environmental microbiology reports

Environmental Microbiology Reports (2013) **5**(6), 899–903 doi:10.1111/1758-2229.12092

Restructuring of the amphibian gut microbiota through metamorphosis

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Summary

Vertebrates maintain complex symbioses with a diverse community of microbes residing within their guts. The microbial players in these symbioses differ between major taxa of vertebrates, such that fish and amniotes maintain notably different communities. To date, there has not been a culture-independent inventory of an amphibian gut microbial community. Here, we compared gut microbial communities of tadpoles and frogs of the Northern leopard frog (*Lithobates pipiens*). We utilized Illumina sequencing, which allowed us to inventory more than 450 000 microbial sequences. We found that tadpoles and frogs differ markedly in the composition of their gut microbial communities, with tadpoles maintaining a community more similar to fish, whereas the frog community resembles that of amniotes. Additionally, frogs maintain a community with lower phylogenetic diversity compared with tadpoles. The significant restructuring of the microbiota is likely due to changes in diet as well as the large reorganization of the intestinal organ during metamorphosis. Overall, we propose that amphibians represent an important system in which to study regulation and selection of gut microbial communities.

Introduction

Symbioses between animals and microbes have markedly influenced the ecology and evolution of both players (McFall-Ngai *et al*., 2013) by modulating energy balance (Semova *et al*., 2012), immune function (Round and Mazmanian, 2009) and even behaviour (Heijtz *et al*., 2011) of the host. Though these symbioses are ubiquitous, the microbial communities residing within the vertebrate gut differ largely among host phylogenetic classes. Teleost fish host communities are rich in *Proteobacteria* (Rawls *et al*., 2006; Sullam *et al*., 2012), while previously studied amniotes (mammals, birds and diapsid reptiles) maintain communities dominated by *Firmicutes* and *Bacteroidetes* (Ley *et al*., 2008; Scupham *et al*., 2008; Costello *et al*., 2010). These differential communities seem to be selected by the host, though the mechanisms are still unclear (Rawls *et al*., 2006). Amphibians represent an important, intermediate clade between these groups (Kardong, 1995); yet to date, there has not been a culture-independent inventory of an amphibian gut microbiota.

Amphibians undergo many physiological and morphological changes through development. Anuran tadpoles lack fully developed external appendages, breathe with gills and are fully aquatic. Through metamorphosis, frogs complete development of limbs, gain the ability to breathe air and may adopt terrestrial lifestyles. The diets of amphibians also largely change over metamorphosis. As tadpoles, many species consume diets comprised almost entirely of plant material, whereas frogs are primarily insectivorous (Jenssen, 1967; Linzey, 1967; Hendricks, 1973). The digestive tract also undergoes rapid and radical changes between these life stages, from non-acidic stomachs and reduced hindguts in tadpoles, to acidic stomachs, typically shorter small intestines and enlarged hindguts in adults (Stevens and Hume, 1995; Hourdry *et al*., 1996). Likewise, the immune system of the gut is underdeveloped in tadpoles compared with metamorphosed frogs (Du Pasquier *et al*., 2000). These developmental changes may have large implications for determining the microbial community that resides within the guts of tadpoles compared with frogs.

Here, we compared gut microbial inventories of tadpoles and frogs of the northern leopard frog (*Lithobates pipiens*). This study represents the first culture-independent investigation of the gut microbial community of an amphibian. Tadpoles were fed a diet of ground alfalfa (88% of dry mass) suspended in a matrix of agar and gelatin (12%) *ad libitum*, while those allowed to develop through metamorphosis were fed a diet of crickets and mealworms for 16 weeks as frogs. We collected total digesta from the whole intestine (small and large) of tadpoles and frogs and conducted microbial inventories by sequencing the 16S rRNA gene on an Illumina MiSeq platform (Illumina Inc., San

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Table 1. Relative abundances (mean ± SEM) of major bacterial phyla residing in the guts of tadpoles ($n = 7$) and frogs ($n = 8$). *P*-values were calculated with a Student's *t*-test. Significant differences are in bold.

	Tadpoles	Frogs	*P-value*
Firmicutes	36.61 ± 8.08	66.05 ± 8.90	**0.029**
Proteobacteria	54.86 ± 7.55	10.43 ± 3.39	**0.0006**
Bacteroidetes	2.43 ± 1.28	22.82 ± 8.96	0.057
Verrucomicrobia	2.66 ± 0.80	0.03 ± 0.01	**0.016**
Actinobacteria	1.13 ± 0.51	0.08 ± 0.06	0.084
Tenericutes	0.95 ± 0.25	0.03 ± 0.02	**0.011**
Planctomycetes	0.47 ± 0.34	< 0.01	0.21
Fusobacteria	< 0.01	0.32 ± 0.11	**0.028**
Acidobacteria	0.09 ± 0.04	0	0.051

Diego, CA, USA) (Caporaso *et al*., 2012). We predicted that the microbial community structure would vary across developmental stages because of the considerable changes in diet and intestinal morphology/physiology between these groups.

Results and discussion

A total of 462 947 high-quality microbial 16S rRNA sequences were produced through Illimuna sequencing of the gut contents of tadpoles and frogs (13 930 ± 403 sequences per sample). These sequences were classified into 7908 operational taxonomic units based on 97% sequence identity using QIIME (Caporaso *et al*., 2010). Sequences were deposited in GenBank under accession SRP019766. Details regarding animal collection, sequencing and data analysis can be found in Supplementary Methods (Fig. S1).

The anuran gut microbial community exhibited marked differences between tadpole and frog life stages. Relative abundances of five of the nine most dominant phyla in the anuran gut differed significantly with life stage (Table 1). Tadpoles harboured a community dominated by the phyla *Proteobacteria* and *Firmicutes*, while frogs maintained a community rich in *Firmicutes* and *Bacteroidetes*. Through development from tadpoles to frogs, anurans exhibited significant reductions in the relative abundances of *Proteobacteria*, *Verrucomicrobia*, *Tenericutes*, and showed trends for reduction in the abundance of *Actinobacteria* and *Acidobacteria* (Table 1). The phylum *Acidobacteria* was present only in tadpoles, and absent from all frogs. Through metamorphosis, there was also a significant increase in the relative abundance of *Firmicutes* and *Fusobacteria*, as well as a trend for *Bacteroidetes* to increase in abundance (Table 1).

Interestingly, these life stages were more similar to communities observed in disparate host taxa rather than to one another. Tadpoles maintained a community dominated by *Proteobacteria*, which is similar to gut communities harboured by teleost fish (Sullam *et al*., 2012). Conversely, frogs housed a community rich in *Firmicutes* and *Bacteroidetes*, which is more similar to communities in amniotes (Ley *et al*., 2008; Scupham *et al*., 2008; Costello *et al*., 2010). The phylum *Acidobacteria* was detected in roughly half of the tadpole digesta samples, but was undetectable in all frog samples. This transition mirrors differences between fish, which usually harbour *Acidobacteria* (Sullam *et al*., 2012), and amniotes, where *Acidobacteria* are generally undetectable (Ley *et al*., 2008; Costello *et al*., 2010). Recently, Costello *et al.* (2010) conducted the first large-scale inventory of a reptile gastrointestinal microbial community and showed that a *Firmicutes*- and *Bacteroidetes*-rich gut community in adult individuals is a trait of amniotes. Our results represent the first inventory of an amphibian, and suggest that tetrapods in general share this trait.

These trends are further supported when comparing microbial genera specific to certain developmental stages of the northern leopard frog. Tadpole- and frog-specific genera were defined as those that were detected in more than half of the individuals of one group, and completely absent from all samples of the other group (Table 2). Several tadpole-specific genera (e.g. *Shewanella, Hydrogenophaga, Devosia*) are predominant members of invertebrate or fish microbial communities (Grossart *et al*., 2009; Li *et al*., 2009; Navarrete *et al*., 2009), while frog-specific genera (e.g. *Odoribacter, Butyricimonas, Akkermansia*) are largely found in the guts of amniotes (Derrien *et al*., 2008; Sakamoto *et al*., 2009; Nagai *et al*., 2010). It is worth noting that although tadpoles had a higher proportion of microbes belonging to the phylum *Verrucomicrobia*, they lacked the frog-specific genus *Akkermansia* (a member of *Verrucomicrobia*). Rather, this phylum-level difference was due to tadpoles harbouring a higher proportion of unidentified microbes belonging to the family *Verrucomicrobiaceae*. A larger survey of amphibian species, especially across various orders, is warranted to investigate the generality of these differences between developmental stages.

Table 2. Tadpole- and frog-specific genera, defined as those that were detected in more than half of the individuals of one group, and completely absent from all samples of the other group.

Genera found only in tadpoles	Genera found only in frogs
Paenibacillus	*Odoribacter*
Novosphingobium	*Butyricimonas*
Hydrogenophaga	*Dysgonomonas*
Shewanella	*Akkermansia*
Devosia	
Rheinheimera	
Emticicia	
Flectobacillus	
Gemmata	
Aminobacter	

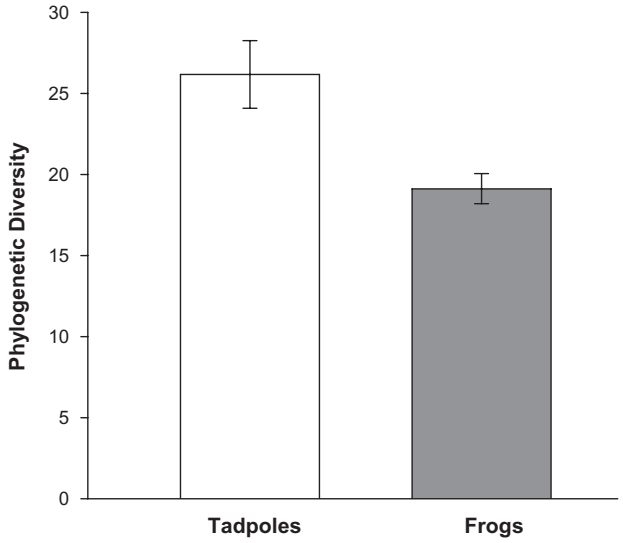

Fig. 1. Faith's phylogenetic diversity index of tadpole and frog gut microbial communities.

Indices of microbial diversity in the gut also differed between tadpoles and frogs. Frogs harboured a community with significantly lower Faith's phylogenetic diversity index than tadpoles ($P = 0.014$, Fig. 1). However, there was no difference in estimated species richness, evenness or the Shannon Index between tadpoles and frogs ($P > 0.25$ for all). Tadpoles and frogs maintained microbial communities with different composition as indicated by Principal Coordinates Analysis of unweighted UniFrac data (Fig. 2). These differences are unlikely to be solely driven by diet, as food sources and water contained very different microbial communities than the anuran gut (Fig. S1).

The change in diet between tadpoles and frogs may drive the observed changes in communities between these two developmental stages. Tadpoles are primarily herbivorous, while frogs are typically insectivorous. Thus, frogs consume a diet higher in protein and chitin, and lower in cellulose compared with tadpoles. Dietary strategy determines microbial community structure in mammals such that herbivores and carnivores have unique communities (Ley *et al.*, 2008). Additionally, changes in content of plant polysaccharides can influence microbial community composition (Turnbaugh *et al.*, 2009). In this baseline study, we aimed to maintain tadpoles and frogs on their typical diets. Future studies may wish to use similar, artificial diets through development to parse out the role of diet on community structure of microbes.

In addition to diet, host factors may select which microbial members flourish within the gut, though the mechanisms are still unknown. When germ-free zebrafishes are inoculated with a *Firmicutes*-rich, mammalian microbial community, the introduced microbial community is resculpted to one rich in *Proteobacteria* (Rawls *et al.*, 2006). Simple physicochemical differences between tadpoles and frogs may explain this difference in community structure. *Proteobacteria* exhibit an increased tolerance to oxygen compared with *Firmicutes* and *Bacteroidetes* (Rawls *et al.*, 2006). Additionally, development of a gastric stomach (absent in tadpoles and present in frogs) and changing gut pH through metamorphosis (Stevens and Hume, 1995; Hourdry *et al.*, 1996) likely alter the microbial community (Duncan *et al.*, 2009). Through metamorphosis, amphibians also undergo rapid degeneration of the flat, primary intestinal epithelium and proliferation of a secondary intestinal epithelium (Hourdry *et al.*, 1996). This secondary epithelia exhibits folded villi, and higher expression of many digestive and innate immunity genes (Hourdry *et al.*, 1996). Epithelial immune function also changes through amphibian metamorphosis, such that the larval gut lacks B cells producing IgM or IgX (Mussmann *et al.*, 1996; Du Pasquier *et al.*, 2000). The types of glycoconjugates produced by the small intestine changes through metamorphosis (Kaptan *et al.*, 2013), which may facilitate colonization by certain microbe species by providing energy sources or binding areas (Hooper and Gordon, 2001). This is further supported by the fact that *Akkermansia*, a genus that specializes on intestinal mucins (Derrien *et al.*, 2008), is found only in frogs and absent from tadpoles. It is likely that these intricate changes in gene expression between tadpoles and frogs result in shifts in microbial diversity. Further studies are necessary to investigate these hypotheses.

Significant restructuring of the gut microbiota has been observed in other systems. The gut microbiota is repeatedly remodelled in pythons during fasting (Costello *et al.*, 2010) and in 13-lined ground squirrels during hibernation (Carey *et al.*, 2013). The guts of several species of insect undergo sterilization and recolonization through metamor-

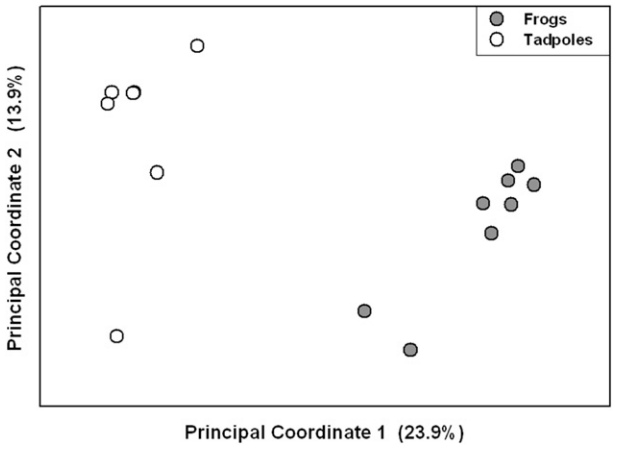

Fig. 2. Principal Coordinate Analysis using unweighted UniFrac scores of the microbial communities from tadpoles and frogs.

phosis because of production of a cocktail of antimicrobial compounds (Russell and Dunn, 1996; Moll *et al*., 2001; Koch and Schmid-Hempel, 2011). Similarly, amphibians produce high levels of lysozymes with antimicrobial activity during the climax of metamorphosis (Hourdry *et al*., 1996). Future studies could conduct microbial diversity and density measurements at various time points throughout metamorphosis to gain better insight in to this process.

Overall, we documented large changes in microbial diversity between tadpoles and frogs. We found that tadpoles and frogs differ significantly in the composition of their gut microbial community, with tadpoles maintaining a community more similar to fish, and frogs resembling amniotes. The changes in diet and gastrointestinal physiology between tadpoles and frogs make amphibians an ideal study system in which to study the host regulators of microbial diversity at the phylum level. Although this study only monitored changes in community structure, the results raise a number of questions and hypotheses that should be addressed to advance our understanding of the mutualisms between vertebrate hosts and gut microbes. Additionally, the physiological, ecological and evolutionary roles of these disparate communities remain to be investigated.

Acknowledgements

We thank Sarah Owens of Argonne National Labs for assistance with 16S rRNA amplification and sequencing. Research was supported by the University of Wisconsin Sea Grant Institute (grant NA16RG2257, project R/EH-2 to W.H.K.) and the National Science Foundation (Graduate Research Fellowship to K.D.K, (Doctoral Dissertation Improvement Grant, DEB 1210094, to M.D.D and K.D.K.).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Principal Coordinate Analysis using unweighted UniFrac scores of the microbial communities from tadpoles and frogs, as well as food and water sources.
Appendix S1. Supplementary methods.