife populations and their microbial symbionts in natural conditions are, therefore, of great importance in order to elucidate the natural responses of microbial communities and their hosts to environmental change.

If microbial flexibility in response to novel environmental change is coupled with microbial functional redundancy, then maintenance of host physiology, health and/or fitness would be expected. However, no studies have demonstrated conclusively that gut microbial responses to changing host environments lead to measurable effects on host health or physiology in fully natural systems (Hauffe & Barelli, 2019; Greyson-Gaito et al., 2020, but see Bletz et al., 2016; van Opstal & Bordenstein, 2019; Gomes et al., 2020; Greenspan et al., 2020). Research on humans and laboratory animals has shown that the gut microbiome acts as an important mediator of the relationship between dietary change and host physiology (Turnbaugh et al., 2006; Sonnenburg & Bäckhed, 2016). To infer direct impacts of the gut microbiome on host

physiology and health, laboratory studies implement faecal microbial transplants (FMT). However, laboratory studies are unable to account for natural environmental variability experienced by the host and its associated gut microbiome (Greyson-Gaito et al., 2020). Moreover, laboratory animals normally have significantly different microbiomes compared to their wild counterparts, which makes it difficult to tease apart the ecology and evolution of wild host-microbial associations (Dethlefsen et al., 2007). To understand the processes impacting natural microbial variation and how the microbiome mediates host physiological responses in changing environments, we need to move beyond laboratory systems and expand to studying microbiomes in wild populations.

Biological invasions provide a valuable opportunity as natural experiments to investigate evolutionary responses of populations and their microbial symbionts to changing environmental conditions. Dietary and intestinal flexibility of introduced populations have been shown to contribute greatly to their success in novel environments (Caut et al., 2008; Banks et al., 2008; Kidera et al., 2008; Ruffino et al., 2011; Redford et al., 2012). The guttural toad (*Sclerophrys gutturalis*) is an invasive amphibian introduced ~20 years ago into periurban Cape Town, from its known source population in Durban, South Africa (Telford et al., 2019). Previously, massive parallel sequencing of invasive guttural toad population gut microbiomes showed that the Cape Town toad population gut microbiome has diverged to become compositionally, phylogenetically and functionally distinct from its source population (see Chapter 2). Furthermore, toads within the Cape Town invasion exhibit increased endurance under dehydrated conditions (Vimercati et al., 2018), sustained resource investments to growth (Vimercati et al., 2019), and behavioural shifts to conserve water (Madelaire et al., 2020). Guttural toads in these areas provide us with a well-studied system to investigate whether gut microbial communities have diverged in their flexibility to respond to changing environmental conditions and how this change impacts predicted microbial functionality and host physiology.

I used this amphibian host system to test (1) the hypothesis that invasive microbiomes will exhibit a higher degree of microbial flexibility or plasticity following environmental change (in this case, a novel dietary challenge) and (2) if a higher degree of microbial flexibility (or lack thereof) will allow the maintenance of similar predicted microbial functionality and host physiology (in terms of resource intake and physiological performance) in response to environmental change. The reciprocal transfer of individuals and/or their microbes between different habitats is a classical technique used in evolution and invasion ecology to ascertain how and by what mechanisms individuals or their microbes adjust to environmental change. Therefore, I used this approach combined with faecal microbial transplants (FMTs) to reciprocally transfer invasive and native gut microbial communities to individual hosts in

Durban (native population) and Cape Town (invasive population) in order to answer these hypotheses.

MATERIALS AND METHODS

Study sites

Adult female guttural toads (*Sclerophrys gutturalis*) were collected in their native range (Durban, 29° 83′ S, 30° 93′ E; 29 May to 10 June 2019), and invasive range (Cape Town, 33° 99′ S, 18° 44′ E; 2 to 2 to 30 March 2019) to complete experiments in the native and invasive areas, respectively. I captured animals of a single sex as intersex microbiome differences may confound results. A total of 48 individuals were collected for experimental trials in each area. A further 34 individuals were collected in each area to serve as faecal material donors. Collections in Cape Town occurred as part of an ongoing eradication programme (Davies et al., 2020) and in Durban under permission from KwaZulu Natal (KZN) wildlife (OP 4353/2018). Ethical clearance for research was obtained from Stellenbosch University Animal Ethics Committee (Protocol Number ACU-2019-9533).

Collection and preparation of donor faecal material

Within each sampling area, adult female toads were captured by hand after sunset (19:00 h). Immediately after capture, toads were weighed (to the nearest 0.01 g; WTB 2000, Radwag, Radom, Poland), and their snout-to-vent length (SVL) was measured using a digital calliper (to the nearest 0.01 mm, Mitutoyo). Toad sex was confirmed through visual inspection for white colouration of the gular region and a greater than 40 mm SVL measurement (Baxter-Gilbert et al., 2020). Female toads were then placed individually in plastic containers (195 x 195 x 180 mm), which had been sterilized with a 10% bleach solution followed by 70% ethanol. Fresh faecal matter was processed and stored within 8 hours of defecation. After 8 hours of capture, native toads were released, whereas invasive toads were euthanized by immersion in a 1 gl^{-1} solution of tricaine ethane sulfonate (MS-222) for 20 minutes.

At least 0.3 g faecal material from each donor was suspended within a 1.0 mL sterile saline solution (0.9% NaCl). Glycerol (90.08%) was then added to obtain a final concentration of 10% (Satokari et al., 2015). Glycerol is used as a cryoprotective agent for frozen faecal samples (Gough et al., 2011; Hamilton et al., 2012) and ensures microbial viability for up to 6 months in -80 °C (Costello et al., 2015). The final suspension was clearly labelled and stored at -80 °C.

Recipient Toad Housing

Recipient toads were housed in mesocosm enclosures created from plastic pools (310 L, 1.98 m in diameter and 0.38 m deep. Two enclosures were placed within each sampling area, Durban and Cape Town (Figure 3.1). Use of outdoor mesocosms increases environmental relevance and maximizes the benefits of field experiments by maintaining relatively controlled environments while incorporating natural elements, such as daily variation in temperature and rainfall (Rowe & Dunson, 1994). Screen mesh was placed on top of mesocosms to prevent toads from escaping and predators from entering the enclosures. Blacklights were suspended 30 cm aboveground inside each enclosure and illuminated each night to attract insects. Toads were, therefore, sustained on a 'natural diet'. Every second day fresh soil and leaf litter was placed inside enclosures.

Before placement in enclosures, toads' snout-to-vent length and mass were measured. Individuals were tagged using 8 mm PIT tags, which are small glass capsules with an electromagnetic coil (Guimaraes et al., 2014). The tag was placed in a 15-gauge hypodermic needle and injected underneath the skin above the dorsal lymph sac (following Donnelly et al., 1994). Afterwards, toads were randomly assigned to each of the two enclosures in each area. Toads were then allowed seven days to acclimate to enclosure conditions (Figure 3.1). Throughout the acclimation and experimental periods, toads' body mass was measured daily.

Faecal Microbial Transplants

After the acclimation period, toads were administered one of three faecal microbial transplant (FMT) treatments: control, invasive and native, for seven days (Figure 3.1). Toads on the control treatments received a 10% glycerol solution dissolved in 1.0 mL sterile saline (0.9% NaCl). Toads under the invasive treatment received approximately 0.3 g invasive toad faecal samples (of Cape Town origin) suspended in a 1.1 mL 10% glycerol and saline solution. Toads under the native treatment received approximately 0.3 g native toad faecal samples (of Durban origin) suspended in a 1.1 mL 10% glycerol and saline solution.

Each day, faecal suspensions were thawed to room temperature. Sterile saline solution (0.9% NaCl) was added to each aliquot to obtain the desired suspension volume. Solutions were fed to toads via a neonatal feeder. These FMT treatments were repeated in each sampling area, Durban (native area) and Cape Town (invasive area), creating six FMT treatment groups across the sampling areas: Durban control, Durban self-transplant (i.e. Durban native toads fed native faecal material), Durban transplant (i.e. Durban native toads fed invasive faecal material), Cape Town control, Cape Town self-transplant (i.e. Cape Town invasive toads fed

invasive faecal material), Cape Town transplant (i.e. Cape Town invasive toads fed native faecal material) (Figure 3.1).

Diet Challenge

After the initial seven days of FMT supplementation to natural diets in mesocosm, toads in each FMT treatment group was exposed to one of two diets along with their normal supplementation of gut samples (Figure 3.1). Toads were either continued on a natural diet or were exposed to a novel diet challenge. Toads exposed to the dietary challenge were fed four large house crickets (*Acheta domesticus* (Linnaeus)) every second day. This presents a novel diet challenge to toads as they have never encountered laboratory crickets in the wild and the diet is monotonous compared to the native diet. The dietary changes were continued for seven days.

Faecal Sample Collection

After completion of experimental novel dietary challenge, faecal samples were collected from all individuals. As before, toads were placed individually in sterilized plastic holding containers (195 x 195 x 180 mm) and at least 0.3 g faecal matter was obtained from each individual. Samples were immersed in 1.0 *RNAlater*™ within 2 ml sterile polypropylene tubes (Ambion, Austin, TX). After approximately 48 hours, faecal samples were centrifuged (2 min at 10 000 x g), the supernatant was removed, and the pellet stored at -80 °C. Empty tubes containing *RNAlater*™ and glycerol solutions were kept as negative controls for DNA processing.

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. DNA extracts were stored at -80 °C until further processing. No template and template from blank filters were included as negative controls throughout the entire process from DNA extraction to PCR amplification.

DNA samples were quantified using the Qubit 4.0 Fluorometer (ThermoFisher Scientific) and the Qubit 1x 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 (GQS) were determined on the LabChip GXII Touch using the DNA Extended Range LabChip and Genimic DNA Reagent Kit (PerkinElmer, Waltham, MA, USA), according to the manufacturer's protocol.

PCR amplification

The V3 and V4 hypervariable regions of the rRNA were targeted during sequencing. Target 16S rRNA sequences were amplified using the universal bacterial primer set, 314F 5' – CCTACGGGNGGCWGCAG – 3' and 785R 5' – GACTACHVGGGTATCTAATCC – 3' (Klindworth et al., 2013). 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 x Phusion HF buffer) with a final concentration of 1.5 mM $MgCl_2$. Polymerase chain reactions (PCRs) were performed on the SimpliAmp™ Thermal Cycler (ThermoFisher Scientific). Initial template DNA denaturation at 98 °C for 30 sec was followed by 25 cycles consisting of 98 °C for 10 sec, 58 °C for 30 sec and 72 °C for 30 sec; with a final product extension at 72 °C for 10 min.

Presence of amplified products were verified on the PerkinElmer LabChip® GXII Touch (PerkinElmer, Waltham, MA, USA), using the X-mark chip and HT DNA NGS 3K reagent kit, according to the manufacturer's protocol. PCR products were then purified with 1.8x 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 1x 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. Approximately 40 µl from each purified PCR product 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 1.8x volume Agencourt™ AMPure™ XP reagent (Beckman Coulter). About 19 µl purified, end-repaired product 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 1.8x 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 (11 865 157) were imported into QIIME2 (version 2020.2) for pre-processing (Bolyen et al., 2019). The divisive amplicon denoising algorithm (DADA2) plugin was used to de-noise sequencing reads (Callahan et al., 2016). 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 using default DADA2 parameters. The resulting sequences were then used to generate amplicon sequence variants (ASVs) for downstream analyses. This resulted in 6 973 959 sequences ranging from 53296 to 154 822 sequences per sample representing a total of 13 986 unique ASVs. ASV sequences were aligned with mafft (Katoh & Standley, 2013; q2-alignment plugin), high entropy positions were filtered from the resulting alignment (Lane, 1991), an unrooted tree was constructed with FastTree 2 (Price, Dehal & Arkin, 2010; q2-phylogeny plugin) 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) (Quast et al., 2013; q2-feature-classifier plugin). The ASV table, phylogenetic tree and assigned taxonomy table was used in all downstream analyses.

The ASV table and its corresponding phylogenetic tree was additionally used to predict functional profiles of samples through the PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2, NSTI cut-off = 2) pipeline in QIIME2 (Douglas et al., 2020; q2-picrust2 plugin) 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). Removal of contaminant sequences were, therefore, not required.

Performance

The effect of diet and FMT treatment on performance was tested in 44 toads from Cape Town (no Durban toads were used). Toads were tested on an indoor circular racetrack (4.1 m) using a rubber grip mat as a substrate (Vimercati et al., 2018). Performance trials were completed during the day between 09h00 – 18h00. Each toad was individually placed on the racetrack and stimulated to hop by gently tapping it on the urostyle with a brush. To standardize tapping

time, toads were tapped by a single operator at intervals of 1 s after each hop. For each toad, I counted the number of laps and therefore the distance (4.1 m per lap) moved until it did not voluntarily hop for 10 consecutive taps (i.e. exhaustion). For each lap around the racetrack I also recorded the time taken until exhaustion.

Dissections

After faecal sample and performance data collection, toads from Cape Town (n = 48) and Durban (n = 48) were euthanized by immersion in a 1 gL^{-1} solution of tricaine ethane sulfonate (MS-222) for 20 minutes. The carcasses were frozen (-20° C) in labelled plastic bags until dissection. In the laboratory, after defrosting each specimen at ambient temperature, individuals were weighed (± 0.01 g) and their SVL was measured using digital callipers (± 0.01 mm). Fat bodies and liver were weighed after removing each organ (± 0.01 g). Tissues were patted dry with a paper towel before weighing. The percentage of body mass composed of fat reserves (hereafter, body fat %) was obtained from the ratio between the mass of fat bodies and body mass (Brown et al., 2011). Lastly, individuals were fully eviscerated and weighed to obtain lean structural mass (± 0.01 g).

Statistical analysis

Preliminary analyses showed that body mass was positively correlated with SVL. Therefore, the body condition (or scaled body mass) was calculated following Peig and Green (2009) and Vimercati et al. (2019). Body condition of toads was used as a covariate in all downstream microbiome analyses. Preliminary analyses indicated that faecal microbial transplants were successful as there were no significant differences of microbial composition between control and self-transplant groups within each experimental area (Table S3.1).

All statistical analyses were performed in R version 3.6.2 (R Core Team, 2019). Metadata, ASV table, taxonomy and phylogenetic tree was imported using the qiime2R package (v0.99.13, Bisanz, 2018). A phyloseq object was built from these datasets using the phyloseq package (v1.30.0, McMurdie & Holmes, 2013). Prior to all downstream analyses alpha rarefaction curves were inspected to assess sequencing depth (Figure S3.1). Visual inspection confirmed that sequencing depth was adequate for each sample with regards to number of ASVs detected. ASV counts of each sample were then filtered, removing ASVs present in less than 5% of the samples, and according to the read depth of each sample using the phyloseq and microbiomeutilities packages (v0.99.02, Shetty et al., 2018).

Diversity metrics inverse Shannon diversity, Evenness, Chao1 species richness and Faith's phylogenetic diversity metrics was calculated using the vegan package in R (v2.5.6, Oksanen

et al., 2007). The inverse Shannon diversity metric incorporates both measures of species richness and abundance. Evenness estimates how similar in abundance species in a sample are, while Chao1 estimates the asymptote on a species accumulation curve to determine species richness. Faiths' phylogenetic diversity metric measures the cumulative branch lengths from randomly sampled species from each sample. Generalized linear models (GLM) were used to determine the effect of FMT treatment, dietary change, their interaction and body condition (dependent variables) on alpha diversity metrics (response variables). Prior to analyses, model assumptions (e.g. normality, homogeneity, and independence) were assessed. None of the diversity estimates, except phylogenetic diversity within the Durban area group, met model assumptions of normality. To meet model assumptions, inverse Shannon and phylogenetic diversity metrics were log-transformed and Chao1 and Pielou diversity estimates were 1/x transformed for the Cape Town group. For the Durban group, inverse Shannon and Chao1 estimates were square-root transformed and Pielou diversity estimates were 1/x transformed. Relative variable importance of competing models was evaluated using Akaike information criterion (AIC). Chi-square values and associated p-values were investigated to examine the effect of the response variables on the dependent variables.

The interaction effect of FMT treatment and dietary change on microbiome CLR- and PHILR-composition matrices was examined using PERMANOVA analyses. CLR- and PHILR-metrics are equivalent to the Bray-Curtis and Unifrac beta diversity metrics, but account for the compositional nature of the data (Gloor et al., 2017). Feature tables containing read counts were first subjected to centre log-ratio (CLR)- and PHILR-transformation using phyloseq and philr packages (v1.12.0, Silverman et al., 2017). Euclidean distance matrices were constructed from the transformed ASV count tables through the adonis function (vegan package). Distance matrices were then subjected to PERMANOVA analyses (999 permutations) to evaluate the effect of FMT treatment, diet change, their interaction and body condition on toad gut microbial composition. As PERMANOVA is sensitive to differences in dispersion of data within groups (assumes a homogenous within-group dispersion), I inspected this assumption with the betadisper and permutest functions of vegan. CLR- and PHILR-transformed Euclidean distance matrices were also used in principle component analyses (PCoA) to visualize the responses of population gut microbial communities to novel dietary changes.

To investigate differential abundance of ASVs, likelihood ratio tests (LTR) were employed through the DeSeq2 package (v1.26.0, Love et al., 2014). This test was implemented using a full model with body condition and the interaction effect of FMT treatment and diet change 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 false discovery rate (FDR) was employed with a cut-off 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 between FMT treatments and diet changes, pairwise comparisons were also performed using DeSeq2.

Functional components of bacterial communities were assessed. Prior to all analyses pathway abundances derived from the PICRUSt2 pipeline were filtered for pathways with > 5 counts. Data was then subjected to compositional (beta diversity) and differential abundance analyses similar to those described above.

Body mass, lean structural mass, and liver mass were positively correlated with SVL and thus, I calculated the scaled mass index for these variables following Pieg and Green (2009) and Vimercati et al. (2019). To determine the effect of FMT treatment, diet change, and their interaction on the scaled body mass, lean structural mass, body fat % and scaled liver mass (response variables) of toads in each area (Cape Town and Durban), generalized linear models (GLM) were employed as described above**.** Prior to analyses, model assumptions (e.g. normality, homogeneity and independence) were assessed. Body mass and lean structural mass was log-transformed and scaled liver mass and body fat % was square-root transformed in order to meet model assumptions of normality.

Finally, mixed effects models (GLMM) were used to determine the effect of FMT treatment, diet change, and their interaction on performance measures; endurance (m) and speed ($m.s^{-1}$). Snout-to-vent length (SVL) of toads was included as a covariate in analyses and trial number as random factor. Both performance measures were square-root transformed in order to meet model assumptions of normality. 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) R2 was calculated according to Nakagawa and Schielzeth (2013) using the 'r.squaredGLMM' function in the package MuMIn (v1.43.15, Barton, 2009). F-statistics and associated p-values were investigated to examine the effect of fixed effects on the dependent variables.

RESULTS

Invasive gut microbiomes display microbial flexibility in response to a dietary challenge

The alpha diversity of gut bacterial communities does not differ across toad microbiomes or in response to dietary change (Figure S3.2 and Figure S3.3; Table S3.2). However, different toad gut microbiomes display varying responses (i.e. microbial flexibility) of beta diversity to a novel dietary challenge (significant interaction effect; Cape Town: PERMANOVA, Pseudo-$F_{(1, 41)}$ = 1.33, p < 0.001 and Durban: PERMANOVA, Pseudo-$F_{(1, 41)}$ = 1.27, p < 0.05, Table 3.1,

Figure 3.2A; Figure 3.2B). Toads with invasive gut microbiomes (i.e. Cape Town controls, Cape Town self-transplant, and Durban transplant) significantly shift their gut microbial composition in response to a dietary challenge (PERMANOVA, $p < 0.01$ for all comparisons, Table S3.1). Toads with native gut microbiomes (i.e. Durban controls, Durban self-transplant, and Cape Town transplant), on the other hand, display microbial resistance towards a dietary challenge, with no significant differences observed between toads with native microbiomes on natural or novel diets (PERMANOVA, $p > 0.05$ for all comparisons, Table S3.1). Responses of invasive toads' microbiomes to a dietary challenge is not the result of dispersion variation between microbiomes (BETADISPR, $p > 0.05$, Figure 3.2C; Figure 3.2D). Body condition also has no effect on the gut microbial shifts observed in guttural toad hosts (Table 3.1). Similar patterns are observed for phylogenetic diversity (Table 3.1, Table S3.1, Figure 3.3).

Of the 732 (Cape Town) and 539 (Durban) differentially abundant ASVs, more ASVs' present in the invasive gut microbiome (i.e. Cape Town controls, Cape Town self-transplant and Durban transplant) become differentially abundant in response to a dietary challenge, compared to those present in the native gut microbiome (i.e. Durban controls, Durban self-transplant, and Cape Town transplant) (Table S3.3). The invasive gut microbiome significantly alters microbial abundance of 395 ASVs (Cape Town self-transplant) and 302 ASVs (Cape Town control) in response to a dietary challenge in Cape Town (invasive area) (Table S3.4). Only 185 ASVs (Cape Town transplant) of the native gut microbiome shift in response to a dietary challenge (Table S3.4). In Durban (native area), the invasive gut microbiome significantly alters microbial abundance of 174 ASVs (Durban transplant) while 162 ASVs (Durban self-transplant) and 113 ASVs (Durban control) of native gut microbiomes shifts in response to a dietary challenge (Table S3.4).

Microbial flexibility in invasive toads is coupled with predicted functional flexibility

Guttural toads' predicted microbial functional diversity is determined by both the microbiome (FMT treatments) and diet (Table 3.2, Figure 3.3C; Figure 3.3D). However, significant interaction effects are only present in hosts from Cape Town (Cape Town: PERMANOVA, Pseudo-$F_{(1, 41)} = 1.57$, $p < 0.05$ and Durban: PERMANOVA, Pseudo-$F_{(1, 41)} = 1.01$, $p > 0.05$; Table 3.2). Invasive gut microbiomes (i.e. Cape Town controls, Cape Town self-transplant and Durban transplant) significantly shift their predicted gut microbial functional capabilities in response to a dietary challenge (PERMANOVA, $p < 0.01$ for all comparisons, Table S3.5). Toads with native gut microbiomes (i.e. Durban controls, Durban self-transplant and Cape Town transplant), on the other hand, display no functional variation in response to a dietary challenge (PERMANOVA, $p > 0.05$ for all comparisons, Table S3.5). Changes of predicted functional capabilities is not the result of dispersion variation (BETADISPR, $p > 0.05$, Figure

3.3G; Figure 3.3H). Body condition also has no effect on the microbial functional differences (Table 3.2).

In Cape Town and Durban, 53 and 158 functional pathways, respectively, are differentially abundant across FMT treatments and diets (Table S3.6). The invasive gut microbiome significantly alters the abundance of 36 (Cape Town self-transplant) and 21 (Cape Town control) functional pathways in response to a dietary challenge in Cape Town (Table S3.7). Only 11 (Cape Town transplant) functional pathways of the native gut microbiome shift in response to dietary challenge. In Durban, the invasive gut microbiome significantly alters the abundance of 48 (Durban transplant) functional pathways while 35 (Durban self-transplant) and 46 (Durban control) functional pathways of the native gut microbiomes shift abundance in response to novel dietary challenge (Table S3.7).

Invasive gut microbiomes stimulate increased resource intake

Scaled body mass and lean structural mass does not vary across toad FMT treatments or diets (Table 3.3; Figure S3.4). Body fat % and scaled liver mass, on the other hand, does vary in response to a novel dietary challenge depending on the FMT treatment (or gut microbiome) of toads (significant interactions effects; body fat %: GLM, $p < 0.05$ in both experimental areas; and scaled liver mass: GLM, $p < 0.05$ in both experimental areas; Table 3.3; Figure 3.4). Toads with invasive gut microbiomes (i.e. Cape Town controls, Cape Town self-transplant and Durban transplant) have significantly higher body fat % and scaled liver mass when subjected to a dietary challenge, while toads with native gut microbiomes (i.e. Durban controls, Durban self-transplant and Cape Town transplant) show no response (Table S3.8). Additionally, toads with invasive gut microbiomes have a significantly higher body fat % and scaled liver mass than toads with native gut microbiomes, irrespective of diet (Table S3.9). Pairwise comparisons also show that there were no differences of body fat % and scaled liver mass between toads in their respective control and self-transplant groups (Table S3.8).

The gut microbiome alters physiological performance irrespective of dietary change

The gut microbiome impacts the distance travelled and speed of guttural toads, i.e. only FMT treatment has a significant effect on the performance of guttural toads (Figure 3.5; Table 3.4). Guttural toads with invasive gut microbiomes (i.e. Cape Town controls and Cape Town self-transplant) attain significantly longer distances and higher performance speeds than those with native gut microbiomes (i.e. Cape Town transplant) (Table S3.10). There are no differences of performance between Cape Town control and self-transplant groups (Table S3.10).

DISCUSSION

How complex host-associated microbial communities respond to environmental change is of great interest in the fields of ecology and evolution. In this study, I demonstrate the varying adaptive responses of invasive and native gut microbial communities to novel diets. I show that the invasive *Sclerophrys gutturalis* gut microbiomes exhibit a higher degree of microbial flexibility enabling them to rapidly respond to novel dietary change compared to their native microbiomes. While there are similar studies that provide compelling evidence for microbial flexibility in wildlife populations (reviewed in Hauffe & Barelli, 2019), these case studies are relatively few and lack experimental approaches required to demonstrate that microbial flexibility impacts organismal fitness. My results indicate that increased microbial flexibility of invasive gut microbiomes facilitates an increased flexibility of microbial predicted functional capabilities and resource investments or energy harvesting in toad hosts. Furthermore, I demonstrate that the invasive gut microbiome facilitates increases in resource investment (i.e. organ mass) and physiological performance of hosts. Overall, my experiment is the first to show that the gut microbiome is a major contributing factor to the adaptive physiology of a wild vertebrate host in its natural environment.

Diet is a common driver of intra- and interspecific gut microbiome variation in many taxa (humans: Turnbaugh et al., 2009; de Filippo et al., 2010; mammals: Muegge et al., 2012; Nelson et al., 2013; birds: Waite & Taylor, 2015; reptiles: Kohl et al., 2014a; amphibians: Vences et al., 2016; fish: Sullam et al., 2012; insects: Jehrke et al., 2018). Variation of microbial flexibility in response to diet has also been recorded in some studies (reviewed in Hauffe & Barelli, 2019). However, most of these studies are unable to fully tease apart separate differential responses to diet from host genetics (Bolnick et al., 2014). In this study, host species as a confounding factor can be excluded since toads studied here represent populations of the same species only recently separated (< 20 years; de Villiers, 2006; Telford et al., 2019) for only approximately 6 generations (Vimercati, 2017; Vimercati et al., 2017). Therefore, this data shows that the gut microbiome can diverge its degree of microbial flexibility in response to novel conditions within a species. High microbial flexibility in invasive Cape Town toads can facilitate the rapid adjustment of microbial communities to environmental change when invasive toads spread or colonize new habitats (see Chapter 2). However, given that the other invasive populations of the same species show limited divergence of gut microbial communities from their native counterparts, compared to the invasive population investigated in this study (see Chapter 2), a high degree of microbial flexibility might, therefore, not be present in all invasive populations. It is possible that the introduction of tadpoles, rather than adults, has resulted in distinct microbial communities in the Cape Town invasive toad population with a unique ability to facilitate colonisation of

beneficial microbial symbionts when exposed to environmental change (see Chapter 2). However, many other mechanisms, such as evolution in response to new environments, could potentially produce similar outcomes.

In many cases, hosts are dependent on their symbiotic gut microbiota to degrade complex substrates useable by the host (Bäckhed et al., 2005; Turnbaugh et al., 2006). Previous studies have found that individuals displaying divergent taxonomic responses to change are able to maintain predicted functional features (i.e. functional redundancy) (Lozupone et al., 2008; Sanders et al., 2015; Bletz et al., 2016). Contrastingly, this study found taxonomic microbial flexibility is coupled with predicted functional flexibility. Differential predicted functional features, such as increased lipid and carbohydrate metabolic pathways, in toad hosts with invasive gut microbiomes, indicate that functional flexibility allows invasive gut microbiomes to upregulate resource absorption, biosynthesis and degradation. Although I only estimated predicted functional pathways, I observed that the predicted functional flexibility was coupled with flexibility of energy investments seen in toads with invasive gut microbiomes (i.e. increased body fat % and liver mass when placed on a novel diet). Similar findings in laboratory studies with germ-free mice have demonstrated that gut microbial change is coupled with functional microbial change, but also changes in resource investments (Turnbaugh et al., 2006; Lozupone et al., 2012; Morgan et al., 2012; Heintz-Buschart & Wilmes, 2018). Microbial flexibility in wild populations can, therefore, act as a source of adaptive potential (in terms of increasing energy investment) in response to novel environmental change, as demonstrated in this study. However, future studies investigating functional genomic pathways will be valuable in order to demonstrate whether this flexibility of microbial taxa and host energy resources is coupled with the true functional potential of the gut microbiome.

Increasingly, symbiotic microbes are recognised as having a fundamental role in host phenomic plasticity (i.e. the ability of a single genotype to adjust its expression in order to display varying phenotypes), and may be influencing host adaptation to environmental change (Chevalier et al., 2015; Alberdi et al., 2016; Voolstra & Ziegler, 2020). Vertebrates' adaptation to novel environments can be impacted by not only the interaction of hosts' genotype with its environment but also the interaction of the hosts' hologenome (i.e. the collection of the host and its symbiotic microbes genomes) with the environment (Alberdi et al., 2016; Shapira, 2016). A host's gut microbial composition and/or functional gene expression can, therefore, improve the capacity of hosts to acclimate and adapt physiologically to environmental change (Alberdi et al., 2016). Within this framework, the present study's results on host physiology (resource investment and physiological performance) provides evidence that gut microbial changes are coupled with a hosts' ability to adapt physiologically to novel environments. A

previous study has shown that invasive guttural toads outperform native individuals, possibly indicating physiological adaptation to its novel introduced environment (Vimercati et al., 2019). The results of the present study provide evidence that the gut microbiome mediates this ecological adaptation in an invasive amphibian, potentially explaining why the changes appeared so quickly (< 20 years) in this population. On the other hand, studies on plant invasions highlight enemy release (i.e., release from native pathogens in the introduced range) as a possible explanation for improved host physiology in invasive regions (Chun et al., 2010). Absence of native gut bacterial pathogens in invasive gut microbiomes could, therefore, also lead to the increased physiological performance observed in the guttural toads' invasive region. Investigations into existing laboratory models, such as rats (*Rattus norvegicus*), the house mouse (*Mus musculus*), African clawed frog (*Xenopus laevis*) and three-spined sticklebacks (*Gasterosteus aculeatus*), could be combined with studies of their invasive populations to provide more insights into the link between host physiological adaptation and the gut microbiome.

Although this study demonstrates that gut microbiome mediates resource uptake and physiological performance, I found no effects on of body mass and lean structural mass. Variation of lean structural mass and body mass between the invasive and native guttural toad populations has been reported in previous studies (Vimercati et al., 2018; Vimercati et al., 2019). It is possible that the study period (only three weeks of FMTs) was too short to induce marked changes in these organs. Long-term FMT experiments are recommended to determine whether gut microbial communities can alter other physiological attributes of the host and if FMTs can bring about long-term benefits in host physiology.

Symbiotic interactions with bacteria enhance invasions of many alien plant (Richardson & Pyšek, 2012; Traveset & Richardson, 2014) and insect species (Lu et al., 2016). However, the contributions of symbiotic bacterial communities to the success of vertebrate invasions has received little attention (see Chapter 2). Most studies investigating host-microbial relationships in vertebrate invasions centre around the impact of pathogen loss (i.e. enemy release hypothesis) on invasive populations (Colautti et al., 2004; Phillips et al., 2010; Prior et al., 2015). It is evident, from my results, that host-associated bacterial communities can have large impacts on host adjustments or adaptation to new environments and ultimately, host fitness. Novel interactions between newly acquired symbionts and their hosts can lead to enhanced performance of invasive species and facilitate establishment in non-native areas (i.e. enhanced mutualism hypothesis; Sun & He, 2010; Coats & Rumpho, 2014). However, I show that the likely acquirement of new symbionts in the hosts' invasive region has led to a unique invasive microbiome enhancing their hosts' ability to respond to novel conditions functionally and physiologically. Invasive populations are generally known to experience a lag phase

between colonisation and expansion, during which time they are thought to evolve adaptations that determine invasion success (Keller & Taylor, 2008). If it takes some time to acquire novel relationships with native microbiota, invasive populations might, therefore, experience a 'microbial lag phase'. However, it is still possible that release from native pathogenic gut bacteria could have led to the observed physiological differentiation in the invasive population, as have been observed in plant invasions (Chun et al., 2010). Whether perceived host physiological variation is the result of either mutualistic, commensalistic or parasitic relationships between bacterial communities and their invasive hosts is an important point to consider when conducting future studies investigating the role of symbiotic bacteria on host responses to environmental change. Nevertheless, I highlight the imperative to identify not only the invasion potential of an introduced vertebrate population but also their microbial symbionts, as is already recognized in plants.

Both animals and plants harbour microbes that affect their physiology and subsequently fitness. This study is the first to demonstrate that microbial symbionts are important mediators of a vertebrate organisms' physiological responses to environmental change with rigorous reciprocal transplant experiments. The importance of microbial communities in facilitating plant and insect invasions has been researched extensively (Traveset & Richardson, 2014; Lu et al., 2016). However, this study is the first to show that the vertebrate microbiome can impact an invasive hosts' physiology and ultimately increase its invasion potential. In some of the most destructive invasions, the invader is not a single species but a mutualistic complex, and its invasion ecology cannot be understood without considering the interactions between the hosts and its microbial symbionts, for example *Chromolaena odorata* and *Fusarium* species spores (Mangla et al., 2008), common reed *Phragmites australis* (Nelson & Karp, 2013) and Chinese tallow *Triadica sebifera* (Yang et al., 2013). Considering the pronounced impact of gut microbial communities on host physiology and fitness demonstrated in this study and other laboratory studies, it is surprising that less than 20 studies currently consider the impact of gut microbial communities on invasive host health and physiology. My findings emphasize the importance and unique opportunity invasive systems provide us to explore host-microbiome evolution.

FIGURES AND TABLES

Figure 3.1. Experiments were completed on native and invasive guttural toads (Sclerophrys gutturalis) in their native, Durban (blue), and invasive, Cape Town (pink), respectively. Toads were captured in the respective regions and allowed to acclimate to mesocosms for seven days while sustained on a natural diet (blue or pink ants). After seven days of acclimation to mesocosms, guttural toads were colonized with the gut microbiome of native toads (blue vials, of Durban origin) and invasive toads (pink vials, of Cape Town origin). Additionally, a final group of toads acted as a control (grey vials). This created six FMT (faecal microbial transplant) treatment groups across experimental areas: Cape Town control, Cape Town self-transplant, Cape Town transplant, Durban control, Durban self-transplant and Durban transplant. After seven days, toads were then subjected to one of either two diets: a continuation of their natural diets (blue or pink ants) or a novel dietary challenge (yellow crickets), while the FMT treatments continued for each group as before. Gut microbial composition, predicted functional capabilities, body mass, lean structural mass, body fat % and liver mass of all toads was measured after experimental trials. Endurance and speed were additionally measured in the invasive region.

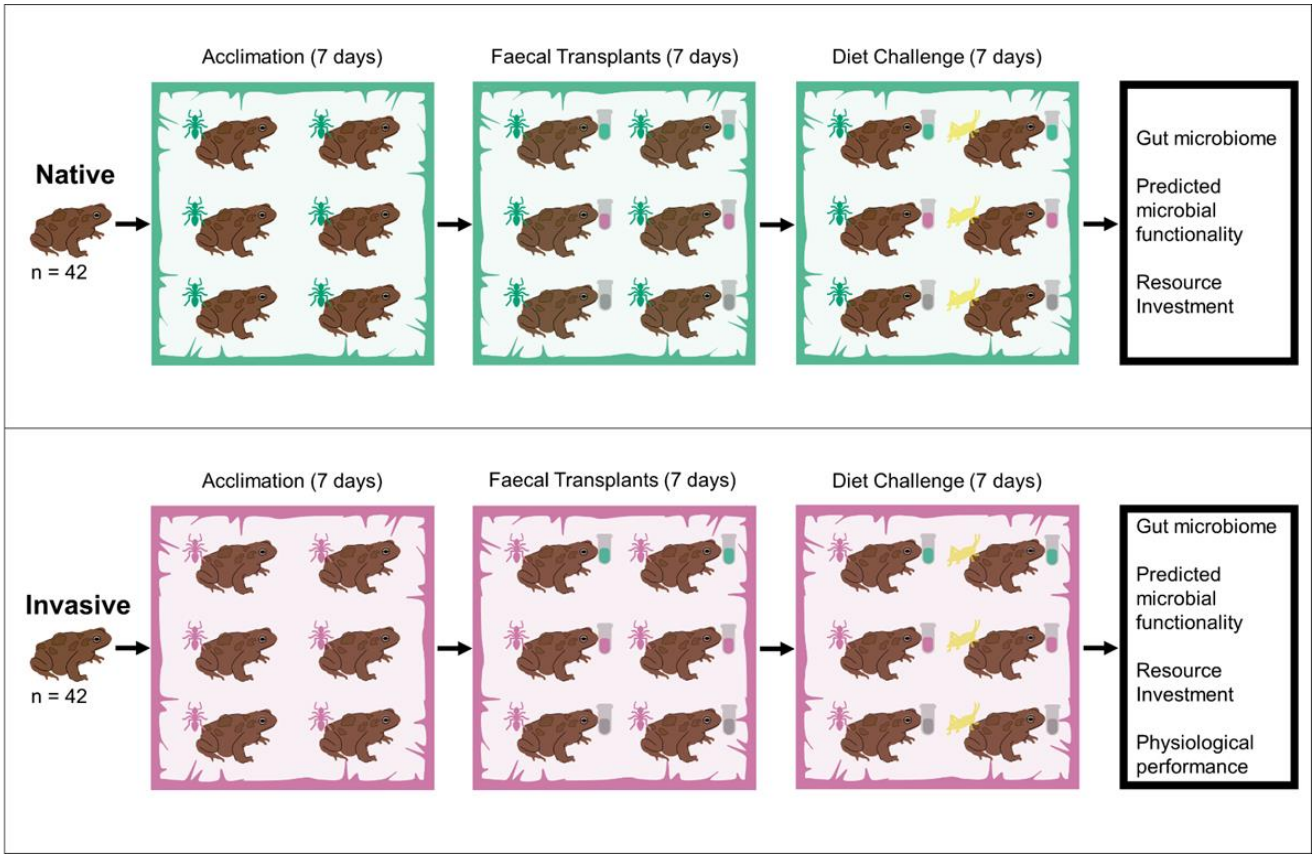

Figure 3.2. Principle Coordinates Analysis (PCoA) of CLR-Euclidean compositional beta diversity demonstrating responses of guttural toads (Sclerophrys gutturalis) colonized by native (blue), invasive (pink), and control (glycerol, grey) toad gut microbial communities to natural diets (circles) and a novel dietary challenge (triangles). Experiments were completed in the toads' native range, Durban (A, C) and invasive range Cape Town, South Africa (B, D). PERMANOVA tests indicated that invasive gut microbiomes (shaded ellipses: Cape Town self-transplant, Cape Town control and Durban transplant) significantly shift their gut microbial composition in response to a dietary challenge, whilst native gut microbial communities (empty ellipses: Durban self-transplant, Durban control and Cape Town transplant) show no response. Permutational test of dispersions (PERDISP) showed responses were not the result of variation in dispersion.

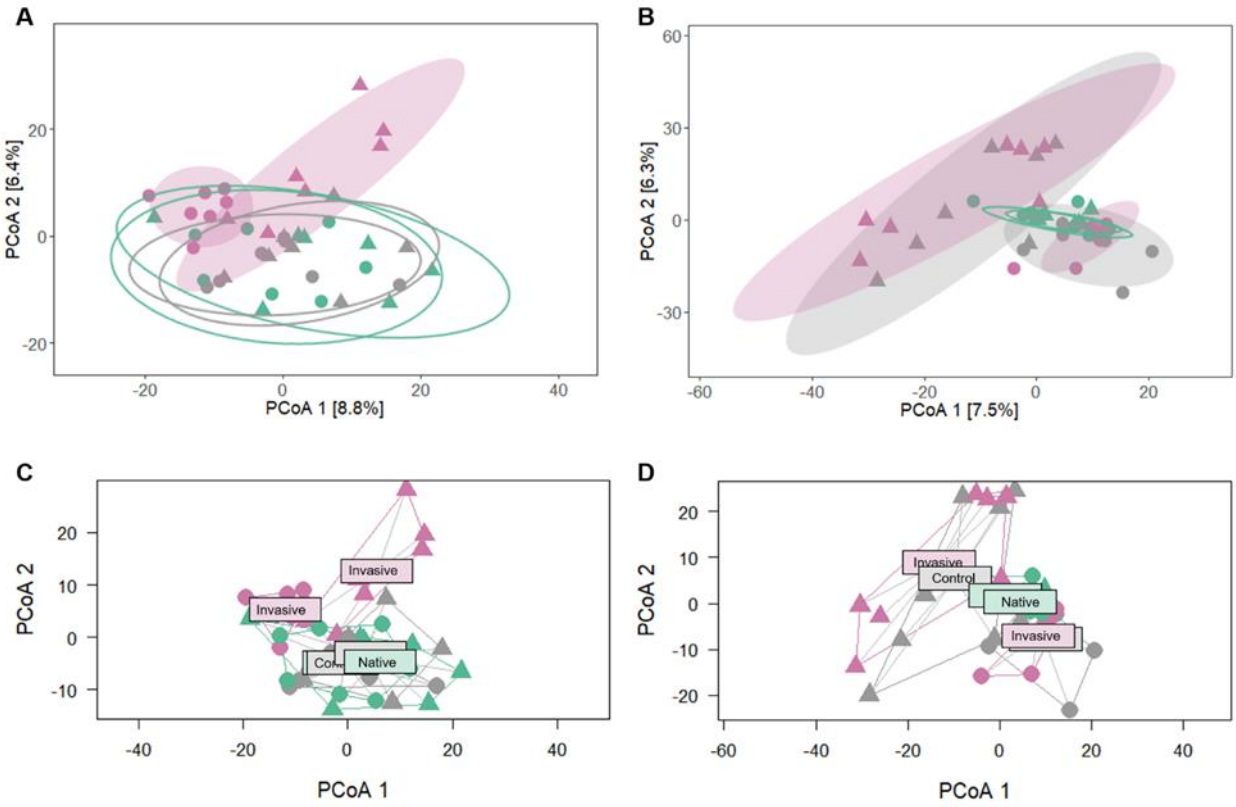

Figure 3.3. Principle Coordinates Analysis (PCoA) of PHILR-Euclidean phylogenetic (A, B, E, F) and CLR-Euclidean predicted functional (C, D, G, H) beta diversity demonstrating responses of guttural toads (Sclerophrys gutturalis) colonized by native (blue), invasive (pink) and control (glycerol, grey) toad gut microbial communities to natural diets (circles) and novel dietary challenge (triangles). Experiments were completed in the toads' native range, Durban (A, C, E, G) and invasive range Cape Town, South Africa (B, D, F, H). PERMANOVA tests indicated that invasive gut microbial communities (shaded ellipses: Cape Town self-transplant, Cape Town control and Durban transplant) significantly shifted their gut microbial phylogenetic and predicted functional diversity in response to novel diets, whilst native gut microbial communities (empty ellipses: Durban self-transplant, Durban control and Cape Town transplant) showed no response to novel diets. Permutational test of dispersions (PERDISP) showed responses were not the result of variation in dispersion.

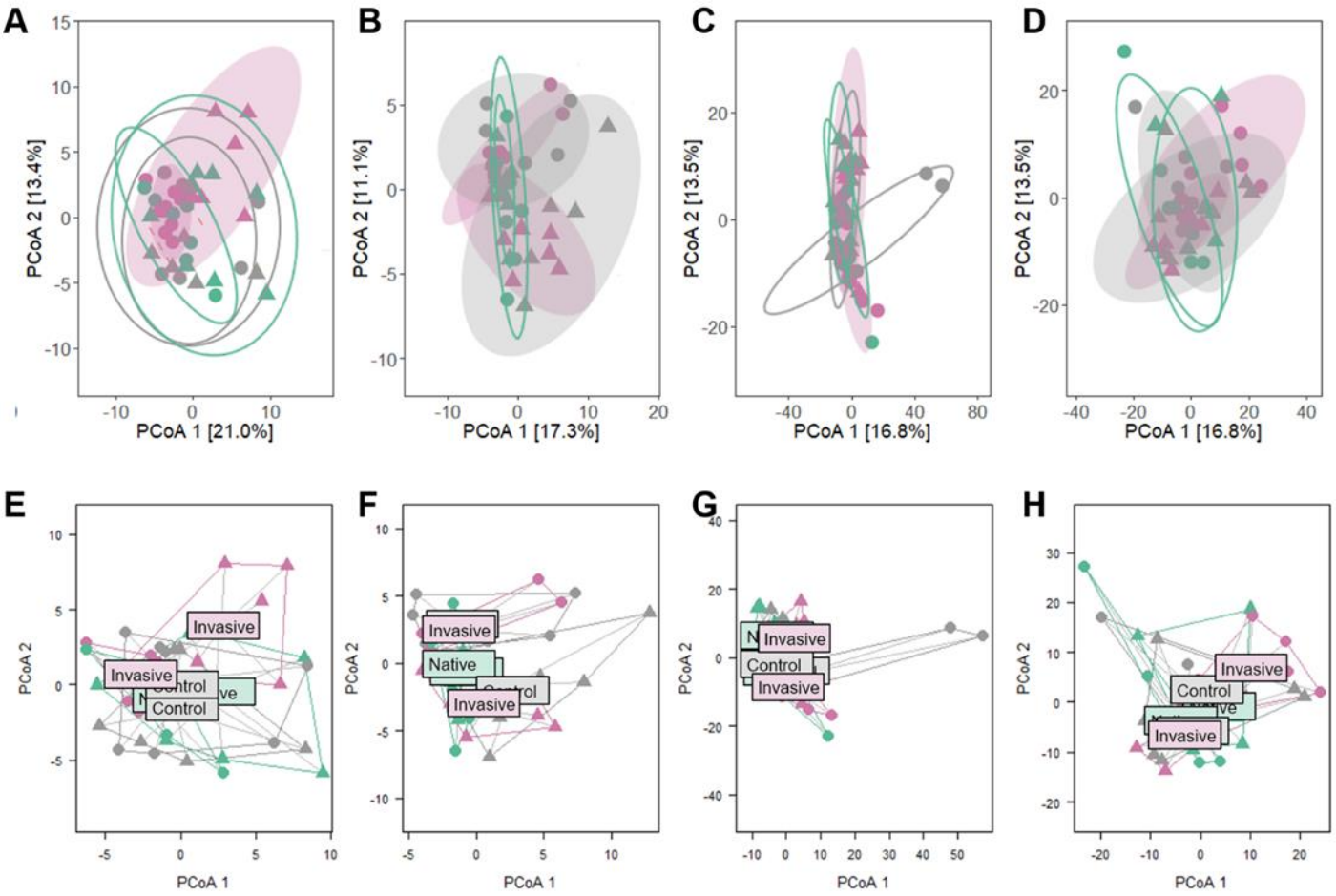

Figure 3.4. Body fat % and liver mass of guttural toads (Sclerophrys gutturalis) colonized by native (blue), control (glycerol, grey) and invasive (pink) toad gut microbial communities and subsequently subjected to two diets, natural (native blue and invasive pink ants) and novel dietary challenge (yellow crickets). Experiments were completed in the toads' native range, Durban (A, C) and invasive range Cape Town, South Africa (B, D). Toads with invasive gut microbiomes significantly increase body fat % and liver mass when subjected to a dietary challenge, while toads with native gut microbiomes show no significant differences (GLM, $p < 0.05$). Toads with invasive gut microbiomes also has a higher overall body fat % and liver mass compared to toads with native gut microbiomes (GLM, $p < 0.05$).

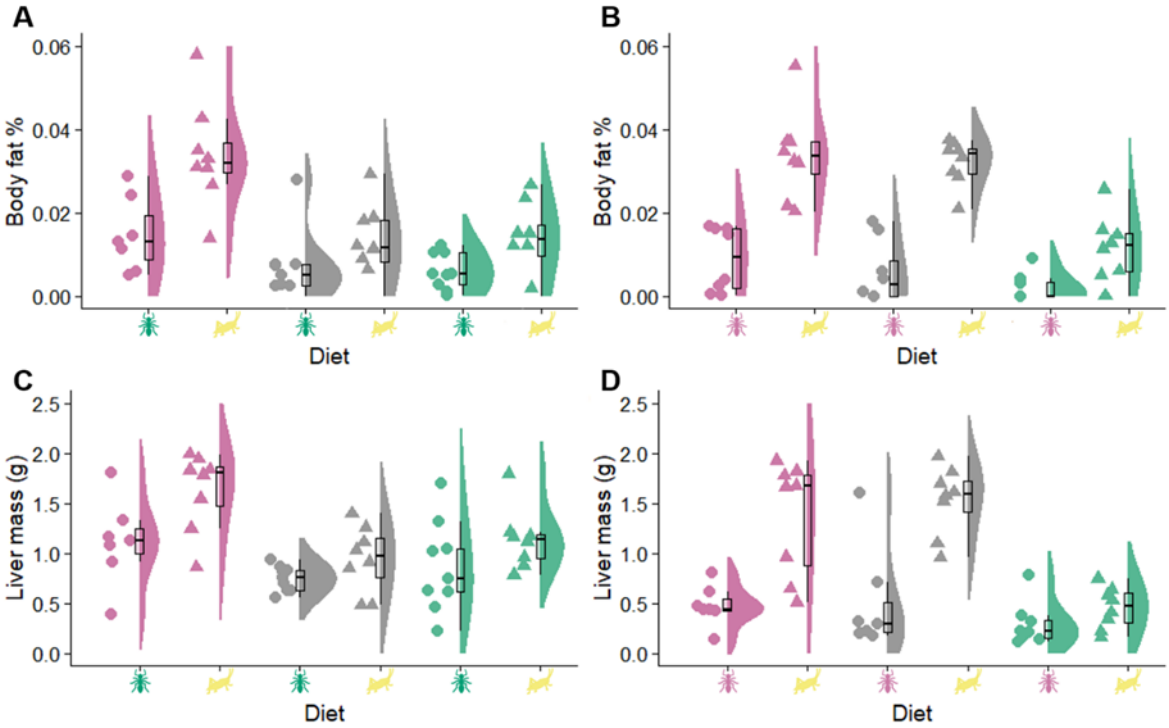

Figure 3.5. Physiological performance, total distance travelled (m) (A) and speed (m.s-1) (B), of guttural toads (Sclerophrys gutturalis) colonized by native (blue), control (glycerol, grey) and invasive (pink) toad gut microbial communities and subsequently subjected to two diets, natural (native blue and invasive pink ants) and novel dietary challenge (yellow crickets). Toads display no significant difference of physiological performance between diets (GLM, $p > 0.05$). However, toads with invasive gut microbiomes have significantly higher physiological performance compared to toads with native gut microbiomes (GLM, $p < 0.05$).

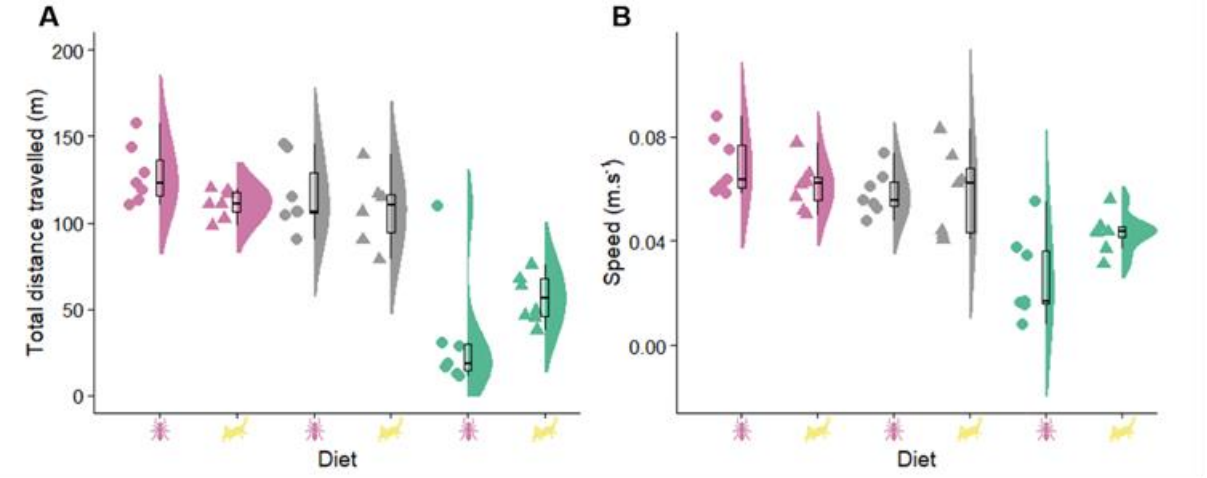

Table 3.1. PERMANOVA results analysing the effect of faecal microbial transplant FMT treatments (glycerol control, native faecal recipients and invasive faecal recipients) and dietary change on the gut microbial composition as measured by compositional CLR- and phylogenetic PHILR-Euclidean metrics in guttural toads (*Sclerophrys gutturalis*) from two experimental areas, Cape Town and Durban, South Africa. For each comparison, dependent and explanatory variables, degrees of freedom (d.f.), sum of squares (SS), pseudo-F-statistic, r-squared values(R2) and *p*-values are reported.

Experimental Area	Dependent variable	Explanatory variable	d.f.	SS	Pseudo-F	R^2	*p*-value
Cape Town	CLR-Euclidean	FMT treatment * diet	2	5369	1.33	0.06	< 0.001
		FMT treatment	1	5129	1.27	0.06	< 0.005
		Diet	1	6424	1.69	0.04	< 0.005
		Body condition	2	2258	1.12	0.03	> 0.05
		Residuals	35	70806		0.81	
		Total	41	86986		1.00	
	PHILR-Euclidean	FMT treatment * diet	2	266	1.44	0.07	< 0.05
		FMT treatment	1	271	1.47	0.07	< 0.05
		Diet	1	185	2.00	0.05	< 0.05
		Body condition	2	90	0.97	0.02	> 0.05
		Residuals	35	3239		0.80	

		Total	41	4051		1.00	
Durban	CLR-Euclidean	FMT treatment * diet	2	3065	1.56	0.06	< 0.05
		FMT treatment	1	3765	1.48	0.07	< 0.001
		Diet	1	1788	1.35	0.03	< 0.005
		Body condition	2	1626	1.27	0.06	> 0.05
		Residuals	35	42217		0.80	
		Total	41	52462		1.00	
	PHILR-Euclidean	FMT treatment * diet	2	3065	1.56	0.06	< 0.05
		FMT treatment	1	3765	1.48	0.07	< 0.05
		Diet	1	1788	1.35	0.03	< 0.05
		Body condition	2	1626	1.27	0.06	> 0.05
		Residuals	35	42217		0.80	
		Total	41	52462		1.00	

Table 3.2. PERMANOVA results analysing the effect of faecal microbial transplant FMT treatments (glycerol control, native faecal recipients and invasive faecal recipients) and dietary change on the gut microbial predicted functional pathways as measured by compositional CLR-Euclidean metrics in guttural toads (*Sclerophrys gutturalis*) from two experimental areas, Cape Town and Durban, South Africa. 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.

Experimental Area	Explanatory variables	d.f.	SS	Pseudo-F	R^2	*p*-value
Cape Town	FMT treatment * diet	2	2027	1.57	0.07	< 0.05
	FMT treatment	1	1337	1.04	0.05	> 0.05
	Diet	1	1045	1.62	0.04	< 0.05
	Body condition	2	637	0.99	0.02	> 0.05
	Residuals	35	22526		0.82	
	Total	41	27572		1.00	
Durban	FMT treatment * diet	2	1704	1.01	0.05	> 0.05
	FMT treatment	1	2228	1.38	0.06	< 0.05
	Diet	1	1942	2.41	0.06	< 0.001
	Body condition	2	592	0.74	0.02	> 0.05
	Residuals	35	28167		0.81	
	Total	41	34634		1.00	

Table 3.3. GLM results analysing the effect of faecal microbial transplant FMT treatments (glycerol control, native faecal recipients and invasive faecal recipients) and dietary change on the scaled body mass, scaled lean structural mass, body fat % of guttural toads (*Sclerophrys gutturalis*) from two experimental areas, Cape Town and Durban, South Africa. For each comparison, dependent and explanatory variables, degrees of freedom (d.f.), ChiSq (x^2), ΔAIC, r-squared values (R^2_m) and *p*-values are reported.

Experimental Area	Dependent variable	Explanatory variable	d.f.	x^2	ΔAIC	R^2_m	*p*-value
Cape Town	Scaled body mass	FMT treatment * diet	42	1.0	8.9	0.02	> 0.05
		FMT treatment + diet	44	0.3	5.7	0.01	> 0.05
		FMT treatment	45	0.1	3.9	0.00	> 0.05
		Diet	46	0.2	1.8	0.00	> 0.05
		Null	47		0.0	0.00	> 0.05
	Scaled lean structural mass	FMT treatment * diet	42	1.5	8.3	0.0	> 0.05
		FMT treatment + diet	44	1.2	4.7	0.0	> 0.05
		FMT treatment	45	0.9	3.0	0.0	> 0.05
		Diet	46	0.4	1.6	0.0	> 0.05
		Null	47		0.0	0.0	

	Body fat %	FMT treatment * diet	42	94.0	0.3	0.67	< 0.001
		FMT treatment + diet	44	88.0	0.0	0.65	< 0.001
		FMT treatment	45	9.8	41.2	0.17	< 0.005
		Diet	46	43.7	16.7	0.48	< 0.001
		Null	47		46.7	0.00	
	Scaled liver mass	FMT treatment * diet	41	52.8	0.0	0.54	< 0.001
		FMT treatment + diet	43	25.6	0.9	0.50	< 0.001
		FMT treatment	44	15.8	18.7	0.26	< 0.001
		Diet	45	15.7	17.1	0.25	< 0.001
		Null	46		29.1	0.00	
Durban	Scaled body mass	FMT treatment * diet	42	1.0	8.9	0.02	> 0.05
		FMT treatment + diet	44	0.3	5.7	0.00	> 0.05
		FMT treatment	45	0.1	3.9	0.00	> 0.05

		Diet	46	0.2	1.8	0.00	> 0.05
		Null	47		0.0	0.00	
	Scaled lean structural mass	FMT treatment * diet	42	1.5	8.3	0.0	> 0.05
		FMT treatment + diet	44	1.2	4.7	0.0	> 0.05
		FMT treatment	45	0.9	3.0	0.0	> 0.05
		Diet	46	0.3	1.6	0.0	> 0.05
		Null	47		0.0	0.0	
	Body fat %	FMT treatment * diet	43	34.1	2.4	0.43	< 0.001
		FMT treatment + diet	45	13.1	0.0	0.42	< 0.001
		FMT treatment	46	18.9	9.4	0.28	< 0.001
		Diet	47	9.7	15.0	0.17	< 0.001
		Null	48		22.2	0.00	
	Scaled liver mass	FMT treatment * diet	41	52.8	0.0	0.5	< 0.001
		FMT treatment + diet	43	45.6	0.9	0.5	< 0.001

	FMT treatment	44	15.8	18.7	0.3	< 0.001
	Diet	45	15.7	17.1	0.3	< 0.001
	Null	46		29.1	0.0	

Table 3.4. GLM results analysing the effect of faecal microbial transplant FMT treatments (glycerol control, native faecal recipients and invasive faecal recipients) and dietary change on the physiological performance, total distance travelled (m) and speed ($m.s^{-1}$) of guttural toads (*Sclerophrys gutturalis*) in Cape Town, South Africa. For each comparison, dependent and explanatory variables, degrees of freedom (d.f.), ChiSq (x^2), ΔAIC, r-squared values (R^2_m) and *p*-values are reported.

	Explanatory variable						
Dependent variable	Fixed	Random	d.f.	x^2	ΔAIC	R^2_m	*p*-value
Total distance travelled	FMT treatment * diet + SVL	Trial Number	9	1.8	2.3	0.48	> 0.05
	FMT treatment * diet	Trial Number	8	0.4	2.1	0.46	> 0.05
	FMT treatment + diet + SVL	Trial Number	7	1.7	0.4	0.46	> 0.05
	FMT treatment + diet	Trial Number	6	0.9	0.1	0.44	< 0.001
	FMT treatment + SVL	Trial Number	6	1.0	1.0	0.44	> 0.05
	Diet + SVL	Trial Number	5	1.0	26.4	0.02	> 0.05
	FMT treatment	Trial Number	5	26.4	0.0	0.42	< 0.001
	Diet	Trial Number	4	1.0	24.4	0.02	> 0.05
	SVL	Trial Number	4	0.0	25.3	0.00	> 0.05

	Null	Trial Number	3		23.4	0.00	
Speed	FMT treatment * diet + SVL	Trial Number	9	0.6	1.4	0.60	> 0.05
	FMT treatment * diet	Trial Number	8	7.7	0.0	0.59	< 0.001
	FMT treatment + diet + SVL	Trial Number	7	0.9	5.7	0.51	> 0.05
	FMT treatment + diet	Trial Number	6	0.5	4.5	0.50	< 0.001
	FMT treatment + SVL	Trial Number	6	1.3	5.0	0.50	> 0.05
	Diet + SVL	Trial Number	5	0.5	32.1	0.07	> 0.05
	FMT treatment	Trial Number	5	27.8	4.3	0.49	< 0.001
	Diet	Trial Number	4	0.9	32.5	0.21	< 0.001
	SVL	Trial Number	4	1.9	30.1	0.06	> 0.05
	Null	Trial Number	3		31.3	0.00	

SUPPLEMENTARY INFORMATION

Figure S3.1. Diversity index “observed species” of species rarefaction curve. Curves are labelled by sample number.

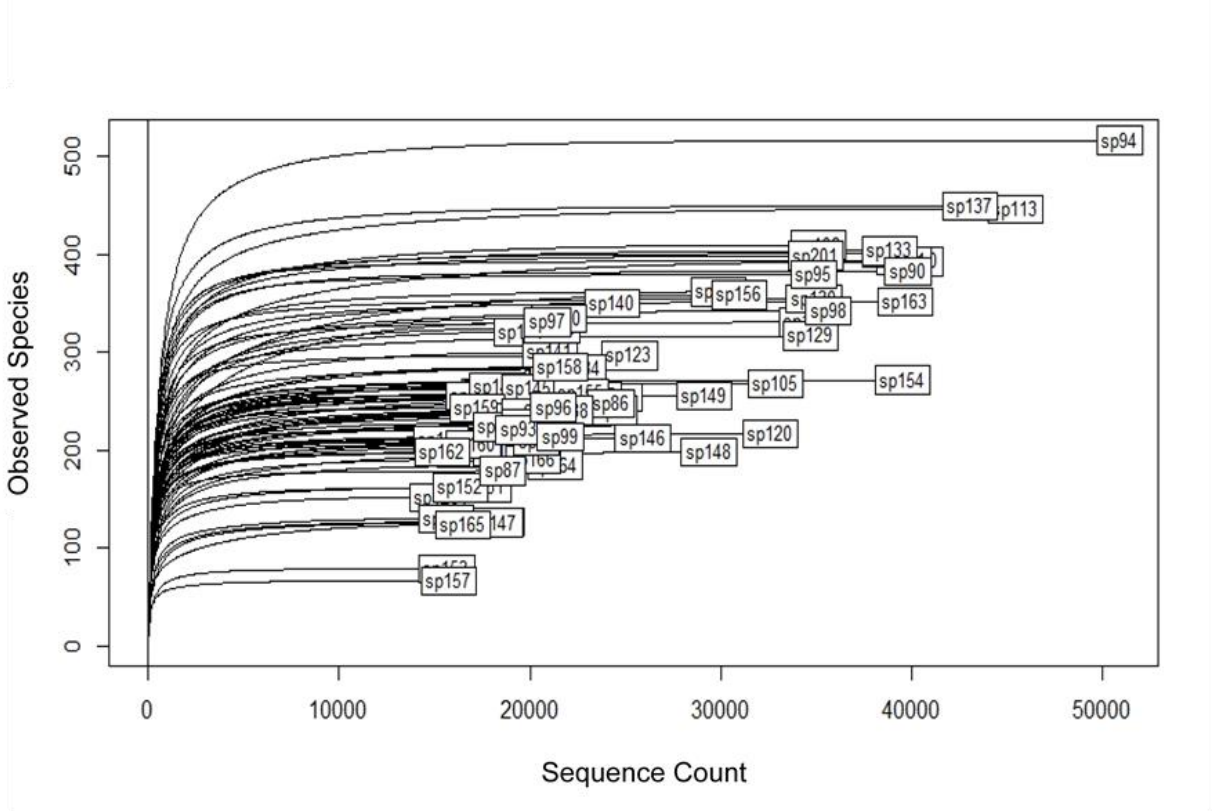

Figure S3.2. Gut bacterial alpha diversity of guttural toads (Sclerophrys gutturalis) on three faecal microbial treatments; native (blue), control (grey), and invasive (pink) exposed to two diets: natural or novel dietary challenge. Experiments were conducted in the native region of guttural toads, Durban, South Africa. Alpha diversity metrics (A) Shannon inverse, (B) Pielou evenness, (C) Chao1 and (D) were not significantly different between faecal microbial treatments or diets (GLMM, $p > 0.05$). The black line and whiskers in the box plots represent the medians and range of the lower quartile (25th percentile) and upper quartile (75th percentile).

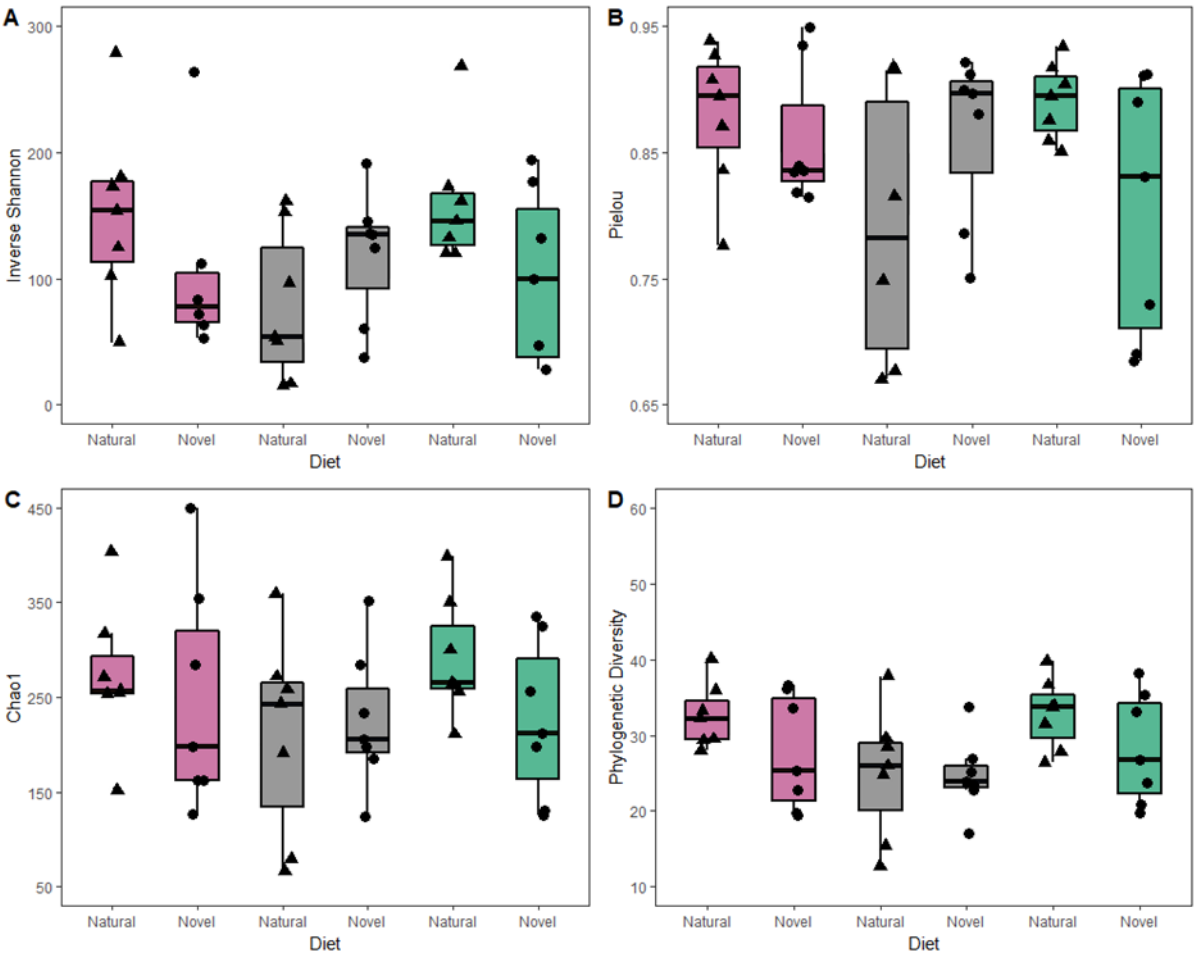

Figure S3.3. Gut bacterial alpha diversity of guttural toads (Sclerophrys gutturalis) on three faecal microbial treatment groups; native (blue), control (grey), and invasive (pink) exposed to two diets; natural or novel dietary challenge. Experiments were conducted in the invasive region of guttural toads, Cape Town, South Africa. Alpha diversity metrics (A) Shannon inverse, (B) Pielou evenness, (C) Chao1 and (D) were not significantly different between faecal microbial treatments or diets (GLMM, $p > 0.05$). The black line and whiskers in the box plots represent the medians and range of the lower quartile (25th percentile) and upper quartile (75th percentile).

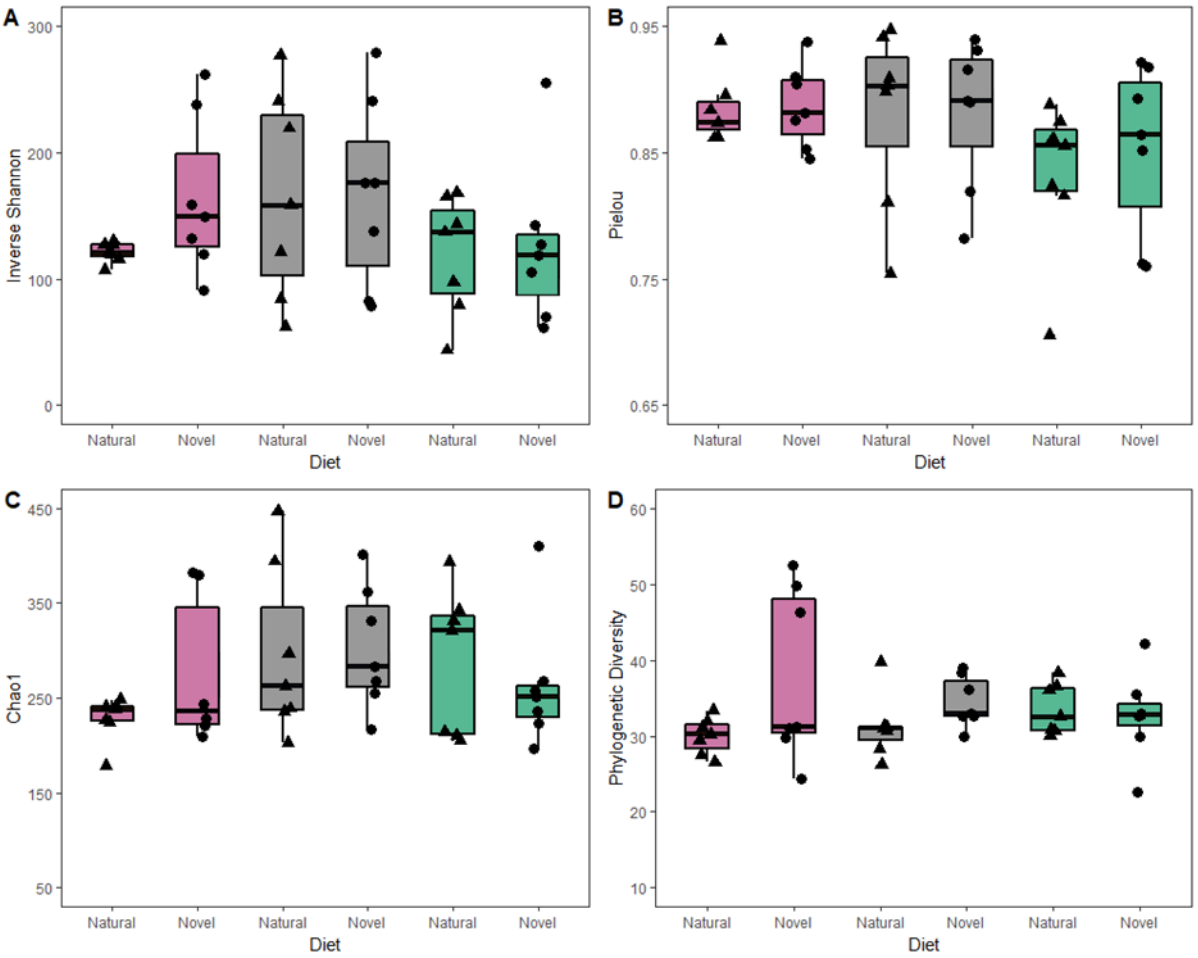

Figure S3.4. Body mass and lean structural mass of guttural toads (Sclerophrys gutturalis) colonized by native (blue), control (glycerol, grey) and invasive (pink) toad gut microbial communities and subsequently subjected to two diets, natural (native blue and invasive pink ants) and novel dietary challenge (yellow crickets). Experiments were completed in the toads' native range, Durban (A, C) and invasive range, Cape Town (B, D). Body mass and lean structural mass did not vary significantly between faecal microbial treatments or diets (GLM, $p > 0.05$).

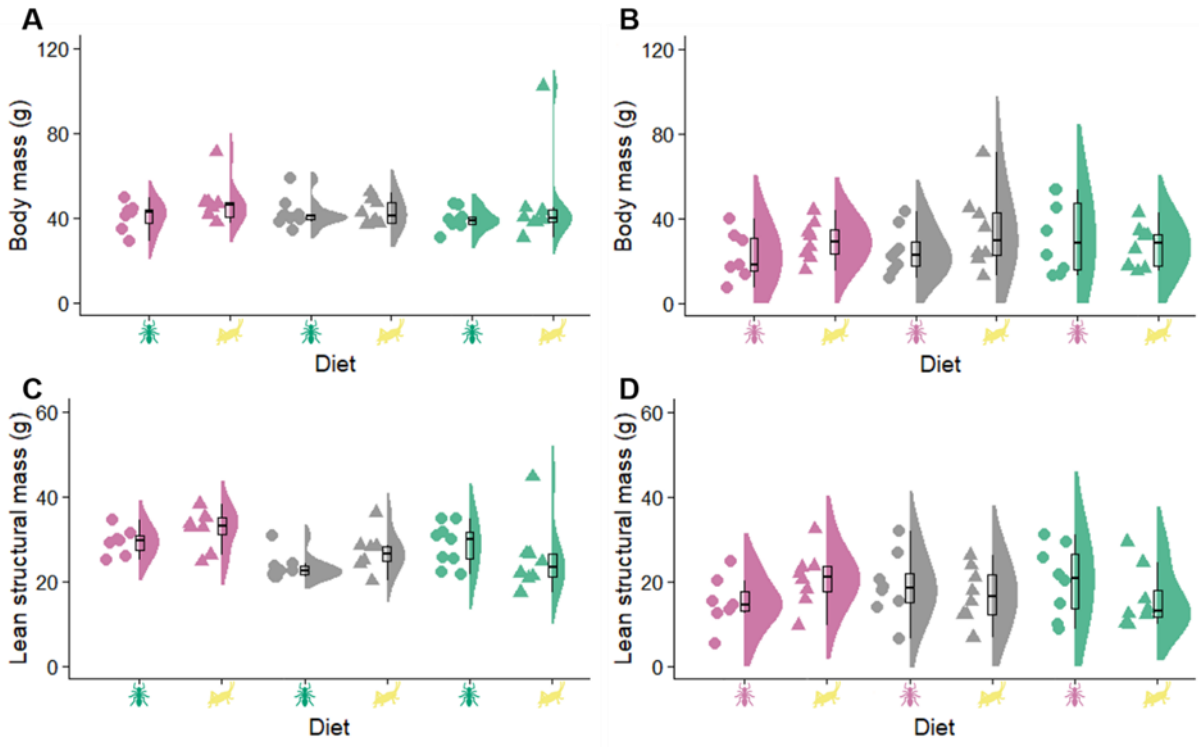

Table S3.1. PERMANOVA pair-wise comparisons analysing differences between toad gut microbial composition and phylogenetic diversity as measured CLR- and PHILR-Euclidean distance matrices. Toads were subjected to either three faecal microbial treatments (FMTs); glycerol control, native faecal recipients and invasive faecal recipients and thereafter exposed to one of two diets; natural or novel dietary challenge. Experiments were conducted in the tpads' invasive (Cape Town) and native (Durban) region. 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.

Experimental area	Pairwise-Comparison	Dependent variable	d.f.	SS	Pseudo-F	R^2	*p*-value
Cape Town	Invasive and control FMTs on dietary challenge	CLR-Euclidean	13	2582.8	1.08	0.08	> 0.05
		PHILR-Euclidean	13	197.5	1.65	0.12	> 0.05
	Invasive and control FMTs on natural diet	CLR-Euclidean	13	2033.9	0.93	0.07	> 0.05
		PHILR-Euclidean	13	141.3	0.69	0.06	> 0.05
	Invasive FMTs on natural and dietary challenge	CLR-Euclidean	13	3838.2	1.74	0.13	< 0.005
		PHILR-Euclidean	13	399.2	1.91	0.14	< 0.05
	Control FMTs on natural and dietary challenge	CLR-Euclidean	13	3144.0	1.30	0.10	< 0.05
		PHILR-Euclidean	13	482.2	1.67	0.12	< 0.05

	Native FMTs on natural and dietary challenge	CLR-Euclidean	13	1471.4	0.94	0.07	> 0.05
		PHILR-Euclidean	13	69.8	0.70	0.05	> 0.05
Durban	Native and control FMTs on dietary challenge	CLR-Euclidean	13	1188.7	1.00	0.08	> 0.05
		PHILR-Euclidean	13	141.4	1.53	0.11	> 0.05
	Native and control FMTs on natural diet	CLR-Euclidean	13	139.0	0.90	0.07	> 0.05
		PHILR-Euclidean	13	141.3	0.69	0.06	> 0.05
	Invasive FMTs on natural and dietary challenge	CLR-Euclidean	13	2175.0	1.79	0.13	< 0.005
		PHILR-Euclidean	13	428.9	2.61	0.18	< 0.01
	Control FMTs on natural and dietary challenge	CLR-Euclidean	13	1241.6	1.10	0.08	> 0.05
		PHILR-Euclidean	13	128.0	0.68	0.05	> 0.05
	Native FMTs on natural and dietary challenge	CLR-Euclidean	13	1426.4	1.13	0.09	> 0.05
		PHILR-Euclidean	13	261.5	1.69	0.12	> 0.05

Table S3.2. Summary of best-fit mixed models analysing the gut bacterial alpha diversity differences between *Sclerophrys gutturalis* (guttural toad) on three faecal microbial treatment groups; invasive faecal recipients, native faecal recipients and controls. Toads were subsequently subjected to one of two diets; natural or novel dietary challenge. Experiments were conducted in toads' invasive (Cape Town) and native (Durban) areas. For each model, fixed and random explanatory variables, degrees of freedom (d.f.), *chi*-square, Akaike's information criterion (AIC), ΔAIC, marginal (R^2_m) and conditional (R^2_c) r-squared values and *p*-values are detailed.

Experimental Area	Dependent variables	Explanatory variables	d.f.	x^2	ΔAIC	R^2	*p*-value
Cape Town	Inverse Shannon	FMT treatment * diet + body condition	35	4.26	7.19	0.09	> 0.05
		FMT treatment + diet + body condition	37	3.95	3.77	0.09	> 0.05
		FMT treatment * diet	36	4.37	5.19	0.10	> 0.05
		FMT treatment + diet	38	4.01	1.79	0.09	> 0.05
		FMT treatment + body condition	38	3.32	2.47	0.08	> 0.05
		Diet + body condition	39	0.58	3.37	0.01	> 0.05
		FMT treatment	39	3.42	0.47	0.08	> 0.05
		Diet	40	0.60	1.37	0.01	> 0.05
		Body condition	40	0.01	1.99	0.00	> 0.05

		Null	41		0.00		
	Chao1	FMT treatment * diet + body condition	35	5.38	5.87	0.12	> 0.05
		FMT treatment + diet + body condition	37	2.80	4.92	0.06	> 0.05
		FMT treatment * diet	36	5.57	3.96	0.12	> 0.05
		FMT treatment + diet	38	2.70	3.12	0.06	> 0.05
		FMT treatment + body condition	38	1.95	3.84	0.05	> 0.05
		Diet + body condition	39	1.08	2.93	0.02	> 0.05
		FMT treatment	39	1.95	1.95	0.05	> 0.05
		Diet	40	0.76	1.21	0.02	> 0.05
		Body condition	40	0.18	1.82	0.00	> 0.05
		Null	41		0.00		
	Pielou	FMT treatment * diet + body condition	35	5.31	7.38	0.12	> 0.05
		FMT treatment + diet + body condition	37	5.22	3.76	0.11	> 0.05
		FMT treatment * diet	36	5.55	5.38	0.12	> 0.05

	FMT treatment + diet	38	5.47	1.76	0.12	> 0.05
	FMT treatment + body condition	38	5.10	1.99	0.11	> 0.05
	Diet + body condition	39	0.26	5.10	0.01	> 0.05
	FMT treatment	39	5.36	0.00	0.12	> 0.05
	Diet	40	0.20	3.20	0.00	> 0.05
	Body condition	40	0.12	3.28	0.00	> 0.05
	Null	41		1.41		
Phylogenetic diversity	FMT treatment * diet + body condition	35	3.29	8.20	0.07	> 0.05
	FMT treatment + diet + body condition	37	1.36	6.47	0.03	> 0.05
	FMT treatment * diet	36	3.40	6.21	0.08	> 0.05
	FMT treatment + diet	38	1.40	4.48	0.03	> 0.05
	FMT treatment + body condition	38	0.30	5.66	0.01	> 0.05
	Diet + body condition	39	1.17	2.79	0.03	> 0.05
	FMT treatment	39	0.31	3.66	0.01	> 0.05
	Diet	40	1.14	0.82	0.03	> 0.05

		Body condition	40	0.01	1.99	0.00	> 0.05
		Null	41		0.00		
Durban	Inverse Shannon	FMT treatment * diet + body condition	35	10.11	2.04	0.19	> 0.05
		FMT treatment + diet + body condition	37	3.61	4.28	0.08	> 0.05
		FMT treatment * diet	36	8.61	0.99	0.17	> 0.05
		FMT treatment + diet	38	3.25	2.55	0.07	> 0.05
		FMT treatment + body condition	38	3.31	3.62	0.07	> 0.05
		Diet + body condition	39	0.33	3.63	0.01	> 0.05
		FMT treatment	39	3.03	0.86	0.07	> 0.05
		Diet	40	0.28	1.71	0.01	> 0.05
		Body condition	40	0.09	1.91	0.00	> 0.05
		Null	41		0.00		
	Chao1	FMT treatment * diet + body condition	35	5.03	6.41	0.11	> 0.05
		FMT treatment + diet + body condition	37	2.85	4.77	0.07	> 0.05
		FMT treatment * diet	36	4.88	4.66	0.11	> 0.05

	FMT treatment + diet	38	2.98	2.83	0.07	> 0.05
	FMT treatment + body condition	38	2.08	3.58	0.05	> 0.05
	Diet + body condition	39	0.85	3.07	0.02	> 0.05
	FMT treatment	39	2.28	1.62	0.05	> 0.05
	Diet	40	0.72	1.25	0.02	> 0.05
	Body condition	40	0.21	1.79	0.00	> 0.05
	Null	41		0.00		
Pielou	FMT treatment * diet + body condition	35	12.95	1.19	0.23	> 0.05
	FMT treatment + diet + body condition	37	2.62	7.04	0.06	> 0.05
	FMT treatment * diet	36	11.86	0.00	0.22	> 0.05
	FMT treatment + diet	38	2.63	5.15	0.06	> 0.05
	FMT treatment + body condition	38	2.66	5.06	0.06	> 0.05
	Diet + body condition	39	0.15	5.80	0.00	> 0.05
	FMT treatment	39	2.69	3.16	0.06	> 0.05
	Diet	40	0.01	3.95	0.00	> 0.05

		Body condition	40	0.15	3.81	0.00	> 0.05
		Null	41		1.96		
	Phylogenetic diversity	FMT treatment * diet + body condition	35	8.79	4.69	0.20	> 0.05
		FMT treatment + diet + body condition	37	7.94	1.96	0.19	> 0.05
		FMT treatment * diet	36	10.62	2.70	0.21	> 0.05
		FMT treatment + diet	38	9.71	0.00	0.19	> 0.05
		FMT treatment + body condition	38	4.98	2.86	0.14	> 0.05
		Diet + body condition	39	3.76	3.54	0.09	> 0.05
		FMT treatment	39	6.66	0.93	0.14	< 0.05
		Diet	40	2.45	3.06	0.06	> 0.05
		Body condition	40	1.55	3.86	0.04	> 0.05
		Null	41		3.56		

Table S3.3. Differential abundance of bacterial ASVs in response to dietary change across guttural toads (*Sclerophrys gutturalis*) exposed to either of three faecal microbial transplant treatments (invasive faecal material, native faecal material or glycerol control). See http://doi.org/10.5281/zenodo.4164856 for table.

Table S3.4. Pairwise comparisons of differential abundance of bacterial ASVs in response to dietary change across guttural toads (*Sclerophrys gutturalis*) exposed to either of three faecal microbial transplant treatments (invasive faecal material, native faecal material or glycerol control). See http://doi.org/10.5281/zenodo.4164856 for table.

Table S3.5. Summary of PERMANOVA pairwise comparisons of *Sclerophrys gutturalis* (guttural toad) gut microbial predicted functional diversity as measured by CLR-Euclidean metrics. Guttural toads on three different faecal microbial treatments (invasive faecal recipients, native faecal recipients and control) was subjected to one of two diets; natural diet or novel dietary challenge. Experiments were conducted in the toads' invasive (Cape Town) and native (Durban) region. For each comparison, dependent variable, degrees of freedom (d.f.), sum of squares (SS), pseudo-*F*-statistic, r-squared values(R^2) and *p*-values are reported.

Experimental area	Pairwise-Comparison	Dependent variable	d.f.	SS	Pseudo-F	R^2	*p*-value
Cape Town	Invasive and control FMTs on dietary challenge	CLR-Euclidean	13	1860.2	1.54	0.06	> 0.05
	Invasive and control FMTs on natural diet	CLR-Euclidean	13	1732.0	1.22	0.11	> 0.05
	Invasive FMTs on natural and dietary challenge	CLR-Euclidean	13	1401.0	2.72	0.18	< 0.01
	Control FMTs on natural and dietary challenge	CLR-Euclidean	13	2103.2	1.88	0.13	< 0.05
	Native FMTs on natural and dietary challenge	CLR-Euclidean	13	625.3	1.16	0.02	> 0.05
Durban	Native and control FMTs on dietary challenge	CLR-Euclidean	13	1565.8	1.23	0.07	> 0.05
	Native and control FMTs on natural diet	CLR-Euclidean	13	1599.3	1.44	0.06	> 0.05

	Invasive FMTs on natural and dietary challenge	CLR-Euclidean	13	1151.9	1.65	0.11	< 0.05
	Control FMTs on natural and dietary challenge	CLR-Euclidean	13	1702.4	1.63	0.12	> 0.05
	Native FMTs on natural and dietary challenge	CLR-Euclidean	13	792.2	1.20	0.09	> 0.05

Table S3.6. Differential abundance of bacterial predicted functional pathways in response to dietary change across guttural toads (*Sclerophrys gutturalis*) exposed to either of three faecal microbial transplant treatments (invasive faecal material, native faecal material or glycerol control). See http://doi.org/10.5281/zenodo.4164856 for table.

Table S3.7. Pairwise comparisons of differential abundance of bacterial predicted functional pathways in response to dietary change across guttural toads (*Sclerophrys gutturalis*) exposed to either of three faecal microbial transplant treatments (invasive faecal material, native faecal material or glycerol control). See http://doi.org/10.5281/zenodo.4164856 for table.

Table S3.8. Summary of pairwise comparisons of *Sclerophrys gutturalis* (guttural toad) body fat % and liver mass. Guttural toads on three different faecal microbial treatments (invasive faecal recipients, native faecal recipients and control) was subjected to one of two diets; natural diet or novel dietary challenge. Experiments were conducted in the toads' invasive (Cape Town) and native (Durban) region. For each comparison, dependent variable, degrees of freedom (d.f.), mean and standard deviation (*SD*), F-, *t*- and *p*-values are reported.

Experimental area	Pairwise Comparison	Dependent variable	d.f.	Mean (±*SD*)	F-value	*t*-value	*p*-value
Cape Town	Invasive and control FMTs on dietary challenge	Body fat %	15	0.034 (± 0.011) and 0.032 (± 0.005)	3.90	0.30	> 0.05
		Liver mass	15	1.370 (± 0.572) and 1.530 (± 0.344)	2.77	-0.79	> 0.05
	Invasive and control FMTs on natural diet	Body fat %	15	0.009 (± 0.008) and 0.006 (± 0.007)	1.11	1.01	> 0.05
		Liver mass	15	0.472 (± 0.204) and 0.753 (± 0.860)	0.06	-0.54	> 0.05
	Invasive FMTs on natural and dietary challenge	Body fat %	15	0.034 (± 0.011) and 0.009 (± 0.008)	1.98	4.89	< 0.001
		Liver mass	15	1.370 (± 0.572) and 0.472 (± 0.204)	7.88	4.04	< 0.01
	Control FMTs on natural and dietary challenge	Body fat %	15	0.032 (± 0.005) and 0.006 (± 0.007)	1.98	6.11	< 0.001

		Liver mass	15	1.530 (± 0.344) and 0.753 (± 0.860)	7.88	2.85	< 0.05
	Native FMTs on natural and dietary challenge	Body fat %	15	0.002 (± 0.003) and 0.011 (± 0.008)	0.25	0.12	> 0.05
		Liver mass	15	0.451 (± 0.207) and 0.290 (± 0.218)	0.91	1.66	> 0.05
Durban	Native and control FMTs on dietary challenge	Body fat %	15	0.013 (± 0.009) and 0.131 (± 0.009)	0.93	0.04	> 0.05
		Liver mass	15	1.140 (± 0.308) and 0.939 (± 0.333)	1.17	-1.29	> 0.05
	Native and control FMTs on natural diet	Body fat %	15	0.006 (± 0.004) and 0.007 (± 0.008)	3.69	0.13	> 0.05
		Liver mass	15	0.859 (± 0.456) and 0.745 (± 0.126)	0.08	-0.41	> 0.05
	Invasive FMTs on natural and dietary challenge	Body fat %	15	0.034 (± 0.013) and 0.015 (± 0.009)	2.06	3.49	< 0.01
		Liver mass	15	1.630 (± 0.394) and 1.110 (± 0.428)	0.85	2.31	< 0.05
	Control FMTs on natural and dietary challenge	Body fat %	15	0.013 (± 0.009) and 0.007 (± 0.008)	2.06	1.37	> 0.05

		Liver mass	15	0.939 (± 0.333) and 0.745 (± 0.126)	6.96	1.43	> 0.05
	Native FMTs on natural and dietary challenge	Body fat %	15	0.013 (± 0.009) and 0.006 (± 0.004)	4.59	1.53	> 0.05
		Liver mass	15	1.140 (± 0.308) and 0.859 (± 0.456)	0.46	1.61	> 0.05

Table S3.9. Summary of pairwise comparisons of *Sclerophrys gutturalis* (guttural toad) physiological performance; body fat % and liver mass (g) across three different faecal microbial transplant treatments; invasive faecal recipients, native faecal recipients and control. Experiments were conducted in the toads' invasive (Cape Town) and native (Durban) region. For each comparison, dependent variable, degrees of freedom (d.f.), mean and standard deviation (*SD*), F-, *t*- and *p*-values are reported.

Experimental area	Pairwise-Comparison	Dependent variable	d.f.	Mean (±*SD*)	F-value	*t*-value	*p*-value
Cape Town	Invasive and control FMTs	Body fat %	15	0.02 (± 0.02) and 0.02 (± 0.02)	0.63	0.12	> 0.05
		Liver mass	15	0.95 (± 0.63) and 1.14 (± 0.075)	0.72	0.37	> 0.05
	Invasive and native FMTs	Body fat %	15	0.02 (± 0.02) and 0.01 (± 0.01)	3.05	1.66	< 0.05
		Liver mass	15	0.95 (± 0.63) and 0.37 (± 0.22)	4.28	2.65	< 0.05
	Control and native FMTs	Body fat %	15	0.95 (± 0.63) and 0.37 (± 0.22)	3.10	1.75	< 0.05
		Liver mass	15	0.95 (± 0.63) and 0.37 (± 0.22)	4.47	2.34	< 0.05
Durban	Invasive and control FMTs	Body fat %	15	0.02 (± 0.01) and 0.01 (± 0.01)	4.56	3.00	< 0.05
		Liver mass	15	1.39 (± 0.48) and 0.84 (± 0.26)	3.48	2.57	< 0.05
	Invasive and native FMTs	Body fat %	15	0.02 (± 0.01) and 0.01 (± 0.01)	4.21	2.51	< 0.05
		Liver mass	15	1.39 (± 0.48) and 0.99 (± 0.41)	3.20	2.49	< 0.05
	Native and control FMTs	Body fat %	15	0.01 (± 0.01) and 0.01 (± 0.01)	0.42	0.31	> 0.05

	Liver mass	15	0.99 (± 0.41) and 0.84 (± 0.26)	0.99	0.63	> 0.05

Table S3.10. Summary of pairwise comparisons of *Sclerophrys gutturalis* (guttural toad) physiological performance; total distance travelled (m) and speed ($m.s^{-1}$) across three different faecal microbial transplant treatments; invasive faecal recipients, native faecal recipients and control. Experiments were conducted in the toads' invasive (Cape Town). For each comparison, dependent variable, degrees of freedom (d.f.), mean and standard deviation (*SD*), F-, *t*- and *p*-values are reported.

Pairwise-Comparison	Dependent variable	d.f.	Mean (±*SD*)	F-value	*t*-value	*p*-value
Invasive and control FMTs	Distance	15	136.77 (± 6.82) and 132.31 (± 7.78)	1.20	0.61	> 0.05
	Speed	15	0.06 (± 0.01) and 0.06 (± 0.01)	1.09	0.87	> 0.05
Invasive and native FMTs	Distance	15	136.77 (± 6.82) and 45.40 (± 2.77)	19.89	4.21	< 0.001
	Speed	15	0.06 (± 0.01) and 0.04 (± 0.01)	6.99	1.29	< 0.05
Control and native FMTs	Distance	15	132.31 (± 7.78) and 45.40 (± 2.77)	21.63	5.21	< 0.001
	Speed	15	0.06 (± 0.01) and 0.04 (± 0.01)	8.14	1.11	< 0.05

Chapter 4: General Discussion

The wildlife microbiome

To date, very few studies have examined the gut bacterial communities of invasive vertebrate species (Kowalski et al., 2015; Eichmiller et al., 2016; Bahrndorff et al., 2016; Shanmuganandam et al., 2020). This thesis contributed new insights into what factors impact gut microbial variation and how adaptive potential of microbiota impacts the performance of a wild species. The work presented in this thesis undoubtedly show that the microbiome acts as a mediator between environmental change and host physiology.

Ecological theory, such as dispersal, local diversification, environmental selection and ecological drift, has been applied extensively in human microbiome sciences to provide a framework for understanding the compositional variability within and between hosts (Costello et al., 2012; Gilbert, 2015; Benson, 2016). However, few efforts have been made to integrate microbiome sciences into the field of ecology (Trevelline et al., 2019). Here, I show how microbiome and ecological sciences can be integrated to understand patterns of microbial distribution across wild populations. Causes of microbial variation across populations have rarely been comprehensively investigated (Hauffe & Barelli, 2019). Despite the complexity of the processes shaping microbial communities, other studies have also demonstrated that population-level variation of wild gut microbiomes is low (Cahill et al., 2016; Blyton et al., 2019; Le et al., 2020). Previous studies have proposed that, low population variation could indicate a lack of flexibility in response to environmental change impairing the adaptive ability of animals to respond to environmental change (Hauffe & Barelli, 2019). However, whether microbial resilience to environmental change has negative impacts on host physiology remains to be tested (but see Chapter 3).

While previous studies have correlated environmental change with gut microbial changes and subsequent changes in host physiology and health, in this thesis I demonstrate conclusively that microbial responses to a dietary challenge regulates responses of host physiology to this environmental change. Although I used an invasive species as a model system to investigate these host-microbial relationships, the results presented in this thesis haves far-reaching implications beyond invasion ecology. Establishing a causal link between microbial disturbance and animal fitness is essential to identify beneficial microbes for desired conservation objectives. Numerous reviews have identified the microbiome as the ‘missing link’ in our overall strategy to conserve and maintain the health of threatened species (Bahrndorff et al., 2016; Jiménez & Sommer, 2017; Trevelline et al., 2019; Hauffe & Barelli, 2019; Banerjee et al., 2020; Guo et al., 2020). In fact, some studies have suggested that microbial transplantation should be implemented in vulnerable populations to advance the

development of healthy gut microbiomes and facilitate subsequent positive effects on host health (Trevelline et al., 2019; Blyton et al., 2019). However, without necessary studies characterising microbial taxa with definitive links to host health and physiology, we cannot effectively conserve these microbes. Invasive species can be used as model systems to determine the true impact of microbes on host health and physiology in changing environments and eventually further effective application of faecal microbial transplants for the preservation of vulnerable species.

The invasive microbiome

In this thesis, I highlight factors shaping gut bacterial diversity across native and invasive populations. Additionally, I emphasize how varying population dynamics can produce significant divergence in gut bacterial diversity in an expanding invasive population. Furthermore, this thesis shows that adaptive microbial flexibility facilitates host phenotypic flexibility. I demonstrate that the invasion success of guttural toads in Cape Town may, in part, be due to the important role gut bacterial communities play in facilitating host physiology. The results in this thesis, thus, emphasise that invasion success and potential of invasive animals cannot be fully understood without considering what factors impact their symbiotic microbes and how these microbes impact host performance and fitness.

In Chapter 2 I show that gut bacterial communities and their predicted functional capabilities in older invasive populations (Mauritius and Réunion) remain conserved even 100 years after introduction from Durban. In many plant invasions, the introduction of novel microbes has been shown to have devastating impacts on native symbiotic networks and native host fitness (Elias et al., 2006; Mangla et al., 2008; Klepzig et al., 2009; Nelson & Karp, 2013; Yang et al., 2013). Non-native species can, additionally, act as source for gut bacterial pathogens (Coats & Rumpho, 2014). In fact, recent studies show that gut microbes show low host specificity and can be transferred across species (Song et al., 2013). This might exacerbate the impact of introducing novel gut bacteria into environments as they can be transferred to a variety of hosts. Therefore, the persistence non-native microbes in the Mauritius and Réunion could have devastating effects on native species.

Chapter 3 demonstrates that the novel gut microbiota in an invasive population is a key component of host physiology and fitness. Similar to previous studies on plant invasions, the results from this thesis demonstrates that microbial flexibility enhances the likelihood of favourable host physiological adaptations to new environments which could increase the invasion establishment probability of the host (Pringle et al., 2009; Coats & Rumpho, 2014). Higher phenotypic plasticity is often linked to an invasive populations' ability to adapt and establish in novel environments (Chown et al., 2007; Caño et al., 2008; Davidson et al., 2011).

Here, I show that the microbiome might be the underlying factor producing this phenotypic plasticity. By associating with novel bacterial assemblages, the guttural toad can gain a competitive advantage over endemic species like the western leopard toad, *Sclerophrys pantherina*. Studies investigating the gut microbial communities of western leopard toads present in the sites examined in this thesis will help answer these questions. Nevertheless, I demonstrate that beneficial microbial associations with invasive hosts can improve their ability to respond to novel environmental change.

As demonstrated in this thesis, and previous studies (Coats & Rumpho, 2014), not all hosts are able to shift their bacterial communities when introduced into novel environments. Previous studies indicate that hosts with flexible microbial relationships are more capable of establishment in a non-native habitat (Coats & Rumpho, 2014). However, naturalisation of invasive species by microbial symbionts are also dependent on whether beneficial microbial symbionts are present in the introduced range (Pringle et al., 2009). If host-microbial relationships are flexible and the native habitat possess sufficient beneficial microbes for the host to associate with, new symbiosis can improve the performance and fitness of the host. These patterns have been documented for many plant invasions (van der Putten et al., 2007; Pringle et al., 2009; Berendsen et al., 2012; Bakker et al., 2013; Yang et al., 2013) and is demonstrated for the first time in this thesis for an invasive vertebrate. Consequently, if hosts and their new symbionts are introduced into other regions, this novel relationship can further enhance the development and spread of new invasive populations. Identifying similar populations with unique host-microbial relationships, is important to focus management efforts on these populations to prevent their spread to other non-native areas.

Conclusion

Currently, microbial communities are being investigated in unprecedented detail across human, laboratory and domestic animal populations. However, little is still known about the consequences of microbiome variation for host processes, particularly across different spatial and temporal scales. Here, I demonstrate how biological invasions can be used as natural experiments to investigate host-microbial ecology and evolution. Many of the hypotheses and ideas I investigated and discussed in this thesis have already been underlined in the plant invasion literature (Richardson et al., 2000; Coats & Rumpho, 2014; Traveset & Richardson, 2014). As we can learn from microbiome science, we have much to gain from integrating already established hypotheses and ideas from plant invasions into our understanding of invasive vertebrate hosts and their microbial communities.

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