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RESEARCH ARTICLE

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Manipulation of gut microbiota during critical developmental windows affects host physiological performance and disease susceptibility across ontogeny

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Abstract

1. Colonization of gut microbiomes during early life can shape metabolism and immunity of adult animals. However, most data are derived from antibiotic-treated or germ-free laboratory mammals. Furthermore, few studies have explored how microbial colonization during critical windows influences a suite of other fitness-related traits in wild animals.
2. This study tested whether hatching constitutes a critical developmental window for gut microbiome colonization in wild-caught amphibians and whether perturbations to gut microbiota at hatching shape fitness-related traits of larval growth, metabolism, metamorphosis and disease susceptibility.
3. We sterilized wood frog eggs and then inoculated them with microbes from differing sources, including from another species (bullfrogs) that differ in disease resistance and life history. We measured development, growth and metabolic rates through metamorphosis among individuals from each microbial treatment. A separate group was exposed to an LD_{50} dose of ranavirus—an emerging disease—to test for microbiome effects on disease susceptibility. We also quantified rates of deformities to test for microbial treatment effects on overall health.
4. Manipulation of microbiota on eggs altered the trajectory of gut microbiome communities across larval ontogeny, though disruption appeared to be transitory. While microbiome structure converged among the treatments by metamorphosis, the effects of disruption on host phenotypes persisted. Larvae inoculated with the bullfrog gut microbiota exhibited accelerated growth and development rates compared to controls. By contrast, sterilized larvae maintained in sterile water for several days after hatching exhibited greater disruption to their gut microbiota across ontogeny, as well as altered metabolism, more tail deformities, and were more likely to die when exposed to an LD_{50} dose of ranavirus compared to the other treatments.
5. These results suggest perturbations to the microbiota during critical developmental windows can alter the trajectory of the gut microbiome, and have long-term effects on fitness-related traits in larval amphibians. These results suggest that explicit tests of how changes in the composition and abundance of the microbial community shape phenotypes across ontogeny in amphibians could shed light on

host–microbe interactions in wildlife, as well as inform conservation efforts to mitigate emerging diseases.

KEYWORDS
amphibian microbiome, carry-over effects, emerging disease, fluctuating asymmetry, intestinal microbiota resilience, phenotypic plasticity, ranavirus

1 | INTRODUCTION

While gut microbiomes can have profound effects on their hosts, we are only beginning to understand how variation in the source and timing of initial colonization shapes the function and health of animals across ontogeny. Initial colonization during developmental windows such as birth appears to shape the gut microbial community in adults and may have lifelong effects on immunity and metabolic function of animals (Arrieta, Stiemsma, Amenyogbe, Brown, & Finlay, 2014; Borre et al., 2014; Cox et al., 2014; Funkhouser & Bordenstein, 2013). For example, caesarian birth in humans, as well as perturbations of microbial communities by antibiotics in newborn mice, are associated with increased risks of metabolic syndromes and obesity (Arrieta et al., 2014; Cox et al., 2014). Furthermore, while microbial communities often appear to recover after disruptions caused by antibiotics or other factors, the altered host metabolic phenotypes can persist if the disruption occurred during early life, supporting the potential for critical developmental windows to shape microbial and host interactions (Cox et al., 2014; Sommer & Bäckhed, 2013). While the majority of this research is on mammals in the context of human health and agricultural practices, non-model wildlife present unique opportunities to gain insight into microbial and host interactions within ecologically and evolutionarily relevant contexts (Pascoe, Hauffe, Marchesi, & Perkins, 2017; Potti et al., 2002; Videvall et al., 2018; Warne, Kirschman, & Zeglin, 2017). In addition, varied gut microbiome composition across ontogeny could also have profound but poorly explored effects on a suite of other fitness-related traits of hosts ranging from development and growth, to behaviour, and infectious disease susceptibility (Bauer, Huus, & Finlay, 2016; Clarke et al., 2014; Hsiao et al., 2014; Lee & Brey, 2013; Sommer & Bäckhed, 2013; Villarino et al., 2016; Warne, 2014; Zaneveld, McMinds, & Vega Thurber, 2017).

The aims of this study were to explore whether alterations to gut microbiota during critical developmental windows influence development, physiology and disease susceptibility in wild-caught larval amphibians. We recently demonstrated that hatching appears to be a critical window for microbial colonization of the gut in larval amphibians (Warne et al., 2017). Specifically, we found that manipulation of the microbiota on amphibian eggs influences the structure and trajectory of the gut microbiome across ontogeny, similar to patterns found in other host taxa. There is a good reason to expect that such variation in gut microbiota during early life may influence host development and homeostatic function in amphibians, and other vertebrates. This is because symbiotic microbes produce a diversity of metabolites including essential amino acids, vitamins and signalling molecules that are critical to a diversity of host physiological processes (Neuman, Debelius, Knight, & Koren, 2015; Sampson & Mazmanian, 2015; Sommer & Bäckhed, 2013). Furthermore, an emerging paradigm suggests gut microbiota may serve as an understudied endocrine organ that plays a central role in regulating host processes including growth and development (Bauer et al., 2016; Clarke et al., 2014; Neuman et al., 2015). To explore these understudied interactions, we used microbiota manipulation of amphibian eggs to test whether varied gut microbiome composition is associated with variation in larval development and growth rates, metabolic rates and susceptibility to ranavirus—an emerging infectious disease of ectothermic vertebrates (Brunner, Storfer, Gray, & Hoverman, 2015; Duffus et al., 2015; Kirschman, Palis, Fritz, Althoff, & Warne, 2017).

In amphibians, susceptibility to infection and disease-induced mortality from emerging diseases that include ranaviruses is often associated with developmental stages around metamorphosis. Elevated infection rates and pathogenicity in association with metamorphosis suggest that remodelling of diverse amphibian tissues and their microbiota during this transition may constitute a critical window for disease vulnerability, which are periods of greatly diminished resistance to infections that can result in massive die-offs of wildlife of a given lifestage (Johnson, Kellermanns, & Bowerman, 2011; Kirschman, Crespi, & Warne, 2018; Rohr, Raffel, & Hall, 2010; Warne, Crespi, & Brunner, 2011). We suspect varied susceptibility to ranavirus across differing life stages could be influenced by ontogenetic shifts in gut microbiome structure, as suggested by recent work in mammals; where the prevalence and virulence of infections tend to be highest in larva, neonates and juveniles, but decline through adulthood (Arrieta et al., 2014). For ranavirus, the intestines appear to be the primary route of ranavirus infiltration (Robert, George, De Jesús Andino, & Chen, 2011), which suggests disruption of the intestinal epithelia (Ishizuya-Oka, 2011; Schreiber, Cai, & Brown, 2005) and gut microbiome (Abt et al., 2012; Honda & Littman, 2012; Kamada, Chen, Inohara, & Nunez, 2013) during late developmental stages around metamorphosis could play an important but poorly understood role in susceptibility to infection. Emerging evidence suggests that interactions among gut-associated lymphoid tissues and their associated microbiota also play a central but still poorly understood role in shifting disease susceptibility in developing and young animals (Abt et al., 2012; Arrieta et al., 2014; Honda & Littman, 2012). Expanding our understanding of how animal microbiomes contribute to changes in disease susceptibility

could aid in developing new methods for combating pathogens and contribute to conservation efforts of threatened wildlife.

In this study, we sterilized wood frog eggs and then inoculated them with microbiota from either the intestines of wild-caught bullfrog larvae, environmental sources or homogenized wood frog eggs (control). Here, we used bullfrogs as a donor because these taxa are generally resistant to ranavirus, and we aimed to test whether resistance to infection could be mediated by transplanted microbiota. Bullfrogs are also much larger in body size and slower growing and developing as larvae, compared to the smaller wood frogs that metamorphose quickly to escape drying ephemeral ponds. We then quantified growth, metabolism and developments rates through metamorphosis among individuals from each treatment to assess whether transplanted microbiota between species affected these fitness-linked traits. A separate group was exposed to a known LD_{50} dose of ranavirus to test for microbiome associated shifts in susceptibility to this pathogen. We also quantified rates of morphological asymmetry among the developing larvae as another means of assessing the potential effects of microbial treatments on health. Through these efforts, we tested the hypothesis that perturbations to amphibian microbiomes at hatching alter the trajectory of the gut microbiota across ontogeny. We also tested the hypotheses that larval development, growth and metabolic rates would vary in association with altered gut microbiome trajectories. Finally, we also tested the hypothesis that altered gut microbiomes would influence susceptibility to ranavirus infection.

2 | MATERIALS AND METHODS

2.1 | Animal collections

We collected one wood frog egg mass, 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).

2.2 | Microbiome manipulation by egg sterilization and inoculation

We sterilized the wood frog egg mass using a protocol adapted from Rendueles et al. (2012) designed for generating gnotobiotic zebrafish from eggs, followed by inoculation with varied sources (Warne et al., 2017). We used a single egg mass to minimize genetic effects. The egg mass was separated into 18 sterile centrifuge tubes (approximately 30 eggs/tube) with 40 ml of autoclaved, carbon-filtered water, then gently mixed and rinsed three times. The eggs were then sterilized by a 4-hr incubation on a nutator with 500 μl penicillin-streptomycin (10,000 U/ml; Life Technologies #15140-122), 200 μl of kanamycin sulphate (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 distributed into 12 autoclaved Mason jars (approximately 50 eggs/jar) kept in a pre-sterilized UVB chamber and aerated with air filtered by a HEPA inline filter disk (0.3 μm pore, Whatman Inc.).

The sterilized eggs were split into three treatments: control, gut microbiota transplant from bullfrogs or environmental inoculation. For controls, wood frog eggs from the original wild-collected clutch that were not exposed to the antibiotic treatment (unmanipulated) were homogenized and added to the respective jars (four per treatment). For the +bullfrog inoculation, intestinal tracts were dissected from three larval wild-caught bullfrogs (Gosner stage 38), homogenized and added to the respective jars. Finally, for the no inoculation treatment, we simply placed the sterilized eggs in sterile water, but they were not maintained germ-free and thus were likely colonized by microbes remaining on the eggs or from the air and water. The hatched larvae were allowed to feed on the inoculated egg jelly for two days, after which they were transferred with their egg contents to autoclaved, plastic containers with 20 L of carbon-filtered water sterilized by a circulating UV system (four containers per treatment). Subsequent larval densities were variable across these tubs because of variation in hatching success (we estimate 50%–90% hatched per jar), and early larval survival past the first few days. To account for this variance, the free-swimming larvae from the relevant treatments were combined seven days after hatching in larger autoclaved 60-L plastic tubs (one stock tub per treatment), and then, individuals were randomly removed for the experiments. The advantage of tracking individuals as a sample unit, rather than tubs, is that co-housed larvae stratify in response to conspecifics, which influences their physiology, growth and behaviour (Araujo, Kirschman, & Warne, 2016; Warne & Crespi, 2015; Warne, Kardon, & Crespi, 2013). Individual housing allowed us to exclude these confounding factors and thus restrict phenotypic responses across ontogeny to early life microbial inoculations. Sixty larvae from the mixed groups were separated into individual 750-ml containers (20/treatment) for monitoring of growth, metabolic and development rates (see below). Another 54 larvae were separated for exposure to an LD_{50} dose of ranavirus; sample size was limited by the amount of virus stock we had at the time (see below). Finally, larvae were also separated for destructive sampling of gut microbiomes, whereby three to four larvae per treatment were sampled at three time points: within 24 hr of hatching at stage 22, two weeks after hatching at stage 27 and at stage 35 (see below). Larvae remaining in the stock tubs were saved for separate experiments. All larvae were fed powdered rabbit pellets and turtle food ad libitum for 1 week and then whole rabbit alfalfa hay pellets thereafter.

2.3 | Microbial sample collection and sequencing

Wood frog larvae were sampled within 24 hr of hatching at stage 22 (four larvae/treatment were sampled, but we had to combine two/four samples to provide sufficient DNA that resulted in a final sample size of two/treatment), two weeks after hatching at stage 27 (n = 3/treatment) and at stage 35 (n = 3/treatment). The wild-caught donor bullfrog larvae (n = 3) that were dissected at stage 38 were also included in analysis. Note, because our study was exploratory, we only

sampled three individuals per treatment and sample period, which provided for a general characterization of the microbial treatment effects on gut microbiomes. Whole intestines were dissected using single autoclaved instruments and frozen at −80°C for later DNA extractions and gut microbial sequencing. Larvae and samples were rinsed (3 ×) with sterile water before and after dissection to limit the microbial sample to gut content. DNA was extracted following the Puregene DNA extraction protocol (Life Technologies). Total DNA in each sample was quantified with a Take3™ Microvolume Plate on a Microplate Spectrophotometer (BioTek Instruments INC). PCR products of the bacterial 16S rRNA gene V4 region 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, Wang, Waythomas, Rainey, & Talbot, 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 1 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 (Bokulich et al., 2013).

The resulting dataset from pre-processing included a mean (*SD*) and median number of reads per sample of 12,480 (3,570) and 12,263, respectively, but all subsequent data analysis was run on a random subset of data using an equal number of reads per sample (5,100 reads, to include all samples in the analysis). The mean (*SD*) and median number of OTUs per sample at this rarefaction depth were 744 (389) and 624, respectively, reflecting OTU collection curves that reached a saturation point. Results for all samples were exported from QIIME for further analysis.

2.4 | Development, growth and metabolic measurements

We measured the microbial treatment effects on larval wood frog development, growth and metabolism on the 60 larvae housed individually in 750-ml plastic tubs and maintained through metamorphosis. Growth and development rates were assessed by weight and Gosner stage measurements at days 11, 25 and 39 after hatching. Metabolic rates were measured twice on days 25 (mean stage ± *SE* = 35 ± 0.3) and 39 (stage = 39 ± 0.3). During the second metabolic measurement period, larvae were undergoing metamorphosis, which is when differences in energetic costs among individuals and treatments are most apparent (Kirschman, McCue, Boyles, & Warne, 2017). Metabolic rates were measured by oxygen consumption at time 0 and again at 60 min, at room temperature, via closed respirometry in centrifuge tubes with 58 ml of oxygen-saturated (>7.5 ppm) water using a multi-parameter DO probe (Hach HQ40d, Loveland, CO, USA).

2.5 | Disease interactions with microbiome treatments

We challenged larvae from each microbial treatment with an LD50 dose of ranavirus (*n* = 18/treatment) to test for interactions between the gnotobiotic inoculation treatments and viral infections (Araujo et al., 2016; Kirschman et al., 2018; Warne et al., 2011). Infections took place in 200 ml baths containing $10^{2.5}$ (pfu/ml) of ranavirus for 24 hr (Warne et al., 2011) using an FV3-like virus strain (AEC37-2; Brunner et al., 2015). We monitored daily for survival and euthanized survivors at metamorphosis (stage 46) by benzocaine overdose (0.1%). Dissected livers were analysed by qPCR on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) to assess infection status (detailed methods in Kirschman et al., 2018).

We also quantified rates of morphological asymmetry among the developing larvae as another means of assessing the potential effects of microbial treatments on health. This was an unplanned assessment that was opportunistically measured once we observed high rates of tail deformity. All larvae with tail deformities universally exhibited moderately to extremely bent tails as shown in Figure 4. We counted the frequency of tail deformity among all of the larvae housed individually for the developmental and ranavirus LD_{50} experiments.

2.6 | Statistical analysis

To evaluate variation in bacterial community composition explained by different developmental stages and transplant treatment groups, a non-metric multidimensional scaling (NMDS) ordination, using the Bray–Curtis distance among samples (a non-phylogenetically explicit metric of difference in relative abundance of all taxa), was run using the "metaMDS" command (Vegan package in R). Alpha diversity assessed as both the total observed OTU richness and the Shannon diversity index and the relative abundance of reads among all represented taxonomic groups were estimated. We then 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 both the UniFrac distance and the Bray–Curtis distance comparisons of OTU relative abundances among samples, and 9,999 permutations. We used pairwise tests to assess differences in relative abundances of the predominant microbial phyla between treatment groups; here, we analysed development stages separately.

Growth rates among individually housed larvae were quantified by changes in body mass across three measurement periods (days 11, 25 and 39 after hatching). We compared growth rates among the treatments using mixed linear models with body mass as the response variable, and treatment, sample day (as an ordinal factor), and their interactions as fixed effects; individual was included as

a random factor to account for repeated measures. We tested for differences in metabolic rates among treatments also using a mixed linear model with treatment, body mass, sample day (days 25 and 39 as ordinal) and their interactions as fixed effects; individual was included as a random factor to account for repeated measures. We used a competing risks model to test for microbial treatment effects on larval development and mortality through metamorphosis (cmprsk package in R, Gray, 2015). This model uses the cumulative incidence function to account for an outcome when two competing and dependent outcomes are possible (i.e. metamorphosis or death; Kirschman et al., 2018). We also used a proportional hazards model to estimate risk ratios for metamorphosis and death, rather than rely on sub-hazards of the competing risks model as post hoc tests because sub-hazards are more descriptive and not pairwise tests of cause-specific hazards (Gray, 2015).

We also used a competing risks model along with a proportional hazards model to estimate risk ratios for the larvae exposed to an LD_{50} dose of ranavirus, to test for the effects of microbial treatments on mortality and metamorphosis following exposure. We then used a logistic fit to test whether viral load predicted survival. We also tested for viral load and treatment effects on disease progression, specifically time to death among larvae that died, and time to metamorphosis among survivors. Here, we used separate generalized linear models (Poisson error) that included days to death or metamorphosis as the response variable and viral load, microbial treatment, and their interaction as fixed effects; estimates were derived from the Firth bias-adjusted maximum-likelihood approach to account for overdispersion. We then used contrasts for post hoc tests of differences among the treatments. Finally, a chi-square test was used to test for differences in the frequency of tail malformations in association with microbial treatments among larvae reared individually (development and LD_{50} animals combined); risk ratios were calculated for post hoc group comparisons. Analyses were conducted using JMP Pro 13, unless otherwise noted.

3 | RESULTS

Gastrointestinal microbial community structure in larval wood frogs shifted during development and differed between inoculation treatments (Figure 1). PERMANOVA evaluation showed direct and interactive effects of microbial treatment and development stage on gut microbiota (UniFrac and Bray–Curtis distance, respectively: $F = 2.2, 9.8$; $R^2 = 0.21, 0.24$; $p < 0.0001$), stage ($F = 4.3, 26.1$; $R^2 = 0.36, 0.43$; $p < 0.0001$) and their interaction ($F = 2.9, 5.7$; $R^2 = 0.17, 0.19$; $p < 0.0001$); these results reflect the contrasting microbiomes among +bullfrog and the other treatments at stage 27. While control, +bullfrog and no inoculation wood frog larvae sampled the day after hatching (stage 22) had similar microbial communities (Figure 1, open symbols), the structure of their microbiota differed within two weeks of hatching, at stage 27 (Figure 1, light grey symbols). The +bullfrog larvae at stage 27 had a gut microbial composition distinct from the other treatments (Figure 1, light grey squares). By stage 35 (40 days after hatching), microbial communities among the treatments converged.

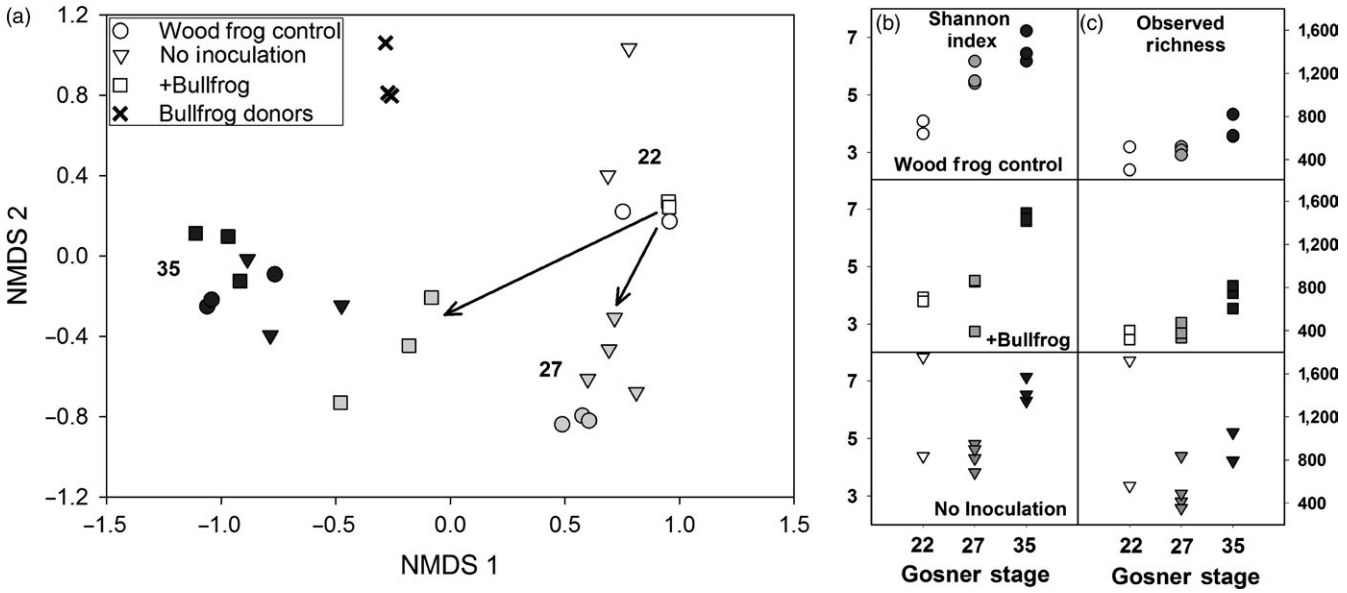

FIGURE 1 Non-multidimensional scaling (NMDS) plot based on Bray–Curtis distances of gastrointestinal microbiota in larval wood frogs across three developmental stages (numbers are Gosner stage); shading of shapes represent stage (a). Clear shapes are Gosner stage 22, light grey are stage 27 and black are stage 35. Differing symbols are microbial inoculation treatments of controls inoculated with wood frog eggs (circles), +bullfrog are wood frog larvae inoculated with bullfrog guts (squares) and wood frog that received no inoculation except environmental sources (triangles). Arrows show differing shifts between +bullfrog and the other treatments at stage 27. The donor bullfrogs used for inoculation of the +bullfrog larvae were at Gosner stage 38 (crosses). Shannon diversity indices calculated for developmental stages by treatment (b), and observed richness (c). Figure layout inspired by (Risely, Waite, Ujvari, Hoye, & Klaassen, 2018)

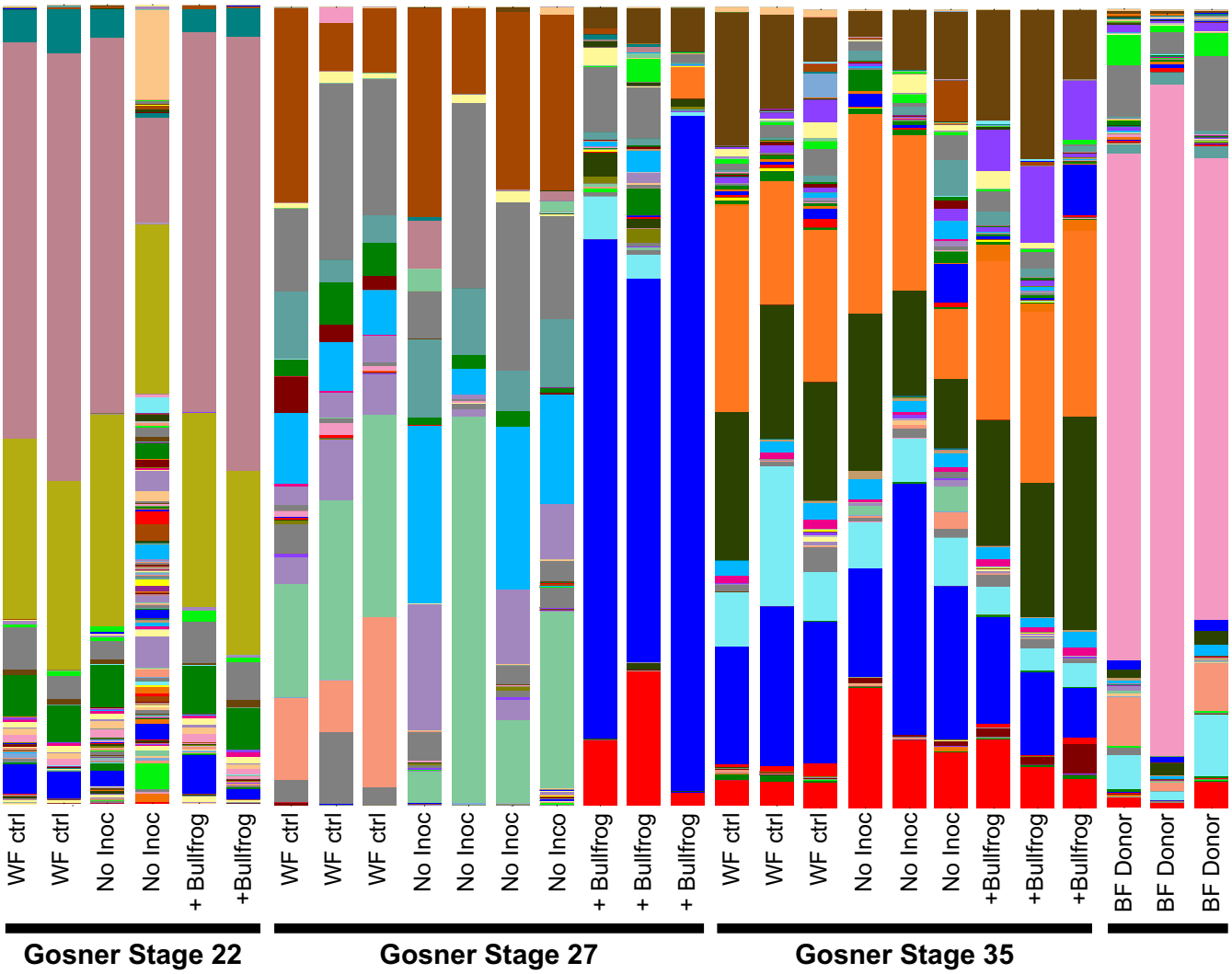

FIGURE 2 Bacterial composition of wood frog larvae across developmental stages and inoculation treatments. Bacterial taxonomy is grouped by family. (Colour figure available on digital version)

Relative abundance among the bacterial phyla varied across host developmental stages (Figure 2). At stage 22, a day after hatching, Gammaproteobacterial families Moraxellaceae and Enterobacteriaceae were dominant in all individuals ($p < 0.05$), and the no inoculation treatment showed greater variation compared to the other treatments. At stage 27, two weeks after hatching, family Bacteroidaceae (Bacteroidetes phylum) was dominant among +bullfrog individuals, while the other treatments had a higher relative

abundance of alpha-, beta- and gammaproteobacterial families (Rhizobiaceae, Comamonadaceae and Xanthomonadaceae, respectively; Figure 2, p < 0.05). At stage 35 (40 days after hatching) when early metamorphic transition begins, the dominant families in all treatments were Lachnospiraceae, Ruminococcaceae (both phylum Firmicutes) and Bacteroidaceae, as well as taxonomically unassigned sequences (Figure 2, p < 0.05).

Gut microbiome treatment influenced growth, metabolic and development rates in wood frog larvae. Growth rate as measured by change in body mass across three sample periods was associated with time (Figure 3a; Supporting Information Table S1; $F_{2,111.1} = 329$, $p < 0.001$), and a treatment × sampling day interaction ($F_{4,111} = 3$, $p = 0.02$); treatment alone did not affect growth rates. The +bullfrog treatment exhibited faster growth by the last sample day when they neared metamorphic climax (Figure 3a; Tukey's HSD $p < 0.05$). Metabolic rates were associated with treatment (Figure 3b; Supporting Information Table S2; $F_{2,96.7} = 9.2$, $p < 0.001$), a treatment × sampling day interaction ($F_{2,59.6} = 17.7$, $p < 0.001$) and body mass ($F_{1,98} = 4.5$, $p = 0.4$). While both control and +bullfrog larvae exhibited increased metabolism in association with growth, larvae from the no inoculation treatment by contrast exhibited an initially high and then declining metabolic rate across the sampling periods (Figure 3b). Microbial treatments also influenced development rates and the competing risks of metamorphosis and mortality (Supporting Information Table S3; $p < 0.01$). Among these larvae, 2/20 died prior to metamorphosis in both the control and +bullfrog treatments, whereas 6/20 died in the no inoculation treatment. Larvae from the +bullfrog treatment metamorphosed at a rate three times that of the other treatments (risk ratio; Supporting Information Table S3; $p < 0.001$). The +bullfrog larvae had a faster median time to metamorphosis of 44 days, compared to the other treatments that took over 49 days. Mass at metamorphosis varied among the treatments (mean ± *SE*, +bullfrog larvae at 0.28 ± 0.02 g; controls 0.25 ± 0.02 g; no inoculation at 0.23 ± 0.02 g); however, these differences were not significant ($p > 0.05$).

Microbial treatment also affected overall health and disease patterns in wood frogs. First, tail malformations varied with treatment among larvae housed individually for both the growth and ranavirus assessments (Figure 4, $\chi^2 = 6.8$, $p = 0.03$, $N = 114$). Among the no inoculation treatment, 26% of larvae had deformities (10/38) and had an odds ratio six times greater of having tail deformities compared to the 5% (2/38) of controls with deformities (odds ratio $p = 0.02$, CI = 1.3–31.7). Rates of deformity among the +bullfrog larvae (16%, 6/38) were not significantly different from controls. Finally, microbial treatment influenced the competing risks of metamorphosis and mortality after exposure to an LD_{50} dose of ranavirus (Figure 5a; Supporting Information Table S4; $p < 0.001$, $N = 54$). Larvae from the no inoculation treatment were three times more likely to die than controls (risk ratio; Supporting Information Table S4; $p < 0.01$), and five times more likely to die than the +bullfrog treated larvae (risk ratio; Supporting Information Table S4; $p < 0.001$). While the +bullfrog larvae appeared to have higher survival, this difference was not significant (Figure 5a; Supporting Information Table S4; $p > 0.05$). All larvae were positive for ranavirus and log viral load affected survival status; larvae with higher viral loads were more likely to die ($\chi^2 = 53.2$, $p < 0.001$). Microbial treatment also influenced disease progression

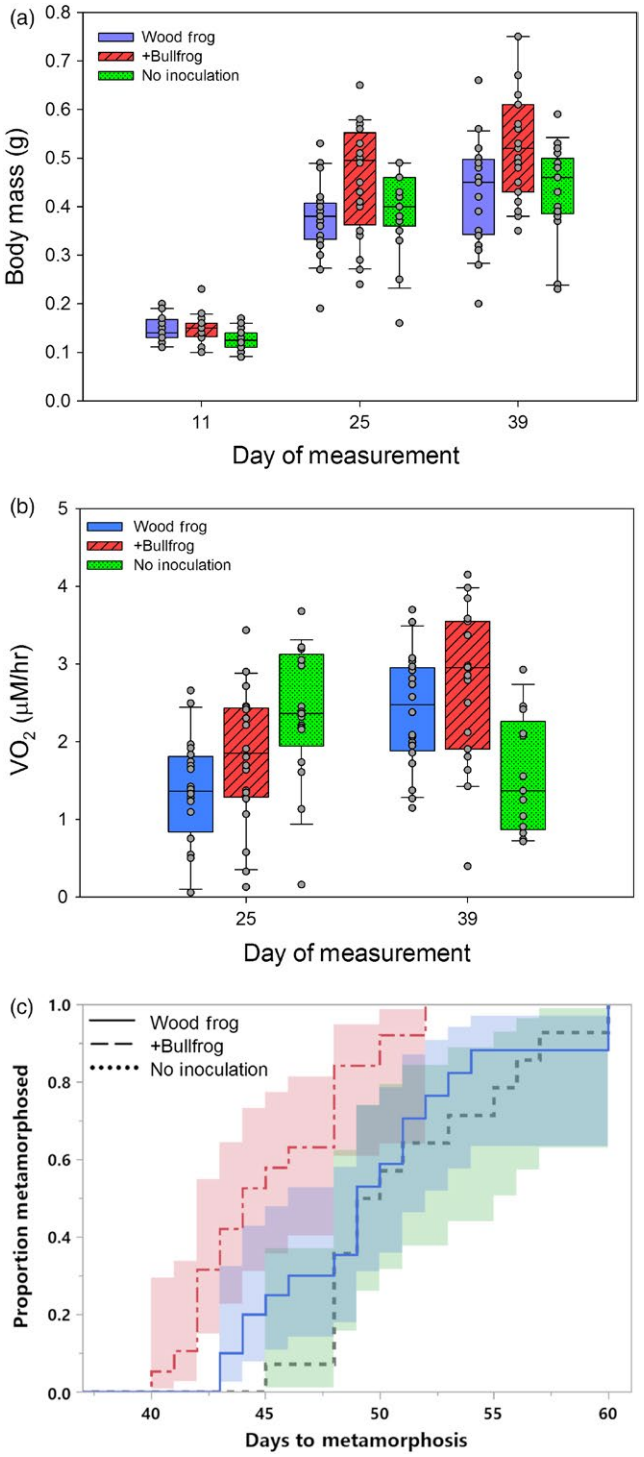

FIGURE 3 Wood frog larvae exposed to egg sterilization and microbial inoculation treatments ($N = 60$) exhibited differences in growth (a), metabolism (b) and development rates (c). Coloured bands represent 0.95 confidence intervals. Days are relative to the day of hatching. Mortality prior to metamorphosis varied among treatments but was not significant (see results for details)

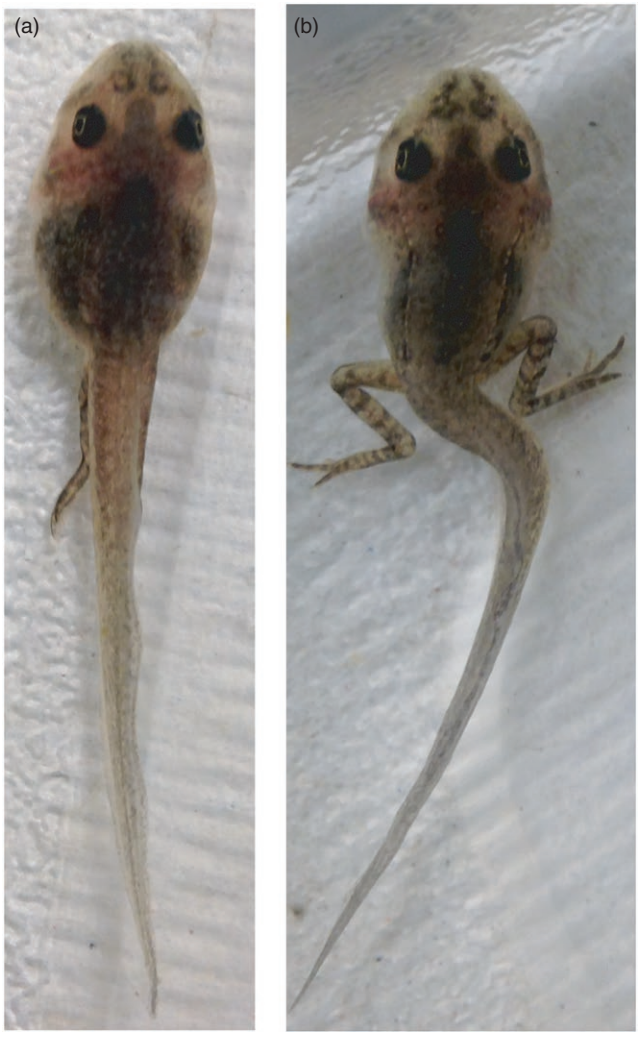

FIGURE 4 Frequency of normal (a) and tail deformities (b) varied among control (5%), +bullfrog (16%) and no inoculation (26%) microbial treatments (N = 114). Deformities were more common among larvae from the no inoculation treatment compared to controls (odds ratio = 6.4, p = 0.02, CI = 1.3–31.7), and +bullfrog were not different from controls

among the larvae that died. Treatment affected the association between viral load and the time to death (Supporting Information Table S5; GLM χ^2 = 6.6, p < 0.05); larvae from the no inoculation treatment died 0.5 times faster at an average viral load compared to the other treatments (post hoc contrasts, p < 0.05). However, time to metamorphosis among ranavirus exposed larvae that survived was not associated with viral load or treatment (GLM p > 0.05).

4 | DISCUSSION

Manipulation of the microbiota on eggs influenced the trajectory of gut microbiome communities across larval ontogeny, suggesting hatching constitutes a critical window in amphibians. While the relative abundance of microbial communities across our three inoculation treatments was similar in larval wood frogs within a day of hatching (Gosner stage < 22), they diverged within 2 weeks (stage 27): larvae inoculated with gut microbes from late-stage bullfrogs were distinct from control and no-inoculate treatments (Figures 1 and 2). However, as these larvae neared metamorphosis (stage 35), relative community composition of the gut microbiomes converged among the three treatments. This convergence may be a result of colonization by microbes from dietary and ecological sources, whereby as amphibian gut physiology and morphology change across ontogeny, environmental microbes may displace early colonizers (Bletz et al., 2016; Knutie et al., 2017). While the above speculation is tentative at the moment, because of our small sample size for microbial sequencing, these findings are supported by our earlier study. We used a similar microbiome manipulation protocol but in a different species, green frogs, in which we found microbial colonization and community trajectories followed similar dynamics across ontogeny, lending support to the concept that hatching is a critical window for gut microbiome colonization (Warne et al., 2017). Our current study extends these findings and suggests that initial microbial colonization of larvae at hatching appears to have long-term consequences for amphibian development, physiology and health.

We found microbial manipulation at hatching was associated with differences in growth and development rates, metabolism, and disease susceptibility in larval wood frogs. Wood frog larvae inoculated with bullfrog gut microbiota (+bullfrog) exhibited accelerated growth rates compared to controls and the other treatment (Figure 3a). While we can only speculate how differences in gut microbial community structure influences larval amphibians, we suspect the +bullfrog treatment could have access to greater concentrations of bacterially produced metabolites that fuelled greater somatic growth rates and potentially fat storage. This is because a primary way microbes affect host condition is by producing metabolites that serve as essential nutrients and energy substrates for their hosts (Arrieta et al., 2014; Borre et al., 2014; Cox et al., 2014; Funkhouser & Bordenstein, 2013; Warne, 2014). Symbiotic microbes produce a diversity of metabolites including essential amino acids, vitamins and short-chain fatty acids (SCFAs) that are critical to host nutrient processing, metabolism and neuroendocrine pathways regulating growth (Neuman et al., 2015; Sampson & Mazmanian, 2015; Sommer & Bäckhed, 2013). For example, variation in the relative abundance of fermentative bacteria (e.g. Bacteroidetes, see Figure 2 stage 27) may have translated into increased production of SCFAs, as has been seen experimentally and observationally shown in mice and humans (Arumugam et al. 2011; Turnbaugh et al. 2006). Furthermore, the coiled guts of detritivorous larval frogs allow for significant bacterial fermentation and production of SCFAs in the small intestine and colon, which account for up to 20% of larval daily energy requirements (Pryor & Bjorndal, 2005). Recent work in other wildlife has also shown complex associations between growth in juvenile animals and microbial diversity. In ostriches, for example, bacterial groups in Bacteroidaceae were positively correlated with growth in juveniles, while bacteria in Enterobacteriaceae, Enterococcaceae, and Lactobacillaceae had negative correlations with growth between individuals of differing ages (Videvall et al., 2018). A powerful approach for future tests of

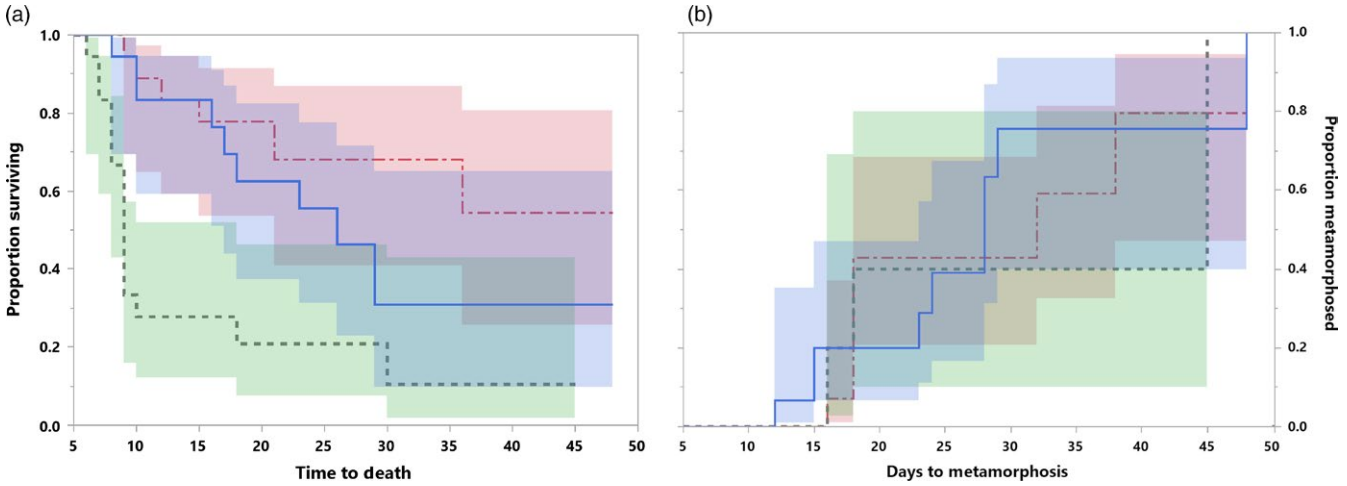

FIGURE 5 Wood frog larvae exposed to an LD_{50} dose of ranavirus (N = 54) exhibited variance in survival (a) in association with microbial treatments. Time to metamorphosis did not vary with treatment (b). Coloured bands represent 0.95 confidence intervals

microbial effects on amphibian growth would be to characterize gut bacterial community structure and metabolite profiles, and to link these to differences in substrate metabolism, growth and lipid storage using stable isotopes (Frost et al., 2014; Kirschman, McCue et al., 2017; Lee et al., 2014 ; Warne, 2014).

The +bullfrog treatment also exhibited the fastest development rates compared to the other treatments. These results are of significant importance for amphibians because both growth and development are fitness-associated traits. Moreover, variation in development rates are often considered adaptive responses to environmental stressors, whereby accelerated development can enable individuals to escape poor conditions and mortality associated with competition, predation, pond drying and infection by diseases including ranavirus (Denver, 1997, 2009b; Kirschman et al., 2018; Warne et al., 2011). Another important point of context is that while growth and development are often considered coupled in amphibians (i.e. faster growth allows for faster development), our recent work demonstrates development is a separate physiological process (Kirschman et al., 2018; Warne & Crespi, 2015). While we can only speculate as to how the +bullfrog treatment influenced development in wood frogs, a reasonable hypothesis is that differences in microbial community composition caused shifts in the concentration of metabolites that may have influenced development via neuroendocrine interactions. The hypothalamus–pituitary–thyroid (HPT) and hypothalamus–pituitary–adrenal (HPA) neuroendocrine axes regulate development, growth and a suite of physiological traits including metabolism in vertebrates (Crespi & Denver, 2005; Denver, 2009a; Warne & Crespi, 2015; Warne et al., 2011). Although there are insufficient data in the microbial literature to directly link gut microbial communities and their metabolites to the differences we observed in development rates, growing speculation and a few experiments in mammals suggest metabolites derived from gut microbes could interface with the central and peripheral nervous system (Bauer et al., 2016; Clarke et al., 2014; Neuman et al., 2015). For example, SCFAs can act as signalling molecules that bind receptors on host cells (e.g. free fatty acid receptors 2 and 3, formerly GPR41 and GPR43), which are expressed in the thyroid and sympathetic ganglion in rodents (Frost et al., 2014; Kimura et al., 2011; Layden, Angueira, Brodsky, Durai, & Lowe, 2013; Perry et al., 2016). Given these tentative findings, we suggest future research that explicitly tests for interactions between gut microbial metabolites and host receptors in neuroendocrine regulatory organs, such as the hypothalamus, could provide new insight into how microbiomes interface with their hosts and influence developmental phenotypic plasticity via endocrine regulation (Lee & Brey, 2013).

Larvae in the no inoculation treatment, by contrast, appeared to have experienced dysbiosis that affected their metabolic processes and health. These larvae exhibited elevated metabolic rates early in their development that later declined, and this pattern was counter to the increasing metabolic rates exhibited by the control and +bullfrog larvae (which are expected with growth). While we do not know how dysbiosis may have influenced this metabolic imbalance, as suggested above, variation in microbial metabolites could have altered neuroendocrine function (e.g. thyroid) and regulation of metabolism; this is because some of the known primary effects of microbial metabolites on neuroendocrine function are related to energy homeostasis (Clarke et al., 2014; Frost et al., 2014; Lam et al., 2005). In addition, the high rates of tail deformities and disease susceptibility in the no inoculation treatment lend further support for dysbiosis in the no inoculation larvae. First, we found a significantly larger proportion of larvae in the no inoculation treatment exhibited asymmetrical tail deformities compared to both control and +bullfrog larvae (Figure 4). A potential cause of these deformities was deficiencies in essential nutrients. In fish, for example, deficiencies in essential amino acids (e.g. tryptophan) and vitamins (e.g. riboflavin—vitamin B2) are associated with scoliosis and spinal deformities (Tacon, 1992). Furthermore, gut microbes vary in their capacity to both synthesize and metabolize these essential nutrients (LeBlanc et al., 2013; O'Mahony, Clarke, Borre, Dinan, & Cryan, 2015). Taken together, our results

suggest variation in gut microbial composition during critical developmental windows in larval amphibians may lead to unstable microbial community states over an animal's life and contribute to variation in physiological function, performance and overall health (Zaneveld et al., 2017).

We also found variation in susceptibility to ranaviruses among the microbial treatments. In our LD_{50} study, we found larvae from the no inoculation treatment were more likely to die compared to controls and +bullfrog larvae. The gut microbiota could have influenced amphibian susceptibility to infection via a few pathways. First, ranavirus infection and mortality in larval amphibians occur during a critical disease window around metamorphosis (Kirschman et al., 2018), during which the energetic costs of tissue remodelling may impose a survival trade-off (Kirschman, McCue et al., 2017). Changes in the gut microbiome may thus have influenced mortality via shifts in energy stores. For example, if dysbiosis resulted in reduced metabolite concentrations (e.g. SCFAs) in the no inoculation larvae, then they may have had insufficient energy stores to fuel both the costs of metamorphosis as well as mount a robust immune response to ranavirus infection (Kirschman et al., 2018; Warne et al., 2011). This could be supported by the time to death we observed in the no inoculation larvae, which died faster than the other treatments regardless of viral load. Another way that gut microbiomes may have influenced these larvae is through gut bacteria interactions with ranavirus. Research in mice suggests some bacteria can amplify viral replication, and thereby contribute to infections in hosts (Kane et al., 2011; Kuss et al., 2011). Gut bacteria can also constrain pathogen growth via localizing populations to intestinal niches, and the induction of host immune responses (Abt et al., 2012; Kamada, Chen et al., 2013). For example, microbiota can promote production of type I interferon, which is integral in the immune response to ranavirus (Grayfer, Edholm, De Jesús Andino, Chinchar, & Robert, 2015; Kamada, Seo, Chen, & Nunez, 2013). While speculative at the moment, these results suggest promising future research could test whether gut microbes influence disease susceptibility in amphibians by directly or indirectly affecting viral dynamics, host immunity and energy processes.

In conclusion, our study contributes to a growing body of research suggesting that establishment of gut microbiota at birth has lasting effects on microbiome communities and their hosts, which can alter disease susceptibility and general health across the life of animals (Cox et al., 2014; Kerr et al., 2015; Rodriguez et al., 2015; Sommer & Bäckhed, 2013). Taken together, our results suggest that establishment of amphibian gut microbiomes occurs during a critical window of hatching and shapes larval development and functional performance. Whether these effects persist through the adult frog life stage remains to be tested. However, as found in other taxa, our results suggest that while microbial communities may appear to recover after disruption, the effects on altered host phenotypes may persist if the disruption occurred during a critical window (Cox et al., 2014; Sommer, Anderson, Bharti, Raes, & Rosenstiel, 2017; Sommer & Bäckhed, 2013). Greater exploration and tests of the importance of critical developmental windows for microbial colonization, and how these shape both amphibian microbiomes and adult phenotypes promise to shed needed light on host–microbe interactions in wildlife.

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AUTHORS' CONTRIBUTIONS

R.W.W. and L.J.K. conceived and conducted the experiment and data analysis; L.H.Z. sequenced and analysed the microbial data. All authors wrote the manuscript.

DATA ACCESSIBILITY

Data are accessible through the OpenSIUC institutional repository at opensiuc.lib.siu.edu/zool_data/15. The representative microbial OTU sequence data are available via BioProject (#PRJNA387290) at NCBI's Sequence Read Archive.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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