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A comparison of non-lethal sampling methods for amphibian gut microbiome analyses

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Abstract

Non-invasive sampling methods for studying intestinal microbiome are widely applied in studies of endangered species and in those conducting temporal monitoring during manipulative experiments. Although existing studies show that non-invasive sampling methods among different taxa vary in their accuracy, to date, no studies have been published comparing non-lethal sampling methods in adult amphibians. In this study, we compare microbiomes from two non-invasive sample types (faeces and cloacal swabs) to that of the large intestine in adult cane toads, *Rhinella marina*. We use 16S rRNA sequencing to investigate how microbial communities change along the digestive tract and which non-lethal sampling method better represents large intestinal microbiota. We found that cane toads' intestinal microbiota was dominated by Bacteroidetes, Proteobacteria, Firmicutes and, interestingly, we also saw a high proportion of Fusobacteria, which has previously been associated with marine species and changes in frog immunity. The large and small intestine of cane toads had a similar microbial composition, but the large intestine showed higher diversity. Our results indicate that cloacal swabs were more similar to large intestine samples than were faecal samples, and small intestine samples were significantly different from both non-lethal sample types. Our study provides valuable information for future investigations of the cane toad gut microbiome and validates the use of cloacal swabs as a non-lethal method to study changes in the large intestine microbiome. These data provide insights for future studies requiring non-lethal sampling of amphibian gut microbiota.

Keywords: amphibian, cane toad, gut microbiota, Illumina, 16S rRNA

Introduction

Accumulating evidence indicates that variation in gut microbial assemblages can significantly affect the host phenotype (Mu, Yang, & Zhu, 2016; Stilling, Dinan, & Cryan, 2014). However, the majority of the work investigating this idea has been descriptive in nature and limited to the identification of correlative associations between microbial community assemblages and phenotypic traits of hosts. The identification of causality requires the use of experimental interventions (e.g., antibiotic administration, faecal microbiota transfer, co-housing, and cross-fostering or rederivation) (Ericsson & Franklin, 2015) that enable the comparison of the phenotypic trait of interest in response to manipulations of intestinal microbiota in the same individuals across time. Therefore, non-lethal methods are needed to further this field of research by providing representative information to enable the assessment of the intestinal microbiota before and after interventions.

Faecal samples and cloacal swabs are two commonly used non-lethal sampling methods; however, the accuracy with which each sample represents the intestinal microbiota is often untested (Bassis et al., 2017). In different hosts, these methods may differ in their representation of the intestinal microbiota, yielding distinct compositions of bacterial taxa. In birds, faecal samples have been shown to provide a more accurate assessment of the large intestinal (colon) microbiota than cloacal samples (Videvall, Strandh, Engelbrecht, Cloete, & Cornwallis, 2018). In another study of birds testing only swabs, the authors found that cloacal swab samples shared similar microbial species with large intestinal (caecal) samples, but displayed different relative abundances (Stanley, Geier, Chen, Hughes, & Moore, 2015). In lizards, faecal samples were very similar to the large intestinal (hindgut) microbiota, yet were less representative of the stomach or small intestinal microbiota (Kohl et al., 2017); in bats, the faecal sample microbiota did not provide an accurate representation of large intestinal microbiota (Ingala et al., 2018). Faeces has been demonstrated to be a robust proxy for the gut microbiota in tadpoles (Song, Song, Song, Zeng, & Shi, 2018). In summary, different species show different patterns regarding the comparison of the large intestinal microbiota to different non-lethal sampling methods and there are no studies in adult amphibians.

Cane toads are one of the most successfully invasive species globally. In Australia, cane toads have spread from the original sites of introduction in Queensland (introduced in 1935), westward to Western Australia and southward to New South Wales (Alford, Brown, Schwarzkopf, Phillips,

& Shine, 2009; Gruber, Brown, Whiting, & Shine, 2017), with increasing annual spreading rates from 10-15 to 55-60km per annum (Tingley et al., 2017; Urban, Phillips, Skelly, & Shine, 2008). This increasing invasion speed may be caused by environmental factors as well as through the development of dispersal-related traits following their introduction (Hudson, Brown, & Shine, 2017; Urban et al., 2008). Morphological and physiological traits underlie this accelerated expansion, yet it is also clear that behavioural traits have evolved across this invasion; western cane toads exhibit bolder behaviour than eastern toads (Gruber et al., 2017). Notably, these changes have occurred despite low genetic diversity in Australian toads (Lillie, Shine, & Belov, 2014; Selechnik, et al., 2019a; Slade & Moritz, 1998). Environmental and intrinsic factors (e.g., genetic components) that may affect behavioural traits have been previously studied (Rollins, Richardson, & Shine, 2015; Selechnik, Richardson, Shine, Brown, & Rollins, 2019b; Urban et al., 2008). Although there is direct evidence of the impact of host gut microbiome on adaptation in novel environments (Hauffe & Barelli, 2019), gut microbiota has never been characterized in cane toads; therefore, the impact of gut microbiota on the changes in invasive behaviour across the invasion range has not been investigated.

In order to explore the intestinal microbiota in cane toads, it is essential to answer the following questions: (1) what microbes are found inside the intestinal tract? (2) which non-destructive sampling method (cloaca or faeces) better represents the intestinal microbiota? and (3) which host factors contribute to the identified differences in the intestinal microbiota? In order to answer the questions, we used 16S rRNA sequencing to characterize microbial communities from samples of the large intestine, small intestine, faeces and cloacal swabs. Because it is well known that microbial communities are affected by host sex and body size (related to differences in diet and age) (Kozik, Nakatsu, Chun, & Jones-Hall, 2017; Muegge et al., 2011), we also examined whether intestinal microbiota varies with sex and body size in cane toads. These findings will inform future microbiome studies of adult amphibians.

Materials and methods

Sample collection

Eighteen adult cane toads were sampled from a captive breeding colony. These individuals were originally collected from wild populations located in Mary Pool, Western Australia (WA; nine males) and Port Douglas, Queensland (QLD; three males, six females) in April 2018. Individuals

were sexed by external morphological characteristics (males possess nuptial pads on the thumbs, rugose dorsal skin and yellow colouration), and vocalizations (males can be observed calling or producing release calls upon handling) (Hudson, Brown, & Shine, 2016). All individuals were held in captivity for one week before being injected with leuporelin acetate to stimulate breeding (for another experiment). For the following three months, animals were housed in sex-specific and sampling location-specific outdoor bins (1,165 x 1,165 x 780mm) fitted with insect-attracting lights and water sprinklers (bin A = males from the WA, bin B = females from the QLD, bin C = males from the QLD). During this period, toads fed on local insects attracted to lights installed over their bins.

After three months in captivity, body weight and body length (SUL = snout-urostyle length and SVL = snout-vent length) were measured and then toads were placed in individual 1L containers with 1mm water in the bottom, fed commercial crickets and housed overnight. The next morning, faecal samples were collected from containers and toads were euthanized using the injection of MS-222 (Sigma Aldrich). Then we collected cloacal swabs, large intestine and small intestine samples from each individual immediately after death to avoid potential differences due to the time of collection. Tools and work surfaces were sterilised between each sample. Faecal and cloacal samples were collected using sterile cotton applicators (FLOQSwabs, Copan Diagnostics Inc.); all large intestine and small intestine samples were collected by squeezing entire contents of each part of intestine separately into clean tubes (free of detectable RNase, DNase, DNA and PCR inhibitors, Scientific Specialties, Inc. SSIBio). All samples were preserved in 95% ethanol (Song et al., 2016) and stored at 4°C before shipping back to the laboratory. We used sterile cotton applicators to sample air above the lab bench before and after sampling and processed these to characterize any contaminants present in the sample collection process (environmental controls) (Eisenhofer et al., 2019).

DNA extraction and 16S amplicon library preparation

Prior to DNA extraction, individual ethanol preserved samples were homogenised by thoroughly mixing them, and then dehydrated using a SpeedVac Concentrator (Thermo Scientific SAVANT DNA 120) at medium temperature for 50 mins. Total DNA was extracted using the DNeasy PowerSoil kit (Qiagen) following the manufacturer's protocol. DNA concentration was estimated

using a Qubit™ ds DNA HS Assay kit (ThermoFisher Scientific) on an Invitrogen Qubit 4 Fluorometer.

We prepared 16S rRNA amplicon libraries by following guidelines for the Illumina MiSeq System. We used Zymo isolated DNA (D6305) as community positive controls and MilliQ water as a PCR negative control to determine contamination during the library preparation process (Eisenhofer et al., 2019). A total of 76 DNA samples (18 faeces samples, 18 cloaca samples, 18 large intestine samples, 18 small intestine samples, 2 environmental controls, 1 community positive control and 1 PCR negative control) were used for Next Generation Sequencing library preparation. We amplified the hypervariable (V3-V4) region of the 16S rRNA gene from each sample using primers 341F (5'-

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 785R (5'-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') (Herlemann et al., 2011) (Illumina's overhang transposase adapter sequence shown in bold).

Briefly, the initial PCR was set up in 25ul reactions using 2.5ul (>1.0ng/ul) of input DNA, 0.75ul (10uM) forward and reverse primers, as well as 12.5ul of EconoTaq® PLUS and EconoTaq PLUS GREEN 2X Master Mixes (Lucigen). The PCR protocol included: an initial denaturation at 94°C for 2 mins, 30 cycles of 94°C (30 sec), 55°C (30 sec), and 72°C (40 sec), and a final extension at 72°C for 7 mins. PCR products were cleaned with Zymo DNA clean and concentrator (Zymo Research). Amplification success and product concentration was determined using a Fragment Analyzer with dsDNA Reagent kit (35-1500bp) (DNF-910) (Agilent Technologies, Inc.). Cleaned amplicons were sent to the Ramaciotti Centre for Genomics (University of New South Wales, Kensington, Sydney), where all libraries were standardized for DNA concentration, indexed using a Nextera XT DNA library preparation kit (Illumina®-Nextera™), and sequenced on the Illumina MiSeq platform targeting 2x300bp paired-end sequence reads.

Data analysis

We downloaded demultiplexed FASTQ data from Illumina's BaseSpace cloud storage, then created amplicon sequence variants (ASVs) using the open-source QIIME2 pipeline (Bolyen et al., 2018). Demultiplexed sequence counts from samples and the positive control ranged between 82,108 and 216,977; the counts of environmental controls and the PCR negative control ranged between 24,644 and 58,785. 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 220 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). ASVs provide finer resolution of amplicon sequences resulting in more novel bacterial taxa than OTUs (operational taxonomic units) which are dependent on assignment to a reference database and clustering to retain taxa that meet an arbitrary similarity cut-off (usually 97%) (Callahan, McMurdie, & Holmes, 2017). After quality filtering, reads from samples and the positive control ranged between 65,184 and 167,363 counts; the reads from environmental controls and the PCR negative control ranged between 10,867 and 26,754 counts. 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 = 2), Chloroplast (N = 4), and 53 unassigned ASVs implemented in the package ‘phyloseq’ in the R statistical program version 1.26.1 (McMurdie & Holmes, 2013). We also removed the ASVs with prevalence less than four, which makes the logged counts per sample more evenly distributed (Figure S1). The remaining 5,298 taxa were classified to the Kingdom Bacteria with 69.03% assigned to phylum level. We visualized relative abundance of bacteria (abundance >2%) in different sample types, classified to the phylum and genus level.

We calculated observed ASVs (DeSantis et al., 2006) and evenness (Pielou, 1966) indices through QIIME2 for all samples, including environmental controls, the PCR negative control and the community positive control. To compare the alpha diversity (within sample) of the communities between different sampling methods, we used two metrics calculated through QIIME2: the Shannon Index (Shannon, 1948), which accounts for both abundance and evenness of the taxa present. Boxplots of alpha diversity indices, generated using the *boxplot* command in base R (R Core Team, 2018), displayed asymmetrical boxes across the medians. Also, our data have potential outliers and were not normally distributed ($p < 0.001$) according to a Shapiro-wilk test to assess multivariate normality using RVAideMemoire package in R (Herve, 2018). Therefore, we used nonparametric tests, Wilcoxon signed-rank test, for pairwise comparisons of medians, using the command *wilcox.test* (Bauer, 1972) in base R to compare alpha diversity between different sampling methods.

For beta diversity, we used a Hellinger transformation implemented in the package “microbiome” in R (Valverde, Makhalanyane, & Cowan, 2014) and then calculated and plotted Bray-Curtis dissimilarity matrix visualized using Principle Coordinate Analysis (PCoA) plots using commands from the package “Phyloseq” (McMurdie & Holmes, 2013). We used the *adonis2* command from the package “Vegan” in R (Oksanen et al., 2013) to perform permutational multivariate analysis of variance (perMANOVA) to check whether the microbial communities of each sample type were significantly different and to identify differences between individual toads. We used the command *betadisper* in the package “Vegan” in R to check the homogeneity of group variances, an assumption of perMANOVA. After finding significant differences between sample types, we performed pairwise comparisons between groups using the command *pairwise.perm.manova* function in “RVAideMemoire” package with the Wilks test (Nath & Pavur, 1985) and corrections for multiple testing were conducted using the Benjamini & Yekutieli (2001) (“BY”) procedure (Yekutieli & Benjamini, 2001). To identify the significant differences in ASVs between groups, we performed differential abundance testing using the function *DESeq* in the package “DESeq2” (Love, Huber, & Anders, 2014). We performed differential abundance testing between non-lethal samples (faeces and cloaca) and intestine samples (large intestine and small intestine samples) to identify taxa that differed significantly and only the 69.03% taxa that assigned to phylum level were counted.

To identify correlation between host characteristics, SUL, SVL, body weight and sex, with the microbial community we performed distance-based redundancy analysis (dbRDA).

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 host characteristics such as SUL, SVL, body weight and sex. A Bray-Curtis dissimilarity matrix of ASVs was ordinated and the results were analysed using redundancy analysis with constraining variables that was not highly correlated to estimate their explanatory proportion. Results for all statistical tests were considered significant where p-values <0.05.

Results

Data quality analysis

The number of distinct ASVs and the diversity from abundant data in the negative controls (environment and PCR) were lower compared to the samples. The number of observed species in

PCR (55) and environmental controls (mean = 36 ± 2.8) were lower when compared to toad samples (mean = 257 ± 121.2), which indicates minimal contamination in our sampling, DNA extraction and library preparation processes. Sample blanks have the highest evenness (Pielou's evenness) when compared with experimental samples (see Table S1).

The number of observed species ($N = 28$) in the community positive control was low compared to experimental samples (mean $N = 257 \pm 121.2$). The six genera used as microbial community standards constituted 99.8% of our sequenced community positive control, as shown in Figure S2, indicating minimal PCR bias in our library preparation process.

Within sample microbiota composition and diversity

Among all samples, the dominant phyla present in samples were from the Bacteroidetes ($35.55 \pm 18.8\%$), Proteobacteria ($26.91 \pm 15.0\%$), Firmicutes ($24.03 \pm 17.1\%$), and Fusobacteria ($11.55 \pm 10.3\%$), which accounted for 98.13% of assigned phyla. The dominant phyla were consistently present in each of the four sample types (Figure S3). The toad large intestine had a greater abundance of Bacteroidetes (Figure S4A), where the small intestine contained more Firmicutes (Figure S4B). At the lower taxonomic level, the genera *Bacteroides*, *Cetobacterium*, *Plesiomonas*, *Clostridium* and *Epulopiscium* were consistently present in each of the four sample types with different ratios (Figure 1A). Large intestine samples tended to have more *Bacteroides* and *Epulopiscium* (Figure S4C), but less *Clostridium* than small intestine sample (Figure S4D).

There were no significant compositional differences between non-lethal sampling methods (cloaca and faeces) and large intestinal samples according to observed species and evenness (Figure S5A, S5B). Compared to the large intestine, cloaca samples had a similar abundance of dominant phyla, with a higher abundance of Proteobacteria and reduced abundance of Fusobacteria. However, faecal samples had a higher abundance of Proteobacteria and lower abundance of Firmicutes and Fusobacteria (Figure S3). Compared to the small intestine, cloacal samples had similar abundance of dominant phyla too, with a higher abundance of Bacteroidetes and reduced abundance of the other three phyla. Faecal samples had a higher abundance of Bacteroidetes and Proteobacteria and lower abundance of Firmicutes and Fusobacteria (Figure S3). Wilcoxon signed-rank test, for pairwise comparisons of medians of observed ASVs showed that large intestinal samples did not differ significantly from small intestine ($p=0.95$), faeces ($p=0.09$), cloacal samples ($p=0.17$); small intestinal samples did not differ significantly from faeces ($p=0.15$), cloacal samples ($p=0.35$).

(Figure S5A). Pairwise comparison of species evenness showed large intestinal samples were not different from cloacal ($p=0.79$) and faecal samples ($p=0.14$) (Figure S5B). However, evenness of the small intestine was significantly different from samples from the large intestine ($p=0.006$), cloaca ($p=0.004$) and faeces ($p<0.001$).

We characterized differences in bacterial community (alpha diversity) with the Shannon index. The pairwise comparison of the Shannon index showed that neither the cloacal ($p=0.4$) or faeces ($p=0.085$) samples had significantly different bacterial communities when compared to samples of the large intestine (Figure 1B). Small intestine samples had significantly different bacterial communities than those taken from the large intestine ($p=0.048$), cloaca ($p=0.007$) and faeces ($p=0.003$).

Microbial community comparison between sample type

We used Bray-Curtis dissimilarity matrix to calculate beta diversity and used perMANOVA to test the dissimilarities in microbial community composition among cloacal, faecal and intestinal samples. The dissimilarities in community composition were clustered by sample types (Figure 2; perMANOVA: $F=3.003$, $p<0.001$). Significant result was checked for homogeneity of variance ($F=1.0619$, $p=0.37$). The further pairwise comparison between communities showed that cloacal samples were similar in microbial community composition to large intestine samples (Wilcoxon, $p=0.50$) and samples from the small intestine had similar microbial community composition to those from the large intestine ($p=0.57$). The cloacal microbial community was significantly different from that of the small intestine ($p=0.03$). The faecal microbial community was significantly different from those of the cloaca, the large intestine and the small intestine (Wilcoxon, $p=0.01$, $p<0.001$ and $p<0.001$, respectively).

We performed differential abundance testing between faecal and large intestine samples (Figure 3A), between cloacal and large intestine samples (Figure 3B), between faecal and small intestine samples (Figure 3C) and between cloacal and small intestine samples (Figure 3D) to identify ASVs that differed significantly ($p=0.05$). The figures show the significant differences between each pair of sample types as the log fold change of a taxa in one sample type compared to the control sample type. In this case, we set taxa abundance of large (or small) intestine as the control and the ASVs with significant abundance were shown in the figures: the taxa with higher

abundance in large intestine shown at the right, the taxa with higher abundance in other sample types shown at the left. Among the 69.03% taxa that assigned to phylum level, 125 ASVs in faecal samples and 60 ASVs in cloacal samples were significantly different from large intestine samples (Table S2, S3), and 116 ASVs in faecal samples and 64 ASVs in cloacal samples were significantly differed from small intestine samples (Table S4, S5). We then compared the taxa that differed significantly between non-lethal samples (cloacal and faecal samples) to large intestine samples. A large number of ASVs in the phylum of Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria were significantly more abundant in both faecal and the cloacal samples, including the classes Sphingobacteriia (all genus *Sphingobacterium*), Gammaproteobacteria (mainly genera *Acinetobacter* and *Pseudomonas*), Flavobacteriia (mainly family Flavobacteriaceae), Betaproteobacteria (mainly family Comamonadaceae), Bacilli (mainly family Bacillaceae), Alphaproteobacteria (mainly family Caulobacteraceae), Actinobacteria (mainly families Microbacteriaceae and Nocardiaceae), and [Saprospirae](family Chitinophagaceae). Both cloacal and faecal samples displayed lower log₂ fold abundances in the class Clostridia (family Clostridiaceae). The comparison between faecal and large intestine samples showed lower abundances of some taxa from the classes Bacteroidia (family Rikenellaceae) and Bacilli that was not seen in the comparison between cloacal samples and those from the large intestine. Further, in the class Thermomicrobia, one taxa were less abundant in in faecal samples as compared to those of the large intestine but such lowered abundances were not seen in cloacal samples. Faecal samples had more taxa with higher abundance than large intestine in the class Epsilonproteobacteria (genus *Arcobacter*) than did cloacal samples.

Host factors associated with microbial community assemblage

We found significant microbial dissimilarity between individuals (Figure S6) (perMANOVA: $F=4.5254$, $p<0.001$) and there was no significant difference in the homogeneity of variance ($F=0.4103$, $p=0.98$). We hypothesized that host factors, such as sex, body weight, and body length (Table S6), would impact microbial communities. Cane toad SUL, SVL and body weight were correlated (>0.80). We found sex was significantly associated with microbial variation (Figure 4A). Because all female toads in this study were from eastern Australia and male toads were from both regions, we further looked at the microbial difference between male and female toads in eastern toads to exclude the influence of sampling location. Since we are more interested in large intestine samples, and cloacal samples were found to be better at representing the large intestinal

microbial community, we plotted the PCoA of eastern toads in both large intestinal and cloacal samples. We found that the microbial communities were grouped by sex in both sample types (Figure 4B), with increased relative abundance of genera *Bacteroides*, *Comamonas*, *Flavobacterium*, *Microvirgula*, *Parabacteroides*, *Pseudomonas* and decreased relative abundance of *Cetobacterium*, *Clostridium*, *Epulopiscium*, *Plesiomonas*, *Vibrio* in female toads (Figure S7). The combination of sex and body weight explained the greatest variation (14.7%) in microbiota through model selection (Figure 4A).

Discussion

This is the first study to compare non-lethal sampling for the study of gut microbiota in adult amphibians, which is important information when repeated sampling or non-lethal sampling is needed. Further, this is the first study to characterise cane toad intestinal microbiota, which is fundamental for understanding the adaptive potential of behaviours that may be influenced by microbiota across the Australian invasion. Here, we characterised intestinal tract microbial composition and diversity, explored the similarities and differences in the microbial community between the non-destructive sampling methods, and examined the potential impact of host factors that may contribute to the differences in intestinal microbiota. Our data show that in cane toad, cloacal samples are more representative of the large intestinal microbiota than are faecal samples. This finding is likely to be useful to other studies of adult amphibian gut microbiome. Similar to other studies of gut microbiome across taxa, we found that both sex and body weight were important factors accounting for inter-individual variation in community assemblage. However, further work is needed to identify the effect of metamorphic transition in cane toad gut microbiomes since previous studies have shown that this is a factor regulating the composition of the microbiome in amphibians (Demircan et al., 2018).

We found that the cane toad's intestinal microbiota were dominated by four phyla: Bacteroidetes, Proteobacteria, Firmicutes, and Fusobacteria. The first three taxonomic groups are commonly identified in the intestine of frogs and toads (Chang, Huang, Lin, Huang, & Liao, 2016; Huang, Chang, Huang, Gao, & Liao, 2017; Kohl, Amaya, Passemment, Dearing, & McCue, 2014; Wiebler, Kohl, Lee, & Costanzo, 2018). On the contrary, although Fusobacteria have also been found as dominant phyla in the intestinal microbiota of Asiatic toad (Chai, Dong, Chen, & Wang, 2018), it has not been reported as a dominant phyla in other frogs and toads (Chang et al., 2016; Huang et

al., 2017; Kohl et al., 2014; Wiebler et al., 2018). Proteobacteria, Bacteroidetes and Firmicutes are generally identified in the intestines of terrestrial mammals, marine taxa and amphibians while Fusobacteria are commonly found in marine species (e.g., fish and seals) (Larsen, Mohammed, & Arias, 2014; Nelson, Rogers, Carlini, & Brown, 2013). The cane toad's microbiota showed similarity to both amniotes and fish, although the adult frog microbial community was previously found to be more similar to amniotes rather than fish (Kohl, Cary, Karasov, & Dearing, 2013). The Proteobacteria genus *Plesiomonas* were found consistently in cane toads' intestine, which is widely found in different hosts and nature (especially aquatic environments) (Santos, Rodríguez-Calleja, Otero, & García-López, 2015). The Bacteroidetes genus *Bacteroides* and Firmicutes genus *Clostridium* were also found consistently in the cane toads' intestine, which were reported in other studies of amphibian intestinal microbiota (e.g., slimy salamander (*Plethodon glutinosus*) and frogs) (Chang et al., 2016; Okelley, Blair, & Murdock, 2010; Wiebler et al., 2018). Another Firmicutes, genus *Epulopiscium*, were also abundantly existing in our samples. However, it is interesting to note the presence of *Epulopiscium* spp, which are large bacteria and the most well-known species, *Epulopiscium fishelsoni*, has a unique symbiotic relationship with the tropical marine, herbivorous brown surgeonfish and has not been observed elsewhere (Angert, Clements, & Pace, 1993; Clements, Sutton, & Choat, 1989; Fishelson, Montgomery, & A. Myrberg, 1985). Fusobacteria are anaerobic gram-negative bacilli (Bennett & Eley, 1993) that ferment carbohydrates and produce butyrate (Potrykus, Mahaney, White, & Bearne, 2007; van Gylswyk, 1980). Butyrate or butyric acid is a short chain fatty acid with known positive effects on the control of enteric pathogens and gut health (Bedford & Gong, 2018). The most abundant Fusobacteria genus in our samples were *Cetobacterium*, which has been found in both mammals and fish (Finegold et al., 2003; Tsuchiya, Sakata, & Sugita, 2008). Interestingly, the *Cetobacterium* were observed in the gut of juvenile Cuban tree frogs and were positively correlated with parasite resistance in adults (Knutie, Wilkinson, Kohl, & Rohr, 2017). The *Cetobacterium* may contribute to the host immune response by producing vitamin B₁₂ (cobalamin) (Degnan, Taga, & Goodman, 2014; Yoshii, Hosomi, Sawane, & Kunisawa, 2019).

We found that the cane toad's large and small intestines were similar in terms of abundances of phyla and observed species. However, bacterial communities in the large intestine have greater diversity (richness and evenness) than small intestine. This could be due to slower intestinal motility and longer transit time in large intestine which increases the probability of microbial

colonization (Berg, 1996; Hillman, Lu, Yao, & Nakatsu, 2017). Large intestine samples tended to have more *Bacteroides* and less *Clostridium* than those from the small intestine, which has also been observed in mice (Onishi et al., 2017). Both genera contain anaerobic bacteria (Wells & Wilkins, 1996; Wexler, 2007) and both contain commensals and pathogens; interestingly, these taxa have also been implicated in the maintenance of host gut physiology, including SCFA production (Lopetuso, Scaldaferri, Petito, & Gasbarrini, 2013; Wexler, 2007). *Bacteroides* are important in fermenting soluble carbohydrates in the human large intestine (Louis, Scott, Duncan, & Flint, 2007). The genus *Clostridium*, a Firmicutes, performs most of their metabolic functions through the release of butyrate that is essential as fuel for colonocytes and maintains gut homeostasis (Lopetuso et al., 2013).

We found that cloacal samples are better than faecal samples for representing the large intestinal microbiota of cane toads. There were no significant compositional differences between cloacal, faecal and large intestine samples with respect to relative abundances of phyla and alpha diversity (observed species, evenness and Shannon index). Shannon's index accounts for both abundance and evenness of the taxa present; therefore, it is unsurprising that this metric (Figure 1B) showed similar patterns of the observed species (Figure S5A) and evenness plots (Figure S5B) among different sample types. However, the beta diversity results showed that cloaca samples were not significantly different to the large intestine microbiota, while faeces samples were significantly different to the large intestine microbiota. One important variable driving the observed differences between faeces and large intestine microbiota could be that faeces had been deposited overnight and collected in the morning. This exposure to aerobic conditions could have a significant effect on their microbial profiles as discussed below. Previous studies in humans have showed that faecal and rectal swab microbiota from the same individual were similar (Bassis et al. 2017). However, collecting "clean" and fresh faeces samples from humans is relatively easy; it is much more difficult to accomplish this in wild animals, such as cane toads. Interestingly, opposite results were found in birds: faeces samples were significantly better than cloacal swabs in representing the colon bacterial community of juvenile ostriches (Videvall et al., 2018). As mentioned above, the representativeness of large intestine microbiota from different non-lethal sampling types varies between species. Our results highlight the importance of validating non-lethal sampling methods for each taxonomic group. It is, however, important to highlight that literature describing the changes in post-mortem gut microbiome composition in hot-blooded animals identifies time since

death as a factor for gut microbiome changes (Brooks 2016). Although no obvious variation is observed immediately post-mortem in the rectum samples in mice and human (Guo et al. 2016; DeBruyn and Hauther 2017), clear changes can be found as fast as 5 minutes after death (Heimesaat et al., 2012). One factor affecting these changes is the loss of body temperature that occurs after death in hot-blooded animals. Even though there is no study on the effect of time since death on the gut microbiome of cold-blooded organisms, it is unlikely that loss of body temperature has a significant effect on microbiome composition when samples are collected immediately after death.

In our results, the majority of ASVs that were found to be significantly more abundant in both faecal and cloacal samples as compared to the large intestine were aerobic, except for a few ASVs that are facultatively anaerobic or commonly found in diverse 187-288s. Moreover, faecal samples had more aerobic taxa than cloacal samples. This is indicative of a rapid depletion of anaerobic taxa in both types of non-lethal sampling approaches, especially faecal samples, that should be taken into account in future studies. For example, the class Clostridia (family Clostridiaceae) from which we found fewer ASVs in both cloacal and faecal samples than in large intestine samples is, unsurprisingly, anaerobic and actively involved in energy metabolism with Enterobacteriaceae (Wiegel, Tanner, & Rainey, 2006; Wüst, Horn, & Drake, 2011). Compared to cloacal samples, faecal samples had lower abundance of family Rikenellaceae and this family had been found in increased abundance in healthy humans than in non-alcoholic fatty liver disease patients (Jiang et al., 2015).

Sex was a major contributor to the differences in the intestinal microbial community of cane toads in this study and body weight was less influential. Both of these factors were found to influence intestinal microbiota in humans (Borgo et al., 2018). Sex hormones were found to mediate the changes in mice intestinal microbiota composition (Bray Curtis distances) by gonadectomy and testosterone hormone replacement (Org et al., 2016). Also, Org et al. (2016), showed that hormonal changes and gender differences strongly affected bile acid profiles, which is prominent in response to a high-fat/high-sugar diet and have been shown to affect gut microbiota (Islam et al., 2011; Li & Chiang, 2015). While it is possible that the interaction of diet and sex may be important in microbiome studies of amphibians because male toads spend more time near water than females, our animals were housed in a common environment with access to the same diet.

However, it is important to note that the intestinal microbiota has previously been strongly associated with long-term diets in humans (Wu et al., 2011).

Conclusion

This study provides important information about non-lethal sampling of gut microbiota in adult amphibians and broadens our scant knowledge of amphibians' intestinal microbiota. Further, we show that cloacal samples are a better choice than faecal samples for the accurate characterisation of cane toad intestinal microbiota. Having a reliable non-invasive method will allow the same animal to be sampled repeatedly across time, enabling manipulative experiments to investigate the role of microbiome in influencing behaviours important to invasion. This capability opens an entirely new dimension to the field of invasion ecology.

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[dataset] Zhou, J., Nelson, T. M., Lopez, C. R., Sarma, R. R., Zhou, S. J., Rollins, L. A. (2019) *Rhinella marina* gut microbiota Raw sequence reads. NCBI Sequence Read Archive (accession number: PRJNA592448).

Data Accessibility Statement

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

1. Supplementary Figures S1-S7.
2. Supplementary Tables S1-S6.

The data that support the findings of this study are openly available in NCBI Sequence Read Archive (accession number: PRJNA592448).

Author Contributions

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

Figures

Figure 1. Bacterial community composition and alpha diversity of different samples types. Relative abundance bar plots display genera (>2%) (A). Boxplots show the Shannon index (B). Pairwise testing between non-lethal sample groups (cloaca, faeces) and intestine sample groups (small intestine and large intestine), and between small intestine and large intestine samples were conducted using Wilcoxon signed-rank test with p-values indicated.

Figure 2. Beta diversity by sample types. Principle coordinate analysis plot of Bray Curtis distances of cloaca, faeces, large intestine and small intestine samples from 18 cane toad individuals.

Figure 3. Significantly different bacterial taxa between sample types in cane toads. Dot plots display the significant difference between ASVs in faeces and large intestine samples (A), cloacal and large intestine samples (B), faeces and small intestine samples (C), and, cloacal and small intestine samples (D). Significant differences were identified between sample types via differential abundance testing based on a negative binomial distribution. The dots represent the average log-2 fold change (x axis) abundance of bacterial taxa classified to the taxonomic level of class (y axis) and coloured by taxonomic level of phylum, where *Actinobacteria* = “red”, *Bacteroidetes* = “blue”, *Chloroflexi* = “green”, *Firmicutes* = “yellow”, *Proteobacteria* = “pink”, *TM7* = “purple”, and *Fusobacteria* = “orange”. Positive log₂ fold changes signify increased ASV abundance in either faeces or cloaca, and negative

$\log_2$ fold changes display increased abundance in large intestine or small intestine. Family name in bracket is proposed taxonomy by Greengene. NA indicates the ASVs could not match to a known bacterial class and may represent novel taxa. The taxa underlined are the class that do not have significant different taxa from both large intestine and small intestine when comparing to non-lethal samples.

Figure 4. Main host factors that affects cane toads' large intestinal and cloacal microbiota differentiation among individuals. CAP (*capscale*) plot displays the combination of variables that explained the greatest variation in the microbiota through model selection, where cloacal = red, faeces = green, large intestine = blue and small intestine = purple (A). The variables implemented in the final model were sex and body weight, which explained 14.7% of variation in the microbiota. Principle coordinate analysis plot of Bray Curtis dissimilarity matrix of the large intestinal and cloaca samples from 9 eastern Australian cane toads, where cloacal = red, large intestine = blue, female = circle, and male = triangle (B).