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SYMPOSIUM

Manipulation of Gut Microbiota Reveals Shifting Community Structure Shaped by Host Developmental Windows in Amphibian Larvae

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Synopsis Exploration of the importance of developmental windows for microbial colonization in diverse animal taxa, and tests of how these shape both animal microbiomes as well as host phenotypes promise to shed needed light on host-microbe interactions. The aims of this study were to explore how gut microbiota diversity of larval amphibians varies among species and across ontogeny, and to test if manipulation of gut colonization can reveal how microbiomes develop. We found that gut microbiomes differ among species and change across larval ontogeny, with distinctive differences between larvae, metamorphic animals, and juvenile frogs. Through applying a gnotobiotic protocol to eggs and cross-inoculating gut microbiomes between species, we demonstrated that microbiota can be transplanted among species and developmental stages. These results also demonstrated that microbial colonization at hatching is potentially formative for long term composition and function of amphibian gut microbiomes, suggesting that hatching may be a critical developmental window for colonization, similar to the effects of birth mode on human microbiomes. Specifically, our results suggest that either the egg jelly and/or capsules surrounding amphibian eggs are likely important sources for initial microbiome inoculation. Furthermore, we speculate these results suggest that vertical transmission may be important to amphibian microbiome establishment and development, as is common among many animal taxa. Taken together, our results suggest that explicit tests of how host developmental windows influence microbial colonization, and shape amphibian microbiomes across life stages promise to provide insight into the ecological and evolutionary dynamics of host-microbe interactions.

Introduction

Microbes colonize virtually all epithelial surfaces, mucosa, and lumen of animal bodies where they often vastly outnumber the somatic cells of their hosts. While we have long recognized that these commensal and symbiotic microbes provide nutritional benefit to their hosts, an explosion of research over the past two decades has revealed a diversity of other effects they have on their host (McFall-Ngai 2015). Initial microbial colonization in larvae and neonates, in particular, often have life-long consequence for processes including the development and function of a host's immunity, metabolism, and health (McFall-Ngai et al. 2013; Borre et al. 2014; Cox et al. 2014; Funkhouser and Bordenstein 2013). Indeed, the source and mode of microbial colonization during critical periods of host development may have profound effects for the diversity and stability of a microbiome, with concomitant effects on their hosts (Cox et al. 2014). Despite our rapidly growing knowledge of host-microbe interactions, however, the majority of these data are derived from studies of mammals in lab settings. Developing both a deeper and broader taxonomic understanding of host-microbe interactions promises to provide insight into not only the effects microbes

have on animal physiology but their ecological and evolutionary dynamics (Warne 2014; Kohl and Carey 2016). In this study, we take a manipulative approach to explore how gut microbiomes vary among amphibian species, and to test if microbial colonization during hatching influences microbiome structure across ontogeny.

While animal microbiomes are dynamic and often variable between species, as well as individuals within a population, recent studies have endeavored to detail universalities as well as document the factors that drive variance and disruption of microbiomes (Bashan et al. 2016). Studies comparing domesticated and wild animals for example, show that higher order taxonomic community structure of intestinal microbiomes often have a similar core set of phyla within a species (Cox and Gilmore 2007; Scupham et al. 2008; Roeselers et al. 2011). However, while a core microbiome may be common, Turnbaugh et al. (2009) demonstrated, through comparing lean and obese twins, that moderate changes in community structure can have pronounced functional effects that impact hosts. Furthermore, a growing set of literature suggests the initial acquisition of microbiota during early life, such as at birth, can have potentially profound effects on community structure and stability across life stages (Arrieta et al. 2014; Cox and others 2014). Taken together, these studies suggest greater exploration of how microbiome structure is shaped by host developmental processes in conjunction with host phylogeny and ecology could greatly expand our understanding of host-microbe interactions.

While associations of symbiotic microbes are ubiquitous among vertebrates, gut microbiome composition varies with broad host phylogeny, life stage, ecology, and at finer scales with factors including diet. For example, mammals generally maintain gut microbiomes dominated by *Firmicutes* and *Bacteroidetes* (Ley et al. 2008), while fish microbiomes are dominated by *Proteobacteria*, and these communities appear to be selected by their hosts as demonstrated by transplant experiments between fish and mice (Rawls and others 2006). However, amphibians which are phylogenetically basal to mammals, exhibit shifts in their gut microbiomes in association with life stage. Aquatic larval anurans have gut microbiomes more similar to fish, while the more terrestrial adult frog have a microbiome, after metamorphosis, more similar to that of mammals (Kohl et al. 2013). These patterns suggest that shifts in diet, ecology (aquatic versus terrestrial), or even amphibian physiology could be driving these ontogenetic changes in microbiome structure. Testing how these factors shape amphibian–microbiota interactions could not only provide greater knowledge of amphibian and vertebrate evolution, but also provide a model system to explore how microbiomes influence diverse physiological and ecological processes in wildlife as well as humans.

Gnotobiotic approaches, or experimental manipulation whereby all symbionts of a host are known such as in axenic hosts, are commonly employed to explore microbiome and host interactions. For example, through the generation of germ-free mice and subsequent microbial inoculation from either lean or obese humans, Ridaura et al. (2013) demonstrated that the structure and function of the gut microbiota influences mouse-host metabolic phenotypes. Rawls et al. (2006) also used reciprocal microbiota transplants between zebrafish and mice to test the importance of hosts (germ-free initially) for shaping microbiome community composition and stability. In amphibians, Rebollar et al. (2016) took a different approach aimed at bioaugmentation, in which they used a probiotic bacterium to test if inoculation onto the intact skin microbiome of larval amphibians would allow for colonization by a select microbe, and if community structure was subsequently altered. These examples of microbiome manipulation demonstrate both the diversity of approaches in this still emerging field (Mueller and Sachs 2015), as well as the potential value of using manipulative approaches to explore microbe and host interactions.

The aims of this study were to use a manipulative approach to explore how gut microbiomes vary among amphibian species, and to test if microbial colonization during hatching influences microbiome structure across ontogeny. We compared the gut microbiota of larval wood frogs (*Lithobates* [*Rana*] *sylvaticus*), green frogs (*L. clamitans*), and bullfrogs (*L. catesbeianus*) at varied developmental stages from the egg, through metamorphosis to the unfed juvenile frog stage. We sought to expand upon the work of Kohl et al. (2013) by comparing closely related species with differing ecology to explore how diet and ecology contribute to ontogenetic shifts in gut microbiomes. Second, we also aimed to test if gut microbiomes can be reliably manipulated in larval amphibians, in order to provide a new model system to explore gut microbiota and host interactions. Towards these ends, we adapted a gnotobiotic protocol developed for zebrafish (Rendueles et al. 2012) to first sterilize amphibian eggs, and then used gut content from varied species to cross-inoculate and potentially transplant microbiota between species. By combining this manipulation with the ontogenetic

shifts in gut microbiomes we tested the hypotheses that microbiota differed among species, and that microbiota transplants between species would provide insight into the factors influencing amphibian microbiome colonization, community composition, and stability.

Methods

Animal collections

We collected wood frog and green frog egg masses, as well as bullfrog larvae (as an inoculate source) from local wetlands, by permit from the Illinois Department of Natural Resources (NH15.5778). The Institutional Care and Use Committee at Southern Illinois University approved all experimental procedures (15-018).

Ontogenetic shifts in gut microbiomes

To track ontogenetic microbiome shifts, wood frogs were sampled 2 weeks after hatching (Gosner stage-GS 27) (Gosner 1960), early metamorphosis (GS 38), metamorphic climax (GS 43), as metamorphs (GS 45), and as juvenile frogs 1 week after metamorphosis. Whole intestines were dissected and frozen prior to DNA extractions and pyrosequencing (see below). All dissections were carried out with autoclaved materials, that were only used for a single larva.

Microbiome transplant experiments: 1 and 2

To explore how transplants by cross-inoculation influences microbiome establishment and structure, we conducted two separate experiments with three species. First, we sterilized egg masses using a protocol adapted from Rendueles et al. (2012) designed for generating gnotobiotic zebrafish from eggs. Egg masses were separated into sterile centrifuge tubes with 40 mL of autoclaved, carbon-filtered water, then gently mixed and rinsed (3×). The eggs were then sterilized by a 4 h incubation with 500 µL penicillin-streptomycin (10,000 U/mL; Life Technologies #15140-122), 200 µL of kanamycin sulfate (25 µg/ML; Life Technologies #11815-032), and 50 µL of amphotericin B solution (250 µg/ML; Sigma-Aldrich #A2942). They were then rinsed (3×) with autoclaved water, and housed in autoclaved Mason jars (150–200 eggs/jar) kept in a pre-sterilized UVB chamber.

The sterilized eggs were split into two treatments: control, or cross-species gut microbiota transplant. For controls, eggs from the focal species were homogenized and added to each jar. For cross-species inoculation, intestinal tracts of donor species were dissected from three larvae, homogenized and added to the respective jars. Wood frog guts were obtained from larvae hatched and reared in the lab (i.e., the ontogenetic stock tank), while bullfrog guts were obtained from wild-caught larvae. Hatched, free-swimming larvae were allowed to feed on the inoculated egg jelly for 2 days, after which they were transferred to autoclaved, plastic containers with 20 L of carbon- and UV filtered water (four containers per treatment). All larvae were fed powdered rabbit pellets and turtle food *ad libitum* for 1 week and then whole rabbit pellets thereafter.

Experiment 1 aimed to test the potential for gut microbiota transplant in green frogs; here homogenized wood frog guts collected from GS 27-30 larvae were added to the sterilized green frog eggs. In conjunction with the ontogenetic sampling of wood frogs, donor wood frog larvae at GS 27 were compared to green frog controls and green frogs inoculated with wood frog guts 2 weeks after hatching at GS 27.

Experiment 2 aimed to test how host development influenced transplant colonization and community dynamics of gut microbiota in wood frogs; here homogenized bullfrog guts were added to sterilized wood frog eggs. In Experiment 2, control wood frog larvae, and wood frog larvae inoculated with bullfrog guts were sampled within 24 h of hatching GS 26, 2 weeks after hatching at GS 27, and at prometamorphosis-GS 35. They were compared to the wild-caught donor bullfrog larvae that were dissected at GS 38.

Microbial sample collection and sequencing

For all gut microbiome dissections and sequencing, whole intestines were dissected and frozen prior to DNA extractions. All dissections were carried out with autoclaved materials, and only used on only a single larva. DNA was extracted from the dissected intestines following the Puregene DNA extraction protocol (Life Technologies). Total DNA in each sample was quantified with a Take3^{TM} Microvolume Plate on a Microplate Spectrophotometer (BioTek Instruments INC). PCR products of a portion of the bacterial 16S rRNA gene were prepared from each sample of genomic DNA in triplicate and pooled into one amplicon library using bacterial universal primers (515F/806R) and Earth Microbiome Project protocols (Caporaso et al. 2012; Zeglin et al. 2016), with minor modifications: PCR was run for 30 cycles instead of 35% and 0.04% Bovine Serum Albumin (BSA) was included in each reaction. The library was spiked with 10% PhiX and sequenced through 2×150 paired-end cycles using the Illumina MiSeq

at the Kansas State Integrated Genomic Facility. Raw sequence data were processed using the QIIME software package (Caporaso et al. 2010): Sequences were quality filtered, joined and demultiplexed, and assigned to operational taxonomic units (OTUs) of 97% DNA sequence similarity using the open-reference workflow. The RDP classifier was used to assign taxonomy, representative OTU sequences were aligned to the GreenGenes v. 13.8 16S rRNA gene reference database, and non-aligned OTUs, singletons and doubletons were removed prior to further analysis.

The dataset coming out of pre-processing included a mean (SD) and median number of reads per library of 12480 (3570) and 12263, respectively, but all subsequent data analysis was run on a random subset of data using an equal number of reads per sample (5100 reads, to include all samples in the analysis). The mean (SD) and median number of OTUs per sample at this rarefaction depth was 744 (389) and 624, respectively, reflecting OTU collection curves that reached a saturation point. The representative OTU sequence data are available via BioProject (#PRJNA387290) at NCBI's Sequence Read Archive. The principal coordinates analysis (PCoA) ordination analysis was undertaken using QIIME to explore patterns of heterogeneity among all samples included in the study. The output model for experiment one 36.1% and 20.5% of the variation among all samples on Axis 1 and Axis 2, and for experiment two represents 48.5% and 14.2% of the variation among all samples on Axis 1 and Axis 2, respectively. Also, the relative abundance of reads among all represented taxonomic groups was exported from QIIME for further analysis.

Statistical analysis

To evaluate variation in bacterial community composition explained by different developmental stages and transplant treatment groups, we used the Adonis function in the Vegan package (Oksanen et al. 2013) for permutation-based multivariate analysis of variance (PERMANOVA) in R (R-Core-Team 2013). PERMANOVA tests were run using Bray-Curtis distance based comparison of OTU relative abundances among samples, and 9999 permutations. To test for differences in relative abundances of the predominant microbial phyla between larval amphibian species, treatment groups, and development stages we used linear models with Tukey's HSD post-hoc tests. For experiment two, we analyzed the stages separately in order to compare bullfrog donors to the swapped treatment.

Results

Gut microbiota varies with host development and species

Diversity of the gastrointestinal microbiota (GIM) in larval wood frogs varied across ontogeny as indicated by PCoA of weighted UniFrac data (Fig. 1A). Gut microbial composition shifted across development, metamorphosis (stage 43) and through the juvenile frog stage (PERMANOVA for stage in wood frogs, $F = 5.99$, $R^2 = 0.60$, $P < 0.0001$; $n = 21$). Gut microbial diversity also differed among frog species. Larval green frogs, collected as eggs and sampled at stage 27 (2 weeks after hatching) had gut microbiota distinct from wood frogs at the same stage (PERMANOVA comparison of green frogs and wood frogs at stage 27, $F = 8.36$, $R^2 = 0.54$, $P < 0.0001$; $n = 8$), despite similar lab rearing conditions and food (Fig. 1A, open circle).

Experiment 1 test of gut microbiota transplant in green frogs

Differences among species allowed for a test of the potential to transplant gut microbiomes. Sterilized green frog eggs inoculated with homogenized larval wood frog gut contents exhibited a shift in microbial diversity towards that of wood frogs (Fig. 1A, gray circle). Green frog larvae inoculated with homogenized green frog eggs (Fig. 1A, halved circle), also exhibited shifts in gut microbiota diversity relative to unmanipulated larvae (Fig. 1A, open circle). Inoculation treatment had a significant effect on green frog gut microbiota (PERMANOVA of inoculation treatment in green frogs $F = 9.89$, $R^2 = 0.71$, $P < 0.0001$; $n = 11$). Relative abundance among the most abundant phyla differed (Fig. 1B; $F_{2,\ 65} = 61.7$, $P < 0.0001$), and there was an interaction with amphibian host species ($F_{10,\ 65} = 24.7$, $P < 0.0001$). The bacterial phyla that accounted for differences were predominantly (delta)Proteobacteria, Firmicutes, and Planctomycetes (Tukey HSD $P < 0.05$).

Experiment 2 test of host development on transplant colonization of gut microbiota in wood frogs

In the second experiment, we colonized wood frog eggs with the gut content of donor larval bullfrogs (+BF) collected at stage 38 (Fig. 2). Transplant (+BF) and unmanipulated wood frog larvae sampled the day after hatching (stage 26) had similar microbiota. However, within 2 weeks of hatching, at stage 27, the +BF transplant larvae had a microbial composition shifted to be similar to that of bullfrogs and wood frog larvae at stage 35 (red triangle), but different from the wood frog controls at stage 27 as

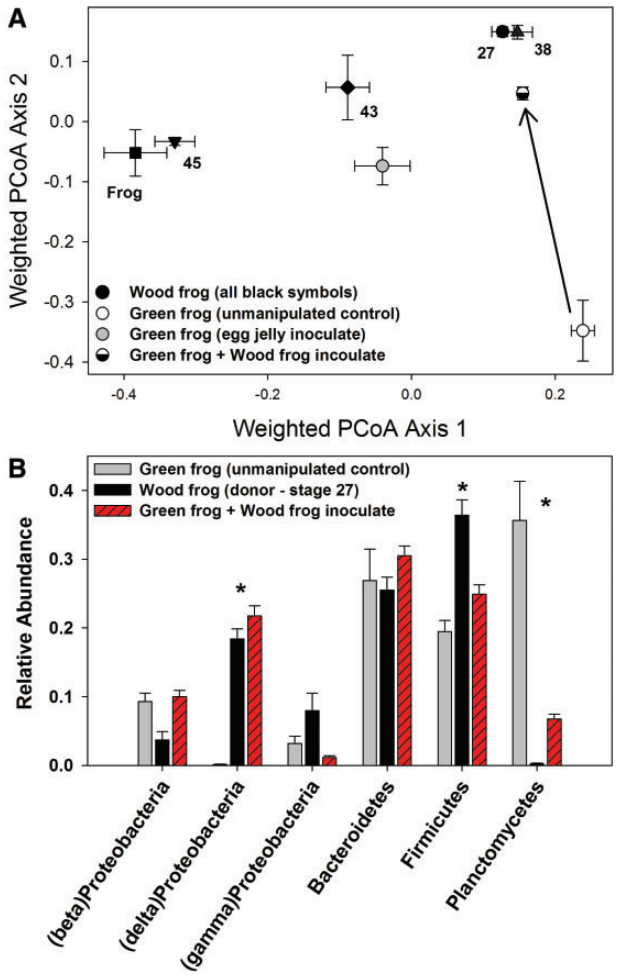

Fig. 1 (**A**) Ordination of mean±SE GIM from larval wood frogs across developmental stages (black shapes), numbers, and shapes are for differing Gosner stages. Sample size is four per group. Green frog eggs were exposed to a gnotobiotic protocol followed by inoculation with either homogenized larval wood frog guts (halved-circle and arrow), green frog egg jelly (gray circle), or unmanipulated control (white circle); green frog larvae were sampled at Gosner stage 27, 2 weeks after hatching. (**B**) Relative abundance (proportion) of phyla that were >10% among transplant green frog larvae treatments and the wood frog donor. Asterisks represent significant differences among treatments in microbial abundance within a phyla (Tukey HSD, $P < 0.05$)

indicated by PCoA of weighted UniFrac distance among bacterial OTUs (Fig. 2). PERMANOVA to evaluate the direct and interactive effects of transplanting and development stage ($n = 21$) showed the significant effect of transplant treatment ($F = 7.86$, $R^2 = 0.066$, $P < 0.0001$), stage ($F = 27.2$, $R^2 = 0.69$, $P < 0.0001$), and their interaction ($F = 8.42$, $R^2 = 0.142$, $P < 0.0001$; reflecting the contrasting microbiome at stage 27).

For stage 27 larvae, relative abundance among the most abundant phyla differed (Fig. 3A; $F_{2,\ 54} = 62.4$, $P < 0.0001$), and there was an interaction with amphibian host species ($F_{16,\ 54} = 32.4$, $P < 0.0001$). Similarly, relative abundance at stage 35 also differed (Fig. 3B; $F_{2,\ 54} = 66.7$, $P < 0.0001$), and there was an interaction with amphibian host species ($F_{16,\ 54} = 42.9$, $P < 0.0001$). The microbial phyla that accounted for these differences at stage 27 were (beta)Proteobacteria, (gamma)Proteobacteria, Bacteroidetes, and Fusobacteria (Fig. 3A; Tukey HSD $P < 0.05$), and at stage 35 Firmicutes became more predominant (Fig. 3B).

Discussion

In this study, we explored how gut microbiota diversity of larval amphibians varies among species and across ontogeny, and tested whether gut microbiome manipulation at hatching influences community structure across host life stages. We found that gut microbiomes differ among larvae of three amphibian species: wood frogs, green frogs, and bullfrogs. Furthermore, we found that relative abundance of gut microbial phyla change across larval ontogeny, with distinctive differences between larvae, metamorphic animals, and juvenile frogs. Finally, we demonstrated that through applying a gnotobiotic protocol to eggs (Rendueles et al. 2012), and subsequently cross-inoculating with gut contents from differing species, that transplanting microbiota between species is a feasible approach to manipulate amphibian microbiomes. Below we explore the implications of these data, as well as discuss the value of this approach for examining interactions between hosts and their microbiomes, and as a potential tool for amphibian conservation.

Across the three amphibian species queried, microbial richness was generally similar but relative abundance varied. Nine microbial phyla were consistently detected among all samples and the three larval amphibian species surveyed (Figs. 1 and 3), with another six phyla rarely detected (Acidobacteria, Actinobacteria, Armatimonadetes, Cyanobacteria, Gemmatimonadetes, Tenericutes). Relative abundance among the commonly detected phyla was dominated by Bacteroidetes, Firmicutes, Proteobacteria, and to a lesser extent Planctomycetes and Fusobacteria. These diversity and dominance patterns are in accordance with the few previously published studies of gut microbiota in amphibians (Kohl et al. 2013; Chang et al. 2016; Vences et al. 2016; Weng et al. 2016). Fusobacteria had the highest relative abundance in bullfrog larva collected from local ponds at Gosner stage 38, whereas control wood frog and green frog larvae were hatched in pond water that included pond debris in the lab, and fed the same rabbit

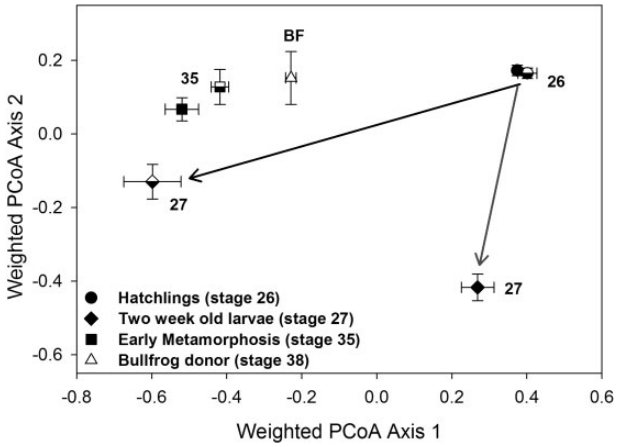

Fig. 2 Ordination of mean±SE GIM in larval wood frogs across three developmental stages (differing shapes), numbers are for Gosner stages. Sample size is three-four per group. As eggs, wood frog larvae were exposed to the gnotobiotic sterilization followed by inoculation with either homogenized wood frog eggs (Control; black shapes) or guts of larval bullfrogs (+BF; halved shapes). Note the bullfrog guts used for inoculation were from stage 38 larvae (triangle). Hatchling wood frog larvae (stage 26, circles) were sampled within a day of hatching, whereas stage 27 larvae (diamonds) were sampled within 2 weeks of hatching. Note, arrows highlight unique shifts at 27 in +BF larvae

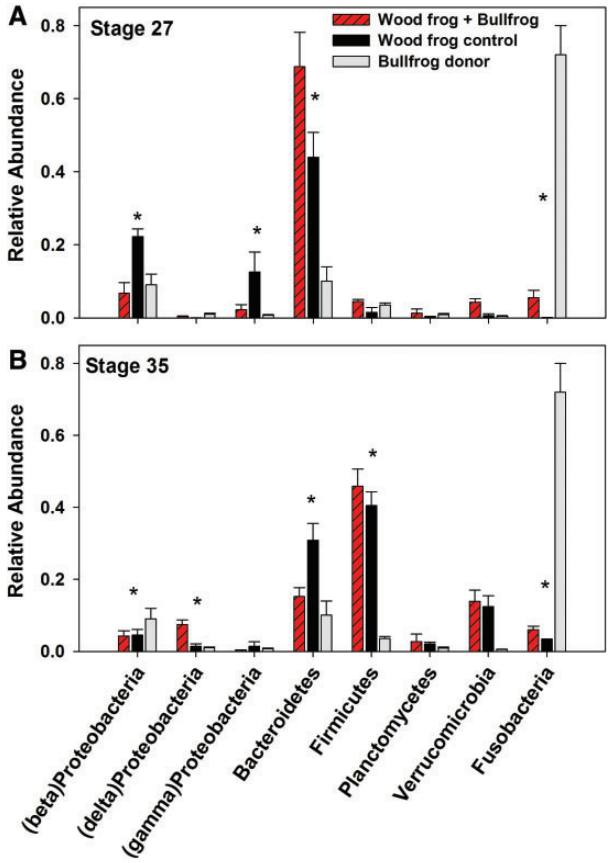

Fig. 3 Relative abundance (mean ± SE) of microbiota that were >10% among the transplant wood frog larvae across Gosner stages 27 and 35 (as in Fig. 2) exposed to the gnotobiotic protocol. Wood frogs that inoculated with bullfrog gut content (+BF, hatched bars) are compared to wood frogs inoculated with eggs (black bars) and the donor bullfrogs (GS 38; gray bars). Note the data presented for bullfrogs is duplicated in panels (**A**) and (**B**) for reference

pellet food. Fusobacteria include facultative aerobic and obligate fermentative anaerobes that are and found in sediments as well as intestines of diverse animal taxa (Krieg et al. 2010). The relative dominance of Fusobacteria in these bullfrog larvae may thus reflect the anoxic conditions in their natal ponds. However, the predominant microbial phyla across all three amphibian species were all likely fermenters. Indeed, the coiled guts of detritivorous larval frogs allows for significant microbial fermentation in the small intestine and colon, where fermentative metabolites such as short-chain fatty acids are produced and account for up to 20% of larval daily energy requirements (Pryor and Bjorndal 2005). Together, these results suggest that while diet and host morphology directly shape microbiome structure, ecology suggest as variation in pond conditions like anoxia levels likely also plays a role as well.

Our results also suggest that in addition to species level effects, developmental shifts across ontogeny strongly influence amphibian gut microbiota. As larval amphibians metamorphose, their coiled guts undergo massive tissue remodeling (Schreiber et al. 2009; Ishizuya-Oka 2011) in association with remodeling of limbs, tail, and the immune system in order to transform into terrestrial frogs. As Kohl et al. (2013) demonstrated, the gut microbiota of larval amphibians also undergoes restructuring. Our results further detail this restructuring, and demonstrate that microbiota remodeling is not a result of shifts in diet but fundamentally associated with remodeling of the coiled gut of a detritivore to that of a terrestrial, insectivorous frog. These shifts in relative abundance among gut microbiota could result from the obligate fasting that larval amphibians undergo during metamorphic climax. Microbial phyla could shift in dominance due to community interactions including competition and cross-feeding, as well as selection for microbes that metabolize differing energy substrates (Kohl et al. 2014; Koh et al. 2016). Additionally, the amphibian host immune system may influence and even regulate shifts in microbial community composition during metamorphosis, similar to host control in metamorphic insects (Johnston and Rolff 2015). For example, phagocytic immune cells play a critical role in amphibian tissue remodeling whereby macrophages are repurposed for phagocytizing apoptotic larval tissues

(Ishizuya-Oka 2011). Lymphocytes and the adaptive immune system also undergo regulatory shifts during metamorphosis to limit conflicts in self-recognition, and diverse immune cells that regulate the gut-associated lymphoid tissue (GALT) mature. Growing evidence in mammals suggest that while gut microbes play a critical role regulating GALT development and immune function, the host immune system also likely actively regulates gut microbiome composition and function (Shulzhenko et al. 2011; Brown et al. 2013; Zhang et al. 2015). Clearly further work is required to test how host specific factors shape such ontogenetic shifts and variation in microbial diversity in larval microbiomes.

Our gut microbiota transplant experiments provide some insight into how initial microbial colonization influence the trajectory of gut communities and potentially shape microbiome structure across ontogeny in amphibians. In two transplant experiments between three species, we found that larvae inoculated with another species gut contents had microbiome composition that shifted away from their native community and towards that of the donor community (Figs. 1 and 2). These results suggest that amphibians may have a critical developmental window at hatching during which microbial colonization and the trajectory of a core microbiome is established, as is common among humans and other animals (Arrieta et al. 2014; Cox et al. 2014; Rodriguez et al. 2015). Our second experiment is most revealing in regards to potential developmental windows for microbiome establishment. Here, wood frogs colonized with bullfrog gut contents were initially similar in community composition just after hatching (Fig. 2; GS 26), but then diverged within 2 weeks (GS 27) to be more similar to bullfrogs (GS 38) and late stage wood frogs (GS 35). These results suggest that community interactions among microbial competitors, agonists, and antagonists during the early periods of community establishment take time to develop. This shift towards a microbiome more similar to late staged larvae also suggests that conditions at hatching or very early larval development may shape the community trajectory over host ontogeny. Presumably, such microbiome changes impact their amphibian hosts and may play a role in shaping their development as well as have lasting effects on their function, as is common in other taxa (Sommer and Bäckhed 2013; Cox et al. 2014; Kerr et al. 2015; Rodriguez et al. 2015). Indeed, in previous studies we have found that environmental and maternal conditions at hatching can have profound effects on wood frog developmental trajectories (Warne et al. 2013; Warne and Crespi 2015). Taken together, these results suggest acquisition of gut microbiomes during potential critical developmental windows in amphibians could have a strong but little tested effect on amphibian life histories.

These results showing that initial inoculation at hatching influences amphibian gut microbiomes, also have implications for bioaugmentation of amphibians in the context of conservation. Specifically, our results suggest that establishment of amphibian gut microbiomes in particular, but potentially skin microbiota as well, occur during a critical window at hatching. In regards to conservation, Rebollar et al. (2016) for example, found that bioaugmentation using a probiotic bacterium in adult frogs, rather than larva, induced a transitory effect on the presence of the select bacteria, and the skin microbiome as a whole. Such bioaugmentation of the skin microbiome in amphibians is often transitory and may be a result of factors associated with skin, but potentially could also be shaped by critical developmental windows. These findings demonstrate that researchers interested in bioaugmentation to counter emerging pathogens such as chytrid fungus (Bletz et al. 2013; Woodhams et al. 2016) or ranavirus (Warne et al. 2011; Brunner et al. 2015) could explore manipulating amphibian microbiomes during potential critical developmental windows.

Finally, our results for microbiome colonization at hatching also suggest that either the egg jelly or the capsule surrounding amphibian eggs is likely an important source for microbial inoculation. The importance of these egg structures for symbiosis in amphibians is apparent in the interactions of *Oophila amblystomatis*, green algae, and salamander and frog eggs. This algae grows within individual egg capsules of the salamander *Ambystoma maculatum*, as well as ranid frogs including wood frogs, and has recently been reported to invade embryonic tissues and cells (Kerney et al. 2011; Kim et al. 2014). Amphibian embryos benefit from increased oxygen concentrations associated with the algae, whereas algae are thought to benefit from nitrogenous wastes released by the embryos (Kerney 2011). Most relevant for our study however, is that Kerney et al. (2011) found strong evidence for oviductal transmission of this algal symbiont. Thus, we believe it is probable that initial inoculation and colonization of amphibian microbiomes also occurs by vertical transmission, which is common among many animal taxa (Walke et al. 2011; Funkhouser and Bordenstein 2013). While explicit tests are required to confirm vertical microbial transmission in amphibians (Walke et al. 2011), our results clearly demonstrate a strong potential for both maternal and

environmental effects to shape the structure and function of amphibian gut microbiomes. Last, greater exploration and tests of the importance of developmental windows for microbial colonization, and how these shaped both amphibian microbiomes and adult phenotypes promise to shed needed light on host-microbe interactions as well as provide to new tools for conservation.

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