Microbial function is related to behavior of an invasive anuran

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Running title: Microbial function relates to host behavior

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Abstract

Invasive species cause negative environmental and economic impacts worldwide. Their management may be improved by clarifying the role of behavior in advancing invasions. Gut microbial communities are known to affect behavior of wild populations, but their impact on behavior underlying invasiveness remains unexplored. Invasive populations of the cane toad (*Rhinella marina*) in Australia have expanded across the continent and exhibit variation in behavioral traits along their expansion trajectory, making this an ideal system to investigate the relationship between gut microbes and behaviors. We collected wild female toads from six locations in Queensland (n = 30) and Western Australia (n = 30), and conducted simple tests on behavioral traits previously associated with invasion ability. We investigated the relationships between toad gut microbiota, behavioral traits and the presence and intensity of co-introduced lungworms (*Rhabdias pseudosphaerocephala*) in toad samples from both ends of their Australian range. Based on 16S rRNA sequencing data, we found that microbiota in cane toad colons were dominated by the phyla Bacteroidetes, Proteobacteria, Firmicutes, Verrucomicrobia, and Fusobacteria. We found significant differences in microbiota composition (p-value < 0.001) between regions and in predicted microbial functional groups (p-value = 0.002). The occurrence of lungworms was strongly associated with variation in both microbial composition and microbial functions. However, the behavioral traits were associated with microbial functional variation, but not microbial compositional variation. These results support the “holobiont concept” (investigating the assemblage associated with a host) to fully understand drivers of invasion and highlight the need for experimental manipulations to detect causal relationships between microbiota, parasites and host behavior.

Keywords: gut microbiota, invasive behavior, Cane toad, 16S rRNA, predicted microbial functions, parasite

Introduction

An animal's behavior can be influenced by the bacterial microbiome within its intestines. To understand this, scientists have experimentally manipulated the microbiome in host species. For example, gut microbiota transfer has been found to modify exploratory behavior of germ-free mice to resemble that of the donors (Bercik et al., 2011), and to influence emotional reactivity in Japanese quail (*Coturnix japonica*; Kraimi et al., 2019). Further, altering gut microbiota by treatment with a sterile diet or with antibiotics changed chemical social cues and triggered aggressive behavior in leaf-cutting ants (*Acromyrmex echinatior*; Teseo et al., 2019). Remarkably, host behavioral changes can be due to only an individual bacterial species. Mono-colonization with one dominant species, *Lactobacillus brevis*, can decrease walking speed and daily activity of germ-free or antibiotic-treated fruit flies (*Drosophila melanogaster*; Schretter et al., 2018). Dietary administration with the probiotic *Lactobacillus rhamnosus* IMC 501 to zebrafish (*Danio rerio*) caused shoaling behavior to be more uniform and exploratory than that of the control groups (Borrelli et al., 2016) and administration of *Bifidobacteria* reduced stress-related behavior of BALB/c mice (Savignac, Kiely, Dinan, & Cryan, 2014). The gut microbiota can contribute to host adaptability (Hauffe & Barelli, 2019) through mating choices (Sharon et al., 2010) and food resource usage (Vogel et al., 2017). For example, gut microbiota influenced foraging decisions in fruit flies (*Drosophila melanogaster*; Wong et al., 2017) and were associated with different behavioral tasks, such as foraging, food processing and nursing in honey bees (Jones et al., 2018).

Empirical evidence for the role of intestinal microbiota on host behavior is increasing, yet to date have focused primarily on human or domesticated models. Studying wild models in-situ

can elucidate the microbial functional value of this biological phenomenon, particularly how it relates to host fitness and subsequent ecological or evolutionary processes (Davidson, Raulo, & Knowles, 2020). This also allows us to understand the impact of microbes on host invasive behavior (Stuart, Shine, & Brown, 2019), particularly when encountering novel environmental stressors (Sampson & Mazmanian, 2015). While this idea is not well-tested, there is evidence that native and invasive populations may differ in their microbiota in unexpected ways. For example, despite sharing the same environment, microbial communities in the hindguts of invasive Asian silver carp (*Hypophthalmichthys molitrix*) differ significantly from those in indigenous planktivorous gizzard shad (*Dorosoma cepedianum*). The invasive species has much higher abundance of the order *Bacteroidales*, with the genera *Bacillariophyta* and *Clostridium* being significantly more abundant in native than invasive fish (Ye, Amberg, Chapman, Gaikowski, & Liu, 2014). It is not clear how the observed differences in gut bacteria contribute to invasiveness, especially the comparison of gut microbiota regarding host invasiveness has only been conducted in two different species (Fontaine & Kohl, 2020a; Qu et al., 2020). Research on gut microbiota variation between populations with different invasion characters in the same species would help to elucidate such patterns.

Host behavior appears to be more closely linked to the microbiota of the colon rather than that of the small intestine. From a mechanistic perspective, it is known that the microbial community in the colon can ferment complex carbohydrates, such as dietary fibre, into short chain fatty acids (SCFAs) that are known to have neuroactive properties (Dinan, Stilling, Stanton, & Cryan, 2015). Certain microbial species in the colon can cause changes to brain metabolites: in pigs, *Ruminococcus* spp. can influence brain N-acetylaspartate through the mediation of serum cortisol (Mudd, Berding, Wang, Donovan, & Dilger, 2017). In the cane toad, reactive nerve cell bodies (which may be caused by the different microbial

communities) were observed to be common in the submucosa of colon and less common in small intestine (Z. S. Li, Furness, Young, & Campbell, 1992). In addition to these explicit links to behavior, there is evidence that colon microbiota may have a greater effect on hosts than microbiota in other parts of the digestive system. The ‘stationary phase’ is the point at which the maintenance of the bacterial population size reaches equilibrium. Following regular nutrient provisioning, microbial communities replicate quickly and reach the stationary phase after only 20 minutes in the colon as compared to more than 2.2 hours in other organs of the digestive system. Microbiota also have longer transit times in the colon (10-60 hours versus less than 3 hours in all other digestive organs; Fetissov, 2017), increasing their opportunity to impact their host.

Intrinsic factors such as genetics, age, sex and parasite load can interact to maintain and shape the host’s gut microbiota community (Kozik, Nakatsu, Chun, & Jones-Hall, 2017; Org et al., 2016; Wang et al., 2016). In mice, significantly different microbiota are observed in narrow age ranges, such as between 4-5 weeks and 6-7 weeks (Kozik et al., 2017). Males and females can also possess different microbiota (in mice: Kozik et al., 2017, and cane toads: Zhou et al., 2020). Further, host health is associated with gut microbiota and the presence of some intestinal parasites can be predicted by studying gut microbial composition. For example, *Blastocystis* colonization is negatively associated with *Bacteroides* (Stensvold & van der Giezen, 2018). Because parasites are also known to impact host behaviors (Herbison, 2017; Klein, 2003), it is important to consider interactions of parasites and gut bacteria host behavioral variation.

Extrinsic environmental factors such as habitat and diet also influence the composition and variation of gut microbiota (Bletz et al., 2016; Carmody et al., 2015; Marques et al., 2010). Amphibians are good models to study these changes because they utilize both aquatic and

terrestrial habitats and their skin is permeable, making them highly sensitive to environmental variation (Hopkins, 2007). For example, the gut microbiota of fire salamander (*S. salamandra*) larvae differs depending on whether they live in ponds or streams (Bletz et al., 2016). Translocating animals to the opposite habitat type shifts their gut microbiota to resemble that of residents. Further, gut microbiomes can be similar across phylogenetically distinct, but sympatric amphibian species (such as *Fejervarya limnocharis* and *Babina adenopleura* inhabiting both farmlands and forests) and mirror environmental conditions (in both species bacterial diversity was higher in farmland populations; Huang, Chang, Huang, Gao, & Liao, 2017). Because diet is a key environmental factor affecting gut microbial community assemblages, these changes are likely to be influenced by differences in diet across environments (Scott, Gratz, Sheridan, Flint, & Duncan, 2013; Zmora, Suez, & Elinav, 2019). In summary, both environmental factors and host factors should be considered when studying intestinal microbiota variation.

Previously, drivers of variation in invasion-related behaviors have been studied, including genetics, morphology, habitat, diet, prior experience and parasites (Brown, Kelehear, Pizzatto, & Shine, 2016; Child, Phillips, & Shine, 2008; Hudson, Brown, & Shine, 2017; Selechnik et al., 2019; Stuart et al., 2019). However, there have been no investigations of gut microbiota as a potential driver of behavioral shifts across an invasive range in any species. Cane toads are an ideal system for these investigations because they display significant differences across Australia in behaviors likely to be related to increased capacity for dispersal ability; invasion front toads are bolder and more exploratory (Gruber, Brown, Whiting, & Shine, 2017; Stuart et al., 2019). A range of differences in dispersal-related morphology and performance metrics also have been shown: for example, wild-caught invasion-front toads have longer legs and toads with longer legs move greater distances (Benjamin L Phillips, Brown, Webb, & Shine, 2006) and toad morphology and performance

are linked (Hudson, Vidal-García, Murray, & Shine, 2020), endurance is greater in invasion-front adult toads held in captivity (Llewelyn, Phillips, Alford, Schwarzkopf, & Shine, 2010), invasion-front toads move in a more consistent direction than those from the range-core (Brown, Phillips, & Shine, 2014). However, the relationships between these traits are complex and may be affected by factors such as sex (Hudson et al., 2020) or rearing environment (Stuart et al., 2019). Understanding how microbiota may contribute to invasion success may be key to successful management of these species.

Here, we investigated the intestinal microbiome of invasive cane toads in Australia to determine whether there were significant differences in the identity or relative composition of gut microbiota in cane toads from range-core (i.e. the introduction site) versus invasion-front populations. We predicted that extrinsic and intrinsic factors would impact microbial communities, including environmental conditions and host parasite prevalence and intensity. We also investigated how microbial variation correlated with cane toad behavior. Given our knowledge of differences in cane toad behavior across the Australian range, we predicted that the microbiota of animals captured from the invasion-front populations would differ to that of the range-core populations, and that this would be correlated with their behavior. We discuss the potential implications of these impacts on invasion ability in this iconic invader.

Methodology

Study species, sample collection and behavioral assays

Cane toads (*Rhinella marina*) are large anurans (measuring up to 23 cm, 1.25 kg) introduced into Australia in 1935 as a biocontrol for pests of sugar cane crops (Shine, 2010). They are actively invading northern Australia and critically threaten many native predator species who are fatally poisoned when they attempt to eat them (Shine, 2010). We hand captured 60 wild

adult females from three sites at the invasion front edge in Western Australia ('invasion-front': Kununurra 15.776566° S, 128.744293° E, Old Theda 14.790795° S, 126.497624° E, Mary pool 18.72528° S, 126.870096° E. N=30, collected November 2018) and three sites in Queensland (QLD) ('range-core', Rossville 15.697069° S, 145.254385° E, Croydon 18.207536° S, 142.245702° E, Lucinda 18.530149° S, 146.331264° E. N=30, collected December 2018) for this study. The University of Adelaide Animal Ethics Committee approved all animals that had been used in this research (approval number: S-2018-056).

We conducted brief behavioral assays while collecting toads based on the methodologies described in a common-garden experiment on this species (Stuart et al., 2019) including: (1) struggle score (number of arm or leg kicks after being picked up until toad remains still for 5 seconds and struggle likelihood) and (2) righting behavior (within two minutes after the toad is placed on its dorsal side: time to right itself, number of limb kicks ('righting effort'), and righting effort likelihood). In that common-garden experiment, righting time was correlated with speed (r^2=0.09, p=0.004) and stamina (r^2=0.07, p=0.01), and righting effort was correlated with speed (r^2=0.05, p=0.02) after body size correction (Stuart, pers. comm.); these data demonstrate the relevance of the simple assays used here to traits more clearly linked to invasion potential in this species. After behavioral assays were conducted, we placed the animals into individual, moist, calico bags and weighed, measured (snout urostyle length; SUL) and euthanised them by injecting tricaine methanesulfonate (MS222) buffered with bicarbonate of soda to balance pH. After euthanasia, we used a clean surface and clean tools to dissect each animal. From each animal, we preserved 0.3cm of colon near the cloaca (including gut content) in 95% ethanol. We also collected data on two toad parasites: an encysted gut parasites (*Physalopterinae*: physalopterine larvae; Kelehear & Jones, 2010) and the co-introduced toad lungworm, which passes into the toad gut as eggs and then hatches into larvae in the gut (Baker, 1979). We examined the gut for evidence of the former. To

investigate the latter, we dissected and inverted the lungs (right lobe when facing the ventral side) and counted lungworms. Body size and parasite data are hereafter collectively referred to as 'host characteristics'.

Analyzing morphological and ecological data

We compared host characteristics, host behavioral traits and environmental factors between range-core and invasion-front toads. Within each of these data sets, we conducted correlation tests to exclude highly corrected variables for downstream analyses. Because body length (SUL) and weight were significantly correlated, we only included SUL in further analyses. For host characteristics and behavioral traits, we used SUL as a covariate in generalized linear models (GLM). We compared count and occurrence data (i.e. absence=0, presence=1) for host parasite infection and behavioral traits. To analyze occurrence data, we used GLM with logistic regression (R version 4.0.2, R Core Team, 2020) and with negative binomial regression *glm.nb* in the "MASS" package (Venables & Ripley, 2002) to analyze over-dispersed count data, when the conditional variance exceeded the conditional mean. We obtained environmental data for 19 bioclimatic variables for each sampling site from WorldClim (Table S1, Fick & Hijmans, 2017). We analyzed these data using the Shapiro-Wilk test to assess multivariate normality using "RVAideMemoire" package (Herve, 2020) and then we used a nonparametric Wilcoxon signed rank test to compare the median of the difference between groups because the data were not normally distributed.

DNA extraction, library preparation and sequencing

We extracted microbial DNA from colon contents following the manufacturer's protocol for the DNeasy PowerSoil kit (Qiagen) and prepared 16S rRNA amplicon libraries by following guidelines for the Illumina MiSeq System. We used Zymo isolated DNA (D6305) as a

community positive control and MilliQ water as a PCR negative control to identify environmental contamination during the library preparation process (Eisenhofer et al., 2019). Libraries were prepared based on the hypervariable (V3-V4) region of the 16S rRNA gene from each sample using primers 341F (5' – **TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGNGGCWGCAG-3') and 785R (5'-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACTACHVGGGTATCTAATCC-3') (Herlemann et al., 2011). The library preparation details have been reported previously (Zhou et al., 2020). The libraries were sequenced at the Ramaciotti Centre for Genomics (University of New South Wales, Kensington, Sydney) on the Illumina MiSeq platform targeting 2x300bp paired-end sequence reads.

Identifying and classifying bacteria community

We downloaded demultiplexed FASTQ data from Illumina's BaseSpace cloud storage, then created an amplicon sequence variants (ASVs) abundance table using the open-source QIIME2 pipeline (Bolyen et al., 2018). Demultiplexed sequence counts from samples and the positive control ranged between 138,989 and 305,662; the count of the PCR negative control was 67,506. The DADA2 pipeline (Callahan et al., 2016), implemented in QIIME2, was used to filter and trim the first 20 bases from each read and truncate sequences to 200 bases. The remaining sequences were dereplicated, then forward/reverse reads were merged, chimeras were removed, and finally ASVs were generated for downstream analysis (Callahan et al., 2016). After quality filtering, reads from colon samples and the positive control ranged between 103,245 and 245,059 counts; the PCR negative control yielded 6,727 reads. The taxonomic assignment of ASVs was performed using Greengenes version 13_8 (DeSantis et al., 2006). Data were pruned to remove representatives classified to Archaea (N = 28),

chloroplast (N = 17), mitochondria (N = 186), and 151 unassigned ("kingdom") ASVs implemented in the package 'phyloseq' (McMurdie & Holmes, 2013). We also removed the ASVs with prevalence of less than four, which makes the logged counts per sample more evenly distributed. The remaining 9,878 taxa were classified to the Kingdom Bacteria with 62.62% assigned to phylum level and 39.65% assigned to family level.

Characterizing diversity and microbial function of bacteria communities

We calculated observed ASVs (DeSantis et al., 2006), evenness (Pielou, 1966), and Shannon (Shannon, 1948) indices through QIIME2 for alpha diversity (a measure of diversity within individuals). We used a Wilcoxon signed-rank test to compare Shannon Index of toad microbiomes from different locations/sites and used a GLM model to check the correlation between Shannon Index and locations/sites with consideration of SUL as a covariate.

In order to explore the bacterial taxa that may contribute to the observed differences in community, the Core50 gut community (Bletz et al., 2016) was calculated as follows: the ASV table was filtered to include only the ASVs present in a minimum of 50% of individual toads from each site. This calculation was performed separately for three sites from invasion-front toads: Kununurra (gut Core50: n = 111 ASVs), Old Theda (gut Core50: n = 118 ASVs), and Mary Pool (gut Core50: n = 129 ASVs); three sites from range-core toads: Rossville (gut Core50: n = 148 ASVs), Croydon (gut Core50: n = 86 ASVs), and Lucinda (gut Core50: n = 117 ASVs). Then we subsequently compiled filtered ASVs of six sites to avoid excluding ASVs that may be specific to only one site. In combination, the gut Core50 contained 325 unique ASVs. We visualized relative abundance of bacteria (abundance >2%) in different sample types, classified to the phylum and family level.

To compare microbial communities between samples, we used a Hellinger transformation implemented in the package "microbiome" (Valverde, Makhalanyane, & Cowan, 2014) in R and then calculated and generated a weighted UniFrac dissimilarity matrix, which was visualized using Principal Coordinate Analysis (PCoA) plots using commands from the package "Phyloseq" (McMurdie & Holmes, 2013). We used the *adonis* command from the package "Vegan" to perform permutational multivariate analysis of variance (perMANOVA) to check whether the microbial communities of toads from each location were significantly different. We used the command *betadisper* in the package "Vegan" (Oksanen et al., 2019) to check the homogeneity of group variances, an assumption of perMANOVA. After finding significant differences between invasion-front and range-core toads, we performed pairwise comparisons between six sites from both locations using the command *pairwise.perm.manova* function in "RVAideMemoire" package with the Wilks Lambda (Nath & Pavur, 1985) and corrections for multiple testing (Herve, 2020) were conducted using the Hochberg procedure (Hochberg, 1988).

We used PICRUST2 (Douglas et al., 2019) to predict microbial functions for Core50 ASVs and generated pathway abundances considering the taxonomic contributions of ASVs. We removed all pathways with less than 0 prevalence and 474 out of 484 pathways remained for analysis. Then we used a Hellinger transformation and calculated and plotted Bray-Curtis dissimilarity matrix through package "Phyloseq". We performed permutational multivariate analysis of variance (perMANOVA) to check whether the pathway abundance of toads from each location and site were significantly different and we used the command *betadisper* to check the homogeneity of group variances.

Correlating intrinsic and extrinsic factors with host microbiota

To identify the association of individual host characteristics, host behavioral traits and

environmental factors with bacterial community and predicted microbial functions, statistical analysis was conducted using the function envfit in the package "Vegan" on Bray Curtis dissimilarities. Bray Curtis dissimilarities were visualized with non-metric multidimensional scaling (nMDS) plots. Host characteristics and behavioral traits were fitted to the ordination plots using the function envfit. Environmental variables that were significantly associated with microbiota taxonomy and microbial function were also fitted to the ordination plots using the function envfit and smooth surfaces of the same environmental factors were added on the nMDS plot with the ordisurf function in package "Vegan".

After analysing all variables individually, we also conducted distance-based redundancy analysis (dbRDA) combining all host characteristics and behavioral traits to identify relationships between variables with respect to their impact on gut microbiome. dbRDA performs constrained ordination directly on a distance or dissimilarity matrix with the function *capscale* in the "Vegan" package in R. We checked the correlation between each pair of traits. Bray-Curtis dissimilarity matrixes of ASVs and predicted microbial functions were ordinated and the results were analysed using redundancy analysis with constraining variables that were not highly correlated (correlation coefficient < 0.85 and > -0.85) to estimate their explanatory proportion. We did not include environmental factors in the *capscale* analysis because these data are not independent (toads from the same site have the same environmental factors).

Identifying differences in microbial taxa and their predicted microbial functions in range-core versus range front toads

To identify the significant differences in ASVs and predicted microbial functions between range-core and invasion-front toads, we performed differential abundance testing using the function *DESeq* in the package "DESeq2" (Love, Huber, & Anders, 2014). We report

significant differences as the log fold change of a taxa in range-core toads versus those from the invasion-front. We set ASVs and microbial functions abundance in invasion-front toads as the control group. Results for all statistical tests were considered significant where p-values <0.05.

Results

Morphological characteristics and parasite load in range-core versus invasion-front toads

The SUL (body length) of wild-caught invasion-front toads was longer than that of range-core toads (edge: mean = 103.51mm, SD = 9.42; core: mean = 96.24mm, SD = 12.5; t = -2.543, df = 53.897, p-value = 0.014). There was no significant differences in lungworm and gut parasite prevalence or intensity between the range-core and invasion-front toads we collected (Tables 1, S2): in all sampled toads, we report the number of lungworms (mean = 2.56, SD = 5.37), the occurrence of lungworms (mean = 0.52, SD = 0.50), the number of gut parasites (mean = 3.26, SD = 5.35) and occurrence of gut parasites (mean = 0.5, SD = 0.51).

Behavioral traits of range-core versus invasion-front toads

Range-core toads were found to have higher struggle scores when compared with invasion-front toads (Table 1, S2). We found range-core toads were more likely to struggle than invasion-front toads (core: mean = 0.83, SD = 0.37; edge: mean = 0.27, SD = 0.43; p-value = 0.008) and, in those who did struggle, the number of struggle movements was significantly higher for range-core toads than for invasion-front toads (core: mean = 2.63, SD = 2.52; edge: mean = 2.1, SD = 4.54; p-value = 0.002). The righting effort likelihood was higher for range-core toads than for invasion-front toads (core: mean = 0.73, SD = 0.45; edge: mean = 0.5, SD = 0.51; p-value = 0.036) (Tables 1, S2). The righting effort and righting time did not differ between range-core and invasion-front toads (Table 1, S2): across all sampled toads,

we report the righting effort (mean = 2.2, SD = 3.56) and righting time (mean = 18.88sec, SD = 41.32).

Environmental conditions experienced by range-core and invasion-front toads

Cane toads at the invasion-front experienced significantly hotter (six temperature measurements, see Table 1, S2 for statistics) and dryer (seven precipitation measurements, see Table 1, S2 for statistics) conditions year round than those in the range-core. Moreover, cane toads at the invasion-front experienced more changeable weather than those in range-core (three temperature variation measurement and one precipitation variation measurement, see Table 1, S2 for statistics). However, one temperature measurement (min temperature of coldest month: mean = 14.65, SD = 1.77) and one temperature variation measurement (isothermality, higher isothermality refering to more variable temperature: mean = 55.17, SD = 2.29) were not different in both environments included here (Table 1).

DNA sequence data quality

The number of observed species in the PCR negative control (N = 36) was much lower compared to that of toad colon samples (mean = 336, SD = 137.8), which indicated minimal contamination throughout the library preparation processes (Table S3). The number of observed species (N = 19) in the community positive control was also low compared to experimental samples. The eight species used as microbial community standards constituted more than 99.8% of ASVs from our sequenced community positive control, as shown in Figure S1, indicating minimal PCR bias in our library preparation process.

Comparison of microbial composition and diversity within individuals

The alpha diversity (diversity within individual toads) was not significantly different between range-core and invasion-front toads: observed species (*wilcox.test*, p-value = 0.631), Pielou's evenness (*wilcox.test*, p-value = 0.307), Shannon (*wilcox.test*, p-value = 0.230). Lucinda had the highest Shannon diversity, followed by Old Theda, Mary Pool, Rossville, Croydon and Kununurra (Figure S2B). Shannon diversity in individual samples was not correlated with measured behavioral traits (*glm*, covariate = SUL, p-values > 0.050).

Relative abundance of core50 microbiota in large intestine

A total of 89.23% ASVs were assigned to the level of phylum and dominant phyla (average abundance > 2%) were Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, and Verrucomicrobia (Figure S3, Table S4). We discuss the microbial functional significance of these groups in the discussion. Across all individuals, we found that: (1) the most dominant phyla were Firmicutes (average abundance = 47.89%, SD = 19.25%) and Bacteroidetes (average abundance = 39.05%, SD = 19.26%) (Table S4); (2) the phylum Bacteroidetes were consistently present in most individuals, except for two toads (Toad 14, 21) from the invasion-front and two toads (Toad 43, 48) from the range-core (Figure 1); and (3) there was one invasion-front toad (Toad 21) that showed a higher abundance of Proteobacteria (77.35%) when compared with other individuals (average abundance = 5.13%, SD = 7.99%).

A total of 70.77% ASVs were assigned to the family level and dominant families (average abundance > 2%) included Bacteroidaceae, Lachnospiraceae, Porphyromonadaceae, Clostridiaceae, Enterobacteriaceae, Erysipelotrichaceae, Fusobacteriaceae, Bacillaceae, Ruminococcaceae, Verrucomicrobiaceae, Turicibacteraceae, and Veillonellaceae (Figure S4, Table S5). Toad 21, who had a high abundance of Proteobacteria, also had a higher abundance of Enterobacteriaceae (77.34%) when compared with other individuals (average abundance = 4.55%, SD = 7.86%) (Figure S5).

A total of 40.62% ASVs were assigned to the genus level and dominant genera (average abundance > 2%) included *Bacteroides*, *Parabacteroides*, *Clostridium*, *Epulopiscium*, *Akkermansia*, *Turicibacter*, *Bacillus* (Figure S6, Table S6).

Comparison of microbial community taxonomy and microbial function between individuals with Core50 microbiota

Microbial taxonomic communities were significantly different between locations (Figure 2A, R^2 = 0.095, F = 6.092, p-value < 0.001). Microbial communities were marginally different between most sites (p-values < 0.050), except that Old Theda was not significantly different from Kununurra, Mary Pool, Croydon and Lucinda (Figure 2A). Among 230 ASVs that assigned to family level, there were 124 ASVs that significantly differed between range-core and invasion-front toads' colon (Table S7). The number of significantly different ASVs in each phylum were: Bacteroidetes (60 ASVs), Firmicutes (55 ASVs), Proteobacteria (7 ASVs), Actinobacteria (1 ASVs), Verrucomicrobia (1 ASV) (Table S7, Figure 4A).

Among the identified 474 predicted microbial functions, we found significant differences between invasion-front and range-core toads (Figure 2B; R^2 = 0.064, F = 4.110, p-value= 0.002). Pairwise tests between sampling sites indicated that toads from Kununurra had significantly different microbial functions to toads from Rossville (p-value= 0.009) and Lucinda (p-value = 0.046), and the other sites were not significantly different (p-values > 0.050) (Figure 2B). The top six most abundant pathways were pentose phosphate pathway (non-oxidative branch), adenosine deoxyribonucleotides de novo biosynthesis II, guanosine deoxyribonucleotides de novo biosynthesis II, pyruvate fermentation to isobutanol (engineered), Calvin-Benson-Bassham cycle, and adenine and adenosine salvage III (Figure 3). There were 84 out of 474 microbialfunctions that were significant different between invasion-front and range-core toads (Table S8, Figure 4B). Range-core toads have

significantly higher abundance of microbial function in superpathway of pyrimidine ribonucleosides degradation (log2FoldChange = 5.98) and lower abundance of microbial functions in phosphopantothenate biosynthesis III (log2FoldChange = -4.98), superpathway of sialic acids and CMP-sialic acids biosynthesis (log2FoldChange = -4.89) and factor 420 biosynthesis (log2FoldChange = -4.72) than invasion-front toads (Table S8, Figure 4B). Out of the top 30 abundant microbial functions, range-core have significantly lower abundance of microbial function in urate biosynthesis/inosine 5'-phosphate degradation (log2FoldChange = -0.10) than invasion-front toads (Figure 3, Table S8).

Associations between host, environment and intestinal microbiota

We assessed which host or environmental factors are strongly associated with microbiota taxonomy and microbial function. When comparing the association between individual host characteristics and behaviors with microbiota variation, only the occurrence of lungworms was significantly associated with the microbial taxonomic community (R^2 = 0.128, p-value = 0.023) (Tables S9, S10; Figure S7A, B). Among the 21 environmental factors investigated here, longitude (R^2 = 0.194, p-value = 0.001), isothermality (R^2 = 0.193, p-value = 0.002), and mean temperature of driest quarter (R^2 = 0.146, p-value = 0.015) were strongly associated with microbial taxonomic community. The mean temperature of driest quarter (R^2 = 0.187, p-value = 0.004) and isothermality (R^2 = 0.175, p-value = 0.007) were also strongly associated with microbial functions (Table S10). Because longitude is a proxy for sampling region, we only present mean temperature of driest quarter and isothermality in figure S7C, D. Host characteristics and behaviors are independent measurements, yet environmental factors are the same for all 10 toads from each site. The variable isothermality was identified as having a possible correlation with microbial taxa and microbial functions at the range of 54.5 – 57.5 (Figures S7C, D). The contour lines of mean temperature of driest quarter show

less linear relationship than it does for isothermality with microbial taxa, which is further supported by envfit results (Figure S7C). The contour lines of mean temperature of driest quarter show the strongest linear relationship with microbial functions (Figure S7D). However, the deviance explained by this ordisurf model is the lowest (18.3%) compared to other three ordisurf models (isothermality x microbial taxa, isothermality x microbial functions, and mean temperature of driest quarter x microbial taxa).

We calculated correlations between the factors that were significantly associated with microbial taxa and microbial functions as: (1) the occurrence of lungworms is correlated with isothermality (t = 2.3234, df = 58, p-value = 0.02369, coefficient = 0.292, method = "pearson"), (2) the occurrence of lungworms is not correlated with mean temperature of the driest quarter (t = 1.0534, df = 58, p-value = 0.2965, coefficient = 0.137), (3) isothermality is highly correlated with mean temperature (t = 8.3496, df = 58, p-value = 1.594e-11, coefficient = 0.739).

We used a redundancy analysis to assess the association of all host variables. The best model which was associated with the most variation in the microbiota taxonomic community included only the occurrence of lungworms (AIC = 178.58), whereas the best model which was associated with the most variation in predicted microbial functions included righting effort likelihood (AIC = 53.613), the occurrence of lungworms (AIC = 54.297) and righting time (AIC = 56.912). The combination of these three factors was associated with 17.8% of variation in the predicted microbial functions (Figure 5).

Discussion

Our research is the first of its kind to investigate how the gut microbiome of an invasive species changes 1) as a function of intrinsic (host) and extrinsic (environmental) factors, 2)

with invasion context (established resident versus invading populations), and 3) importantly, with relation to aspects of animal behavior that influence dispersal, and thus wider invasion success. There is growing acknowledgement that microbiota influences animal behavior in diverse ways (Davidson, Cooke, Johnson, & Quinn, 2018; Davidson et al., 2020) yet until now, the links between microbiota and behaviors that influence invasiveness remain unexplored. We found that whilst the alpha diversity in microbiota was similar in the invasion-front and range-core individuals, there were significant differences in both the composition and microbial function of gut microbiota in toad populations across the Australian invasive range. Microbial functions better explain cane toad righting behavior than does microbial composition. Interestingly, the presence of the co-introduced parasitic lungworm and environmental factors related to temperature could contribute to the variation of both gut microbial community composition and microbial functions, highlighting the importance of studying how these factors interact.

Microbial taxa

Four phyla (Firmicutes, Bacteroidetes, Proteobacteria, and Fusobacteria) were found to be dominant (Mean > 2%) in toad samples from both ends of the range, similar to results of a previous study of cane toads from both of these regions held in captivity in a common facility (Zhou et al., 2020). The previous three phyla are commonly observed as dominant in the intestine of frogs and toads (Chang, Huang, Lin, Huang, & Liao, 2016; Kohl, Amaya, Passement, Dearing, & McCue, 2014; Wiebler, Kohl, Lee, & Costanzo, 2018), which suggests these three phyla may play important roles for maintaining the health of toads, and that these taxa are not dependent on particular host or environmental conditions. Even though not described as dominant phyla, Fusobacteria were found in the intestine of other frogs and toads (Chai, Dong, Chen, & Wang, 2018; Kohl et al., 2014). Fusobacteria are dominant phyla

in the fish intestine (Colombo, Scalvenzi, Benlamara, & Pollet, 2015; Kohl et al., 2014) and Fusobacteria (*Cetobacterium*) were previously reported to be associated with changes in immunity of Cuban tree frogs (Knutie, Wilkinson, Kohl, & Rohr, 2017). Interestingly, we found a higher abundance of the phylum Verrucomicrobia in range-core toads than in toads from the invasion-front (core: mean = 3.98%, SD = 7.39%; edge: mean = 1.29%, SD = 2.62%) (Table S4, Figure S3). Species within the phylum Verrucomicrobia are commonly found in the intestinal microbiota of frogs or toads (Jiménez & Sommer, 2017; J. Li et al., 2019) and one dominant genus within this phylum in anurans is *Akkermansia* (Chai et al., 2018; Kohl, Cary, Karasov, & Dearing, 2013), which is also identified in range-core wild-caught toads (Figure S6, Table S6). One particular species (*A. muciniphila*) is common in the human intestinal tract, specializing in the degradation of intestinal mucins (Derrien, Collado, Ben-Amor, Salminen, & de Vos, 2008) and therefore often considered a to be associated with a 'healthy' metabolic status in humans (Dao et al., 2016). The reduction in members of the phylum Verrucomicrobia in invasion-front toads could be a result of an altered habitat or different available dietary options. Previously, members of the phylum Verrucomicrobia were not found to be dominant in the gut of captive toads from the invasion-front and range-core (Zhou et al., 2020).

Range-edge cane toads, despite their ability to spread rapidly, are known to experience health problems, such as severe spinal arthritis (Brown, Shilton, Phillips, & Shine, 2007). Emerging research in humans has highlighted the association of the gut microbiome on inflammation and the development of musculoskeletal disorders such as arthritis which has a significant impact on mobility (Szychlinska, Di Rosa, Castorina, Mobasheri, & Musumeci, 2019). In the study of cane toad skin microbiota, a higher proportions of bacteria with antifungal properties (fungal pathogen: *Batrachochytrium dendrobatidis*-inhibitory) were detected in toads from range-core than those from invasion-front, which known to be shaped by site-specific

pathogen pressures (Weitzman et al., 2019). Interestingly, even though cane toads are known to eat their skins, the 14 core *Batrachochytrium dendrobatidis*-inhibitory bacteria found on cane toad skin were not observed in its core gut bacteria, except family Enterobacteriaceae (*Bufotyphonius*-inhibitory_9).

We found significant differences in the diversity and composition of microbial taxonomy between range-core and invasion-front toads even though their dominant phyla and alpha diversity were similar. ASVs in the family Veillonellaceae were found to be significantly higher in invasion-front toads, compared to range-core toads (Figure 4A). ASVs in this family have been found to influence host metabolic regulation. For example, in an experiment that altered air temperature for Brandt's voles (*Lasiopodomys brandtii*); in colder temperatures, voles which huddled, had a higher abundance of Veillonellaceae as well as higher concentrations of short-chain fatty acids (SCFAs) in their intestines when compared with non-huddling voles (Zhang et al., 2018). This family is known to produce SCFAs, such as propionic acid (Gamage et al., 2017; Louis & Flint, 2017), and then contribute to increased locomotor activity (Thomas et al., 2012). The relationship between the bacterial members of this family and host metabolic regulation, suggests that invasion-front toads may use the SCFAs to fuel their invasion by producing SCFAs, such as propionic acid and increasing locomotor activity (Llewelyn et al., 2010). ASVs from another family Clostridiaceae (Hugenholtz et al., 2018) that belongs to SCFA-producing bacteria were also higher in invasion-front toads than those from the range-core. Furthermore, the family Veillonellaceae may be associated with host sociality. A significant reduction of Veillonellaceae has been observed in children with Autism Spectrum Disorder, often known for desiring social isolation, when compared to a neurotypical group of children (Kang et al., 2013). Higher abundance of Veillonellaceae in invasion-front toads could foster their "bolder" personality,

retaining a higher propensity for exploration and risk-taking (González-Bernal, Brown, & Shine, 2014; Gruber et al., 2017).

Several other ASVs that differed across the cane toad's range have previously been related to behavior. ASVs from family Peptococcaceae were found to be significantly higher in invasion-front toads than in range-core ones (Figure 4A). Peptococcaceae have been found to be related with host neurotransmitter (noradrenaline linking visual awareness to external world events, (Gelbard-Sagiv, Magidov, Sharon, Hendler, & Nir, 2018): for example, Peptococcaceae in the caecum showed a significantly positive correlation with noradrenaline levels in mice (Houlden et al., 2016). ASVs from family Bacillaceae were found to be significantly lower in invasion-front toads than range-core (Figure 4A). Bacillaceae have been found to be related to host anxiety: for example, this species was found in higher abundance in methamphetamine-treated rats versus the control (Ning, Gong, Xie, & Ma, 2017) and in higher abundance in excercised mice versus sedentary group (Choi et al., 2013). Higher abundance of Bacillaceae could contribute to higher anxiety-like behaviors thus heightening the stress response (Ning et al., 2017) and may be correlated with a decreased exploratory behavior in new environments (Golla, Østby, & Kermen, 2020). In summary, invasion-front toads possessed gut microbial communities that in other studies have been associated with SCFAs production and neurotransmitters, suggesting the potential to increase locomotor ability, visual awareness to external world events and propensity for exploration and risk-taking. In comparison, range-core toads possessed taxa are associated with higher anxiety-like behavior, suggesting decreased propensity to explore.

Predicted microbial functions

Variation in the predicted microbial functional groups observed between locations and cites was less obvious than in community composition (Figure 2). Other studies have found similar

results, suggesting that microbial function is more likely to be preserved than composition (e.g. in fire salamanders, Bletz et al., 2016). This could result from different gut microbiota harboring similar microbial functions and may contribute to increasing resilience and persistence of microbial functional stability of gut microbiota (Bletz et al., 2016; Huang et al., 2017; Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012). For invasive species, this can also contribute to its adaptive potential in novel environments. We found significant differences in microbial functions between range-core and invasion-front toads even though their most abundant microbial functions were similar. There are significant differences in microbial functional pathways that contribute to the microbe food sources and metabolism. As for microbe food sources, invasion-front toads have significantly lower abundance of microbial function in the superpathway of pyrimidine ribonucleosides degradation. This pyrimidine ribonucleosides degradation provides nitrogen source for microbes (West, 1996) and plays an important role in perturbations in the uridine monophosphate (UMP) biosynthetic pathways. This allows the bacterial cell to sense signals such as starvation, nucleic acids degradation, and availability of exogenous pyrimidines, and to adapt the production of the extracellular matrix to the changing environmental conditions (Garavaglia, Rossi, & Landini, 2012). This may be related to the disappear of Verrucomicrobia as a dominant taxon. As for microbe metabolism, invasion-front toads have a higher abundance of microbial functions in factor 420 biosynthesis; cofactor 420 is critical to bacterial metabolism and mediates a variety of important redox transformations involved in bacterial persistence, antibiotic biosynthesis, pro-drug activation and methanogenesis (Bashiri et al., 2019). Moreover, there are significant differences in microbial functional pathways that contribute to host health. Invasion-front toads have higher abundance of microbial functions that are beneficial to general host health and immunity: (1) phosphopantothenate biosynthesis, which is the first step for the bacteria production of coenzyme A (CoA), an indispensable enzyme

cofactor for all living organisms (Sibon & Strauss, 2016); and (2) superpathway of sialic acids biosynthesis, which generates sialic acids that play multifarious roles in immunity including acting as host receptors and pathogen decoys for viruses and bacteria (Varki & Gagneux, 2012) and are especially critical for preventing neural tissue damage (Liao, Klaus, & Neumann, 2020). Despite this higher abundance of microbial functions that could enable invasion-front toads to expand their range, there were some indications that these toads may also face some health challenges. Invasion-front toad microbiota have significantly higher abundance of urate biosynthesis function (urate biosynthesis/inosine 5'-phosphate degradation, the only significantly different one out of the top 30 abundant functions), which plays a significant, but modest, role in determining variation in serum urate levels (Sinnott-Armstrong, Naqvi, Rivas, & Pritchard, 2020). Excessively high levels of urate can result in the formation of needle-like crystals of urate in the joints (gout), which may be related to the finding that invasion-front cane toads have a higher incidence of severe spinal arthritis thought to be caused by high levels of movement in combination with morphological differences found in that population (Brown et al., 2007).

Associations between gut microbiota, environmental isothermaliy and host lungworm

Higher environmental isothermality increased the prevalence of cane toad lungworms, and both factors could contribute to changes in the cane toads' colon microbiota. It is interesting that even though environmental isothermality and lungworm infection intensity are not significantly different between range-core and invasion-front toads, environmental isothermality and occurrence of lungworms are significantly associated with the microbial taxonomic community and predicted microbial function. Environmental temperature can alter microbial taxonomic community and predicted microbial function, resulting in increased phenotypic plasticity and persistence in harsh conditions (Fontaine & Kohl, 2020b). For

example, gut microbiota of invasion bullfrog tadpoles responds more rapidly to environmental temperature than noninvasion green frogs (Fontaine & Kohl, 2020b). Environmental temperature alters the digestive performance and gut microbiota of red-backed salamanders (*Plethodon cinereus*: Fontaine, Novarro, & Kohl, 2018) and in a study of western clawed frogs (*Xenopus tropicalis*) the authors observed a higher prevalence of Verrucomicrobia in the gut of frogs inhabiting a warmer environment, and an opposite pattern in Proteobacteria (J. Li et al., 2019). The effects of temperature on Verrucomicrobia, Proteobacteria, and Akkermansia observed in that study was consistent with previous studies in mammals (J. Li et al., 2019). Pathogens and parasites impact the composition of the host microbiota and have also been shown to modify host behavior in a manner that improves their probability of transmission and survival (Gegear, Otterstatter, & Thomson, 2006; House, Vyas, & Sapolsky, 2011; Poulin, 2010). Lungworms can affect cane toad locomotor performance and reduced host endurance, presumably because of the reduced oxygen supply from infected lungs (Pizzatto & Shine, 2012). Lungworms are also known to alter a cane toad's thermal preference and manipulate the timing and location of defecation, thereby enhancing lungworm egg production and larvae survival (Finnerty, Shine, & Brown, 2018). Lungworms are reported to lag behind their host on the invasion front by 2-3 years (Ben L Phillips et al., 2010) and to affect righting behavior (prolongs righting time; Finnerty, 2017). In the current study, although we collected invasion-front toads in areas where toads had been present for less than one year, we found no difference in lungworm presence or intensity between the invasion-front and range-core toads, nor did we find differences in righting times between these populations. In combination, these data suggests a need for further studies to determine how isothermality and occurrence of lungworms may affect cane toad gut microbiota variation, and whether this influences toad behavior.

Associations between gut microbiota and host righting reflex behavior

Interestingly, behaviors including righting effort likelihood and righting time were not associated with the gut microbial taxonomy composition but were associated with predicted microbial functions. It is possible that multiple identified taxa share the same microbial function or that one taxa contributes to multiple microbial functions, which might obscure the relationship between behavior and taxonomic composition. Nevertheless, these relationships we found between gut microbial function and righting behaviors may be related to toads' health and/or rearing conditions. A dampened stress response (lower level of corticosterone) in invasion-front toads exposed to stressors (Brown, Kelehear, Shilton, Phillips, & Shine, 2015) could be a result of higher abundance of microbial functions that are beneficial to general host health and immunity, especially the superpathway of sialic acids biosynthesis, which generates sialic acids that are especially critical for preventing neural tissue damage (Liao et al., 2020). Further, invasion-front toads respond less to stressful stimuli and to be more reluctant to flee than those from range-core toads in simulated predation trials, exhibiting "bold" anti-predator responses when exposed to novel predators (Hudson, et al., 2017). Studies showed that dampened stress responses are related to higher propensity for exploratory behavior (Golla et al., 2020), which has been linked to dispersal ability (Gruber et al., 2017). Rearing conditions also have been shown to affect righting behavior: juvenile toads whose parents were collected on the invasion-front that were raised in high exercise regimes in a common-garden setting righted themselves more quickly than those whose parents were collected from the range core, whereas the opposite trend was seen in controls (Stuart et al., 2019). It is also possible that the lower likelihood of righting effort we found in invasion-front toads could be caused by the higher incidence of severe spinal arthritis (Brown et al., 2007) described above, which could be related to the higher abundance of microbial functions (urate biosynthesis function) that contribute to the formation of needle-like crystals of urate in the joints (Sinnott-Armstrong et al., 2020). Although manipulative studies are

needed to clarify causal relationships between stress responses, proactive behaviors, and gut microbial functions, our results indicate that behavior and toad colon microbial functions are related, suggesting that gut microbiome should be considered as a potentially important driver of invasion.

Conclusion

In this study, we observed significant differences in the microbial community and predicted microbial functions between range-core and invasion-front Australian cane toad gut microbiota. Both environmental factors (isothermality and mean temperature of driest quarter) and host factors (occurrence of lungworms) were significantly associated with these differences. Also, we found that behavioral traits were not associated with the gut microbiota taxonomy, but were associated with their predicted microbial functions. Owing to the predictive nature of PICRUSt and its use here with a non-model host, metagenomic or transcriptomic sequencing will be an important future research direction for verifying our results related to gut microbial function (Bletz et al., 2016). In model organisms, the gut microbiome has been shown to impact host metabolic activity, brain function and the pathogenesis of disease. Our research demonstrates that the cane toad gut microbiota are linked to behaviors that may be important to invasion, providing a new perspective of species invasions. Manipulative experiments (e.g. fecal transplants) coupled with longer term behavioral measurements would be useful to clarify relationships between microbiota and host behaviors related to invasion ability in this species.

Data Accessibility Statement

All the data and supporting information will be made available online.

1. Supplementary Figures S7.
2. Supplementary Tables S11.

The sequence data that support the findings of this study are openly available in NCBI Sequence Read Archive (accession number: PRJNA670039). The raw data of host, environmental and behavioral measurements, and correlation tests of all factors will be made available on Dryad.

Author Contributions

1. Designed research: JZ, TMN, CRL, SJZ, LAR
2. Performed Research: JZ, CRL, GWF, KS, LAR
3. Analyzed data: JZ, TMN, CRL, SJZ, KS, LAR
4. Wrote the paper: JZ, TMN, CRL, SJZ, GWF, KS, LAR

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Tables and Figures

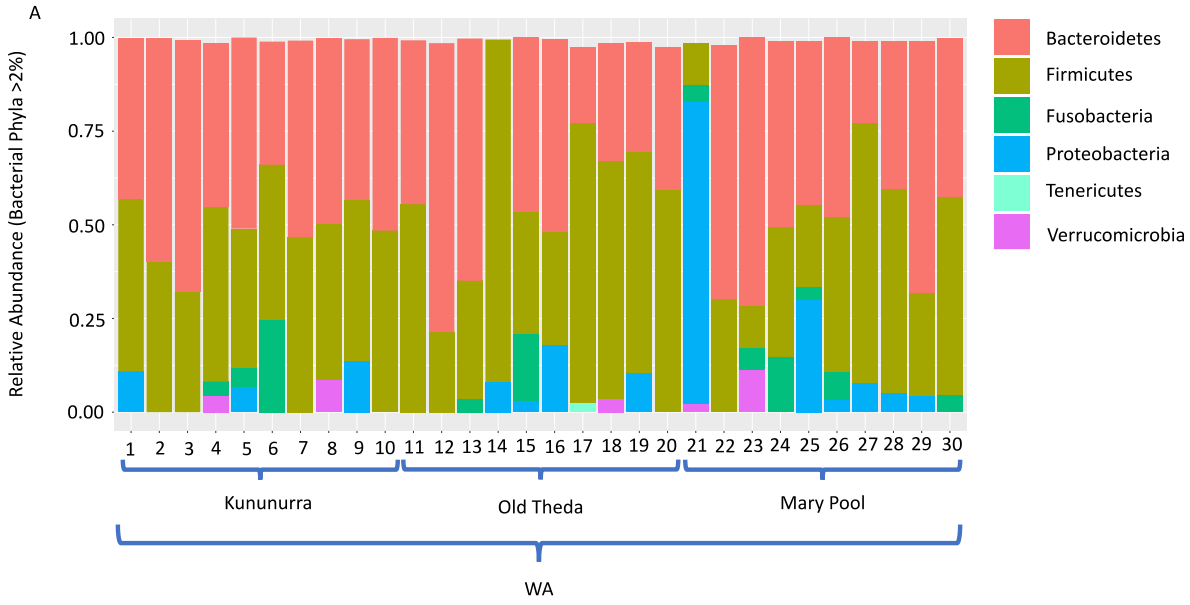
Table 1. Comparison of host characteristics, behavioral traits and environmental factors between range-core and invasion-front toads.

Variables		wilcox.test	GLM (SUL as covariate)		
			glm.nb	glm	binomial
Host characteristics	Body length (SUL)	_	_	0.01099 *	_
	Lungworms	_	0.8600	_	_
	Occurrences of lungworms	_	_	_	0.07783 .
	Gut parasites	_	0.817578	_	_
	Occurrences of gut parasites	_	_	_	0.604488
Behavioural traits	Struggle score	_	0.002196 **	_	_
	Struggle likelihood	_	_	_	0.008477 **
	Righting effort	_	0.3203	_	_
	Righting effort likelihood	_	_	_	0.03569 *
	Righting time	_	0.5067	_	_
Environmental factors	Longitude	1.596e-11***	_	_	_
	Latitude	0.458	_	_	_
	Annual Mean Temperature	1.608e-07 ***	_	_	_
	Mean Diurnal Range	0.0001836 ***	_	_	_
	Isothermality	0.4515	_	_	_
	Temperature Seasonality	0.02501 *	_	_	_
	Max Temperature of Warmest Month	1.608e-07 ***	_	_	_
	Min Temperature of Coldest Month	0.458	_	_	_
	Temperature Annual Range	0.0001836 ***	_	_	_
	Mean Temperature of Wettest Quarter	1.608e-07 ***	_	_	_
	Mean Temperature of Driest Quarter	0.02099 *	_	_	_
	Mean Temperature of Warmest Quarter	1.608e-07 ***	_	_	_
	Mean Temperature of Coldest Quarter	0.0001836 ***	_	_	_
	Annual Precipitation	1.608e-07 ***	_	_	_
	Precipitation of Wettest Month	1.608e-07 ***	_	_	_

Precipitation of Driest Month	8.069e-12 ***	_	_	_
Precipitation Seasonality	0.02501 *			
Precipitation of Wettest Quarter	1.608e-07 ***	_	_	_
Precipitation of Driest Quarter	5.222e-06 ***	_	_	_
Precipitation of Warmest Quarter	1.596e-11 ***	_	_	_
Precipitation of Coldest Quarter	5.222e-06 ***	_	_	_

Note

Negative binomial regression (glm.nb) was used for over-dispersed count data, that is when the conditional variance exceeds the conditional mean. Shapiro-wilk test to assess multivariate normality using RVAideMemoire package in R (Herve, 2018). If it is below 0.05, the data significantly deviate from a normal distribution. Wilcox.test used for the data that's not normally distributed and ttest for the data that's normally distributed. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1. SUL: snout urostyle length (body length).



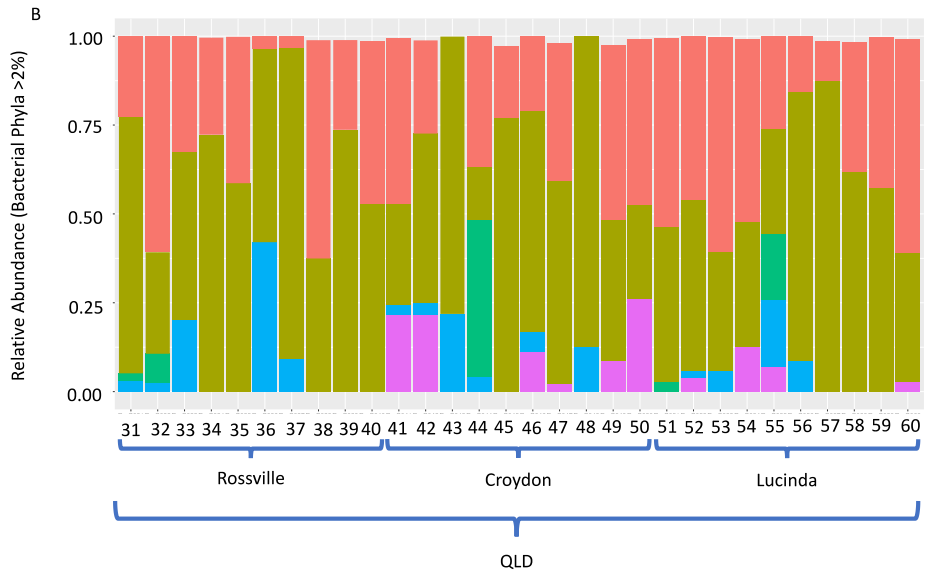
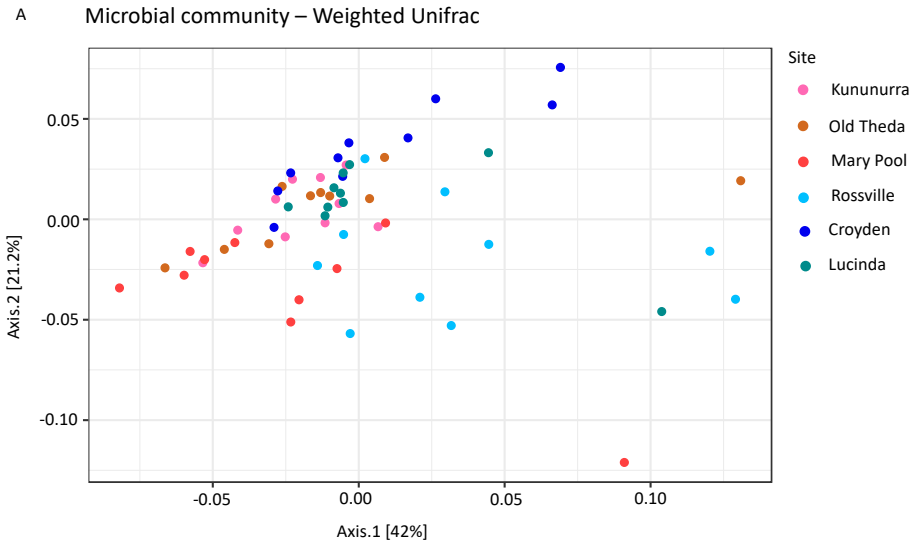


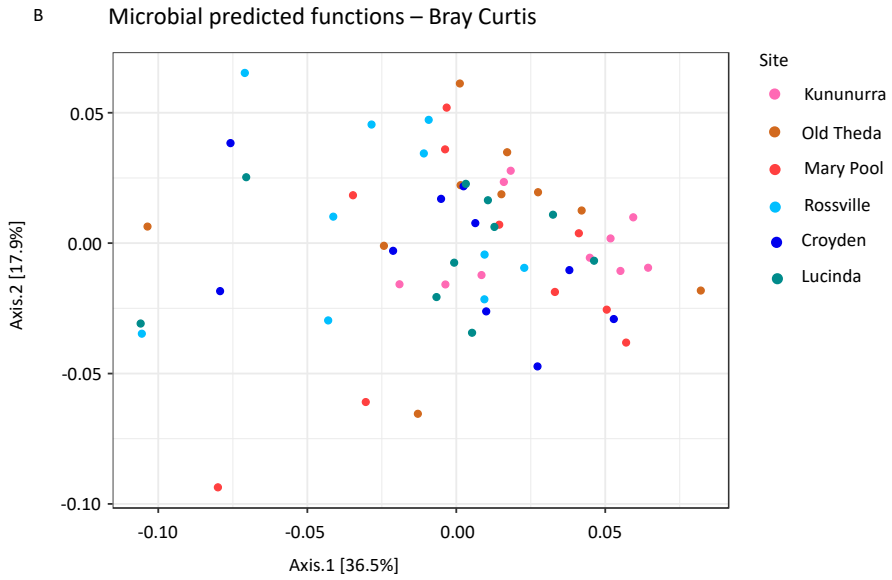
Figure 1. Core bacterial community composition of different individual toads. Relative abundance plot shows phyla (>2%). WA: invasion-front; QLD: range-core.



Pairwise comparisons using permutation MANOVAs on a weighted UniFrac distance matrix of Core50 microbiota

	Kununurra	Old Theda	Mary Pool	Rossville	Croydon
Old Theda	0.0608	-	-	-	-
Mary Pool	0.0045	0.0584	-	-	-
Rossville	0.0011	0.0340	0.0175	-	-
Croydon	0.0020	0.0608	0.0011	0.0011	-
Lucinda	0.0011	0.1352	0.0011	0.0324	0.0175

P value adjustment method: Hochberg (1988)



Pairwise comparisons using permutation MANOVAs on a Bray Curtis distance matrix of Core50 microbial functions

	Kununurra	Old Theda	Mary Pool	Rossville	Croydon
Old Theda	0.298	-	-	-	-
Mary Pool	0.298	0.298	-	-	-
Rossville	0.009	0.298	0.298	-	-
Croydon	0.105	0.298	0.298	0.298	-
Lucinda	0.046	0.298	0.298	0.298	0.298

P value adjustment method: Hochberg (1988)

Figure 2. Beta diversity by locations and sites. Principle coordinate analysis plot of weighted UniFrac distance of microbial community (R^2 = 0.095, F = 6.092, p-value < 0.001) (A) and Bray Curtis distance of predicted functional groups (R^2 = 0.064, F = 4.110 , p-value= 0.002) (B) from 60 cane toad individuals of the invasion-front (Kununurra, Old Theda, and Mary Pool) and the range-core (Rossville, Croydon, and Lucinda). Pairwise comparisons were conducted on both distance matrixes using permutation MANOVAs. P values were adjusted with Hochberg method.

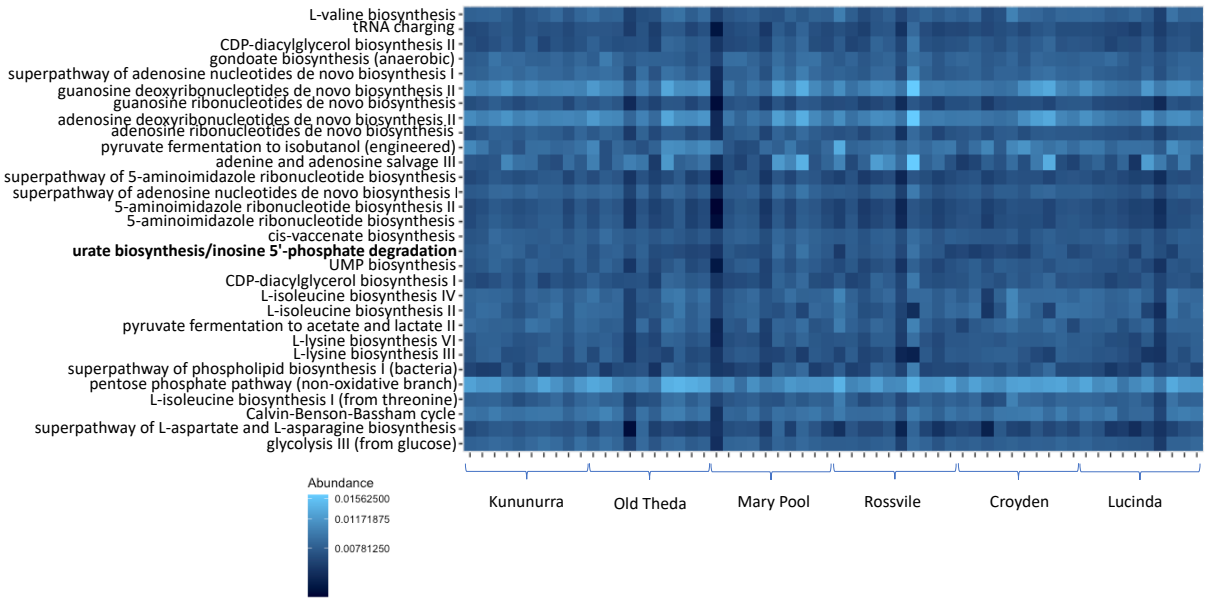
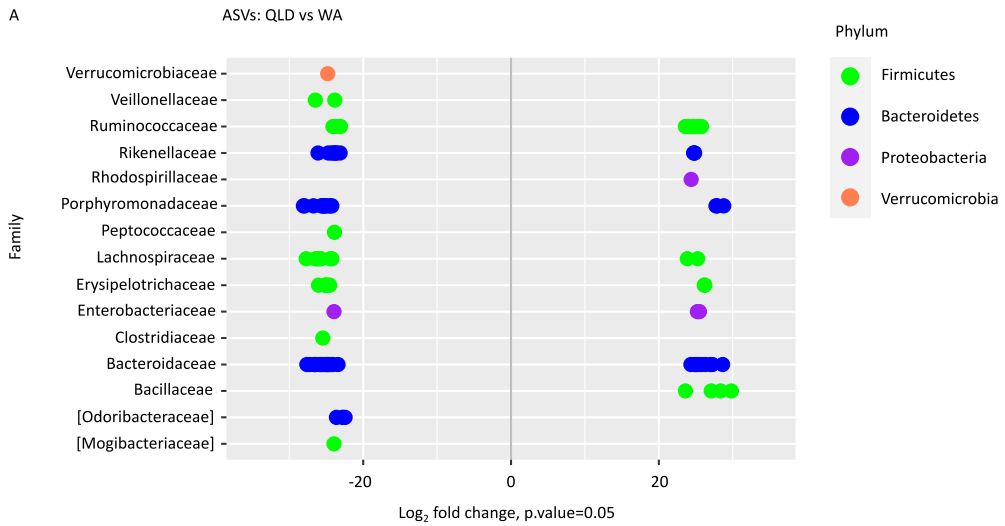


Figure 3. Heatmap for top 30 functional group abundance.
Heatmap indicates the top 30 functional groups in the intestinal samples from range-core and invasion-front toads. Abundance indicates the raw count of functional groups inferred from taxonomic 16S sequences using PICRUSt where light blue is high abundance and dark blue is lower abundance. Functional pathways that differ significantly between range-core and invasion-front toads are highlighted in bold.



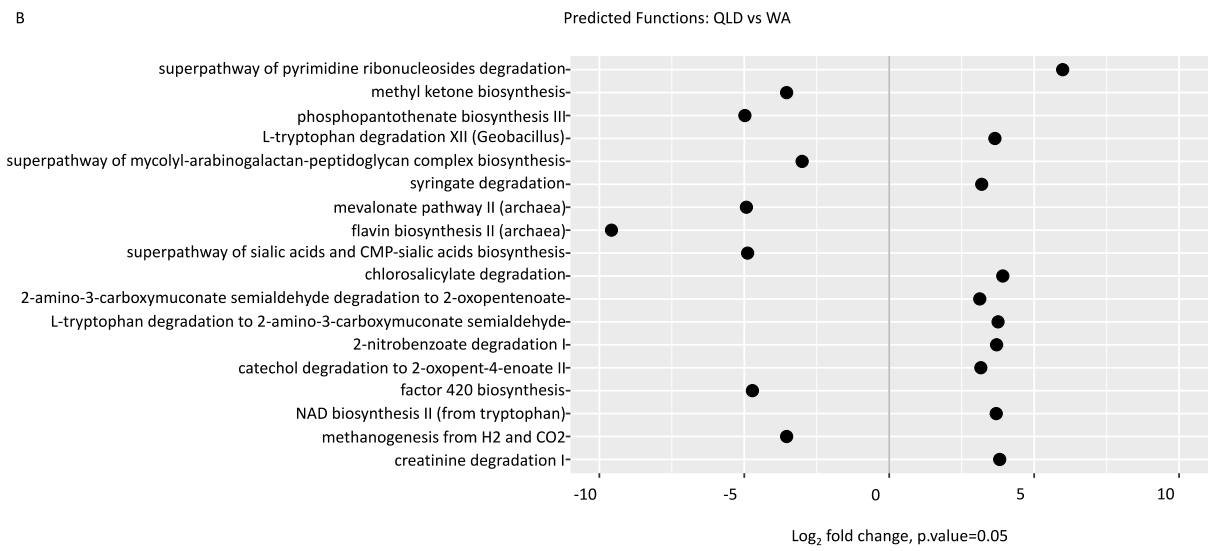


Figure 4. Significant different bacterial taxa and predicted functions between range-core (QLD) and invasion-front (WA) toads' colon. Significant differences were identified between locations via differential abundance testing based on a negative binomial distribution. The dots represent the average log-2 fold change (x axis) abundance and positive log_2 fold changes signify increased abundance in range-core, and negative log_2 fold changes display increased abundance in invasion-front. Bacterial taxa (A) were classified to the taxonomic level of family (y axis) and coloured by taxonomic level of phylum. Family name in bracket is proposed taxonomy by Greengenes. Only ASVs that could be matched to a known bacterial family and with a log2FoldChange value higher than 20 or lower than -20 are presented. Predicted functions (B) with a log2FoldChange value higher than 3 or lower than -3 are presented.

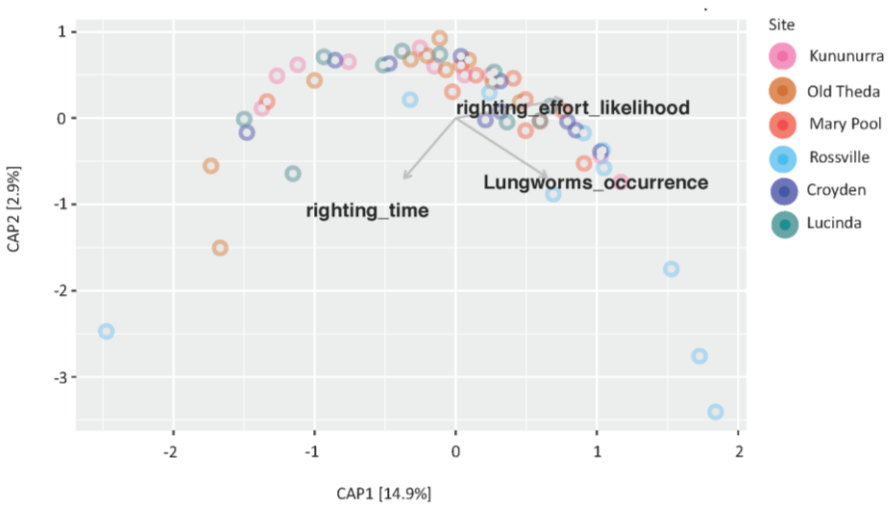


Figure 5. Main variables that affect predicted function differentiation among individuals. CAP (*capscale*) plot displays the combination of variables that explained the greatest variation in the predicted functions through model selection, using 60 cane toad individuals from the invasion-front (Kununurra, Old Theda, and Mary Pool) and the range-core (Rossville, Croydon, and Lucinda). The final model explained 17.8% of variation in the microbial predicted functions, which includes righting effort likelihood (AIC = 53.613),

occurrence of lung worms (AIC = 54.297) and righting time (AIC = 56.912) explained the greatest variation.