Multidirectional interactions between amphibian hosts, the gut microbiome, and environmental temperature

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All vertebrate species harbor dense and diverse microbial communities living in their gastrointestinal tract, called the gut microbiome, that profoundly influence their physiology, ecology, and potentially evolution. These communities are shaped by both the internal and external environment of their host, and in turn, influence how hosts respond to the environment through provisioning of numerous physiological services. Although the gut microbiome benefits all vertebrate groups, the majority of studies have focused on mammalian microbial communities, likely due to their similarities to humans. However, extending study to a wider variety of animals will aid our understanding of the role microbes play in the ecological and evolutionary trajectories of vertebrates. For my dissertation, I have focused on investigating relationships between amphibians, a particularly understudied group in the microbiome literature, their gut microbes, and their external environment. I focus specifically on temperature as an environmental variable because temperature impacts all aspects of amphibian physiology, and rising global temperatures threaten amphibian persistence. Throughout my dissertation, I've investigated these relationships from multiple directions. First, I explored how environmental temperature alters amphibian gut microbial communities, with the potential to impact host physiological function. Specifically, I showed that increasing environmental temperatures alters the composition and diversity of salamander gut microbiota, which is associated with deleterious outcomes for host digestive performance. Additionally, I showed that increases in environmental temperature can cause significant changes to the tadpole gut microbiome on the timescale of hours, indicating that

expected increases in temperature variability could rapidly affect amphibian physiology. Second, I focused on how the microbial community itself could influence amphibian responses to environmental temperature. Here, I found that disrupting the natural microbiome of tadpoles resulted in reductions of the host's acute thermal tolerance, physiological performance at high temperatures, and ultimately survival under prolonged heat stress. Subsequently, using a hologenomic approach, I identified potential interactions between host and microbial functional pathways that could be driving these effects. Ultimately, my dissertation has highlighted the importance of gut microbial communities in shaping interactions between amphibians and their environment, and that these interactions should be considered when assessing amphibian responses to global climate change.

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Preface

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1.0 Introduction

1.1 Background

1.1.1 The vertebrate gut microbiome

Communities of microorganisms—known collectively as 'the microbiome'—inhabit nearly all surfaces in and on animal bodies (Douglas 2018). The vertebrate gut microbiome is a particularly dense and diverse community consisting of viruses, bacteria, archaea, fungi, and protozoa, which outnumber the host in non-redundant genes by up to 150-fold (Qin et al. 2010). Such diversity in genetic content allows microbes to perform an array of unique functions which may directly benefit the host, such as synthesis of essential amino acids and vitamins, or degradation of toxins (Douglas 2018). These biological services mediate proper functioning of host physiological systems, exerting a substantial influence on animal development, metabolism, digestion, immunity, and behavior (McFall-Ngai et al. 2013). Disruption of native gut microbial communities can result in the loss of important functions, leading to negative consequences for host health and physiology (Carding et al. 2015, Trevelline et al. 2019). Gut microbial community composition is shaped by a complex set of host and environmental factors, including host genotype, species, and ontogeny, as well as diet, habitat, geographic location, and anthropogenic disturbance (Ley et al. 2008, Spor et al. 2011, Yatsunenko et al. 2012, Amato et al. 2013, Trevelline et al. 2019).

1.1.2 The theory of hologenomic evolution

Close associations between animals and microorganisms have existed since metazoans arose, resulting in complex and interdependent relationships with the potential to drive evolutionary change in both partners (McFall-Ngai et al. 2013). For example, many species of bacteria are not known to exist anywhere else on Earth but inside the guts of their hosts (Hongoh 2010), suggesting the animal habitat provides an exploitable niche which has influenced the diversification and distribution of microorganisms. On the other hand, microbes have likely shaped the course of animal evolution by mediating the response of individuals to their environment through the provision of vital physiological services or the alteration of host gene expression (Hooper and Gordon 2001). For example, microbial partners may facilitate novel ecological strategies that improve host performance in harsh environmental conditions, such as specialization on typically unpalatable food sources (Kohl et al. 2014b), or winter hibernation (Sommer et al. 2016). The recognition that microbial communities vary within and across host species, and impact whole-animal performance, has given rise to the concept of 'hologenomic evolution', which considers the collection of host and microbial genomes—known as 'the holobiont'—a biological unit of organization upon which natural selection can act (Bordenstein and Theis 2015). In this framework, microbial symbionts may be gained, lost, or change in frequency and abundance within a population, which occurs in parallel with random mutations and changes in allele frequencies in nuclear genes. These events provide the raw genetic variation, at multiple levels, necessary for natural selection to act. Despite similarities between hologenome theory and traditional ideas of evolution, the concept has been met with considerable criticism. For example, questions regarding patterns of transmission in the microbiome, and fidelity among partners remain to be resolved (Douglas and Werren 2016). The true nature of evolutionary relationships

among hosts and microbes is likely to become clear only when study is extended to a wider variety of host taxa.

1.1.3 The field of vertebrate gut microbiome research

The advent of new sequencing technologies has allowed for a surge in microbiome research, providing insights into host-microbe associations at a level of detail that was not previously possible. However, studies of the gut microbiome are not distributed equally among taxa or topic. Meta-analyses of the gut microbiome literature reveal that >90% of analyzed studies focus on mammals, up to 85% focus on domesticated or model organisms, and diet is consistently the most common research topic (Colston and Jackson 2016, Pascoe et al. 2017). Further, of the few studies that do focus on wild animals, the majority are purely descriptive, as opposed to utilizing experimental manipulations to understand microbial community function (Pascoe et al. 2017). We therefore lack a basic understanding of the functional importance of the gut microbial communities of wild, non-mammalian hosts in an ecological and evolutionary context. Extending studies to more diverse groups would aid our understanding of how variation in animal morphology, physiology, ecology, and evolutionary history shapes, and is shaped by, gut microbial community development, structure, and function (Hird 2017). While these are some of the most important and complex questions facing the field (Koskella et al. 2017), they cannot be adequately answered by focusing only on a small group of model organisms (Colston and Jackson 2016).

1.1.4 Amphibians as a model to study, host, gut microbiota, and environmental interactions

Despite their underrepresentation in the gut microbiome literature, amphibians are an ideal system to address these questions for a number of reasons. First, amphibians follow a predictable, trackable developmental pattern, culminating in a dramatic metamorphosis where almost all physiological systems are remodeled, including composition of the gut microbiota (Gosner 1960, Kohl et al. 2013). During this transition, amphibian ecology changes drastically as well, including major shifts in habitat and diet. Therefore, these animals can be used to understand how various biotic and abiotic factors experienced during development impact future phenotypes. Second, amphibians house diverse gut microbial communities which contribute markedly to host physiological function. For example, it is estimated that larval anurans receive up to 20% of their daily energy requirements from gut bacterial fermentation (Pryor and Bjorndal 2005). Further, disruption of gut microbial communities during the larval period may leave adult hosts more susceptible to disease (Knutie et al. 2017). Lastly, amphibians are highly sensitive to a variety of environmental factors (Hopkins 2007). Of these, temperature may have the most pervasive effect on amphibian biology because, like >99% of all species, amphibians are ectotherms and lack an internal thermoregulation mechanism (Atkinson and Sibly 1997). Therefore, environmental temperature shapes virtually all phenotypes of amphibians, including their behavior, physiological function, and gut microbial community composition (Angilletta Jr et al. 2002, Fontaine et al. 2018).

1.2 Dissertation aims

To understand the dynamic interplay between hosts, gut microbiota, and the environment, my dissertation focuses on investigating the relationship between amphibians, their gut microbes, and environmental temperature. Using a novel combination of field, laboratory, and molecular techniques I conducted a set of experiments that addressed two specific aims:

1. **Describe how environmental temperature impacts amphibian gut microbiomes**
2. **Determine if the gut microbiome influences the physiological responses of amphibians to environmental temperature**

1.2.1 Aim 1 background and summary: Describe how environmental temperature impacts amphibian gut microbiomes

Environmental temperature has pervasive effects on multiple facets of amphibian physiology, including development (Goldstein et al. 2017), metabolism (Whitford 1973), digestion (Benavides et al. 2005), and immune function (Raffel et al. 2006). Although temperature-dependent function of physiological systems can maximize performance in environments individuals are well adapted to, significant deviation from these conditions, such as under global climate change, may be deleterious (Huey et al. 2012). In addition to the aforementioned effects, environmental temperature also impacts the composition and diversity of amphibian gut microbial communities (Kohl and Yahn 2016, Fontaine et al. 2018, Fontaine and Kohl 2020a). This effect presents the possibility that the relationship between environmental temperature and amphibian physiology may be driven in part by temperature-mediated alterations to gut microbiota, as opposed to solely direct effects on host biology. For example, in lizards, temperature-induced

changes to gut microbial community diversity and function were correlated with animal survival (Bestion et al. 2017). To investigate this possibility further, in **Chapter 2,** I used salamanders housed under several environmental temperatures to compare the composition and diversity of the gut microbiota and their digestive performance,. I identified links between salamander gut microbiota and host digestion which suggest that temperature-induced alterations to the gut microbiome may indirectly drive relationships between host digestion and temperature.

In addition to understanding how environmental temperature impacts host gut microbial communities and physiology in general, it is also important to understand the temporal dynamics of these effects. For example, how long does it take for temperature to alter the gut microbiome? While climate change is expected to alter mean temperatures over time, it will also produce more variable and short-term extreme weather events, such as heat waves and cold snaps (Jentsch et al. 2007). If gut microbial communities change rapidly with temperature, these events may be expected to have more deleterious effects on hosts than if microbial communities require more time to adjust. Further, these effects may depend on host species identity. It is currently unclear if the effects of temperature on amphibian microbiomes is due to the impacts of temperature on microbes alone (i.e., microbial growth) or mediated through the impacts of temperature on host physiology. Thus, it is possible for temperature to impact microbial communities of different species in unique ways due to their individual differences in microbial community composition and host physiology. To explore these ideas further, in **Chapter 3,** I identified the length of time required for the gut microbiome to change compositionally and functionally in response to increased temperature in two host species of tadpoles. Results of this study reveal that microbial communities can change in response to temperature on rapid time scales, but the degree of microbiome plasticity is dependent on host species identity.

1.2.2 Aim 2 background and summary: Determine if the gut microbiome influences the physiological responses of amphibians to environmental temperature

Ectotherms are expected to be especially vulnerable to the deleterious effects of global climate change on animal fitness (Paaijmans et al. 2013). Predicting how these animals will respond physiologically to increased temperature regimes is a global conservation priority which requires a thorough understanding of the mechanisms that contribute to host thermal tolerance. Thermal tolerance of ectotherms can be assessed in several ways including the measurement of acute thermal minima and maxima (i.e. CT_{min} and CT_{max}), survival time at various sub lethal temperatures, physiological acclimation to varying conditions, and determination of optimal temperatures using metrics of physiological performance (Angilletta Jr 2009, Sunday et al. 2011, Jørgensen et al. 2019). The molecular mechanisms delimiting these outcomes can be complex but often include the structure and composition of phospholipids in cell membranes, and the stability and flexibility of enzymatic function (Angilletta Jr 2009). Further, the oxygen and capacity limitation of thermal tolerance (OCLTT) hypothesis posits that in aquatic ectotherms, thermal tolerance limits are dictated by a mismatch in supply and demand for oxygen to the mitochondria during respiration (Pörtner 2001, Pörtner et al. 2017). Although thermal tolerance is in part a genetic trait (Perry et al. 2001, Kokou et al. 2018), it is plastic, and can be shaped by environmental factors as well, such as time of day, season, oxygen availability, feeding, and infection status (Hu and Appel 2004, Nyamukondiwa and Terblanche 2009, Healy and Schulte 2012, Greenspan et al. 2017).

Gut microbiota can influence the response of animals to their external environments through various mechanisms such as the production of important metabolites, or regulation of host gene expression (Alberdi et al. 2016, Kohl and Carey 2016, Fontaine and Kohl 2020b). While

environmental temperature is known to influence the diversity and composition of the gut microbiome, it is also possible that the microbial community itself could impact host thermal tolerance due to its effects on host physiology. A relationship between symbiotic microbes and host thermal tolerance has been demonstrated in several invertebrates such as bacteria-phage systems (Padfield et al. 2020), algae (Xie et al. 2013), coral (Ziegler et al. 2017), aphids (Russell and Moran 2006), and flies (Jaramillo and Castañeda 2021). Typically, acquisition of particular symbionts increases host tolerance to heat, and allows hosts to persist in otherwise lethal habitats (Russell and Moran 2006, Ziegler et al. 2017). Further, the gut microbiome's impact on energy metabolism has been shown to facilitate cold tolerance in multiple mammalian systems (Chevalier et al. 2015, Sommer et al. 2016, Khakisahneh et al. 2020).

Although there is correlative evidence that thermal tolerance in vertebrate ectotherms could be linked to gut microbiome composition (Moeller et al. 2020), and that thermal tolerance profiles themselves may alter gut microbiota (Kokou et al. 2018), to date there have been no manipulative studies that test the impact of gut microbial communities on host thermal tolerance in vertebrate ectotherms. In **Chapter 4**, I developed a system to experimentally manipulate tadpole gut microbiota to be depleted of diversity and altered in composition, and determined how amphibian gut microbiota contribute to the host's acute thermal tolerance (critical thermal limits, locomotor performance at high temperatures, and survival under heat stress). I also compared several physiological metrics across microbiome treatment groups, including cell membrane phospholipid composition, mitochondrial enzyme activity, and whole organism metabolic rate to uncover putative mechanisms behind these effects. To explore the mechanistic basis of the relationship between gut microbiota and tadpole thermal tolerance even further, in **Chapter 5**, I utilized a hologenomic approach to understand how interactions between host and microbial function govern

the response of tadpoles to heat. Specifically, I compared host transcriptomic and microbial metagenomic profiles of tadpoles with differing microbial community exposures during a period of acute heat stress.

2.0 Environmental temperature alters the digestive performance and gut microbiota of a terrestrial amphibian

The contents of this chapter are adapted from the following publication:

Fontaine, S. S., A. J. Novarro, and K. D. Kohl. 2018. Environmental temperature alters the digestive performance and gut microbiota of a terrestrial amphibian. *Journal of Experimental Biology* 221. DOI: 10.1242/jeb.187559

2.1 Introduction

Environmental temperature is a crucial factor impacting the physiology, development, and behavior of ectotherms (Huey 1979, Gillooly et al. 2002). Specifically, multiple aspects of digestive performance in ectothermic vertebrates are temperature dependent, including foraging rates, energy assimilation, digestive efficiency (McConnachie and Alexander 2004), gut passage time (Waldschmidt et al. 1986), and metabolic response to feeding (Wang et al. 2002). The thermal sensitivity of whole-organism digestive performance traits can be defined using standard thermal performance curves, where performance slowly increases until reaching a thermal optimum and then rapidly decreases until reaching the critical thermal maximum (Huey and Kingsolver 1989). This relationship has been demonstrated in a number of ectothermic taxa such as fish (Nicieza et al. 1994), tadpoles (Benavides et al. 2005), salamanders (Clay and Gifford 2017), lizards (Angilletta 2001), and snakes (Naulleau 1983). However, other abiotic (seasonality, habitat quality; (Ortega et al. 2014)), and biotic factors (prey availability, foraging behavior; (Adams et al. 1982, Ayers and Shine 1997)) may interact with temperature to impact an organism's digestive performance. Understanding the factors that influence the relationship between temperature and

physiological performance in ectotherms is becoming increasingly important because—while already some of the most threatened vertebrate taxa (Gibbons et al. 2000, Stuart et al. 2004)—they are expected to be highly sensitive to the deleterious effects of global climate change (Paaijmans et al. 2013).

Recently, a rapidly growing body of research has demonstrated that microbial communities living in the vertebrate gut have a major impact on many aspects of host physiology, including digestive performance (McFall-Ngai et al. 2013, Kohl and Carey 2016). Gut microbiota can facilitate enhanced digestion through various functions such as fermentation of plant materials (Mackie 2002), detoxification of typically unpalatable food (Kohl et al. 2014b), or provision of an alternative energy supply during food scarcity (Amato et al. 2015). While most studies have focused on the relationship between microbiota and digestion in mammalian hosts, the gut microbiome is important for digestion in ectothermic vertebrate hosts as well. For example, in both tadpoles and lizards, the gut houses diverse microbial communities with high levels of fermentative activity (Mackie et al. 2004, Pryor and Bjorndal 2005).

The ability of gut microbiota to provide digestive services may be dependent on temperature. For example, in mammals, exposure to cold leads to characteristic shifts in the community composition of gut microbiota, resulting in marked impacts to overall energy homeostasis (Chevalier et al. 2015). Additionally, in a controlled laboratory study with tadpoles, environmental temperature was determined to be a significant factor shaping community membership and structure of the gut microbiome (Kohl and Yahn 2016), though the functional consequences of these changes were not studied. Because ectotherm body temperature fluctuates more widely than other organisms, impacts on whole animal performance due to temperature-mediated alterations of gut microbiota may be most pronounced in this group. Indeed, small

increases in temperature resulted in decreased diversity and altered community composition of gut microbiota in lizards, which correlated with reduced animal survival (Bestion et al. 2017). However, the mechanisms driving these associations are unclear.

It is possible that temperature-mediated alterations to gut microbiota composition or function may be an additional factor underlying the relationship between environmental temperature and digestive performance in ectothermic vertebrates. However, studies exploring this possibility are lacking. To address this knowledge gap, we assessed the impacts of environmental temperature on the digestive performance and gut microbiota of a terrestrial amphibian, the eastern red-backed salamander (*Plethodon cinereus*). Additionally, we investigated potential connections between digestive performance and gut microbiota that may mediate the relationship between environmental temperature and digestion. We hypothesized that (1) salamander digestive performance—energy intake, energy assimilation, and digestive efficiency—would be significantly impacted by environmental temperature, (2) the diversity and community composition of salamander gut microbiota would be temperature dependent, and (3) the relative abundance of specific bacterial taxa would be temperature dependent and correlate with aspects of host digestive performance.

2.2 Methods

2.2.1 Animal husbandry

Animals were collected with permission from Virginia Department of Game and Inland Fisheries (permit #056084), interstate transport was permitted under a Federal Fish and Wildlife

injurious species permit (permit #MA90136B-0), and vertebrate research was approved by the University of Maryland (protocol FR-15-72).

We collected 19 sexually mature (>32mm snout-vent length; (Sayler 1966)) eastern red-backed salamanders from the Blue Ridge Mountains of Pembroke, Virginia, USA in October 2015. To avoid the potentially confounding physiological effects of color polymorphism, we only collected individuals that clearly displayed the striped, rather than unstriped, phenotype (Moreno 1989, Fisher-Reid et al. 2013). Based on nocturnal summer surveys, body temperature of this population of salamanders ranges from 7.4-20.9°C in the wild (Novarro 2018).

Upon collection from the field, salamanders were transported to the University of Maryland (College Park, MD,USA). Salamanders were housed individually in plastic containers lined with unbleached paper towels and were provided an additional rolled-up paper towel to use as a retreat. Salamanders were acclimated to a constant temperature of 15°C for four weeks prior to experiments, and held on a 12:12 light:dark cycle for the duration of the study. Salamanders were fed 15-20 live, adult flightless fruit flies (*Drosophila hydei*) weekly and sprayed with spring water as necessary.

2.2.2 Feeding trials and digestive performance metrics

Following acclimation, each individual salamander underwent three temperature controlled feeding trials performed in the following order—10°C, 15°C, and 20°C— following the protocol of Clay and Gifford (2017). At the beginning of each trial, 50 live, adult flightless fruit flies (*Drosophila hydei*) were offered to each salamander. After 24 hours, the number of flies remaining were counted and eaten flies were replenished. Counting and replenishing flies continued for five consecutive days. Remaining flies were counted and subsequently removed

from enclosures on the sixth day. Feces and shed skin were collected from each individual during trials until the digestive tract was clear (3-5 days without fecal production). Following each trial, salamanders were transferred to the next experimental temperature and allowed to acclimate for 7-10 days prior to beginning the next trial. During this time, they were not fed.

Energy assimilation and digestive efficiency were calculated for each individual during each trial as:

$$Energy\ Assimilation = EA - (EF + ES)$$

$$Digestive\ Efficiency = EA - (EF + ES) / EA * 100$$

where EA = total energy acquired through ingestion (kJ), EF= energy lost as feces (kJ), and ES= energy lost as shed skin (kJ). As salamanders shed skin more frequently at higher temperatures, we chose to quantify ES to account for variation in energy expenditure among temperatures (Merchant 1970). All energy measurements were quantified using a Parr 6725 Semimicro Calorimeter (Parr Instrument Company, Moline, IL, USA). Fruit flies were subsampled at different points during the adult life stage and a mean energy content per fly was determined to be 0.064 kJ/fly. To calculate EA, this measurement was multiplied by the number of flies ingested during each trial. Energy contents of fecal and skin samples from individual salamanders were too small to process on their own and therefore, samples from each trial were combined. Combined samples were weighed, dried at 80°C for 24 – 48 hours, pelletized into subsamples, and energy contents were quantified. The mean energy content of fecal and skin subsamples from each trial was multiplied by the mass of each individual's feces and shed skin samples from the same trial to obtain EF and ES for each individual.

2.2.3 Microbiome sample collection and processing

Fecal samples for microbiome analysis were collected from each salamander immediately after each feeding trial ended, before being transferred to the next experimental temperature. Samples were kept frozen at -80°C until processing. DNA was extracted from fecal samples using a PowerFecal DNA isolation kit (MoBio, Carlsbad, CA, USA) following the manufacturer's protocol. Extracted DNA was sent to Argonne National Laboratory (Argonne, IL, USA). At the laboratory, the V4 region of the 16S rRNA gene was amplified using primers 515F and 806R. PCR amplification was conducted in triplicate, and the resulting products were pooled within a single sample. DNA was cleaned using the UltraClean PCR Clean-Up Kit (MoBio, Carlsbad, CA, USA), and amplicons were sequenced on the Illumina MiSeq platform (Caporaso et al. 2012).

Raw sequence data was processed using the QIIME2 pipeline version 2017.8 (Caporaso et al. 2010). Following demultiplexing, using the DADA2 pipeline within QIIME2, forward sequence reads were filtered, processed, and assigned to OTUs (Callahan et al. 2016). Singleton OTUs were removed, and a phylogenetic tree was built using FASTTREE (Price et al. 2010). Taxonomy was assigned to OTUs using the Greengenes Database (McDonald et al. 2012) and sequences identified as chloroplast or mitochondria were removed from downstream analysis. OTU tables were rarefied to 27,285 reads, excluding one sample with fewer than 10 reads from analysis (from the 10°C trial). To measure bacterial community diversity within each rarefied sample, the number of observed OTUs (OTU richness), Shannon diversity, and Faith's phylogenetic diversity were calculated within QIIME2. Shannon diversity is a measure of biodiversity which accounts for OTU richness and evenness (Shannon 1948). Faith's phylogenetic diversity is a measure of biodiversity which compares phylogenetic relatedness among OTUs in a community by taking the sum of their branch lengths (Faith 1992). To compare bacterial

community composition between samples, unweighted and weighted UniFrac distances between samples were calculated in QIIME2 (Lozupone and Knight 2005). Unweighted UniFrac distance compares samples on the basis of presence and absence of bacterial OTUs, which we call community membership. Weighted UniFrac distance compares samples on the basis of presence, absence, and relative abundance of bacterial OTUs, which we call community structure.

2.2.4 Statistical analyses

We used linear mixed effect models (LMMs) with Tukey's post hoc HSD in JMP version 12.0 to test for differences across temperature treatments in digestive performance metrics (total energy intake, energy assimilation, and digestive efficiency) and microbial community diversity (OTU richness, Shannon diversity, and Faith's phylogenetic diversity). We included individual as a random effect in all models, and checked residuals for normality with a Shapiro-Wilk test before proceeding.

To visualize dissimilarity in microbial community composition across temperature treatments, we used Principal Coordinate Analysis (PCoA) with unweighted and weighted UniFrac distances. To test for significant differences in the distance between temperature groups we used permutational multivariate analysis of variance (PERMANOVA) with 999 permutations and false discovery rate (FDR) corrected p-values, calculated in QIIME2.

To identify specific bacterial genera which had relative abundances that were significantly associated with temperature, energy assimilation, or digestive efficiency, we used multivariate association with linear models (MaAsLin) with default settings. MaAsLin uses boosted, additive general linear models to find associations between the relative abundance of specific bacterial taxa and metadata (Morgan et al. 2012). MaAsLin controlled for individual effects and provided FDR

corrected p-values. MaAsLin was run in R version 3.4.3 using the package "Maaslin". Additionally, we used linear discriminant analysis effect size (LEfSe; (Segata et al. 2011)), with default settings, to find non-linear associations between the relative abundances of specific bacterial genera and temperature, controlling for individual effects. LEfSe uses a Kruskal-Wallis test to determine differentially abundant taxa between classes, and subsequently ranks them by their linear discriminant analysis score. LEfSe was run on the Galaxy platform (https://huttenhower.sph.harvard.edu/galaxy/).

2.3 Results

2.3.1 Digestive performance analysis

Salamander digestive performance was significantly reduced at the highest (20°C) and lowest (10°C) experimental temperatures, relative to the intermediate temperature (15°C). Mean total energy intake and energy assimilation were significantly greater at 15°C compared to 10°C and 20°C (Figure 2.1a,b; LMM, energy intake: $F=38.6$, $p<0.001$, energy assimilation: $F=45.7$, $p<0.001$). Mean digestive efficiency was significantly greater at 15°C compared to 10°C, but digestive efficiency at 20°C was not significantly different from 10°C or 15°C (Figure 2.1c; LMM, $F=5.7$, $p<0.01$).

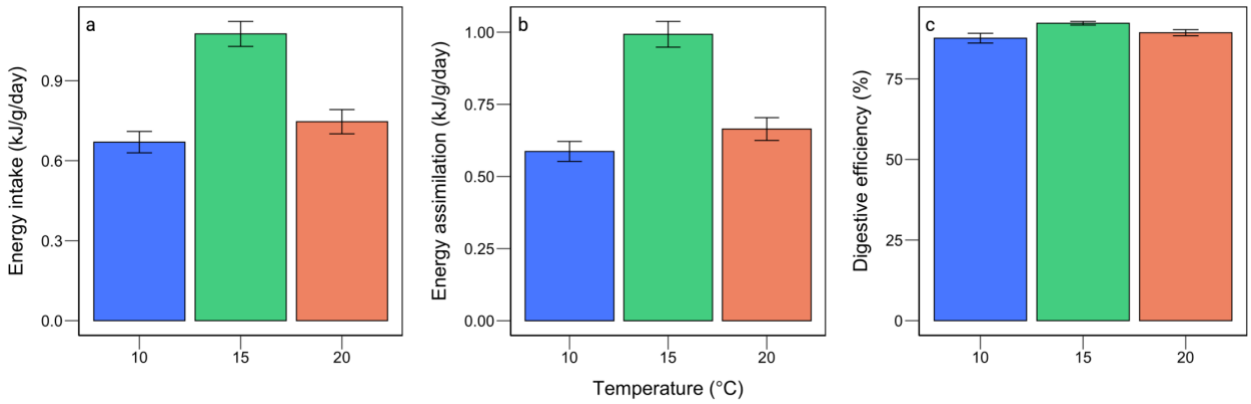


Figure 2.1 Salamander digestive performance at three environmental temperatures.

(a) energy intake per day (b) energy assimilation and (c) digestive efficiency. Bars show mean ± s.e.m. N = 19 animals per treatment.

2.3.2 Microbial community analysis

After DADA2 processing and removal of chloroplast and mitochondrial sequences, we retained 2,671,629 16S rRNA sequences (mean of 47,708 ± 12,374 SD per sample) representing 2,109 unique bacterial OTUs.

Salamander gut microbial diversity was significantly decreased at high temperatures. Bacterial OTU richness decreased by 24.9% at 20°C compared to 10°C and 15°C (Figure 2.2a; LMM, $F=23.5$, $p<0.001$). Similarly, Shannon diversity was 8.3% lower at 20°C compared to 10°C and 15°C (Figure 2.2b; LMM, $F=7.6$, $p<0.01$), and Faith's phylogenetic diversity was 20.5% lower at 20°C compared to 10°C and 15°C (Figure 2.2c; LMM, $F=23.2$, $p<0.001$). Further, salamander gut microbial community composition was distinct at each environmental temperature on the basis of both community membership and structure (Figure 2.3a,b; unweighted UniFrac PERMANOVA $p<0.001$, weighted UniFrac PERMANOVA $p<0.01$).

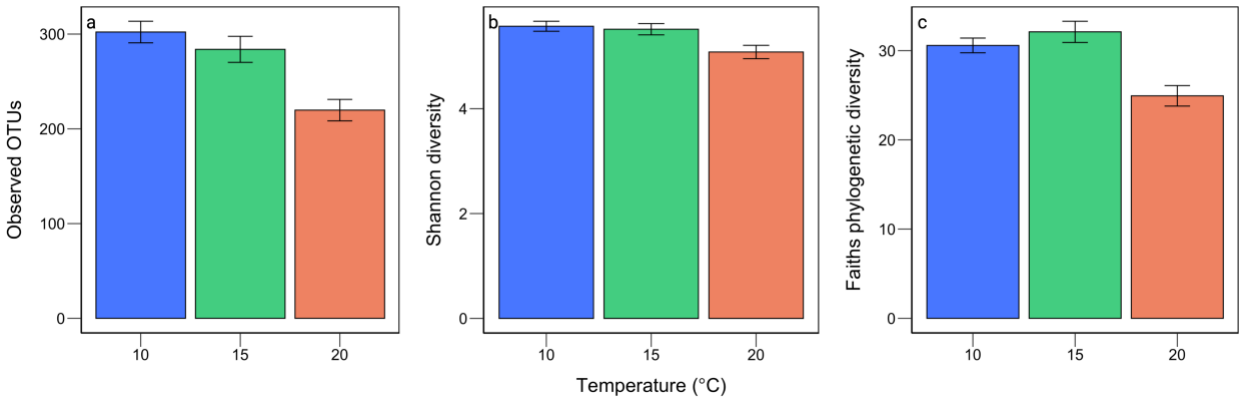


Figure 2.2 Salamander gut microbial community diversity at three environmental temperatures.

(a) Number of bacterial OTUs observed (B) Bacterial Shannon diversity and (C) Bacterial Faith's phylogenetic diversity. Bars show mean ± s.e.m. N = 19 animals per treatment.

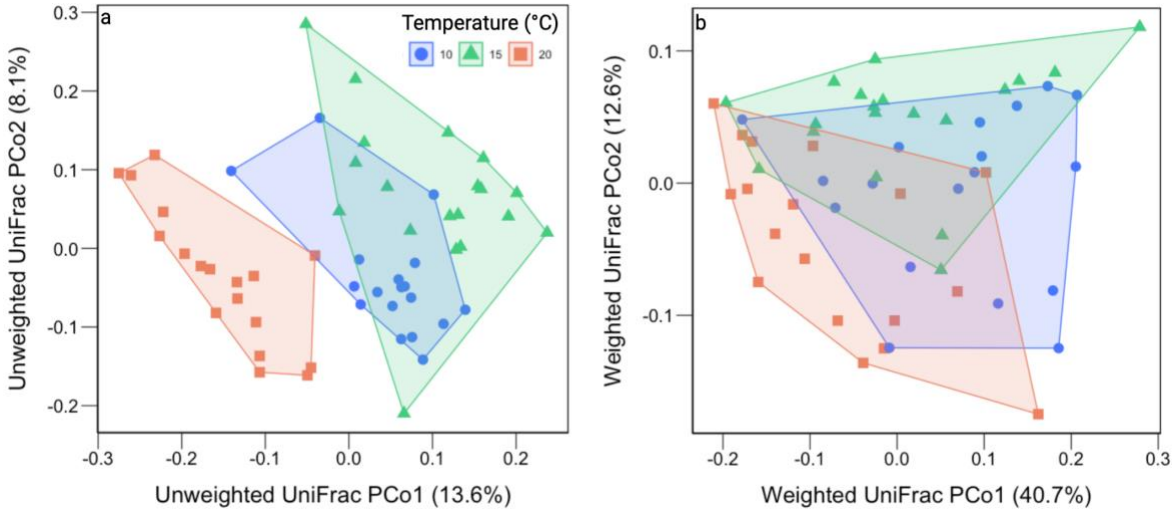


Figure 2.3 Gut microbial community composition of salamanders at three environmental temperatures.

Principal coordinate analysis plots constructed based on (a) Unweighted UniFrac and (b) Weighted UniFrac distance between gut bacterial samples. Percentages represent proportion of total variation explained by each axis.

The relative abundances of 25 bacterial genera were significantly associated with temperature. Specifically, four genera were positively correlated with temperature, 14 genera were negatively correlated with temperature, six genera were enriched at 15°C, and one genus decreased in relative abundance at 15°C (Table 2.1; MaAsLin and/or LEfSe, $p<0.05$). Significant correlations were detected between host energy assimilation and the abundances of four bacterial genera: *Cellvibrio* (Figure 2.4a; MaAsLin, coefficient=0.418, p=0.01), *Stenotrophomonas* (Figure 2.4b; MaAsLin, coefficient=0.091, p=0.01), *Sphingopyxis* (MaAsLin, coefficient=0.025, p=0.013), and *Roseococcus* (MaAsLin, coefficient=0.011, p=0.012). The relative abundances of *Cellvibrio* and *Stenotrophomonas* were significantly enriched at 15°C (Table 2.1), and although *Sphingopyxis* and *Roseococcus* were not statistically associated with temperature, they were also most abundant at 15°C. No significant associations were observed between the relative abundance of any bacterial genera and salamander digestive efficiency.

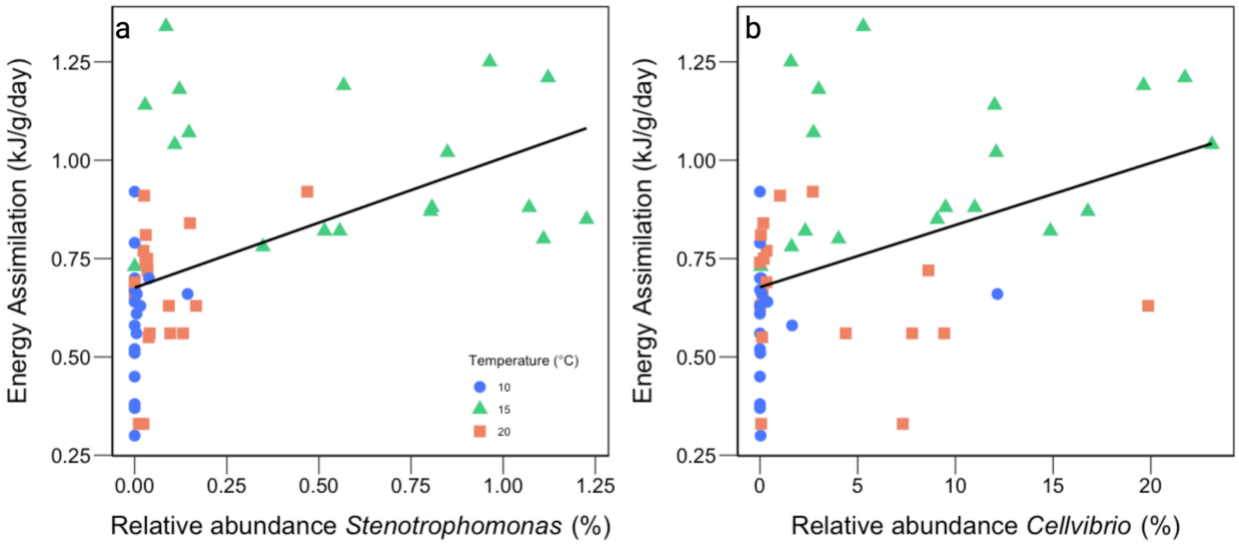
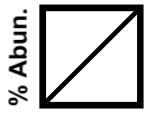


Figure 2.4 Correlations between the relative abundance of bacterial genera in the salamander gut and host energy assimilation.

(a) Relative abundance of *Stenotrophomonas* and (b) *Cellvibrio.* Color and shape of points represent the temperature at which the host salamander was housed**.**

Table 2.1 Relative abundance of gut bacterial genera that differed significantly in salamanders housed at three enivronmental temperatures.

Relative abundances are presented as means ± s.e.m. Genera are organized by the direction of their relationship with temperature. P values are FDR corrected. Highest abundance of each genus is in bold. N.O.= not observed. N.S.= not significant.

		Percent Abundance				
	Genera	10°C	15°C	20°C	LEfSe *P*	MaAsLin *P*
	*Morganella*	0.04 ± 0.02	0.80 ± 0.20	**1.68 ± 0.44**	<0.001	<0.001
	Delftia	N.O.	0.01 ± 0.01	**0.19 ± 0.06**	<0.001	<0.001
	Agrobacterium	0.06 ± 0.02	0.16 ± 0.03	**0.29 ± 0.06**	N.S.	0.016
	Dysgonomonas	<0.01	0.05 ± 0.04	**0.06 ± 0.20**	<0.001	<0.01
	*Pseudomonas*	**13.67 ± 2.35**	7.34 ± 1.73	1.68 ± 0.55	<0.001	<0.01
	Janthinobacterium	**5.81 ± 1.13**	3.35 ± 0.65	0.14 ± 0.06	<0.001	<0.001
	AF12	**0.43 ± 0.10**	0.19 ± 0.05	0.07 ± 0.03	<0.001	<0.001
	Anaerotruncus	**0.35 ± 0.04**	0.17 ± 0.02	0.13 ± 0.03	<0.001	<0.01
	Paenibacillus	**0.20 ± 0.10**	0.09 ± 0.02	<0.01	N.S.	<0.01
	Devosia	**0.08 ± 0.02**	0.02 ± 0.01	<0.01	N.S.	<0.001
	Sedimentibacter	**0.07 ± 0.01**	0.04 ± 0.02	0.02 ± 0.01	N.S.	0.014
	Wohlfahrtiimonas	**0.03 ± 0.01**	<0.01	<0.01	<0.001	<0.001
	Erysipelothrix	**0.02 ± 0.01**	N.O.	N.O.	N.S.	<0.001
	Rhodobacter	**0.02 ± 0.01**	<0.01	<0.01	<0.01	<0.001
	Rhodococcus	**0.02 ± 0.01**	<0.01	<0.01	N.S.	0.022
	Bdellovibrio	**0.06 ± 0.01**	0.02 ± 0.01	0.03 ± 0.01	<0.01	N.S.
	Cytophaga	**0.08 ± 0.05**	<0.01	N.O.	<0.01	N.S.
	Sphingobacterium	**0.04 ± 0.02**	<0.01	<0.01	<0.001	N.S.
	*Cellvibrio*	0.80 ± 0.70	**9.81 ± 1.70**	3.47 ± 1.26	<0.001	N.S.
	Stenotrophomonas	0.01 ± 0.01	**0.58 ± 0.10**	0.10 ± 0.03	<0.001	N.S.
	Methylotenera	0.09 ± 0.01	**0.12 ± 0.01**	0.04 ± 0.01	<0.001	N.S.
	Sphingomonas	0.01 ± 0.01	**0.12 ± 0.04**	<0.01	<0.001	N.S.
	Megamonas	0.06 ± 0.01	**0.07 ± 0.03**	<0.01	N.S.	<0.001
	Prosthecobacter	0.01 ± 0.01	**0.05 ± 0.01**	<0.01	N.S.	0.012
	*Citrobacter*	0.22 ± 0.09	0.03 ± 0.02	**0.28 ± 0.27**	N.S.	<0.01

2.4 Discussion

Our findings demonstrate that environmental temperature significantly impacts digestive performance of salamanders (energy intake, energy assimilation, and digestive efficiency), which is consistent with the understanding that temperature has profound impacts on ectotherm physiology (Huey 1979), and specifically, digestive performance (Waldschmidt et al. 1986, Wang et al. 2002, McConnachie and Alexander 2004). Similar to a recent study of energy assimilation in Plethodontid salamanders (Clay and Gifford 2017), we found that performance of eastern red-backed salamanders is highest at an intermediate temperature (15°C) and is reduced at relatively cool and warm temperatures (10°C and 20°C). Although it is possible our results were influenced by the order of temperature trials, which was consistent among individuals, Clay and Gifford (2017) were able to detect species and population level differences in thermal optima using a similarly repetitive order of trials. Therefore, we expect that our results are generally reflective of the host's physiological response to their thermal environment rather than other factors. Our results contrast with Bobka et al. (1981), who measured energy assimilation of *P. cinereus* fed fruit flies at the same experimental temperatures, and found energy assimilation to be optimal at 10°C, and to decrease significantly with increasing temperature (Bobka et al. 1981). Multiple factors may explain the contradictory findings of these studies, as population-level differences in thermal preferences and physiological optima can be due to differences in geographic locality (Clay and Gifford 2017), habitat (Huey and Bennett 1987), seasonality (Ortega et al. 2014), and morphological or genetic differentiation between populations (Moreno 1989). Ectotherm-associated microbiota may exhibit similar local adaptations, as significant variation in community diversity and composition has been observed in spatially separated host populations (Muletz Wolz

et al. 2018, Zhang et al. 2018). The degree to which such changes in microbial communities are due to differing host thermal environments or other factors would be interesting to test.

Additionally, we found that temperature is a significant factor impacting the diversity and composition of salamander gut microbial communities. These results are consistent with the relatively few other studies that have addressed the relationship between temperature and the gut microbiome of ectotherms (Kohl and Yahn 2016, Bestion et al. 2017). Specifically, we find that increases in environmental temperature are associated with reduced gut microbial diversity and altered bacterial community membership and structure. Underlying these changes is the significant effect of temperature on the relative abundances of 25 bacterial genera. The overall trend was a reduction in relative abundance of these taxa with temperature, with 14 genera significantly decreasing as temperature increased.

Notably, we detected a significant decrease in the genus *Janthinobacterium* at high temperatures (Table 2.1). At 10°C *Janthinobacterium* represented 5.8% of the gut bacterial community (the fifth most abundant bacterial genus), but was diminished to just 0.14% at 20°C. This genus commonly occurs on the skin of *P. cinereus* and, when present, has been shown to protect individuals from the globally devastating fungal disease chytridiomycosis, through production of antifungal metabolites (Becker et al. 2009). Additionally, this genus has been successfully used as a probiotic to protect other susceptible species from chytridiomycosis (Harris et al. 2009, Kueneman et al. 2016). Due to its typically high abundance and ability to survive passage through the gastrointestinal tract, it has been suggested that the gut harbors important reservoirs of *Janthinobacterium*, allowing colonization of the skin upon exit from the cloaca (Wiggins et al. 2011). Our results suggest this reservoir may become depleted under

warming conditions, potentially hindering the ability of salamanders to resist cutaneous pathogens.

Furthermore, we detected shifts related to temperature in the relative abundance of *Citrobacter* (Table 2.1), a genus of pathogenic bacteria associated with bacterial dermatosepticemia (red leg syndrome) in amphibians (Densmore and Green 2007). *Citrobacter* relative abundance exhibited a nonlinear relationship with temperature, decreasing dramatically from 10°C to 15°C, and increasing again to its highest relative abundance at 20°C. A previous study detected significant increases in *Citrobacter* abundance in the gut of brown tree frogs when temperature was decreased due to induced hibernation in the laboratory (Weng et al. 2016). It is possible that the contrasting effects of temperature on this pathogenic bacterium are a function of fluctuating host immune response rather than a direct effect of environmental temperature on bacterial growth. Indeed, temperature shifts can depress the amphibian immune system (Raffel et al. 2006). However, the degree to which temperature induced changes in the microbiome are direct effects, or mediated through the host, remains to be tested.

Lastly, we identified connections between salamander gut microbiota and host digestive performance. Specifically, we found correlations between measurements of energy assimilation and the relative abundances of four bacterial genera— *Cellvibrio, Stenotrophomonas, Sphingopyxis,* and *Roseococcus.* Notably, bacteria within the genus *Cellvibrio* produce numerous carbohydrate-degrading enzymes, including potent chitinases (Forsberg et al. 2016, Monge et al. 2018). Bacteria within *Stenotrophomonas* are also capable of digesting chitin (Ryan et al. 2009). Chitin is the dominant component of arthropod exoskeletons, and the diet of the red-backed salamander consists almost exclusively of arthropods (Maglia 1996), which suggests a potential explanation for the relationship between the abundance of these bacteria and energy assimilation

in these animals. However, explicit testing of this hypothesis through experimental manipulations of gut microbiota is needed.

If *Cellvibrio* and *Stenotrophomonas* do indeed directly facilitate enhanced host energy assimilation, temperature-induced changes in the relative abundances of these genera may have contributed to the relationship observed between temperature and salamander digestive performance. The relative abundance of *Cellvibrio* in the salamander gut was significantly enriched at 15°C, making up almost 10% of the bacterial community (Table 2.1). *Stenotrophomonas* relative abundance was also significantly increased at 15°C, and although still uncommon (<1% of the community; Table 2.1), rare microbes serve important functional roles (Jousset et al. 2017). The relationships between abundances of microbial taxa and salamander digestive performance suggests the possibility that temperature-mediated effects on digestive performance may not only be driven by host physiology, but may also be influenced by alterations to the gut microbiome. Interestingly, in addition to demonstrating a correlation between altered gut microbial communities and reduced animal survival in lizards, Bestion et al. (2017) detected changes in the functional profile of gut microbiota related to energy metabolism at increased temperatures, providing further support for this hypothesis. Global climate change is already expected to hinder ectotherm digestive performance by decreasing animal foraging rates (Sinervo et al. 2010). We suggest another mechanism by which digestive performance may be reduced under increased temperature regimes—compositional and functional changes to the gut microbiome. More accurate depictions of these impacts may be quantified in the future by incorporating temperature variability into studies, as experiments conducted at constant temperature can actually overestimate measures of digestive performance in ectotherms (Ruppert 1980).

Overall, our results demonstrate that temperature is a critical factor impacting ectotherm digestive physiology and structuring of gut microbial communities. Fluctuations or increases in environmental temperature, as predicted under current and future global climate change (Parmesan and Yohe 2003), may negatively impact salamanders as a result of reduced digestive performance, reductions in gut microbial diversity, and alterations to microbial community composition with implications for host health and physiology. Identifying the mechanism by which temperature alters the microbiome, as well as further understanding the direct contribution of microbial alterations to reduced animal performance, will be important directions for future investigation.

2.5 Acknowledgements

We thank Sarah Via for providing incubators and Alexa Bely for providing research facilities, equipment, and technical support. We also thank Sarah Bailey, Matthew Griffin, and Tori Mezebish for assistance with field collection and animal husbandry. This work was supported by start-up funds from the University of Pittsburgh.

3.0 Gut microbiota of invasive bullfrog tadpoles responds more rapidly to temperature than a noninvasive congener

The contents of this chapter are adapted from the following publication:

Fontaine, S. S. and K. D. Kohl. 2020. Gut microbiota of invasive bullfrog tadpoles responds more rapidly to temperature than a noninvasive congener. *Molecular Ecology* 29(13):2449-2462. DOI: 10.1111/mec.15487

3.1 Introduction

Gut microbial communities exert a profound influence on vertebrate physiology, impacting animal development, nutrition, immune function, and behavior (McFall-Ngai et al. 2013). The composition of these communities can be quite plastic, responding to environmental factors such as diet (David et al. 2014), habitat (Amato et al. 2013), and anthropogenic disturbances (Trevelline et al. 2019). In ectotherms, environmental temperature is capable of altering both the diversity and composition of gut microbial communities (Kohl and Yahn 2016, Bestion et al. 2017, Fontaine et al. 2018, Huyben et al. 2018), and these impacts may negatively affect host physiology and fitness. For example, increases in environmental temperature typically result in the loss of microbial diversity, which is associated with reduced digestive performance in salamanders (Fontaine et al. 2018), and decreased survival in lizards (Bestion et al. 2017). It can therefore be predicted that global climate change may indirectly affect the physiology of ectothermic vertebrates through the alteration of gut microbial communities.

However, the significance of these effects may depend on how quickly gut microbial communities are impacted by temperature change. Longer term seasonal shifts in temperature may

result in predictable and adaptive changes to gut microbiota composition, as occurs in mammals during winter hibernation (Carey et al. 2012, Sommer et al. 2016). On the other hand, if gut microbial communities are affected by temperature over shorter temporal scales, such as days or hours, extreme weather events (heat waves, cold snaps etc.), which are expected to become more common under predicted patterns of climate change (Gao et al. 2012), may have the potential to rapidly impact host physiology (Carey and Duddleston 2014). Despite this possibility, the temporal effects of temperature on ectothermic gut microbiota are unknown.

Further, it is unclear whether the impacts of temperature on gut microbial communities are direct effects of temperature on bacterial growth, or mediated through the effects of temperature on host physiology (Kohl and Yahn 2016, Fontaine et al. 2018). If the latter is true, the temporal effect of temperature on gut microbial communities could depend on aspects of the host's biology, and therefore differ across host species. Although altered microbial communities are typically assumed to be a detriment to host physiological performance, plasticity in gut microbial communities may allow animals to adapt to changing conditions more rapidly than the host alone could accommodate (Alberdi et al. 2016). Therefore, if some species harbor more plastic gut microbiota than others, they may exhibit enhanced phenotypic plasticity in novel environments, a trait often observed in invasive species (Davidson et al. 2011). It will thus be important to explore how temperature alters gut microbiota in multiple host species that differ in their colonization abilities.

To understand how quickly temperature can alter ectothermic gut microbial communities, we exposed tadpoles to increased environmental temperature and inventoried their gut bacterial communities at multiple time points post-exposure to determine when differences in these communities first become detectable. We also explored how temperature may alter the function of

gut microbial communities by comparing predicted metagenomic profiles of gut bacterial communities across temperature treatments at the final time point of our experiment. To elucidate the impact of host biology on these patterns, we conducted this experiment in tadpoles of two common North American frog species, the green frog (*Lithobates clamitans*), and its congener, the American bullfrog (*L. catesbeianus*). These species are an ideal model system to test our questions because tadpoles rely on gut microbiota for important physiological functions (Pryor and Bjorndal 2005, Knutie et al. 2017, Warne et al. 2019), and the composition of their gut microbial communities are known to be temperature-dependent (Kohl and Yahn 2016). Additionally, despite the ecological similarities between the two study species, the American bullfrog is globally invasive (Ficetola et al. 2007), while the green frog remains restricted to its native range. This difference allowed us to test the hypothesis that bullfrog gut microbial communities would exhibit more rapid plasticity compared to those of the green frog, potentially contributing to their adaptation and success in novel habitats, as predicted by Alberdi et al. (2016). Although we sampled bullfrogs from within their native range, we reason that pre-existing phenotypic plasticity may facilitate a species' successful colonization of a new habitat upon introduction, as has been discussed regarding the immune defenses of invasive birds (Lee et al. 2005). Our results highlight the rapidity by which ectothermic gut microbial communities can change based on external environmental conditions, and the host species-dependent nature of this process.

3.2 Methods

3.2.1 Animal collections and husbandry

All animal collections, husbandry, and experimentation were reviewed and approved by the University of Pittsburgh IACUC (protocol #10862782). In June 2018, on a single day, we collected wild first-year green frog and bullfrog tadpoles (300 individuals/species) from a single natal pond at Pymatuning Laboratory of Ecology (Linesville, PA, USA), where these species co-occur. All tadpoles were housed communally in large plastic buckets (filled with water from the pond in which they were collected) for a period of two days, prior to return from the field. We then transported the tadpoles back to the laboratory at the University of Pittsburgh and housed each species separately in 3L polycarbonate tanks at a density of 30 individuals/tank (10 tanks/species), inside a walk-in, climate-controlled environmental chamber (Darwin Chambers, St. Louis, MO, USA). Tanks were filled with natural pond water that was obtained from Carnegie Lake in Highland Park (Pittsburgh, PA), collected with permission from the City of Pittsburgh Department of Public Works (General Permit #DPW1807031). Although this water was obtained from a different pond than the experimental animals, both species of frog are known to occur here. Prior to its addition to tanks, we filtered pond water through a 500-micron stainless steel sieve to remove macroorganisms. Tank water was maintained at 24°C with aquarium heaters and the environmental chamber was held at 65% humidity, on a 14 h:10 h light: dark cycle. Each tank of tadpoles was fed three 0.5g blocks of commercial rabbit chow suspended in agar three times weekly, and fecal material was removed from tanks daily with a turkey baster. Tadpoles were acclimated to these laboratory conditions for two weeks prior to experimentation.

3.2.2 Experimental manipulation

To begin the experiment, we increased the water temperature in half of the tanks (5 tanks/species) from 24°C to 29°C (warm treatment) with aquarium heaters. The other half of the tanks remained at 24°C (cool treatment). We chose these two experimental temperatures because in past surveys of amphibian thermal requirements, bullfrog tadpole body temperatures were found to range between 24 and 29.5°C during the summer months, with a mean of 25°C (Brattstrom 1963). Therefore, these temperatures are ecologically relevant to our study species. Additionally, our lower temperature limit is closer to the most common temperature experienced, and our upper limit is closer to a rarer, and potentially more stressful occurrence. Further, we have previously shown that 5°C differences in temperature manipulations can alter amphibian gut microbial communities (Fontaine et al. 2018). We recorded water temperature of each tank daily to ensure it remained within 1°C of our treatment temperatures. At six time points post temperature increase— 12 hours, 24 hours, 48 hours, 96 hours (four days), 168 hours (seven days), and 240 hours (ten days)— we removed 10-13 green frog and 11-15 bullfrog tadpoles per treatment from the experiment (1-3 individuals from each tank at each time point), and euthanized each individual by immersion in buffered MS-222 (10g/L). Following euthanasia, we recorded the Gosner stage (Gosner 1960), mass (g), and total body length (mm) of each tadpole. We then dissected each individual to remove the entire gastrointestinal tract. We changed gloves and washed all dissection instruments and trays with 100% EtOH between individuals. Following dissection, all gut samples were immediately frozen at -80°C until further processing.

3.2.3 Microbiome analyses

We extracted DNA from all gut samples using the QIAamp PowerFecal DNA Isolation Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. We also performed eight blank reactions to serve as controls for potential contaminants found in DNA extraction kits (Salter et al. 2014). Extracted DNA was sent to the DNA Services Facility at the University of Illinois at Chicago, where the V4 region of 16S rRNA gene was amplified using primers 515F and 806R, and the resulting amplicons were sequenced on the Illumina MiSeq platform (Caporaso et al. 2012). A more comprehensive description of the laboratory's library preparation and sequencing methods are available as supplementary information (Appendix A.1). We processed raw sequence data using the QIIME2 pipeline version 2019.7 (Caporaso et al. 2010). First, we used the DADA2 pipeline within QIIME2 to filter reads based on quality, merge paired forward and reverse reads, and assign reads to amplicon sequence variants (ASVs) (Callahan et al. 2016). Each read was trimmed to a length of 220 base pairs, and all singleton reads were removed. Following DADA2 processing, a phylogenetic tree of ASVs was built using FASTREE (Price et al. 2010), and taxonomy assigned to ASVs using the SILVA database classifier (Quast et al. 2012). Subsequently, we removed any reads identified as chloroplast, mitochondria, or archaea from downstream analysis.

To compare gut microbial communities of the tadpole species to one another, we first created an ASV table including samples from both green frog and bullfrog guts, and kit controls. This table was rarefied to 2,257 reads per sample, which excluded one bullfrog sample (from the 24-hour, cool treatment group) and one kit control sample with very few reads from analysis. Only 1 out of 3,867 ASVs detected in samples from our study animals was shared with those detected in kit control samples, and thus there should not be any influence of kit contaminants on our results.

Next, we created ASV tables for each species to be analyzed separately. We rarefied the green frog ASV table to 2,257 reads per sample (the number obtained from the sample with the fewest reads), and the bullfrog ASV table to 3,039 reads per sample, which excluded one sample from analysis (from the 24-hour, cool treatment group) due to an exceptionally low read count.

To measure bacterial community alpha diversity within each rarefied sample, we calculated the number of observed ASVs (richness), Shannon diversity, and Faith's phylogenetic diversity within QIIME2. Shannon diversity is a measure of biodiversity which accounts for ASV richness and evenness (Shannon 1948). Faith's phylogenetic diversity is a measure of biodiversity which computes the sum of the branch lengths connecting ASVs on a phylogenetic tree and subsequently compares these values across samples (Faith 1992). To compare bacterial community composition across samples, we calculated the dissimilarity between samples using Bray-Curtis, unweighted UniFrac, and weighted UniFrac distance matrices within QIIME2. Bray- Curtis and weighted UniFrac metrics compare dissimilarity of samples on the basis of presence, absence, and relative abundance of bacterial ASVs in the community (Bray and Curtis 1957, Lozupone and Knight 2005). Unweighted UniFrac compares dissimilarity of samples on the basis of presence and absence of ASVs only, while both UniFrac measures consider phylogenetic relatedness of ASVs in the community (Lozupone and Knight 2005).

3.2.4 Statistical analyses

3.2.4.1 Tadpole development

We first investigated for effects of host species, temperature, time, and the interaction of temperature and time on tadpole development, specifically on Gosner stage, body mass (g), and body length (mm). Shapiro-Wilk tests revealed that the residuals of these variables were not

normally distributed, and thus, we used generalized linear mixed models (GLMMs) in R version 3.6.3 for these analyses.

3.2.4.2 Comparison of gut microbial communities between species

We next compared the gut microbial communities of the two species of tadpoles to one another. To investigate for differences in alpha diversity (number of observed ASVs, Shannon diversity, and Faith's phylogenetic diversity) by species, regardless of temperature or time, we again used GLMMs in R because the residuals of these variables were not normally distributed based on Shapiro-Wilk tests. To test for significant differences in gut bacterial community composition by host species, we conducted PERMANOVAs in QIIME2 using Bray-Curtis, unweighted UniFrac, and weighted UniFrac distance matrices, after verifying the assumptions for this test were met, as outlined by Alekseyenko (2016). For each PERMANOVA, 999 permutations were used, and FDR corrected p-values were calculated to control for multiple comparisons. This data was displayed visually using Principal Coordinate Analysis (PCoA) plots constructed in R.

To determine which bacterial phyla and genera had relative abundances that were significantly different by tadpole host species, we used t-tests in JMP version 14.1.0 with the relative abundance of each taxa as response variables. We used the response screening function in JMP to obtain FDR corrected p-values to control for multiple comparisons. Prior to this analysis, we normalized the relative abundance data using an arcsine square-root transformation (Shchipkova et al. 2010). We also removed any bacterial taxa from analysis that were either unidentified or present in less than 10% of samples of both host species, to reduce the influence of very rare taxa on our results (Chen et al. 2018). After removing such taxa, we retained all 29 bacterial phyla present and 119 out of 690 genera.

3.2.4.3 Effects of experimental treatments on the gut microbial communities within each species

Lastly, we investigated for effects of temperature and time on the gut microbial communities of each species individually. To detect significant effects of these two variables (or their interaction) on gut microbial community alpha diversity (number of observed ASVs, Shannon diversity, and Faith's phylogenetic diversity), we again used GLMMs in R. To determine how temperature, time, and their interaction impacted gut microbial community composition within each species, we conducted PERMANOVAs in the program PRIMER7, using the PERMANOVA+ add-on, based on Bray-Curtis, unweighted UniFrac, and weighted UniFrac distance matrices. Each model used 999 permutations, and we FDR corrected p-values to control for multiple comparisons. After conducting full models including each of these variables, we sought to determine at what time point a significant effect of temperature could first become detectable. We therefore conducted pairwise PERMANOVA models comparing the two levels of temperature within each time point, using each of the three distance matrices. Because bacterial communities could potentially differ due to tank, we included tank ID as a variable nested within temperature in all PERMANOVA models. The results of these analyses were displayed visually using Principal Coordinate Analysis (PCoA) plots, which we constructed in R.

We next investigated which specific bacterial phyla or genera had relative abundances that were affected by temperature, time, or the interaction of these variables. We used ANCOVA models in JMP with the relative abundance of each taxa as response variables, utilizing the response screening function to obtain FDR corrected p-values to control for multiple comparisons. We again normalized the relative abundance data using an arcsine square-root transformation (Shchipkova et al. 2010), and removed any bacterial taxa from analysis that were either

unidentified or present in less than 10% of samples (retaining all 29 phyla for both species, and 116 out of 553 genera in green frogs, and 108 out of 556 genera in bullfrogs).

Lastly, to determine how increased temperature may affect the function of gut microbial communities, we used the PICRUSt software to predict metagenomic functional profiles from our 16S rRNA data at the final time point of the experiment (240 hours- 10 days) (Langille et al. 2013). To enable compatibility of our data with the PICRUSt software, we created separate operational taxonomic unit (OTU) tables for each host species, using a closed reference OTU clustering method, with the greengenes database as our reference (set to a 99% similarity level) (McDonald et al. 2012). We then imported the OTU tables into PICRUSt on the galaxy platform version 1.1.1 (http://galaxy.morganlangille.com/), and used the Categorize by Function command to identify which KEGG functional pathways (https://www.genome.jp/kegg/) were predicted to be present within each sample, and at what frequency. We subset these results to include only pathways classified within the higher order functional group of metabolism (the majority of affected pathways in both species), and imported these data into the program STAMP version 2.1.3 (Parks et al. 2014). In STAMP, we identified metabolic pathways which had relative frequencies that were significantly affected by temperature using a Welch's two-sample t-test, removing unclassified reads from analysis and correcting p-values for multiple comparisons using the FDR method.

3.3 Results

3.3.1 Tadpole development

We found that there was no effect of host species, temperature, or time on tadpole body mass or body length (Appendix Table 1; GLMM, $p>0.05$). However, we found a significant effect of both host species and time on Gosner stage by the end of the experiment (Appendix Table 1; GLMM, $p<0.05$ for both variables). Specifically, green frogs were significantly more developed than bullfrogs, regardless of time point (Appendix Figure 1), and Gosner stage increased over the time course of the experiment, regardless of host species (Appendix Figure 2).

3.3.2 Comparison of gut microbial communities between host species

After sequence filtering and processing within the DADA2 pipeline, and removal of reads identified as mitochondria, archaea, and chloroplast, we retained a total of 2,631,696 reads from green frog gut samples, with an average of 16,978 reads per sample, representing 3,690 unique bacterial ASVs. From bullfrog gut samples, we retained a total of 2,606,688 reads with an average of 16,926 reads per sample, representing 3,506 unique bacterial ASVs.

We found no significant differences in any metrics of alpha diversity (number of observed ASVs, Shannon diversity, and Faith's phylogenetic diversity) between green frog and bullfrog gut microbial communities (GLMM, $p>0.05$ for all metrics) at the end of the experiment. However, based on Bray-Curtis, unweighted UniFrac, and weighted UniFrac distance matrices, we observed that green frog and bullfrog gut microbial communities were significantly distinct from one another in terms of community composition, regardless of temperature or time (Appendix Figure

3; PERMANOVA, FDR $p<0.01$ for all metrics). Lastly, in comparisons of relative abundance of bacterial taxa (phyla and genera) between the gut microbial communities of the two host species (regardless of temperature and time), we determined that no bacterial phyla had relative abundances that were significantly different between green frogs and bullfrogs (t-test, FDR $p>0.05$ for all phyla). However, we identified 45 bacterial genera that had relative abundances that were significantly different by host species (Appendix Table 2; t-test, FDR $p<0.05$ for all genera). Specifically, 21 of these genera were more abundant in the gut microbial communities of green frogs, and 24 of these genera were more abundant in the gut microbial communities of bullfrogs.

3.3.3 Effects of experimental treatments on the gut microbial communities within each species

In green frogs, we found no significant effects of temperature, time, or the interaction of these variables on any metric of alpha diversity (number of observed ASVs, Shannon diversity, and Faith's phylogenetic diversity) of the gut microbiota (GLMM, $p>0.05$ for all metrics). Although we found no effect of temperature, time, or their interaction on the number of observed ASVs and Faith's phylogenetic diversity of bullfrog gut microbial communities (GLMM, $p>0.05$), we did find a significant effect of time on Shannon diversity of the bullfrog gut microbiome (Appendix Figure 4; GLMM, $p<0.05$). However, there was no effect of temperature or the interaction of temperature and time on this variable (GLMM, $p>0.05$).

In terms of gut bacterial community composition, we found that in green frogs, the gut microbiome was impacted by temperature, time, and the interaction of temperature and time based on Bray-Curtis dissimilarity between samples (Appendix Table 3; PERMANOVA, FDR $p<0.05$ for all variables). However, in terms of UniFrac distance between samples, unweighted UniFrac

distance between green frog samples was impacted by temperature and time (Appendix Table 3; PERMANOVA, FDR $p < 0.05$), while weighted UniFrac distance between samples was affected by time only (Appendix Table 3; PERMANOVA, FDR $p < 0.01$). In contrast, bullfrog gut bacterial community composition was significantly affected by temperature, time, and the interaction of temperature and time on the basis of all three measures of dissimilarity (Appendix Table 3; PERMANOVA, FDR $p < 0.05$ for all).

In pairwise models comparing gut bacterial community composition by temperature within each of the six time points, we discovered that, when considering Bray-Curtis dissimilarity between samples, green frog gut bacterial communities in the two temperature treatments were indistinguishable at the first three time points of the experiment (Appendix Table 4; Figure 3.1a; Appendix Figure 5a,b; PERMANOVA, FDR $p > 0.05$ at 12, 24, and 48 hours), but became significantly different by the fourth time point of the experiment (96 hours- four days), and remained distinct for the remainder of the experiment (Appendix Table 4; Figure 3.1b,c; Appendix Figure 5c; PERMANOVA, FDR $p < 0.05$ at 96, 168, and 240 hours). However, when considering unweighted and weighted UniFrac distance, there were no significant differences in green frog gut microbial community composition between the two temperature treatments at any time point of the experiment (Appendix Table 4; PERMANOVA, FDR $p > 0.05$ at all time points). On the other hand, in bullfrog gut bacterial communities, when considering Bray-Curtis dissimilarity across samples, we observed a significant effect of temperature on community composition at every time

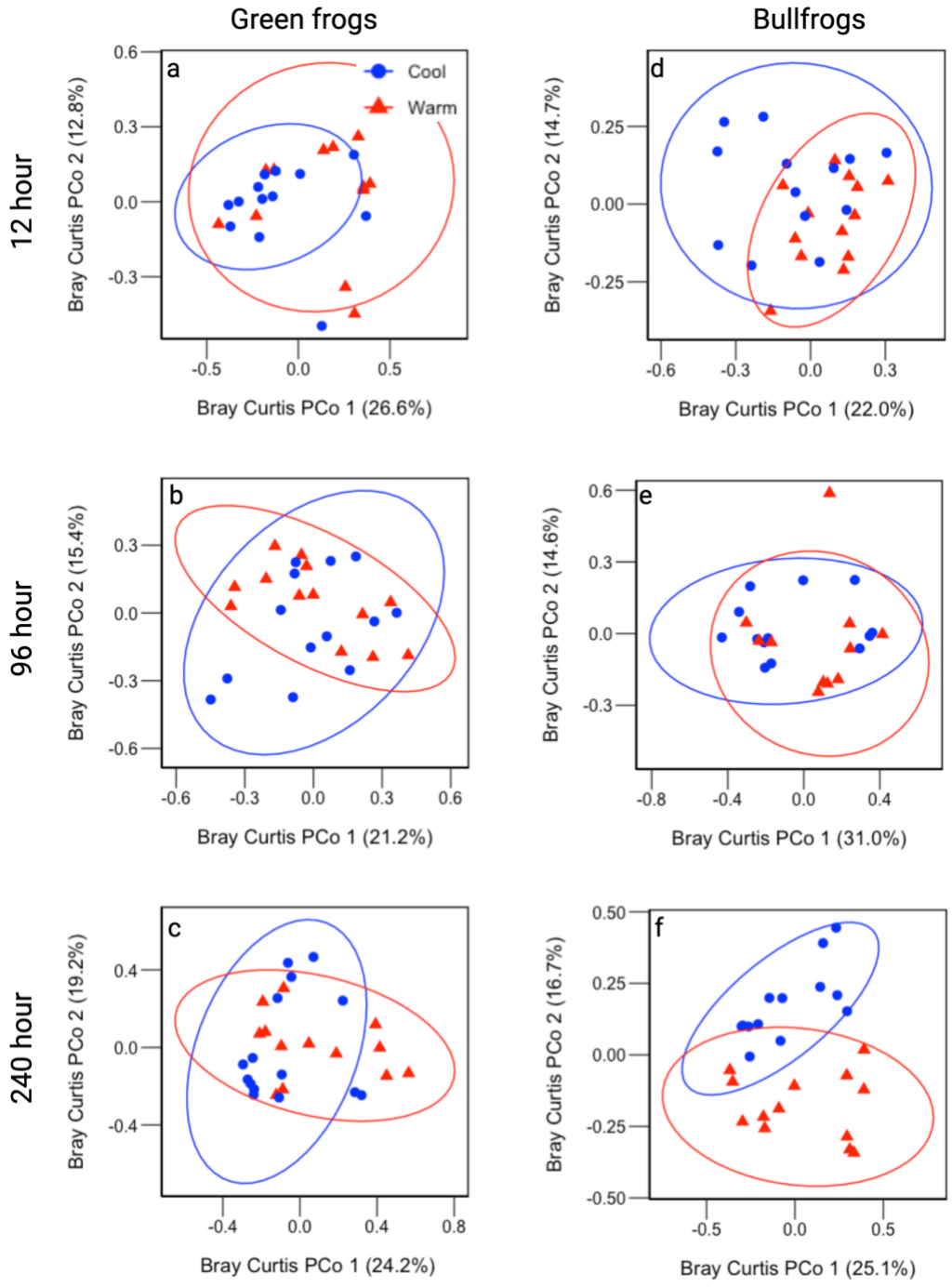


Figure 3.1 Gut microbial community composition of green frogs and bullfrogs across two temperature treatments at three experimental timepoints.

Plots are based on Bray-Curtis distance between samples. (a),(b), and (c) show green frog tadpole communities and (d),(e), and (f) show bullfrog tadpole communities. Percentages represent the proportion of variation explained by each axis. Ellipses represent 95% confidence intervals.

point of the experiment (Appendix Table 4; Figure 3.1d-f; Appendix Figure 5d-f; PERMANOVA, FDR $p<0.01$ at every time point). We also observed a significant effect of temperature at every time point in bullfrog gut microbial communities when considering weighted UniFrac distance between samples (Appendix Table 4; PERMANOVA, FDR $p<0.05$ at every time point). However, we did not observe a significant effect of temperature on bullfrog gut bacterial communities at any time point when we considered unweighted UniFrac distance between samples (Appendix Table 4; PERMANOVA, FDR $p>0.05$ at every time point).

In green frog gut microbial communities, we identified one phylum and nine genera that had relative abundances that were significantly affected by temperature, or by the interaction of temperature and time (Table 3.1; Figure 3.2a; ANCOVA, FDR $p<0.05$ for all taxa). Further, we detected five phyla and 22 genera in this host species with relative abundances that were altered by time alone (Appendix Table 5; ANCOVA, FDR $p<0.05$ for all taxa). In bullfrog gut microbial communities, we identified nine phyla and 14 genera that had relative abundances that were significantly affected by temperature, or by the interaction of temperature and time (Table 3.1; Figure 3.2b-e; ANCOVA, FDR $p<0.01$ for all taxa). In this host species, we identified five phyla and 18 genera with relative abundances that were altered by time alone (Appendix Table 5; ANCOVA, FDR $p<0.01$ for all taxa).

Table 3.1 Bacterial phyla and genera in green frog and bullfrog tadpole gut microbial communities with abundances that were impacted by experimental temperature or the interaction between temperature and time.

A symbol in the temp column indicates an effect of temperature, (+) indicates greater abundance of this taxa in warm temperatures, and (-) indicates greater abundance of this taxa in cool temperatures. A symbol in the temp x time column indicates an effect of the interaction of temperature and time, an arrow indicates the direction of the

relationship of that taxa's abundance with time (increasing or decreasing), and a (W) or (C) indicates that change with time occurred in warm or cool temperatures respectively. Significance was determined with ANCOVA models and the F statistic for each variable in the model is shown, as well as FDR corrected p-values (q-values). Taxa are ordered by their effect size.

		Temp	Temp x Time	F statistic	q-value
Green frogs	**Phyla**				
	Cyanobacteria	+		9.49	0.03
	Genera				
	Pirellula	+		14.01	<0.01
	Reyranella	-		13.62	<0.01
	Methylocystis	-		12.92	<0.01
	Clostridium sensu stricto 13	-	↑(C)	11.92	<0.01
	Phreatobacter	-		11.44	<0.01
	Laribacter	+		9.33	<0.01
	Tyzzerella	-		8.25	<0.01
	Xanthobacter	+		7.94	<0.01
	dgA-11 gut group	+		7.70	<0.01
Bullfrogs	**Phyla**				
	Proteobacteria	+		39.38	<0.01
	Dependentiae		↑(W) ↓(C)	26.85	<0.01
	Actinobacteria	+		21.43	<0.01
	Chlamydiae	+		14.23	<0.01
	Bacteroidetes	+		11.92	<0.01
	Plantomycetes	+		10.45	<0.01
	Gemmatimonadetes	+	↑(W)	9.67	<0.01
	Cyanobacteria	+	↑(W)	9.21	<0.01
	Firmicutes	+	↓(C)	7.96	<0.01
	Genera				
	Reyranella	-	↑(C)	43.57	<0.01
	Clostridium sensu stricto 8	+	↑(W)	29.80	<0.01
	Macellibacteroides	+		28.45	<0.01
	Alsobacter	-		26.83	<0.01
	Clostridium sensu stricto 5	+	↑(W)	23.83	<0.01
	Polynucleobacter	-		17.57	<0.01
	Legionella		↑(W)	14.10	<0.01
	Epulopiscium	+		14.07	<0.01
	Dielma	+		12.48	<0.01
	Romboutsia	+		11.35	<0.01
	GKS98 freshwater group	-		10.47	<0.01
	Aurantimicrobium	-		9.06	<0.01
	ZOR0006	-		8.33	<0.01
	Crenobacter	+		8.06	<0.01

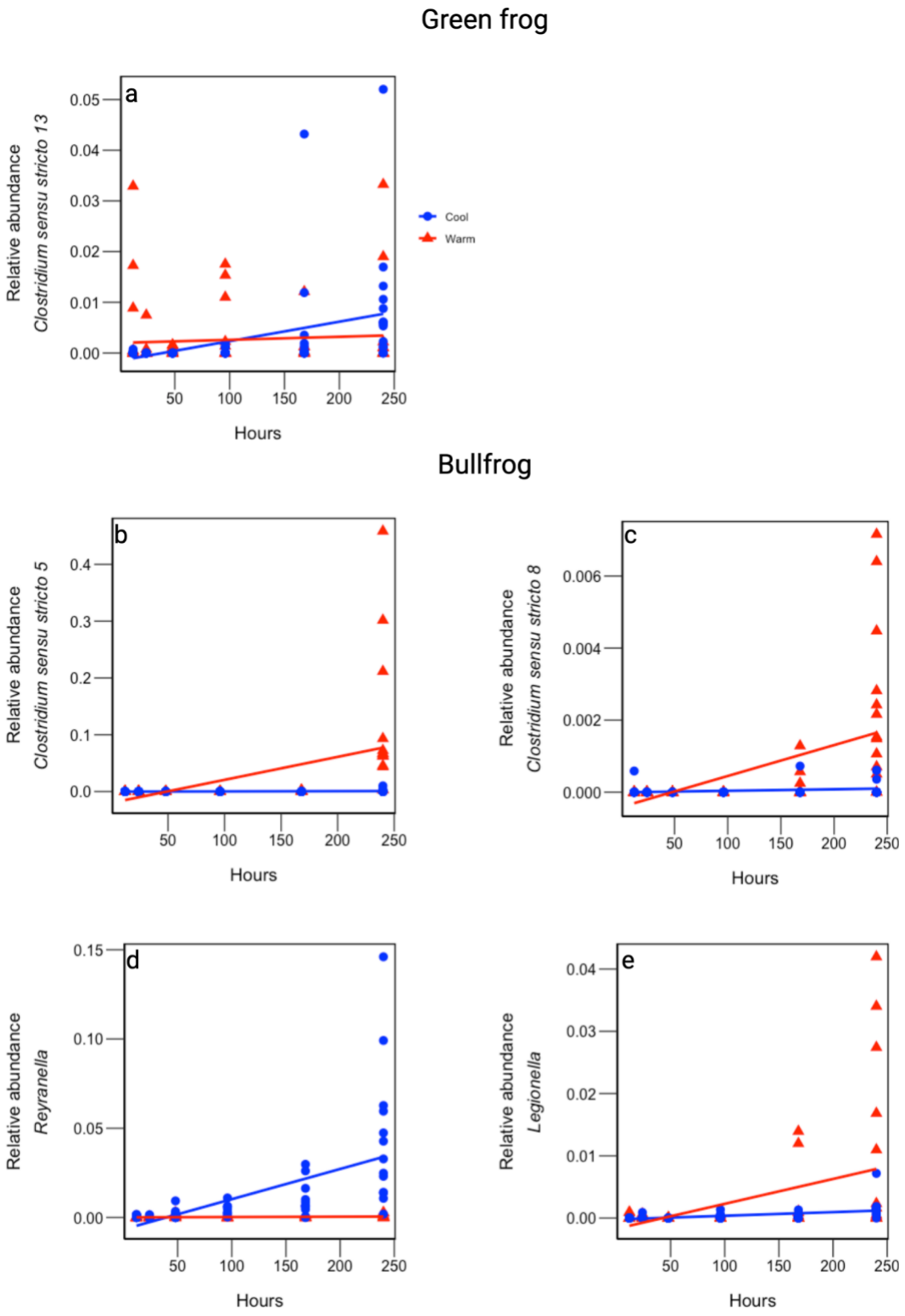


Figure 3.2 Relative abundances of bacterial genera in the gut microbial communities of green frogs and bullfrogs that were significantly affected by the interaction of temperature and time.

Relative abundance of *Clostridium* sensu stricto 13 in green frog gut microbial communities in warm and cool treatments over time. (b) Relative abundance of *Clostridium* sensu stricto 5 in bullfrog gut microbial communities in warm and cool treatments over time. (c) Relative abundance of Clostridium sensu stricto 8 in bullfrog gut microbial communities in warm and cool treatments over time. (d) Relative abundance of *Reyranella* in bullfrog gut microbial communities in warm and cool treatments over time. (e) Relative abundance of *Legionella* in bullfrog gut microbial communities in warm and cool treatments over time.

Using predicted metagenomic profiles from PICRUSt, in green frog gut bacterial communities, we identified two KEGG pathways in the metabolism functional group which differed significantly in relative frequency between our two temperature treatments at the final time point (240 hours- 10 days) of the experiment (Figure 3.3a; Welch's two-sample t-test, FDR $p<0.05$ for both pathways). Both of these identified pathways are classified within the group metabolism of cofactors and vitamins, and were more frequent in individuals within the cool temperature treatment group (Figure 3.3a). In contrast, in bullfrog gut bacterial communities, we identified 26 KEGG metabolic pathways with relative frequencies that were affected by temperature at the final time point of the experiment (Figure 3.3b; Welch's two-sample t-test, FDR $p<0.05$ for all pathways). These effected pathways were classified within the groups metabolism of amino acids, cofactors and vitamins, carbohydrates, lipids, secondary metabolites, xenobiotics, and others (which contained only one affected pathway each). The effect of temperature on the bullfrog predicted metagenome was more variable than in green frogs. For example, a majority of genes involved in carbohydrate metabolism were enriched in the warm treatment group, while those involved in amino acid metabolism tended to be reduced at higher temperatures (Figure 3.3b).

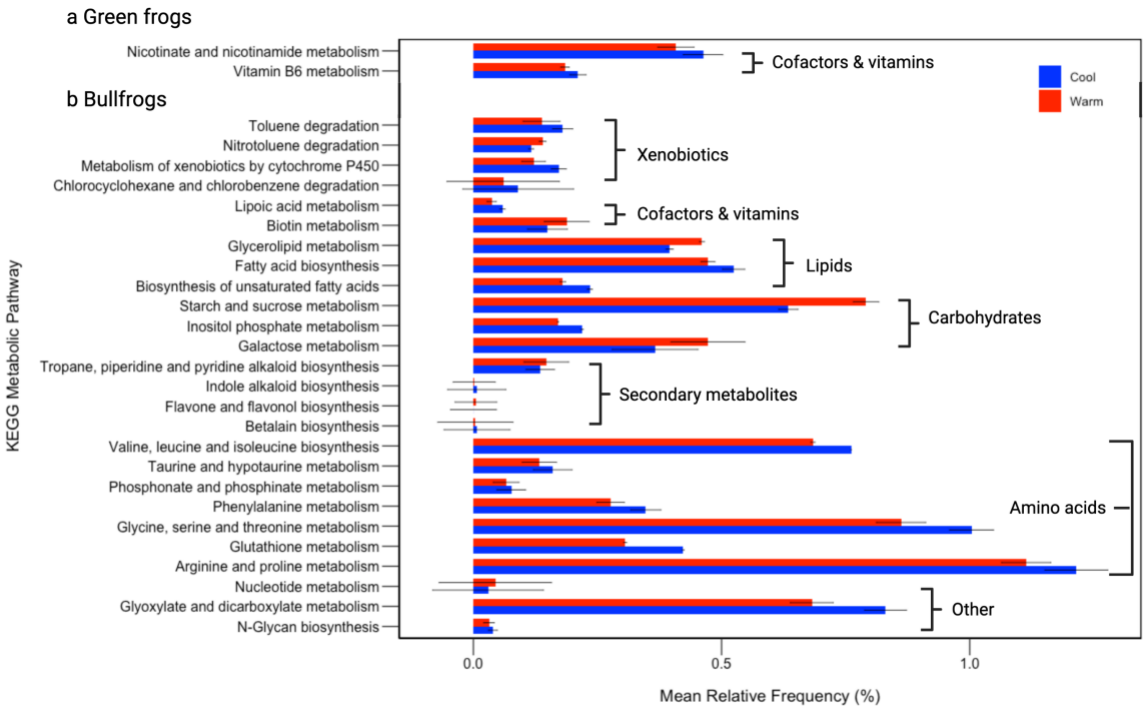


Figure 3.3 Predicted KEGG metabolic pathways that differed in relative frequency in tadpole guts after 10 days of exposure to warm temperatures.

Abundance values are means ± SD of the relative frequency (%) of each pathway in (a) green frogs or (b) bullfrogs.

3.4 Discussion

Ectotherms are expected to be especially vulnerable to the detrimental effects of global climate change, in part due to the impact of daily temperature fluctuations on their performance (Paaijmans et al. 2013). Therefore, it is imperative to understand how short-term changes in environmental temperature impact aspects of ectotherm physiology, including the composition of their gut microbial communities, which serve important functions for the host (McFall-Ngai et al. 2013). To elucidate the temporal effects of temperature on the gut microbial community composition of ectotherms, we inventoried the bacterial communities in the gut of tadpoles of two

frog species—the green frog and American bullfrog—at six time points (ranging from 12 to 240 hours) following an increase in environmental temperature. Our results highlight that the gut microbiota of ectotherms can respond to temperature shifts on rapid time scales, but these effects are host-species specific.

We found that the gut microbial communities of bullfrogs were more plastic than those of the green frog. Specifically, bullfrog gut bacterial communities were compositionally altered by temperature much more rapidly (<12 hours, first time point; Figure 3.1d) than those of the green frog (96 hours (4 days), fourth time point; Figure 3.1b). It is important to note that we did not assess gut microbial community composition of either species prior to the temperature change, and thus cannot definitively say that bullfrog communities were homogenous prior to experimentation. However, all individuals were collected from a single pond and cohoused for a period of time prior to distribution into tanks (with multiple tanks per temperature), and thus, it is unlikely that any other variable would account for the differences we observed in microbial community composition across temperature treatments.

The finding that the gut microbial communities of these two host species differ in their response to temperature is important because although these species are closely related congeners with a similar ecology, green frogs remain restricted to their native range, while bullfrogs have become globally invasive over the past century (Ficetola et al. 2007), recognized as one of the world's worst invasive species (Lowe et al. 2000). The success of bullfrogs as an invasive species has been largely attributed to their relatively large size, generalist dietary habits, high reproductive capacity, and enhanced dispersal capabilities, which allow them to prey upon and outcompete native amphibian species (Snow and Witmer 2010). Invasive species also tend to be more phenotypically plastic than natives, which can enhance their adaptability and success in novel

environments (Davidson et al. 2011). For example, invasive *Xenopus* frogs are exceptionally plastic in their optimal temperature of locomotor performance, which has allowed them to rapidly shift their range into new habitats (Araspin et al. 2020). It has been posed that plasticity in gut microbial communities may further help species acclimate and adapt to new ecological challenges, such as limited food resources or harsh climatic conditions (Alberdi et al. 2016). Therefore, it is possible that the rapid plasticity of bullfrog gut microbial communities in the face of environmental change may play a role in their successful colonization of novel habitats. Indeed, inoculation of heterospecific embryos with bullfrog gut microbiota enhances tadpole growth and development (Warne et al. 2019)—two key traits of invasive species in general (Whitney and Gabler 2008), and bullfrogs in particular (Snow and Witmer 2010)—suggesting a potential connection between gut microbiota and invasion success.

In addition to enhanced plasticity of their microbial community composition, bullfrogs were also more plastic in terms of the function of their gut microbiota. Specifically, over ten times more KEGG metabolic pathways were affected by increased temperature in the bullfrog predicted metagenome as compared to the green frog. Further, the effect of temperature on metabolic pathways in the bullfrog gut microbiome was more variable. In the green frog gut microbiome, only two metabolic pathways were temperature-dependent, and both were decreased in frequency upon exposure to increased temperature (Figure 3.3a). This effect could potentially indicate a metabolic depression, which is similar to what occurs in host metabolic pathways upon chronic exposure to temperature-related stressors in aquatic ectotherms (Sokolova and Lannig 2008). In contrast, in bullfrogs, we detected 26 metabolic pathways that were altered in frequency by increased temperature, and eight of those pathways—roughly one third—were enriched in the warm temperature treatment (Figure 3.3b). This response could indicate a more adaptive change

in bacterial metabolism. For example, we observed enrichment in two of the three affected carbohydrate metabolism pathways in warm conditions, similar to a trend observed in the predicted metagenomes of heat-tolerant corals compared to non-tolerant conspecifics (Ziegler et al. 2017). We also detected a general decrease in amino acid metabolism pathways at high temperatures, and together, these two trends could support the idea that accelerated metabolic rates under increased temperature may lead to an increased host demand for carbon (carbohydrates), compared to nitrogen (amino acids) (Bestion et al. 2019).

It is important to note, however, that this functional data was based on predictions from taxonomic profiles obtained from marker-gene sequencing. Although PICRUSt can perform similarly well to true metagenomic profiles when analyzing an environment well-represented within the database (Xu et al. 2014), we caution that this tool was developed using data from the human microbiome, which we would expect to differ from wildlife species, like the tadpoles studied here. For example, in the gut microbiome of herbivorous woodrats (*Neotoma* spp.), the coefficient of determination (R^2) between relative abundances of functional categories as estimated by PICRUSt and as determined by actual metagenomic sequencing was only 0.5 (Kohl et al. 2018b). Therefore, the degree to which the observed changes in gut microbial community composition and predicted functions are correlated with actual functional changes that enhance bullfrog physiological performance warrants additional study. Further, multispecies studies will be necessary to determine if the differences we observed across species are truly related to invasiveness, or to other biological differences between our study species (Garland Jr and Adolph 1994), especially considering bullfrogs were sampled from within their native range.

Regardless of the relevance to invasiveness, the impacts of temperature on ectotherm gut microbial communities may have implications for animal physiology under climate change. While

it is known that seasonal temperature changes result in characteristic shifts in the gut microbiota of mammals (Carey et al. 2012, Sommer et al. 2016), and longer term (weeks to years) experimental temperature manipulations alter ectothermic gut microbiota in the laboratory (Kohl and Yahn 2016, Bestion et al. 2017, Fontaine et al. 2018, Huyben et al. 2018), we have shown for the first time that gut microbial communities may be altered in response to temperature on rapid time scales, from hours to days. Because short-term extreme weather events are predicted to become more common in the coming decades (Gao et al. 2012), we may also expect gut microbial communities of ectotherms to become less stable. Although the physiological consequences of these changes are unknown, the loss of important bacterial taxa could be detrimental to species that rely heavily on microbial services, such as the herbivorous tadpoles studied here, which obtain a substantial portion of daily energy requirements from gut bacterial fermentation (Pryor and Bjorndal 2005). Indeed, exposure to increased temperature on longer time scales was associated with a loss of digestive performance in salamanders (Fontaine et al. 2018), and altered energy metabolism within the lizard gut microbiota (Bestion et al. 2017). Further, temperature induced changes in gut microbiota could also result in the increase of pathogenic taxa (Fontaine et al. 2018) or loss of immune function. For example, in this study, we detected an increase in the relative abundance of the genus *Legionella* over time in the gut of bullfrogs in the warm temperature treatment only (Figure 3.2e). Although their significance to amphibian health is uncertain, these bacteria are known pathogens of both mammalian and ectothermic invertebrate hosts (Brassinga et al. 2010, Steinert 2011). Additionally, the genus *Reyranella*, which is positively correlated with immune function in zebrafish (Peng et al. 2019), was largely absent from the gut of bullfrogs housed in warm conditions (Figure 3.2d). Lastly, we found that the relative abundance of *Clostridium* tended to increase in warm conditions in bullfrogs (Figure 3.2b,c), although the

opposite trend was observed in green frogs (Figure 3.2a). Interpreting the potential impact of changes within this bacterial genus on animal health is complex because its members are generally considered pathogenic (Bartlett 1994, Collins and East 1998, Myers et al. 2006), but may be commensal in tadpoles (Pryor 2008). Understanding the true consequences of thermally-altered gut microbiota to host physiology will require manipulative strategies, such as isolating the bacteria in question or performing fecal microbiota transplants, because many gut microbial taxa have functions that are currently unknown (Heintz-Buschart and Wilmes 2018).

Previous studies focused on the effects of environmental temperature on ectothermic gut microbial community composition and function have been limited in scope to only one study species (Kohl and Yahn 2016, Bestion et al. 2017, Fontaine et al. 2018, Huyben et al. 2018), and therefore, an outstanding question has remained as to whether these shifts are due to direct effects of temperature on microbial growth, or mediated through temperature-induced changes in host physiology. Because we detected host species-specific differences in the effects of temperature on gut microbial community composition, our findings suggest these changes may be, at least in part, host-mediated. Environmental temperature influences all aspects of ectotherm physiology (Angilletta Jr et al. 2002), and certain physiological functions exert control over microbial community structure. For example, amphibian immunity is tightly linked to temperature, responding on both short and long-term time scales via changes in the transcription of important immune factors, as well as in the capacity for peak performance of immune cells (Rollins-Smith 2017, Ellison et al. 2020), and the immune system is arguably the single most important host regulator of microbial community composition (Hooper et al. 2012). Additionally, temperature can control tadpole digestive processes through impacts on behavior (e.g. feeding rates), morphology (e.g. gut size), and physiology (e.g. intestinal enzyme activity) (Warkentin 1992,

Benavides et al. 2005, Castaneda et al. 2006). Gut microbial community composition is highly sensitive to host dietary traits, shifting in response to all of the aforementioned factors (Stearns et al. 2011, Kashyap et al. 2013, Kohl et al. 2014a). However, thermal plasticity in the same physiological traits can differ across amphibian species (Simon et al. 2015), and thus, the host-specific sensitivity of physiological function to temperature may determine if, how, and when gut microbial communities become affected by temperature change.

Although the effects of temperature on ectotherm gut microbial communities may be host-mediated, we cannot rule out that the initial composition of the gut microbial community may influence the nature of its response to temperature. For example, one species' microbiome may be more plastic in response to temperature if it initially contains more sensitive taxa. Although we did not compare the gut microbial communities of green frogs and bullfrogs prior to experimentation, by the end of manipulations, each host species retained a distinct bacterial community, despite any effects of temperature or time. While a description of the larval green frog gut microbial community appears to be lacking in the literature, at least some aspects of the bullfrog tadpole gut microbial community we observed were consistent with previous research. We observed that in this host species, the phylum Proteobacteria was enriched in relative abundance in warm temperatures (Table 3.1). This was also observed in the gut of leopard frog (*L. pipiens*) tadpoles upon exposure to increased temperature (Kohl and Yahn 2016), and on the skin of adult bullfrogs on a global climatic scale (Kueneman et al. 2019). It would be interesting to test if high abundances of these taxa are adaptive at high temperatures. We also found that bullfrog gut microbial communities were enriched in relative abundance of the bacterial genus Candidatus *Amphibiichlamydia*, which was also reported in the gut of invasive bullfrog tadpoles in the Netherlands, and may be a pathogenic threat to native species (Martel et al. 2013). Further, we

detected the largest difference across host species in the relative abundance of *Cetobacterium* in their gut. This genus was dominant in bullfrog bacterial communities, accounting for 25% of mean relative abundance in the community (Appendix Table 2). This genus was also observed in exceptionally high abundance in previous studies of the gut bacterial communities of larval bullfrogs (Wang et al. 2020). Interestingly, this genus provides important physiological services to other aquatic ectotherms, including B vitamin synthesis, carbohydrate metabolism, and production of short chain fatty acids (Tsuchiya et al. 2008). It has not been explored whether or not the growth of dominant bacteria in the bullfrog gut are simply more responsive to temperature than those within the green frog. However, this investigation would be necessary to demonstrate that the temperature-dependent function of host physiological systems is the true driver of host-specific changes in gut microbiota, and not simpler bacterial community dynamics.

Regardless of temperature, we also noticed large effects of time on the community composition of gut microbiota, as well as the relative abundance of various phyla and genera in these communities, in both host species (Appendix Table 3, Appendix Table 5). Captivity has been consistently shown to alter the associated microbiota of many hosts, including amphibians, potentially via the large differences in diet and available environmental substrates between captive and wild habitats (Antwis et al. 2014, Becker et al. 2014, Loudon et al. 2014). These changes may therefore explain some of the effects of time that we observed. Further, we removed individuals from each tank at each time point, and therefore animal density consistently decreased in tanks over time in the experiment. This could have affected stress levels through decreased levels of competition, or potentially increased food intake—both of which may alter gut microbial communities of aquatic ectotherms (Zha et al. 2018).

The physiological performance of ectotherms is already expected to be challenged in the future by increased short-term fluctuations in environmental temperature (Paaijmans et al. 2013), and we have shown here that these temperature changes may also have implications for the composition of gut microbial communities, which many species rely on for their basic physiological functioning. Understanding how past weather events may have impacted gut microbial communities will be important information for those monitoring the long-term physiology and health of wildlife populations. However, we identified that the effects of temperature on ectotherm gut microbiota can vary across host species, with potentially important implications for how invasive species adjust and adapt to novel environments. Therefore, it will be important to elucidate the degree to which these shifts differentially impact species' physiology, ecological distributions, and persistence under future global climate change.

3.5 Acknowledgements

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4.0 Experimental manipulation of microbiota reduces host thermal tolerance and fitness under heat stress in a vertebrate ectotherm

The contents of this chapter are adapted from the following publication:

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4.1 Introduction

Temperature is a crucial environmental factor impacting the physiological performance and fitness of ectothermic animals, which, are expected to be especially vulnerable to the deleterious effects of global climate change (Clusella-Trullas et al. 2011, Paaijmans et al. 2013). Indeed, global surveys have shown declines in several ectothermic vertebrate groups due to impacts of climate change (Pounds et al. 2006, Sinervo et al. 2010). Continuing to predict how these animals will physiologically respond to changing temperature regimes requires a thorough understanding of the mechanisms that contribute to host thermal tolerance and the impact of temperature on animal performance (Pacifici et al. 2015). Decades of research have assessed the thermal tolerance of ectotherms under a range of conditions by quantifying metrics such as the critical thermal minimum and maximum (CT_{min} and CT_{max}), physiological performance across temperatures (thermal performance curves), and survival at sublethal temperatures (Angilletta Jr 2009, Sunday et al. 2011, Jørgensen et al. 2019). These studies have identified several physiological traits that underlie thermal tolerance including the fluidity and structure of plasma

membranes, the stability and conformation of enzymes, and the maintenance of metabolic capacity at high and low temperatures (Angilletta Jr 2009, Pörtner et al. 2017, Gangloff and Telemeco 2018). Additionally, while thermal tolerance and its physiological underpinnings are in part genetic traits (Perry et al. 2001), they are also plastic, and can be shaped by environmental factors, such as time of day (Healy and Schulte 2012), season (Hu and Appel 2004), oxygen availability (Healy and Schulte 2012), feeding (Nyamukondiwa and Terblanche 2009), and infection status (Greenspan et al. 2017, Padfield et al. 2020).

Host-associated microbial communities, or microbiomes, have recently emerged as a critical factor that regulate how animals respond to their external environment. Interactions between hosts and microbes are bidirectional with microbial communities being shaped by host factors such as the immune system and host genetics (Hooper et al. 2012, Goodrich et al. 2014), while also impacting host physiology (e.g. digestion, immune function) and gene expression (Alberdi et al. 2016, Kohl and Carey 2016, Fontaine and Kohl 2020b). Impacts of the microbiota on host traits like phospholipid profiles (Velagapudi et al. 2010) and metabolism (Donohoe et al. 2011) suggest these communities could also play a role in host thermal tolerance. However, we currently lack a complete understanding of this relationship across ectotherm groups. In invertebrate animal systems, there is indeed a relationship between symbiotic microbes and host thermal tolerance. For example, in sea anemones, corals, and aphids, acquisition of particular symbionts can buffer against the lethal effects of heat shock, allowing hosts to persist in otherwise extreme habitats (Montllor et al. 2002, Russell and Moran 2006, Ziegler et al. 2017, Herrera et al. 2020). Further, axenic flies exhibit decreased survival under heat stress compared to conventional flies (Jaramillo and Castañeda 2021), and microbial transplants can alter fly thermal tolerance (Moghadam et al. 2018).

While there have been no studies that directly manipulate the microbiome to test this relationship in ectothermic vertebrates, there is correlative evidence that host-associated microbial communities may also impact thermal tolerance in this group. Environmental temperature influences the diversity and composition of the gut microbiome in several systems (Kohl and Yahn 2016, Bestion et al. 2017, Fontaine et al. 2018, Fontaine and Kohl 2020a, Zhu et al. 2021). In lizards, exposure to heat alters the gut microbiome, some aspects of microbial composition are associated with the critical thermal maximum of hosts (Moeller et al. 2020), and loss of microbial diversity is correlated with reduced animal survival (Bestion et al. 2017). Additionally, artificial selection for cold tolerance in fish alters microbiome composition and its sensitivity to cold exposure, suggesting a relationship between these phenotypes (Kokou et al. 2018). However, a major challenge of the microbiome field is to move past correlations between the microbiome and host phenotypes, and to truly demonstrate functional effects of the microbiome on host biology through direct manipulations of microbial communities (Hanage 2014). This need is especially apparent in non-model systems, where the vast majority of microbiome studies are purely observational (Pascoe et al. 2017).

Here, we use a series of manipulative experiments to test the hypothesis that the host-associated microbiome influences thermal tolerance in an ectothermic vertebrate. We demonstrate a direct relationship between the microbiome of a vertebrate ectotherm and the host's thermal tolerance, performance, and fitness. Specifically, we find that tadpoles with experimentally manipulated microbiomes exhibit a significant reduction in tolerance to acute thermal stressors, impaired fitness under prolonged heat stress, and a change in the thermal sensitivity of whole-organism performance compared to those with unmanipulated microbial communities. To enhance our understanding of the physiological integration of host-microbe interactions across levels of

biological organization (Mykles et al. 2010, Kohl 2018), we compare several physiological metrics associated with thermal tolerance across microbiome treatment groups, including cell membrane phospholipid composition, mitochondrial enzyme activities, and metabolic rates. Collectively, our study demonstrates that host associated-microbial communities are an important factor contributing to the thermal physiology of ectothermic vertebrates on several scales, from the subcellular to whole-organism level, which may be important information under the threat of climate change and global declines in ectotherm diversity.

4.2 Results and Discussion

4.2.1 Microbiota manipulation altered the tadpole gut microbiome

For all experiments, we used laboratory-reared tadpoles from wild adults of the green frog *(Lithobates clamitans)*. Green frogs are an abundant and widespread anuran amphibian across eastern North America, and can adjust their physiology in response to temperature during both larval and adult lifestages (Brattstrom and Lawrence 1962, Gray et al. 2016). Appendix Figure 6 provides a visual description of the laboratory rearing process, our experimental designs, and sample sizes. To manipulate the tadpole microbiome, we used an environmental water sterilization technique which involves housing developing animals in autoclaved water. This approach has been used previously in tadpoles to disrupt host-associated gut and skin microbial communities, and demonstrate the importance of the microbiome to host development, metabolism, and disease susceptibility (Knutie et al. 2017, Warne et al. 2019). The advantage of this technique is that we can reduce colonizing microbes without inducing off-target effects on host physiology, which can

occur when using other methods to deplete microbiomes, such as antibiotics (Morgun et al. 2015). Using this technique, we generated two groups of tadpoles raised with differing exposure to environmental microbes. Tadpoles in our first treatment, which we call "colonized", were raised in a microbially rich environment. Specifically, these tadpoles were housed in autoclaved laboratory water seeded with 25% natural pond water from their parent's site of capture. Tadpoles in the second group, which we refer to as "depleted", were raised in an environment depleted of environmental microbes, and were housed in autoclaved laboratory water seeded with 25% autoclaved pond water from the same source.

In our first experiment, we raised colonized and depleted tadpoles spawned from adults collected from Louisiana (USA) at three acclimation temperatures: 14°C, 22°C, and 28°C, and used high-throughput 16S rRNA gene amplicon sequencing to compare gut bacterial communities across treatment groups. Additionally, to track the bacterial communities of our stored pond water (kept at 4°C), we collected and froze samples of this water weekly during the experiment and compared these communities to samples collected from the pond and immediately frozen in the field to determine how well our colonized treatment reflected natural pond communities. Appendix B.1.1 Supplementary Discussion and Appendix Figure 7 detail the results of this comparison.

In tadpole gut bacterial communities, the dominant phyla overall were Firmicutes and Proteobacteria (Appendix Figure 8), which appear to be common commensals of larval amphibians, having been documented in high abundance in the guts of several other tadpole species (Kohl et al. 2013, Vences et al. 2016), as well as in the early life-stages of the eastern newt (Fontaine et al. 2021). However, acclimation temperature significantly altered the tadpole gut microbiome such that tadpoles raised at warmer temperatures had less diverse bacterial communities than those raised at cooler temperatures (Figure 4.1a, Appendix Figure 9a, Appendix

Table 6; GLMM, $p<0.001$), and bacterial community composition and variability were distinct across all temperature groups (Figure 4.1b, Appendix Figure 9e-f, Appendix Table 6; PERMANOVA, $p<0.001$; PERMDISP, $p<0.01$). Although significant differences in microbial community dispersion could drive observed differences in microbial community composition, our PCoA plots demonstrate strong clustering by treatment groups, and our balanced study design should minimize this issue (Anderson and Walsh 2013). A single bacterial phylum, Acidobacteria, had a relative abundance that was associated with acclimation temperature, and was most abundant in the 14°C group (Appendix Table 7; MaAsLin2, corrected $p=0.03$), although no bacterial genera differed in relative abundance across temperatures. Our results are consistent with those that find reduced gut bacterial diversity and altered composition at increased temperatures in various ectotherms (Bestion et al. 2017, Fontaine et al. 2018, Moghadam et al. 2018, Sepulveda and Moeller 2020, Arango et al. 2021, Zhu et al. 2021). The causes of these changes are unknown but may involve temperature-dependent growth of bacterial communities, or indirect effects of temperature on host physiology and/or behavior (Bestion et al. 2017, Fontaine et al. 2018).

We found that depleted tadpoles largely had reduced gut bacterial diversity compared to colonized tadpoles (Figure 4.1a, Appendix Figure 9a-c, Appendix Table 6; GLMM, $p<0.001$), but this effect interacted with temperature (Figure 4.1a, Appendix Figure 9a-c, Appendix Table 6; GLMM, $p<0.05$). Because increasing temperatures reduced bacterial diversity even in colonized individuals, tadpoles at the coolest acclimation temperature exhibited larger differences in microbial diversity between colonized and depleted animals than those at warmer acclimation temperatures. Depleted tadpoles also harbored bacterial communities with a distinct composition compared to colonized tadpoles (Figure 4.1b, Appendix Figure 9e-f, Appendix Table 6; PERMANOVA, $p<0.001$). Our results mirror those of a previous study that found raising tadpoles

in sterilized water reduces the diversity and alters the composition of the gut bacterial community (Knutie et al. 2017). Additionally, depleted tadpoles exhibited greater degrees of intraindividual variability within their gut microbial communities than colonized tadpoles (Appendix Table 6; PERMDISP, $p<0.01$). This difference could be due to development in an environment with low microbial diversity, as reduced microbial diversity is associated with increases in host stress hormones (Stothart et al. 2016). Increased stress could then reduce a host's ability to regulate their commensal microbiome, and ultimately lead to greater stochasticity in the community (Zaneveld et al. 2017). Additionally, development in an environment with fewer microbial members may also increase community stochasticity due to ecological drift (Orrock and Watling 2010). Within these communities, the relative abundances of seven bacterial phyla and 11 bacterial genera were impacted by microbial colonization treatment (Appendix Table 7; MaAsLin2, corrected $p<0.05$). The affected phyla included those such as Dependentiae, Plantomycetes, and WPS-2, which are common to aquatic and soil habitats (Deeg et al. 2019, Kaboré et al. 2020, Sheremet et al. 2020), and were also abundant in tadpole guts colonized with natural pond water, but were absent or present at very low abundances in depleted tadpoles (Appendix Table 7). Because green frog tadpoles develop externally to the mother and lack parental care, it is likely a high proportion of their symbiotic microbes come from transmission of environmental microbes (Correa et al. 2020), and therefore, depleting these communities results in a gut microbiome with reduced abundances of these taxa.

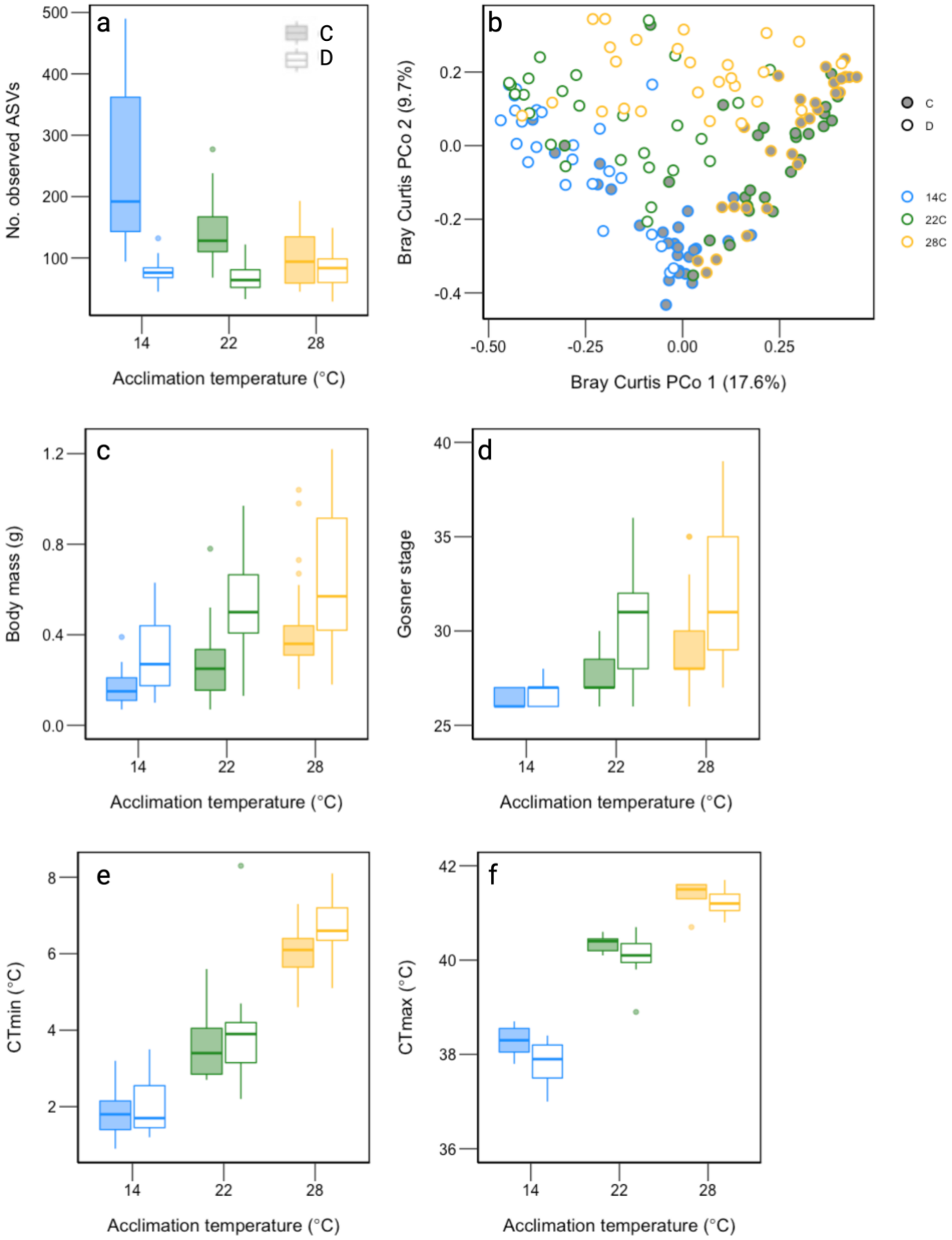
a
No. observed ASVs
500
400
300
200
100
C
D
14
22
28
Acclimation temperature (°C)
b
Bray Curtis PCo 2 (9.7%)
0.2
0.0
-0.2
-0.4
-0.50
-0.25
0.00
0.25
Bray Curtis PCo 1 (17.6%)
C
D
14C
22C
28C
c
Body mass (g)
1.2
0.8
0.4
0.0
14
22
28
Acclimation temperature (°C)
d
Gosner stage
40
35
30
25
14
22
28
Acclimation temperature (°C)
e
CTmin (°C)
8
6
4
2
14
22
28
Acclimation temperature (°C)
f
CTmax (°C)
42
40
38
36
14
22
28
Acclimation temperature (°C)

Figure 4.1 Effects of microbial colonization treatment and acclimation temperature on tadpole gut microbial communities, morphometrics, and acute thermal tolerance.

(a), Number of observed bacterial amplicon sequence variants (ASVs) in tadpole gut microbial communities (b) Principal Coordinate (PCo) analysis plot based on Bray-Curtis dissimilarity of gut microbial communities between samples. Percentages represent the proportion of variation explained by each axis (c) tadpole body mass (d) tadpole developmental stage based on the Gosner system, (e) tadpole critical thermal minimum (CTmin) (f) tadpole critical thermal maximum (CTmax). In each boxplot, the center line represents the median, the length of the box extends through the IQR, and whiskers extend to 1.5x IQR. All points outside this range are plotted individually. C= colonized tadpoles and D= depleted tadpoles. Colors represent tadpole acclimation temperature.

However, neither microbial colonization treatment nor acclimation temperature impacted the total abundance of bacterial cells in the gut based on flow cytometry analyses (Appendix Figure 9d; LME, $p>0.05$). This result supports the idea that there may be host species-specific carrying capacities that set limits to gut microbial population densities, regardless of differences in community composition (Contijoch et al. 2019). While we cannot definitively determine the source of gut microbiota that was able to colonize depleted tadpoles, we speculate that these animals may have been exposed to microbes during egg-laying from their mother's cloaca (Warne et al. 2017), or from routine laboratory contamination because we did not raise individuals under germ-free conditions. However, this result does suggest that opposed to germ-free studies that compare colonized animals with those under the highly artificial state of sterility, our results may more accurately represent dynamics that occur in animal microbiomes in the wild. Although our pond water communities may differ from natural microbial communities (see Appendix B.1.1), in wild populations anthropogenic and environmental impacts can also often change microbial community diversity and composition without reducing the numbers of microbes available for colonization (Trevelline et al. 2019).

4.2.2 Microbiota manipulation altered tadpole thermal tolerance

In our first experiment, after tadpoles had developed for seven weeks post-hatching, their acute thermal tolerance was measured via the critical thermal minimum (CT_{min}) and maximum (CT_{max}). We used Hutchison's dynamic method (Lutterschmidt and Hutchison 1997), in which temperatures are increased or decreased until a loss of the righting response occurs, to measure CT_{min} and CT_{max} in 11 individuals from each of the six treatment groups (acclimation temperature x microbial colonization). All remaining individuals (N = 16 animals per group) were euthanized for morphometric data without measuring thermal tolerance. In each tadpole, we measured body mass (g), body length (mm), body width (mm), Gosner stage (Gosner 1960), and facial symmetry. Greater degrees of asymmetry are associated with reduced developmental stability and stressful conditions (Daloso 2014).

We found that temperature impacted tadpole growth and development such that tadpoles raised at warmer acclimation temperatures were significantly larger and more developed than tadpoles raised at cooler temperatures (Figure 4.1c-d; Appendix Figure 10a-b; Appendix Table 8; GLMM, $p<0.001$). This finding was expected based on the wealth of studies demonstrating the positive relationship between environmental temperature and tadpole growth and development at sublethal temperatures (Marian and Pandian 1985, Harkey and Semlitsch 1988, Alvarez and Nicieza 2002, Goldstein et al. 2017). We also observed that tadpoles raised at warmer temperatures exhibited decreased facial symmetry compared to those at cooler temperatures (Appendix Figure 10c; Appendix Table 8; GLMM, $p<0.001$) indicating our higher developmental temperatures may have increased tadpole stress and developmental instability.

We also found that depleted tadpoles were significantly larger and more developed than colonized tadpoles (Figure 4.1c-d; Appendix Figure 10a-b; Appendix Table 8; GLMM, $p<0.001$).

Specifically, depleted tadpoles were 55% larger in terms of body mass, and more developed by an average of two Gosner stages than colonized tadpoles, regardless of acclimation temperature. Our results are similar to studies in wild, laboratory, and agricultural systems that find enhanced growth and body size in animals treated with antibiotics to deplete gut bacterial communities (Coates et al. 1963, Gaskins et al. 2002, Potti et al. 2002, Kohl et al. 2018a). There are several hypotheses that may explain this phenomenon. For example, animals invest heavily in their immune systems to both defend against pathogens, and regulate populations of commensal bacteria (Hooper et al. 2012), and thus, reducing gut microbial diversity may allow animals to invest more in growth and development as opposed to immune system regulation (Gaskins et al. 2002). Interestingly, the gut microbiome of colonized tadpoles had higher proportions of the bacterial phylum Chlamydiae than depleted tadpoles (Appendix Table 7), which are obligate intracellular microbes known to exploit host nutrients and elicit an immune response (Gitsels et al. 2019). However, not all taxa within the phylum are necessarily pathogenic and we did not notice any symptoms of infection (lethargy, skin lesions or discoloration etc.) in any experimental animals. Additionally, stress hormones are known to stimulate growth and development in tadpoles (Denver 1997), and reductions in microbial diversity can increase animal stress (Stothart et al. 2016). However, we did not measure physiological markers associated with stress, such as glucocorticoids, in our system, and we did not observe a significant effect of microbial colonization treatment on tadpole facial symmetry (Appendix Figure 10c; Appendix Table 8; GLMM, $p>0.05$).

We found that acclimation temperature predictably impacted tadpole thermal tolerance, such that tadpoles raised at warmer temperatures were more tolerant to heat, and tadpoles raised at cooler temperatures were more tolerant to cold. Specifically, animals raised at the coolest temperature (14°C) had a CT_{min} that was on average 4.5°C lower than animals raised at the

warmest temperature (28°C) (Figure 4.1e; GLMM, χ^2= 182.04, p<0.001). Animals raised at the warmest temperature had a CT_{max} that was on average 3.3°C higher than animals raised at the coolest temperature (Figure 4.1f; GLMM, χ^2= 673.25, p<0.001). These results are consistent with studies demonstrating that many anuran amphibian species, including the green frog, can adjust their critical thermal limits adaptatively in response to acclimation temperatures (Brattstrom and Lawrence 1962).

Notably, we observed that depleted tadpoles exhibited reductions in their tolerance to cold temperatures compared to colonized tadpoles. Specifically, these animals had CT_{min} values that were on average 0.4ºC higher than colonized tadpoles after controlling for the effects of acclimation temperature, body size, and Gosner stage (Figure 4.1e; GLMM, χ^2= 5.30, p=0.02). Other studies demonstrating a relationship between gut microbiota and host cold tolerance are scarce in ectothermic systems, however, selection for cold tolerance in fish does alter the gut microbial community (Kokou et al. 2018). In mammalian systems, changes occurring in the gut microbiota upon exposure to cold ultimately lead an increased host tolerance to cold through enhanced energy acquisition (Chevalier et al. 2015, Khakisahneh et al. 2020). The mechanisms underlying tadpole tolerance to cold likely differ, as studies in mice demonstrate that microbial products facilitate host maintenance of internal body temperature during cold exposure, which would not be important in ectothermic systems. Regardless, our result warrants future study and may impact animals in the wild, as green frogs often overwinter as tadpoles and can be exposed to near freezing temperatures (Gray et al. 2016).

Depleted tadpoles were also less tolerant to heat than their colonized counterparts. At each acclimation temperature, depleted tadpoles had CT_{max} values that were an average of 0.3ºC lower than colonized tadpoles (Figure 4.1f; GLMM, χ^2= 18.60, p<0.001). These results echo those of

studies focused on invertebrate ectotherms that have shown microbial symbionts can enhance host heat tolerance (Montllor et al. 2002, Russell and Moran 2006, Xie et al. 2013, Ziegler et al. 2017, Moghadam et al. 2018). The reduction in heat tolerance in microbially depleted tadpoles is not simply a function of the difference in growth and development between the two groups. Body mass and Gosner stage were included as covariates in the above models and did not change the significant relationship between microbial colonization and host thermal tolerance. Further, we observed a significant positive relationship between animal body mass and Gosner stage, and CT_{min} and CT_{max} (GLMM, χ^2= 4.23 and 13.90 for body mass and Gosner stage respectively with CT_{min}, and χ^2= 4.06 and 17.88 for body mass and Gosner stage respectively with CT_{max}, $p<0.05$ for all). For heat tolerance, this trend is the opposite of what would be expected if these factors were driving the relationship between thermal tolerance and microbial colonization treatment.

Taken together, our results show that depleted tadpoles have both higher CT_{min} and lower CT_{max} values than colonized tadpoles. These results demonstrate that manipulating the microbiome reduces the overall window of thermal tolerance in an ectothermic vertebrate, which was previously unknown. Wider thermal tolerance breadths are often observed in populations that experience greater degrees of thermal variability (Guiterrez-Pesquera et al. 2016), and can be important in adapting to fluctuating environments (Litmer and Murray 2019). Although the reductions in thermal tolerance we observed due to microbiota manipulations were generally small, there still may be important implications for these subtle changes. Aquatic environments, like the ponds these tadpoles inhabit, tend to be more buffered and have more stable temperatures diurnally and seasonally compared to terrestrial environments. Therefore, animals in these environments may be adapted to a narrower range of temperatures. Thus, even slight reductions in thermal tolerance due to changes in the microbial community could hinder their ability to cope with

temperature fluctuations expected to occur under climate change. However, we observed a significant degree of plasticity in thermal tolerance, as evidenced by differences in CT_{min} and CT_{max} across acclimation temperatures, which could help animals adjust to changing temperatures. Importantly, we observed this capability for plasticity in both microbial treatment groups, so disruptions to the microbiota do not seem to hinder these processes of physiological adjustment.

In the wild, changes to host-associated microbiota may occur due to various anthropogenic environmental impacts (Trevelline et al. 2019), however, increasing temperatures themselves can also alter gut microbial communities of amphibians as evidenced by our results and others (Kohl and Yahn 2016, Fontaine et al. 2018, Fontaine and Kohl 2020a). Thus, negative impacts of climate warming may compound such that warming-induced changes in bacterial communities may leave animals even more vulnerable to further warming due to potential reductions in their thermal tolerance. However, before extrapolating our results to wild systems, it will be important to follow up our work with additional studies. Future directions should include validating our findings in studies with larger numbers of individuals, in populations with more genetic diversity (single egg clutches were used in these experiments), and across several species. Additionally, as the impact of acclimation temperature on thermal tolerance was generally larger than that of the microbial community, it would also be important to determine if an impact of microbiota on thermal tolerance could still be detected in animals raised under more natural conditions. This idea could be tested using outdoor mesocosms in which environmental microbial communities can still be manipulated but more natural temperature fluctuations would be permitted. Lastly, although we focus solely on thermal tolerance, our results demonstrate effects of the microbiome on body size and development, which could set up important trade-offs in the wild. For example, depleted tadpoles experience a cost of reduced heat and cold tolerance, but could benefit from larger body

sizes when it comes to predator escape (Semlitsch 1990), prey capture (Cabrera-Guzmán et al. 2013), and fecundity (Tejedo 1992), if body size differences are maintained after metamorphosis. However, accelerated development times, also observed in depleted tadpoles, could result in lowered immune function and greater disease susceptibility (Warne et al. 2011). Experiments that capture the full range of biotic and abiotic conditions experienced by tadpoles in nature will help elucidate the true impacts of microbiota disruption on host performance in the wild.

4.2.3 Microbiota manipulation reduced tadpole survival

We conducted an additional study addressing the differences in heat tolerance observed between groups, given that climate warming is a major threat to many species worldwide (Urban 2015), as well as in our study area (Pearce and Paustian 2013). In this second experiment, we sought to determine if depleted tadpoles, which experienced a significant reduction in their acute heat tolerance, would also incur fitness costs, in terms of survival, upon exposure to sublethal heat stress for longer time periods.

Using adult frogs and pond water from a different geographic location (PA, USA), we raised colonized and depleted tadpoles at a single acclimation temperature of 22°C for seven weeks. In a subset of these tadpoles (20 colonized and 20 depleted), we verified that several of our findings from experiment 1, including differences between colonized and depleted tadpoles in terms of gut bacterial community composition and diversity, body size and development, and acute heat tolerance were consistent across both populations of tadpoles. See Appendix B.1.2 Supplementary Discussion, Appendix Tables 6-8, and Appendix Figures 11-12 for these results.

After this verification, 39 of the remaining tadpoles in each microbial colonization treatment group were exposed to an increased temperature of 32°C for ten days, which was then

increased to 34°C for another ten days, and finally increased to 36°C for a final ten days. An additional 39 tadpoles in each group remained at 22°C. We monitored survival of each individual daily for the full 30 days. We found that both temperature and microbial colonization influenced tadpole survival. There was 100% survival in both microbial treatment groups at 22°C, and more variable survival across temperatures in the heat stress treatments (Figure 4.2; discrete-time logistic regression, $z = 4.79$, $p<0.001$). However, colonized animals exhibited significantly greater survival than depleted animals under heat stress conditions (Figure 4.2; discrete-time logistic regression, $z = 2.07$, $p=0.04$). Based on hazard functions from the logistic regression model, the risk of mortality in the depleted group was ~4-5 times greater than in the colonized group at both 32 and 34°C, while there was 100% mortality in both groups at 36°C.

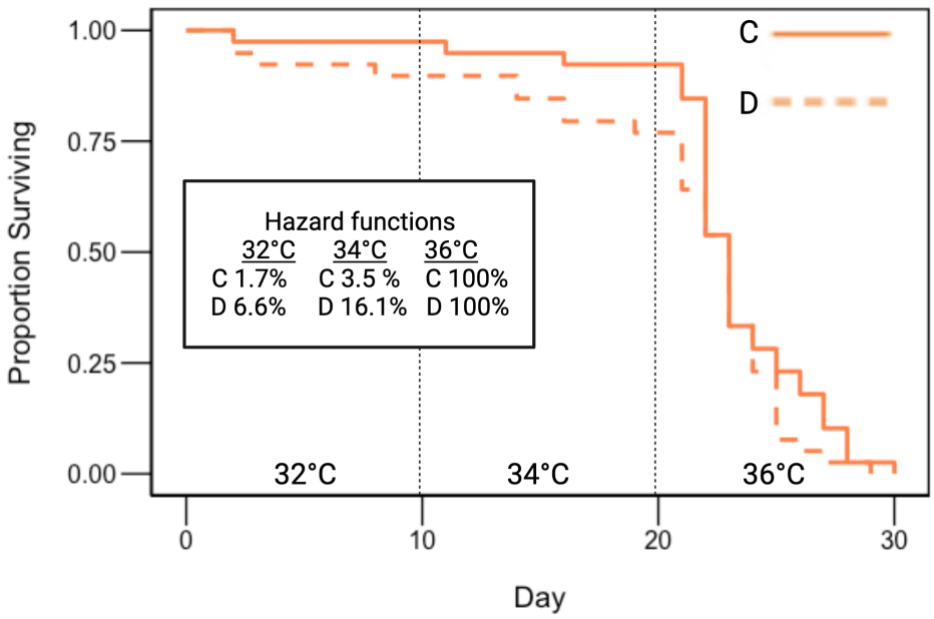

Figure 4.2 Survival of colonized and depleted tadpoles under heat stress conditions.

Labels above the x-axis correspond to the temperature treatment at each timepoint of the experiment. Hazard functions were calculated from a discrete-time logistic regression and represent the risk of mortality of colonized

and depleted tadpoles at each temperature. C= colonized tadpoles and D= depleted tadpoles. A 22°C control group was not included in the analysis because there was 100% survival in this group.

Our results add to a growing body of literature demonstrating the importance of the host-associated microbiome in maintaining host fitness under heat stress. In *Drosophila*, axenic flies exhibit poorer survival compared to conventional flies at lower heat stress temperatures (35-36°C), but mortality is accelerated in both groups at higher heat stress temperatures (37-38°C) (Jaramillo and Castañeda 2021). Taken together, the results of our study along with those of Jaramillo and Castañeda (2021) indicate there may be a window of temperatures at which gut microbial communities can enhance host heat tolerance, but there is an upper limit of temperatures at which animals succumb to heat stress regardless of microbial colonization. Interestingly, the flies used by (Jaramillo and Castañeda 2021) were completely devoid of bacteria, and thus highly artificial, while we were able to obtain similar results using tadpoles that were still colonized with bacterial communities, but had lower diversity and altered composition in these communities. Our results are also similar to those that found an association between losses of gut bacterial diversity and reduced animal survival in lizards (Bestion et al. 2017). However, the lizard study was correlative and thus could not disentangle the direct impact of microbial diversity from other physiological impacts that may affect both bacterial diversity and host survival.

We have thus shown that directly manipulating the host-associated microbiome can impact animal survival under heat stress in an ectothermic vertebrate which was previously unknown. Our results may have implications for animals in the wild because the temperatures we tested are consistent with daily maximum air temperatures in our study area (PA, USA) that are predicted to become increasingly common in future decades (Wolfe et al. 2008). However, most climate change predictions focus on air temperatures, and temperatures in aquatic systems may be more

buffered. Thus, similarly to our first experiment, it will be important to reproduce these findings with more individuals and species, in more wild settings, such as mesocosms, that can accurately replicate natural temperature fluctuations and provide refugia for behavioral thermoregulation. If the microbiome does indeed affect survival under heat stress in wild settings, animals will likely experience declines in fitness in the future because anthropogenic impacts and increasing temperatures are already resulting in widespread disruptions to host-associated microbiota (Trevelline et al. 2019).

4.2.4 Microbiota manipulation altered tadpole locomotion

To determine if the impacts of the microbiome that we observed on tadpole acute thermal tolerance and fitness would also affect the thermal sensitivity of whole-organism performance, we measured locomotor performance of tadpoles across a range of temperatures. Specifically, after the completion of the survival portion of experiment 2, we measured the maximum swimming velocity in 18 colonized and 18 depleted tadpoles from the 22°C control group at six assay temperatures: 5, 15, 22, 26, 30, and 34°C. The velocity measures were then standardized to the body length of each individual to control for body size differences across groups.

As predicted, temperature significantly impacted tadpole locomotor performance (Figure 4.3; GLMM, χ^2= 242.01, $p<0.001$), such that performance tended to increase with increasing temperature. Locomotor performance typically follows the standard thermal performance curve relationship where performance increases steadily with temperature until reaching an optimum and then declines sharply(Huey and Kingsolver 1989, Bennett 1990). However, our data indicates that we did not reach temperatures at which this decline is observed and thus we were unable to calculate the thermal optimum. This may be because we measured up to only 34°C, which is still

several degrees below our observed values of CT_{max} (~40°C), and locomotor performance has been observed to increase until close to the upper lethal limits(Bennett 1990).

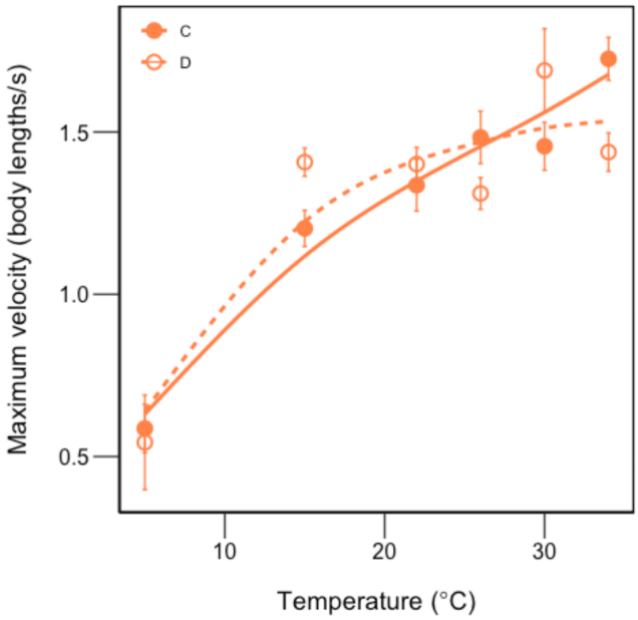

Figure 4.3 Maximum swimming velocity of colonized and depleted tadpoles at six assay temperatures.

C = colonized tadpoles and D = depleted tadpoles. Error bars show means ± s.e.m. and curves were fit to each group using a GAM function.

Nevertheless, we still observed an effect of microbial colonization treatment on the thermal sensitivity of locomotor performance. Specifically, the interaction between temperature and microbial colonization treatment significantly impacted tadpole maximum velocity (Figure 4.3; GLMM, χ^2= 19.89, p=0.02), although we did not observe a direct effect of microbial colonization (GLMM, p>0.05). This interactive effect represents a crossing of colonized and depleted curves at higher assay temperatures (Figure 4.3), such that the performance of depleted tadpoles plateaued at higher temperatures, whereas performance continued to increase with temperature in colonized tadpoles. While the order of assay temperatures could potentially impact

measured performance values due to acclimation to one temperature prior to measurement at subsequent temperatures, the order of assays was kept consistent across treatment groups, and thus differences in performance observed between colonized and depleted tadpoles should represent real differences between the two groups.

Although infection status has been shown to impact the shape of thermal performance curves in bacteria (Padfield et al. 2020), to our knowledge, the host associated-microbial community in an ectothermic animal has not been previously shown to impact the shape of a host's thermal performance curve. Our results suggest that not only does manipulating the microbiome result in reduced thermal tolerance and fitness of tadpoles, but depleted tadpoles may also experience a detriment to locomotor performance at high temperatures compared to colonized tadpoles. In addition to the direct effects of heat on tadpole fitness observed previously, reduced locomotor capabilities may further reduce depleted tadpole fitness under heat stress. Locomotor performance is thought to be strongly linked to animal fitness due to its importance in predator escape, prey capture, and reproduction (Husak et al. 2006, Seebacher and Walter 2012). As tadpoles are mainly herbivores and are non-reproductive, predator escape is likely the most important factor during the larval stage. However, it would be interesting to test if these effects persist in adult frogs after metamorphosis. Despite the possible link between reduced locomotor performance and predator escape in depleted tadpoles, these animals were also larger, which could actually aid them in escaping predators (Semlitsch 1990). Multifactorial experiments that manipulate microbiota, temperature, and predator presence could help identify the most important factors shaping animal performance and predator escape in the wild.

4.2.5 Microbiota manipulation subtly changed phospholipid profiles

After investigating thermal tolerance, fitness, and whole-organismal performance, we tested for differences between colonized and depleted tadpoles in several physiological metrics that may be associated with thermal tolerance to understand how the microbiome may impact thermal physiology at lower levels of biological organization, and what factors might underlie our results. The saturation of phospholipids in cell membranes and resulting changes in membrane fluidity is one biological mechanism that can contribute to thermal acclimation in ectotherms (Gladwell et al. 1976, Angilletta Jr 2009, Gray et al. 2016, Chung et al. 2018, Gangloff and Telemeco 2018, Mineo et al. 2019). Our study species, the green frog tadpole, has been previously shown to modulate phospholipid composition of cell membranes in response to temperature change (Gray et al. 2016). For example, winter acclimated tadpoles have higher proportions of unsaturated fatty acids in their muscles than summer acclimated tadpoles (Gray et al. 2016). To probe if cell membrane composition may contribute to the differences in thermal phenotypes we observed between colonized and depleted tadpoles, we used LC-MS analysis to compare the composition of phospholipids in tail muscle tissue between colonized and depleted tadpoles from our first experiment, from a single acclimation temperature of 22°C.

While we detected differences in the abundances of some phospholipid species between colonized and depleted tadpoles (Appendix Table 9; ANOVA, corrected $p<0.05$), we did not detect differences in traits expected to be associated with host thermal tolerance, such as membrane saturation. A full description of these results is provided in Appendix B.1.3 Supplementary Discussion. In mouse systems, there are differences in tissue and serum lipid profiles, including abundances of specific phospholipids, between germ-free and conventional animals (Velagapudi et al. 2010), which could be due to the role of gut microbiota in phospholipid metabolism (Wang

et al. 2011). Our results demonstrate that in an amphibian system, manipulating the host-associated microbiota can also result in alterations to the abundances of specific phospholipid species. However, these differences are likely not the cause of the altered thermal tolerance we observed across groups.

4.2.6 Microbiota manipulation lowered mitochondrial enzyme activity

The function of enzymes is another important subcellular mechanism impacting thermal performance in ectothermic animals (Angilletta Jr 2009, Gangloff and Telemeco 2018). For example, the loss of enzyme structure and function at high temperatures can underlie thermal tolerance if it occurs at temperatures lower than those impacting function at higher levels of organization (Gangloff and Telemeco 2018). According to the oxygen capacity limitation of thermal tolerance hypothesis (OCLTT), mitochondrial enzymes, which contribute to oxidative capacity, may be particularly important in maintaining thermal tolerance. This hypothesis posits that thermal tolerance limits at extreme temperatures are set by a mismatch in the supply and demand for oxygen, and a resulting loss of aerobic scope, which is the difference between routine and maximal rates of oxygen consumption (Pörtner 2001, Pörtner et al. 2017). Although there is debate and mixed support surrounding this hypothesis (Gräns et al. 2014, Jutfelt et al. 2018), the activity of aerobic mitochondrial enzymes are positively associated with enhanced thermal performance under thermal stress in many ectothermic vertebrate systems including several fish (St-Pierre et al. 1998, McClelland et al. 2006, LeMoine et al. 2008, Grim et al. 2010, Pichaud et al. 2019), alligators (Seebacher et al. 2003), newts (Berner and Bessay 2006), as well as green frog tadpoles (Gray et al. 2016).

To evaluate the importance of mitochondrial enzymes to thermal tolerance in our system, we tested the activity of two enzymes, citrate synthase (CS) and cytochrome c oxidase (CCO), in tail muscle tissue from colonized and depleted tadpoles from our first experiment in the 28°C acclimation group. The tissue mass-specific activity of each enzyme was tested at three assay temperatures, 28°C which corresponds to the animal's acclimation temperature, 34°C which is a temperature at which we observed differential survival between colonized and depleted tadpoles, and 40°C which is close to the CT_{max} values for these animals.

We found that assay temperature had a significant impact on both CS and CCO activities (Figure 4.4a-b; repeated-measures ANOVA, F= 76.14 for CCO and 69.07 for CS, $p<0.001$ for both). For CCO, we found a peak in activity at 34°C, and a significant reduction in activity at 40°C. However, for CS, we found a lower activity at 28°C, with similar levels of activity at 34 and 40°C. The maintenance of CS activity at temperatures close to animal CT_{max} is consistent with the idea that some enzymes are able to maintain function beyond the whole-organism's critical thermal limit (Gangloff and Telemeco 2018). Regardless of the impacts of assay temperature, we found that depleted tadpoles exhibited significantly lower activities of both CCO and CS activity at all temperatures tested, compared to colonized tadpoles, while controlling for differences in body mass (Figure 4.4a-b; repeated-measures ANOVA, F= 5.22 for CCO and 8.75 for CS, $p<0.05$ for both). Specifically, depleted tadpoles had enzyme activities that were on average 15 and 37% lower than those of colonized tadpoles for CCO and CS respectively.

Reductions in mitochondrial enzyme activities in depleted tadpoles could be associated with the lower thermal tolerance we also observed in this group. Activity of CS is positively correlated with mitochondrial density and VO_2 max (Vigelsø et al. 2014), which is the upper limit of aerobic scope, and CCO is thought to be a major regulator of oxidative phosphorylation capacity

and ATP production (Li et al. 2006). In accordance with the OCLTT hypothesis (Pörtner 2001), depletion of microbiota could reduce tadpole aerobic scope, via reductions in mitochondrial enzyme activity and associated VO_2 max, and thus limit thermal tolerance in these animals. There are several possible mechanisms by which microbiome manipulation may impact mitochondrial phenotypes. Gut microbiota can have marked impacts on mitochondrial metabolism mainly through the action of short chain fatty acids (SCFAs) produced by microbial fermentation. For example, there is a 70% drop in oxidative phosphorylation rates in colonocytes of germ-free mice, which can be rescued by the addition of butyrate, a SCFA serving as the primary energy source for cells in the colon (Donohoe et al. 2011). In a congeneric species of the green frog, the American bullfrog, an estimated 20% of daily energy requirements during the tadpole stage come from microbially-produced SCFAs in the gut (Pryor and Bjorndal 2005). Moreover, production of SCFAs and bile acids by gut microbiota can stimulate mitochondrial biogenesis and protect against reactive oxygen species (ROS), which allows for an increase in maximal oxygen consumption by hosts during periods of high demand (Clark and Mach 2017). Future studies measuring levels of SCFAs in the gut contents of colonized and depleted tadpoles are needed to determine if depleted individuals exhibit a loss of mitochondrial enzyme activity due to reductions of important microbial products.

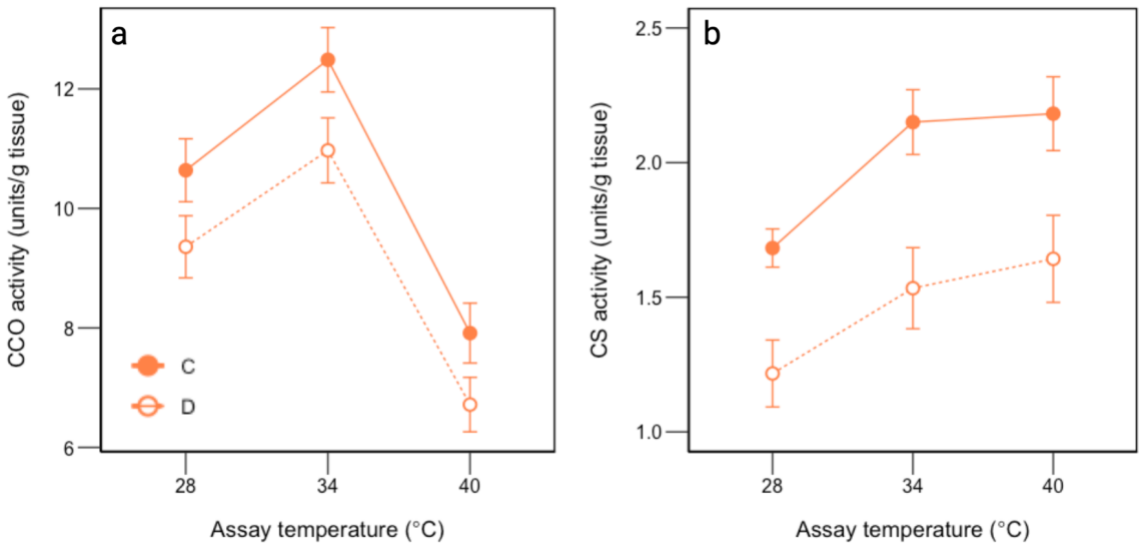

Figure 4.4 Mitochondrial enzyme activities in tail muscle of colonized and depleted tadpoles at three assay temperatures.

(a) cytochrome c oxidase (CCO) activity (b) citrate synthase (CS) activity. C=colonized tadpoles and D=depleted tadpoles. Error bars show means ± s.e.m. On the y-axes, one unit is equal to one μmol of substrate modified per minute.

4.2.7 Temperature, body mass, and microbiota impact metabolic rate

Since our mitochondrial enzyme results suggested that microbial depletion could limit tadpole aerobic scope, and thus thermal tolerance, we explored this idea further by measuring whole-organism resting metabolic rate, which is the lower limit of aerobic scope. A fundamental prediction of the OCLTT hypothesis is that aerobic scope is lost at extreme temperatures when resting metabolic rate meets or exceeds maximal metabolic rate (Pörtner 2001). For heat tolerance, this prediction is based on the idea that routine oxygen consumption tends to increase steeply with rising temperatures, until demand can no longer be met (Van Dijk et al. 1999, Pörtner 2001, Schulte 2015, Payne et al. 2016). While we did not explicitly measure aerobic scope, we tested the impacts

of heat on routine oxygen consumption. Specifically, with the animals remaining in our second experiment from the 22ºC control group, we measured the resting mass-specific metabolic rate (VO_2) of 10 colonized and 10 depleted tadpoles at 22°C and in 10 additional tadpoles per group after a 24-hour exposure to 32°C.

We found that mass-specific metabolic rate was influenced by temperature treatment, such that animals at the warmer temperature had significantly higher resting metabolic rates compared to those at the cooler temperature (Appendix Figure 13; GLM, $F= 67.5$, $p<0.001$). This result was expected, as positive relationships between temperature and metabolism are a generality across ectothermic organisms (Gillooly et al. 2001). However, regardless of temperature treatment, we did not see a direct effect of microbial colonization treatment on tadpole mass-specific metabolic rate (Appendix Figure 13; GLM, $p>0.05$). This result is in contrast to a previous study which found that raising tadpoles in sterile water did alter metabolic rate, although the direction of this effect was dependent on the developmental stage of the animals (Warne et al. 2019).

However, we did find that there was a significant effect of the interaction between microbial colonization state and body mass on mass-specific metabolic rate, that also depended on assay temperature. Specifically, we saw that at the cooler assay temperature (22°C), both colonized and depleted animals exhibited a significant negative relationship between mass-specific VO_2 and body mass (Figure 4.5a; GLM, $F= 5.99$, $p=0.02$). This relationship is consistent with the theory of metabolic scaling (Hoppeler and Weibel 2005), which explains that across a range of animal taxa, larger animals tend to have higher absolute metabolic rates, but lower mass-specific metabolic rates, because mass and whole-animal metabolic rates do not scale linearly. At the higher assay temperature (32°C), there was still a strong negative relationship between mass-specific VO_2 and

body mass in colonized tadpoles, but no relationship between these variables in depleted animals (Figure 4.5b; GLM, mass x treatment interaction, F= 5.48, p=0.03).

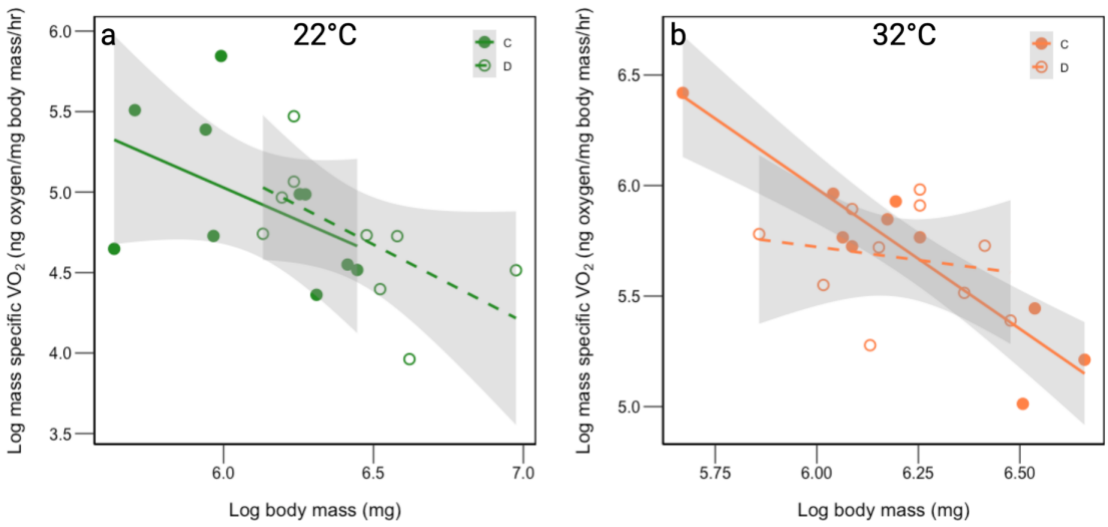

Figure 4.5 Relationship between mass-specific resting metabolic rate and body mass in colonized and depleted tadpoles at two temperatures.

. (a) results from assays performed at 22°C (b) results from assays performed at 32°C. C=colonized tadpoles and D=depleted tadpoles. Solid lines are trendlines created using linear models and gray shading indicates 95% percent confidence intervals. On the y-axes, VO_2 refers to oxygen consumption.

This result suggests that under heat stress, larger depleted tadpoles may have higher mass-specific metabolic rates than colonized individuals of the same size. It is possible this increase in metabolic rate in depleted animals is due to the interaction of several stressors (high temperature and microbial manipulation). Physiological responses to stress can be energy intensive and exposure to several stressors can often result in increases in resting metabolic rates in ectotherms (Hopkins et al. 1999, Sokolova and Lannig 2008, Sokolova 2021). These differences may occur only in larger animals because some studies show that temperature stress can occur more quickly in larger bodied ectotherms (Peralta-Maraver and Rezende 2021). Regardless of the mechanisms,

an increase in oxygen demand in large, depleted animals at high temperatures coupled with a lower oxidative capacity of the mitochondria in these individuals, as evidenced above, can be expected to result in a narrower window of aerobic scope at high temperatures. Ultimately, these effects may result in the reduced heat tolerance we observed in deleted tadpoles as compared to colonized tadpoles. Although this hypothesis is promising, an explicit test of aerobic scope across a range of temperatures in colonized and depleted tadpoles is necessary to support it further.

4.3 Conclusion

We have shown for the first time that experimentally manipulating host-associated microbiota results in reduced host thermal tolerance, impaired fitness under heat stress, and an altered thermal sensitivity of whole-organism performance in a vertebrate ectotherm. Increased temperatures themselves may also disrupt microbial communities, and thus climate warming occurring in wild habitats may further reduce the ability of organisms to cope with increasing temperatures. We additionally present preliminary data assessing the putative mechanisms behind these impacts and suggest a role for microbial depletion-induced changes in mitochondrial performance and metabolism in setting thermal tolerance limits. Future experiments may focus on comparing SCFA production and aerobic scope across groups, as well as assessing any potential global changes in gene expression to further explore the mechanistic basis of these effects.

Importantly, although we have demonstrated an impact of the microbiota on several phenotypes related to host thermal tolerance, we cannot determine what aspects of the microbiome, such as depletion of microbial diversity, altered microbial composition, or loss/gain of individual microbial taxa, is driving our results. Future studies with more focused microbial treatments that

vary degrees of microbial diversity, change community composition without impacting diversity, or manipulate particular microbial strains will help identify important microbial traits that impact host performance. Nevertheless, our results coupled with those from invertebrate studies, suggest gut microbial communities are an important factor impacting the response of animals to environmental temperature. There have been several recent calls to incorporate host-associated microbiota in animal conservation management (Bahrndorff et al. 2016, Jiménez and Sommer 2017, Hauffe and Barelli 2019, Trevelline et al. 2019), and our study demonstrates that relationships with microbes may need to be considered when predicting species' responses to global climate change.

4.4 Methods

Animal research was approved by the University of Pittsburgh IACUC under protocol no. 18062782 and Elmhurst University IACUC under protocol no. FY21-007.

We conducted two separate experiments because we aimed to measure a large suite of traits, which required a similarly large number of tadpoles, and we could not perform all assays simultaneously. For each experiment, to obtain tadpoles for experimentation, we bred single pairs of wild-caught adult green frogs (*Lithobates clamitans*) in the laboratory and raised the subsequently hatched tadpoles in microbially colonized or depleted water conditions. In experiment 1, we used adult frogs and water obtained from Louisiana (USA), and in experiment 2, we used adult frogs and water obtained from Pennsylvania (USA). A full description of frog and water collections, frog spawning, water treatments, and routine tadpole husbandry is available

in Appendix B.2.1-B.2.2 Supplementary Methods. Visual descriptions of each experimental design with sample sizes for each treatment group are available in Appendix Figure 6.

4.4.1 Experiment 1

We distributed 180 tadpoles spawned from frogs collected from Louisiana into 36 1L polypropylene containers (five individuals per container). Colonized tadpoles were assigned to half of the containers which were filled with autoclaved laboratory water (675 mL), seeded with unmanipulated pond water collected from the parent's site of capture (225 mL). Depleted tadpoles were assigned to the other half of the containers which were filled with autoclaved laboratory water (675 mL), seeded with autoclaved pond water (225 mL). We placed 12 containers (six colonized and six depleted) each into three 8.5-gallon polycarbonate water baths. The environmental chamber was set to maintain water temperature at 14°C (the lowest acclimation temperature) and kept at 65% humidity on a 14hr: 10hr light: dark cycle. Water temperature in all three baths was kept at 22°C using aquarium heaters. All tadpoles were allowed to develop at this temperature for four weeks. We then increased temperature in one water bath to 28°C (using aquarium heaters), and decreased temperature in one water bath to 14°C (by removing aquarium heaters). The third water bath remained at 22°C and animals were allowed to acclimate for another three weeks. We additionally collected and froze 1mL samples of our stored pond water weekly, to compare environmental microbial communities in laboratory-stored pond water to samples obtained fresh from the pond during water collections. We monitored water temperature in each tadpole container daily, adjusting aquarium heater temperatures if needed to stay within 1°C of our target treatment temperatures. Ultimately, mean (± s.d.) temperatures across all tanks were 14.4 ± 0.1, 22.0 ± 0.1, and 28.0 ± 0.3 for our cool, moderate, and warm temperature treatments respectively.

4.4.2 Acute thermal tolerance assays and animal dissections

After the acclimation period, we tested tadpole acute thermal tolerance by measuring the critical thermal minimum (CT_{min}) and maximum (CT_{max}) following Hutchison's dynamic method (Lutterschmidt and Hutchison 1997). Specifically, individual tadpoles were placed in 400mL beakers that contained 200mL of room temperature laboratory water. Beakers were then submerged halfway in a refrigerated circulating water bath (Arctic A10B, Fisher Scientific). To measure CT_{min} and CT_{max}, the temperature of the bath was decreased or increased by 0.5°C per minute, respectively. During the assay, tadpoles were monitored continuously and at least once per minute, each tadpole was prodded with a small metal spatula until it no longer responded to the stimulus and could not right itself. At this endpoint, we measured the temperature of the water immediately adjacent to the tadpole using a Traceable Type K thermometer (Fisher Scientific) and recorded this value as the CT_{min} or CT_{max} depending on the assay being conducted. The tadpoles were then placed into another beaker of room temperature water for recovery and monitored for at least one hour prior to euthanasia. All animals successfully recovered. Individual tadpoles underwent only one trial (CT_{min} or CT_{max}) and we conducted 11 trials for both CT_{max} and CT_{min}, during which one animal from each of the six treatment groups was measured. The remaining individuals from the experiment were euthanized and dissected without collecting thermal tolerance data.

Each individual was euthanized by immersion in buffered MS-222 (10g/L). Following euthanasia, we recorded tadpole body mass (g), and placed each individual under a Leica S9i dissecting microscope fitted with a camera attachment to photograph each tadpole and record their Gosner stage. Photographs were later used to measure tadpole body length and width (mm), excluding the tail. As a proxy for developmental stress (Swaddle 2003), we also measured facial

symmetry by measuring the distance (mm) from the center of each eye to the tip of the nose and computing the absolute value, subtracted from 1, of the difference between these two measures. All images were analyzed using Image J v1.52q. We then dissected each individual and removed the entire gastrointestinal tract for gut microbiome and flow cytometry analyses, and the entire tail for mitochondrial enzyme assays and phospholipid composition analyses. Gut samples were immediately frozen at -80°C and tail samples were flushed in N_2 gas, frozen under liquid N_2, and then stored at -80°C. Dissection instruments were wiped with 70% EtOH and flame sterilized between each individual. Our final sample size for morphometrics and tissue collections was 27 individuals per each of the six treatment groups. The 27 individuals per group included 11 tadpoles assayed for CT_{max}, 11 tadpoles assayed for CT_{min}, and 5 tadpoles that did not undergo any thermal tolerance assay.

To assess the effect of acclimation temperature, microbial colonization treatment, and their interaction on tadpole CT_{min}, CT_{max}, body mass, body width, body length, Gosner stage, and facial symmetry, we used generalized linear mixed models (GLMMs) including tadpole tank as a random effect in all models. Body length was included as a covariate in the facial symmetry model, and body mass and Gosner stage were included as covariates in the CT_{min} and CT_{max} models. All models were constructed in R v3.4.3 (Team 2019) using the lme4 package (Bates et al. 2018).

4.4.3 Flow cytometry

We used flow cytometry to calculate the absolute bacterial abundance in gut samples from experiment 1. Whole gut samples were originally minced into smaller sections, and these sections were then divided randomly into two samples. One gut sample per individual was used for microbiome analyses (see below), and the other was used for flow cytometry. We performed flow

cytometry analysis on 9-11 samples per treatment group, except for the 14°C colonized group. In this group, we only retained four samples as most were too small to be divided and we prioritized microbiome analyses. For each sample, we created bacterial cell suspensions from gut contents and sterile PBS and added SYBR Green I dye (Sigma Aldrich) to stain bacterial cells in the sample and CountBright Absolute Counting Beads (Thermo Fisher Scientific) to serve as an internal standard reference. We then counted bacterial cells in each sample on an Attune NxT Flow Cytometer (Thermo Fisher Scientific). See Appendix B.2.3 Supplementary Methods for a detailed description of the flow cytometry sample prep and protocol, and Appendix Figure 14 for a visualization of our gating procedure.

To compare the absolute abundance of bacterial cells across treatment groups, we used a log transformation to normalize the cell counts, and used a linear mixed effect model in the nlme (Pinheiro et al. 2017) package in R with acclimation temperature, microbial colonization treatment, and their interaction as fixed effects, and the date of measure as a random effect. We removed one outlier from the 14°C colonized group from analysis. Throughout, outliers were identified as data points with disproportionate effects on our results that were beyond 1.5x the IQR added to the third quartile or subtracted from the first quartile.

4.4.4 Phospholipids analyses

We identified the phospholipids present in tail muscle tissue of eight colonized and eight depleted tadpoles from the 22°C group only from experiment 1. Tail tissue samples were sent to the University of Pittsburgh's Health Sciences Metabolomics and Lipidomics core facility for LC-HRMS analysis to identify the phospholipids present in each sample. A full description of the facility's LC-HRMS protocol is available in Appendix B.2.4 Supplementary Methods. Using the

data from this analysis, each phospholipid species present in each sample was identified by class (Phosphatidylcholines (PC), Phosphatidylethanolamines (PE), Phosphatidylglycerols (PG), Phosphatidylinositols (PI), or Phosphatidylserines (PS)) and species were grouped together based on their class, their total carbon chain length, and total number of double bonds (combined for both fatty acid tails). We calculated the molar percentage of each phospholipid class, and each phospholipid species out of the total for each sample. We additionally calculated the combined molar percentages of all saturated fatty acids (%SFA; zero double bonds), monounsaturated fatty acids (%MUFA; single double bond), and polyunsaturated fatty acids (%PUFA; >1 double bond) for each sample. We then calculated the unsaturation and peroxidation indices for each sample as described by Hulbert et al. (2007). The unsaturation index computes the number of double bonds per 100 fatty acids, and the peroxidation index determines the susceptibility of membranes to peroxidation. Lastly, we calculated the average chain length for each sample by dividing the molar percentage of each phospholipid species in the sample by its chain length, summing these values, and dividing by 100.

To identify differences between colonized and depleted tadpoles in the molar percentages of individual phospholipid classes or species, we used the response screening function in JMP v14.1 which performs ANOVAs across groups with the abundance of each phospholipid class or species as the response variable and subsequently corrects all p-values using the Benjamini-Hochberg False Discovery Rate (BH FDR) method. For phospholipid species, we included only those that were present at greater than 0.1% relative abundance in at least one treatment group. To compare phospholipid compositions across microbial colonization groups in multivariate space, we created a Bray-Curtis distance matrix using the vegdist function in the vegan package (Oksanen et al. 2013) in R, based off the molar percentage data for each phospholipid species, excluding

species present in abundances of less than 0.1% in both treatment groups. We then used the adonis2 function to perform a PERMANOVA on the distance matrix, with 999 permutations. Lastly, after confirming normality with Shapiro-Wilk tests, we used two-sided t-tests in JMP to compare %SFA, %MUFA, %PUFA, unsaturation indices, peroxidation indices, and average chain lengths, between the two colonization groups.

4.4.5 Mitochondrial enzyme assays

We measured the activities of cytochrome c oxidase (CCO) and citrate synthase (CS) isolated from the tail muscle of eight depleted and eight colonized samples from the 28ºC acclimation group from experiment 1. Most of our colonized tail samples were too small to be analyzed individually, and in these cases, we pooled tail tissues from multiple individuals together in a single sample. Specifically, seven out of eight colonized samples were pooled, and of the seven pooled samples, five samples used tissues from two individuals, and two samples used tissues from three individuals. Activities of each enzyme for each sample were measured at three assay temperatures: 28, 34, and 40ºC following previously published protocols (Gray et al. 2016). A full description of the assay protocol for each enzyme is available in Appendix B.2.5 Supplementary Methods.

To determine the effects of both assay temperature and microbial colonization treatment on CS and CCO activity, we used repeated-measures ANOVAs in R, controlling for the effects of individual sample. In both models, tadpole body mass was included as a covariate, and in the case of pooled samples, the average body mass of the pooled individuals was used instead. Here, we are working under the "biological averaging assumption" which hypothesizes that the measured value of a pooled sample is comparable to an arithmetic average of levels in the individual samples

making up the pool (Mary-Huard et al. 2007). In our CS model, we removed one depleted outlier sample from analysis.

4.4.6 Experiment 2

We distributed 200 tadpoles spawned from frogs collected from Pennsylvania individually into 12oz polypropylene containers. Half of the tadpoles were assigned to a colonized treatment, and their containers were filled with autoclaved laboratory water (225mL) and seeded with unmanipulated pond water from the parent's site of capture (75mL). The other half of the tadpoles were assigned to a depleted treatment and their containers were filled with autoclaved laboratory water (225mL) and seeded with autoclaved pond water (75 mL). The environmental chamber was set to 22°C and kept at 65% humidity on a 14hr: 10hr light: dark cycle.

4.4.7 Acute heat tolerance and animal dissections

After seven weeks of development, we removed 20 tadpoles per treatment from the experiment. In 17 colonized and 17 depleted tadpoles, we assayed CT_{max}, following the protocol described above, to verify repeatability of our heat tolerance results from experiment 1 in a second population of tadpoles. We then euthanized all tadpoles in buffered MS-222 (10g/L), and recorded their mass (g), body length excluding tail (mm) using digital calipers, and Gosner stage. We then dissected each animal and removed the entire gastrointestinal tract, to be used later for microbiome analyses. Dissection instruments were wiped with 70% EtOH and flame sterilized between each individual. All gut samples were stored at -80°C prior to processing. We used generalized linear

models (GLMs) in R to compare body mass, body length, Gosner stage, and CT_{max} between colonized and depleted tadpoles.

4.4.8 Survival under heat stress

The remaining tadpoles developed for an additional week (eight weeks total), and we then moved 78 of these tadpoles (39 from each colonization treatment) into a separate animal chamber kept at 32°C, 65% humidity, and on a 14hr: 10hr light: dark cycle. An additional 78 tadpoles (39 from each colonization treatment) remained in the previous animal chamber at 22°C. After 10 days, we increased the temperature of the heat treatment to 34°C for a duration of 10 days, and to 36°C for a final 10 days. We monitored survival of each individual each day. In the 32-36°C temperature treatment group we observed 100% mortality in both treatment groups by the end of the 30 days. In the 22°C group, we did not observe any mortality in either group over the duration of the 30 days and these animals were then used for additional experimentation (see below).

To compare survival of colonized and depleted tadpoles under heat stress, we performed a discrete-time logistic regression analysis in R using the base glm function with a binomial distribution, including temperature (synonymous with each 10-day time period) and microbial colonization treatment as predictor variables. We computed the hazard function of mortality for both colonization groups at each temperature from our model using the formula established by Singer and Willett (1993). Because no mortality was observed, animals from the 22°C group were not included in the analysis.

4.4.9 Resting metabolic rate

Of the 78 remaining tadpoles in the 22°C group after the survival experiment, we used 40 to assay resting metabolic rate. We measured metabolic rate in 20 tadpoles (10 colonized and 10 depleted) at 22°C. The other 20 individuals (10 colonized and 10 depleted) were placed at 32°C for 24 hours, prior to measuring metabolic rate at 32°C. Resting metabolic rate was measured via oxygen consumption (VO_2) using an intermittent-flow respirometry system (Q-box mini-AQUA aquatic respirometer, Qubit Systems). A detailed description of the respirometry protocol is described in Appendix B.2.6 Supplementary Methods.

We ultimately analyzed one minimum value of VO_2 per animal, and these values were normalized using a log transformation prior to analyses. We used GLMs in R to test for significant effects of assay temperature, microbial colonization treatment, body mass, and interactions of these variables on mass-specific VO_2. Gosner stage was included as an additional covariate in the model. Two depleted tadpoles (one from 22°C, and one from 32°C) were removed from analysis because they did not survive the assay.

4.4.10 Locomotor performance

Of the remaining animals from the 22˚C treatment group of the survival experiment, 36 individuals (18 colonized and 18 depleted) were shipped overnight to Elmhurst University where the thermal sensitivity of locomotor performance was measured. To compare locomotor performance across temperatures, we measured the maximum swimming velocity of colonized and depleted tadpoles at 5, 15, 22, 26, 30 and 34˚C. Prior to locomotor performance assays, the tadpoles were brought from their housing temperature (22˚C) to the appropriate assay temperature at a rate

of 5˚C per hour in a temperature-controlled biological incubator (model I-41 VL, Percival). Tadpoles remained at the assay temperature for 30 minutes prior to assays. The order of assay temperatures was 22, 5, 30, 15, 34, and 26˚C, and velocity was measured at one assay temperature per day with 24 hours between assays at different temperatures. The specific protocol used to measure locomotor performance is available in Appendix B.2.7 Supplementary Methods.

Tadpoles that failed to swim at a particular assay temperature were omitted from our analysis at that temperature (n=1 individual from the colonized group at 5˚C, n=1 individual from the depleted group at 5˚C, n=1 individual from the depleted group at 22˚C, n=1 individual from the colonized group at 26˚C, n=1 individual from the colonized group at 30˚C and n=1 individual from the colonized group at 34˚C). We used GLMMs in the lme4 package in R to determine the effects of assay temperature, microbial colonization treatment, and their interaction on tadpole maximum swimming velocity and included individual as a random effect. Body length was included as an additional covariate in the model. To visualize the relationship between temperature and performance, we fit curves to our data using generalized additive models (GAMs) for both colonized and depleted tadpoles. For GAMs, a k value of 6 was used.

4.4.11 Microbiome analyses

To compare bacterial diversity and composition across samples, we used high-throughput amplicon sequencing of the 16S rRNA gene to create bacterial inventories for gut (N = 25-27 per group) and water (N = 7 total) samples collected from experiment 1 and gut samples (N = 20 per group) collected from experiment 2. Details regarding DNA extraction, library preparation, PCR, Illumina sequencing, and raw sequence processing is available in Appendix B.2.8 Supplementary Methods.

For experiment 1, we first compared the composition of bacterial communities between gut samples, fresh pond water samples, and stored pond water samples using a PERMANOVA with 999 permutations based on the Bray-Curtis distance between samples in QIIME2. Next, focusing on gut samples only, we examined the effects of both acclimation temperature, microbial colonization treatment, or their interaction on tadpole gut bacterial alpha diversity. We used GLMMs in the lme4 package in R, including tank as a random effect, to compare differences in Shannon diversity, Faith's phylogenetic diversity, Pielou's evenness, and the number of observed ASVs. To determine differences in community composition of the microbiome across treatment groups, we used the adonis2 function in the vegan package to perform PERMANOVAs, with 999 permutations, using Bray-Curtis, Unweighted UniFrac, and Weighted UniFrac distance matrices based on both microbial colonization treatment, acclimation temperature, and their interaction. We additionally controlled for the effect of tank using the strata function. We ran additional alpha diversity GLMM and beta diversity PERMANOVA models including an assay treatment variable which described if an individual underwent the CT_{min} assay, CT_{max} assay, or no thermal tolerance assay prior to dissection. This variable did not significantly impact microbiome alpha or beta diversity and did not change the effects of microbial colonization treatment and acclimation temperature on the microbiome, and thus was excluded from the formal analysis. We used the same three distance matrices to compare intraindividual variability in the bacterial community across acclimation temperature and microbial colonization treatment groups using PERMDISP tests with the betadisper function.

For experiment 2, the four metrics of gut bacterial alpha diversity were compared between colonized and depleted tadpoles using Kruskal-Wallis tests in QIIME2. Bacterial community

composition was compared between colonized and depleted tadpoles using the three calculated distance matrices with PERMANOVAs, using 999 permutations, in QIIME2.

For both experiments, we used the MaAsLin2 package (Mallick et al. 2021) in R to identify bacterial phyla and genera in gut bacterial communities with relative abundances that were significantly different across treatment groups. For experiment 1, microbial colonization treatment and acclimation temperature were used as fixed effects. For experiment 2, microbial colonization treatment was used as the fixed effect. MaAsLin2 was run using an arcsin-square root transformation of the relative abundance data, and p-values were corrected using the BH FDR method.

4.5 Acknowledgements

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5.0 The microbiome buffers tadpole hosts from the deleterious effects of heat stress: a hologenomic approach to understand host-microbe interactions under warming conditions

The contents of this chapter are adapted from the following manuscript:

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5.1 Introduction

Increasing global temperatures is one of the largest threats facing animal populations in current times, and the deleterious effects of heat stress have begun to alter organismal physiology (Dillon et al. 2010) and behavior (Beever et al. 2017), ultimately with negative consequences for animal fecundity and survival (Moreno and Møller 2011). For populations to persist, animals must evolve rapidly to these new conditions or employ phenotypic plasticity if temperatures change too quickly for evolution to occur (Seebacher et al. 2015). Plasticity in organismal gene expression can lead to coordinated and conserved responses that allow animals to better tolerate extreme heat events. For example, heat induces the upregulation of several classes of heat shock proteins (HSPs) which perform a variety of functions to prevent cellular damage from heat, including clearing misfolded proteins, repairing DNA damage, and inducing transcription of other stress response pathways (Richter et al. 2010). These responses ultimately act to increase animal survivorship under thermal stress (Harada and Burton 2019). Exposure to heat may lead to variable changes in gene expression, from relatively few genes to large whole-transcriptome wide shifts in expression (Logan and Cox 2020). However, we lack an understanding of how differences in gene expression

may lead to changes in whole-animal performance or fitness. There is not always concordance between the magnitude of gene expression plasticity in response to heat and the degree of change in whole-organism heat tolerance (Logan and Cox 2020), indicating there may be other factors that play a role in regulating animal responses to heat.

In addition to host responses to the environment, all animals harbor dense and diverse communities of microorganisms (bacteria, fungi, archaea etc.), collectively termed the microbiome, that influence their host's physiology, resulting in "emergent phenotypes" (Lynch and Hsiao 2019) that may ultimately enhance phenotypic plasticity and shape how organisms respond to environmental challenges (Alberdi et al. 2016). For example, in mammalian herbivores, gut microbial communities can help hosts break down difficult-to-digest plant material and facilitate feeding on toxic food sources through the breakdown of plant secondary compounds (Kohl et al. 2014b). Gut microbial communities of several invasive amphibian species have been shown to be exceptionally plastic in response to environmental change and may help facilitate host adaptation to novel environments (Fontaine and Kohl 2020a, Wagener et al. 2020, Zhou et al. 2020). Additionally, in hibernating mammals, exposure to cold can change the gut microbiota, and these changes result in increased energy acquisition, cold tolerance of the host, and nutritional balance during hibernation (Chevalier et al. 2015, Sommer et al. 2016, Regan et al. 2022). By altering host phenotypes in these ways, the microbiome can ultimately shape host responses to selective pressures, and the evolutionary trajectories of populations (Henry et al. 2021).

In ectothermic animals, a growing body of literature has demonstrated that host colonization with individual microbial symbionts as well as more complex microbial communities can have protective effects when animals face heat stress (Hector et al. 2022). For example, in invertebrate systems, individual microbial symbionts can increase host growth rates (Russell and

Moran 2006, Harmon et al. 2009), fecundity (Montllor et al. 2002, Hoang et al. 2019), and survival under heat stress conditions (Berkelmans and Van Oppen 2006, Russell and Moran 2006, Brumin et al. 2011, Nakagawa et al. 2016), as well as their behavioral preference for warm environments (Porras et al. 2020) and the upper lethal limits of thermal tolerance (critical thermal maximum, CT_{max}) (Porras et al. 2020). These effects can ultimately facilitate host adaptation to thermally stressful environments (Hoang et al. 2021). In invertebrates with more complex communities of associated microbes, gut microbial transplants from flies reared at cooler temperatures can reduce the CT_{max} of recipient flies (Moghadam et al. 2018), and axenic flies exhibit poorer survival than conventionalized flies under heat stress (Jaramillo and Castañeda 2021). Mechanistically, the protective effects of microbes for invertebrate hosts under heat stress involve the colonization-mediated stimulation of host gene upregulation of oxidative stress response pathways (Nakagawa et al. 2016), cytoskeleton genes that protect cellular integrity (Brumin et al. 2011), and HSPs (Porras et al. 2020). Additionally, bacterially derived metabolites and HSPs may also act to increase their host's heat tolerance (Dunbar et al. 2007, Burke et al. 2010).

In a vertebrate system, we previously demonstrated a direct link between the gut microbiota of an ectothermic vertebrate and the host's heat tolerance (Fontaine et al. 2022) (throughout, this citation refers to Chapter 4 of this dissertation) . Specifically, we raised tadpoles of the green frog (*Lithobates clamitans*) in two conditions which differed in their environmental microbial exposure. Tadpoles we call "colonized" were raised in natural pond water and had gut microbiomes that were significantly more diverse and differed in composition than "depleted" tadpoles, which were raised in autoclaved pond water to reduce the microbial diversity of this environmental source for inoculation (Fontaine et al. 2022). Compared to colonized tadpoles, depleted tadpoles had lower acute heat tolerance measured via CT_{max}, poorer locomotor performance at high temperatures, and

ultimately exhibited lower survival under heat stress conditions (32-34ºC) (Fontaine et al. 2022), indicating that commensal microbial communities can also influence host heat tolerance in vertebrate hosts. These results were consistent with previous correlative studies in lizards showing associations between gut microbiota composition and host CT_{max} (Moeller et al. 2020), and microbiota diversity with animal survival under warming conditions (Bestion et al. 2017). We also found that depleted tadpoles had lower activities of aerobic mitochondrial enzymes (cytochrome c oxidase and citrate synthase) and reduced metabolic rates at high temperatures in large-bodied individuals (Fontaine et al. 2022), suggesting a mismatch in supply and demand for oxygen under heat stress could limit heat tolerance in depleted tadpoles, which would support the oxygen capacity limitation thermal tolerance hypothesis (OCLTT) (Pörtner et al. 2017). Despite this possibility, the mechanisms governing the relationship between ectothermic vertebrate gut microbial communities and host heat tolerance are far less resolved than those among invertebrate hosts.

Here, we use a "hologenomics" approach to identify potential mechanistic pathways that may underlie the relationships we've observed between tadpole heat tolerance and gut microbial communities. Specifically, we compare host transcriptomic and gut microbial metagenomic profiles across colonized and depleted tadpoles under non-stressful or heat stress temperatures to identify how patterns of host and microbial physiological functions change in response to heat in animals with differing microbial exposure histories. Such hologenomic approaches have been called for to answer outstanding questions regarding the evolution of relationships between hosts and microbes (Alberdi et al. 2021), and have been used previously to understand the influence of microbes on various host phenotypes including blood-feeding (Mendoza et al. 2018) and the degradation of complex plant material (Bredon et al. 2018). Here, we expand on this approach by

assaying host gene expression and microbial metagenomics under variable conditions with the major goal of identifying host and microbial pathways that differ in their responses to heat between colonized and depleted tadpoles, as these differences may underlie our observed reductions in heat tolerance in the latter group. We are additionally interested in identifying host and microbial pathways that differ based on temperature alone (regardless of colonization effects) and microbial colonization (regardless of temperature effects). A recent review canvassing the literature for studies examining whole-transcriptome responses to heat in ectotherms found none conducted in amphibians (Logan and Cox 2020), and while some studies have examined the impact of temperature on the predicted gut metagenomic profiles of ectotherms (Ziegler et al. 2017, Kokou et al. 2018, Fontaine and Kohl 2020a), none to our knowledge have utilized true shotgun metagenomic sequencing in an experimental setting (see Littman et al. (2011) for a field-based approach). Further, while we previously demonstrated taxonomic differences in the microbiome between colonized and depleted tadpoles (Fontaine et al. 2022), this study provides an opportunity to identify potential microbial functional differences between these groups.

5.2 Methods

5.2.1 Frog spawning and tadpole development

The tadpoles used in these experiments were generated in parallel with animals used for previously published work, where detailed rearing and development methods can be found (Fontaine et al. 2022). In brief, mating pairs of adult green frogs (*L. clamitans*) were collected from a pond at Pymatuning Laboratory of Ecology (Linesville, PA, USA) in July 2020 and were

transported back to the laboratory at the University of Pittsburgh. Additionally, 100L of pond water was collected monthly from the same pond for the duration of the experiment and stored in the laboratory at 4ºC after filtering through a 500µm sieve. In the laboratory, we induced spawning in a single pair of adult frogs using the method described by Trudeau et al. (2010) and modified as described by Fontaine et al. (2022). The resulting embryos were maintained in autoclaved laboratory water until they developed to Gosner stage 25 (Gosner 1960). At this stage, tadpoles were distributed evenly into two groups with differing environmental microbial community exposure. Colonized tadpoles were maintained individually in 12 oz polypropylene containers filled with 75mL (25% volume) unmanipulated pond water from their parent's site of capture and 225mL (75% volume) autoclaved laboratory water. Depleted tadpoles were maintained individually in 12 oz polypropylene containers filled with 75mL (25% volume) autoclaved pond water from their parent's site of capture and 225mL (75% volume) autoclaved laboratory water. The environmental chamber was set to 22ºC, 65% humidity, and a 14h:10h light:dark cycle. Tadpoles were transferred to fresh water of the appropriate treatment weekly and fed a diet of autoclaved rabbit chow suspended in autoclaved agar and supplemented with a commercial pet vitamin *ad libitum*. Tadpoles developed in these conditions for nine weeks.

5.2.2 Heat stress and sample collection

After tadpole development, ten colonized and ten depleted tadpoles were transferred to a second environmental chamber set to 32ºC with the same humidity and light cycle, while all other tadpoles remained at 22ºC. Tadpoles were exposed to this warmer temperature for a period of 24 hours. After this period, ten colonized and ten depleted tadpoles from both the 32ºC and 22ºC treatments were removed from the experiment and euthanized by double pithing. Chemical

euthanasia agents (e.g. MS-222) were not used because they can alter animal gene expression (Yu et al. 2018). We recorded each tadpole's body mass (g), body length (mm), and Gosner stage. Subsequently, each animal was dissected to remove the entire gastrointestinal tract in its typical coiled structure so that our data represent an integration of all gut sections, rather than focusing on a particular region. We chose to use the gut because it is the site of highest microbial density in vertebrates (Sender et al. 2016), and the gut microbiota are important for tadpole physiological function (Pryor and Bjorndal 2005). Further, heat can induces changes in gene expression in the gut of amphibians (Near et al. 1990), and other vertebrates (Pearce et al. 2015, He et al. 2018). We split each coiled gut sample into two equal sections, with effort to distribute the longitudinal portions of the gut across our tubes. Samples used for transcriptomics were placed in RNAlater for 24 hours at 4ºC, and then frozen until processing at -80ºC after the removal of RNAlater. Samples used for metagenomics were flash frozen with liquid nitrogen and then frozen at -80ºC until processing. All dissection instruments were flame sterilized between individuals. Overall, we obtained samples from 40 individuals which included ten animals per treatment group (crossed design with two microbial colonization treatments and two temperature treatments).

5.2.3 Host transcriptomics sample prep and sequencing

From the samples collected for transcriptomics above, we chose five samples from each treatment group to be used for RNA sequencing. Our previous work demonstrated differences in body size and development between colonized and depleted tadpoles, though observed differences in temperature tolerance were independent of these effects (Fontaine et al. 2022), Regardless, to avoid potential confounding effects of size or development on gene expression, we selected individuals for this experiment that were of similar size and developmental stage (Appendix Table

10) (Row et al. 2016). From these individuals, we extracted RNA from gut samples using the RNeasy Plus Mini Kit (QIAGEN) following the manufacturer's protocol. At step 3 of the protocol, we used 350µL of buffer RLT and a TissueLyser II (QIAGEN) set to 30 Hz for one minute for disruption and homogenization. We quantified the concentration of RNA in each sample using a plate reader and stored samples at -20ºC until sequencing. Extracted RNA samples were sent to the University of Pittsburgh Genomics Research Core (Pittsburgh, PA, USA) for library preparation. Briefly, mRNA was isolated, cDNA libraries constructed, and unique sample indexes added using the TruSeq RNA Library Prep Kit (Illumina) following the manufacturer's protocol. Libraries were pooled within samples and sent to the University of Pittsburgh Medical Center Genome Center (Pittsburgh, PA, USA) for sequencing on the Illumina NovaSeq 6000 platform using an S2 flow cell to generate 2x101bp paired-end reads. Raw RNA-seq reads were trimmed to remove indexes and for quality using Trim Galore (Krueger 2015). Reads were mapped to an available reference draft genome of the green frog's congener, the American bullfrog (*L. catesbeiana*, GenBank accession number GCA_002284835.2) (Hammond et al. 2017), as well as a reference genome of the African clawed frog, (*Xenopus laevis*, GenBank accession number GCA_017654675.1), using BWA (Li and Durbin 2009). Using the *X. laevis* genome, less than 60% of reads per sample were successfully aligned to the reference genome. Using the bullfrog genome, greater than 97% of reads were successfully aligned to the reference genome for each sample and thus, this alignment was used for all downstream analyses. Next, we used StringTie (Pertea et al. 2015) to generate a matrix of read counts across samples for each transcript. We then filtered the count data to remove any transcripts from analysis that were present at an abundance of less than two counts per million or in less than three samples. This reduced the final data set

from 410,385,578 reads distributed across 22,238 transcripts to 392,568,234 reads distributed across 8,477 transcripts.

5.2.4 Microbiome metagenomics sample prep and sequencing

To prepare samples for metagenomics, we first separated gut contents from gut tissues for each sample. Specifically, we placed each gut sample onto a small weigh boat under a dissecting microscope. Using sterile inoculating loops, we divided thawed gut samples into small sections, and scraped the gut contents from the tissue, discarding the tissue. We then flushed the weigh boat with 800µl reagent CD1 from the QIAmp PowerFecal Pro DNA Kit (QIAGEN), transferred the gut contents and reagent mixture to a bead tube, and extracted DNA from the samples following the manufacturer's protocol. We quantified the concentration of DNA in each sample using a plate reader and stored samples at -20ºC until sequencing. If gut samples did not contain a sufficient amount of gut contents for extraction (some guts were nearly empty), the samples were not used. Of the samples we successfully extracted, we selected the five from each treatment group with the highest DNA concentration to be used for metagenomic analysis. Some of these samples were from the same individuals from which we obtained transcriptomic samples (Appendix Table 10), however, we obtained too few paired samples to integrate these data in downstream analyses. The selected samples were sent to Diversigen (New Brighton, MN, USA) for shallow shotgun metagenomic sequencing using their BoosterShot method. Briefly, libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina) and sequenced on the Illumina NovaSeq 6000 platform generating 1x100bp single-end reads. Reads were trimmed and filtered for low quality or length, and using BURST (Al-Ghalith and Knights 2020), were then aligned to Diversigen's Venti database, which contains all RefSeq bacterial genomes and an annotated

bacterial KEGG database (Kanehisa and Goto 2000), at 97% similarity. Count tables of KEGG orthologs and modules were then created for each sample.

5.2.5 Statistical analyses

5.2.5.1 Host transcriptomics

To compare differences in overall gene expression profiles across the four treatment groups, we created a Bray Curtis distance matrix in the R Vegan package (Dixon 2003) from our filtered count data of all expressed transcripts (8,477 total). We visualized these data using a Principal Coordinates Analysis plot and assessed the statistical significance of the effect of microbial colonization, temperature, or their interaction on tadpole gut gene expression with a PERMANOVA using the adonis2 function and 999 permutations. Next, we identified genes that were differentially expressed based on temperature only (warm vs. cool across all tadpoles), colonization only (colonized vs. depleted across all tadpoles), or based on temperature within colonized or depleted tadpoles separately (warm vs. cool within each colonization group).

For all comparisons, to quantify differential expression of genes, we used the R package edgeR (Robinson et al. 2010) which uses negative binomial generalized linear models and normalized read counts to compare across groups. Significance was assessed after p-value correction using the Benjamini-Hochberg false discovery rate (BH FDR). We used Venn diagrams to depict the number of genes that were shared between colonized and depleted tadpoles in response to the heat treatment (upregulated or down regulated). To functionally annotate all differentially expressed genes, for each transcript identified as differentially expressed in any of the comparisons described above, we used Blast2GO (version 6.0.3) (Conesa et al. 2005) to

BLAST the sequence against the NCBI database and identify the associated GO functions using the SWISSPROT database with the blastx-fast function (Blast E-value = .001).

Next, we used enrichment analyses to identify GO terms that were enriched among differentially expressed genes across our comparisons of interest. Because there were very few differentially expressed genes between colonized and depleted tadpoles (without considering temperature), we did not perform an enrichment analysis for this comparison. We created lists of genes that were upregulated or downregulated in response to heat in all tadpoles, colonized tadpoles only, and depleted tadpoles only. We then used Fisher's exact tests with BH FDR corrected p-values in Blast2GO to identify GO terms that were differentially enriched across groups. To identify pathways enriched in response to heat across all tadpoles, we compared gene lists (upregulated or downregulated in response to heat) to all genes in the dataset. To identify pathways differentially enriched between colonized or depleted tadpoles in response to heat, we compared upregulated and downregulated gene lists of colonized and depleted tadpoles to one another. For any significant comparisons, we used the "Reduce to Most Specific" within Blast2GO, which removes more general terms and retains the most specific statistically significant GO terms.

5.2.5.2 Microbiome metagenomics

We removed one depleted sample from the warm temperature group from all analyses due to exceptionally low numbers of KEGG orthologs (KOs) identified in the sample (n KOs = 33 and mean s.e.m. of KOs for all other samples = 10,801 ± 3,709). To identify overall differences in microbial community function across groups, we created a Bray Curtis distance matrix based on KO counts for each sample in R in the vegan package. We then used a PERMANOVA to statistically compare the effects of temperature, microbial colonization treatment, and their

interaction on microbial function using the adonis2 function with 999 permutations. We visualized these data using a Principal Coordinates Analysis plot. Next, KEGG orthologs were collapsed into KEGG modules, and we used the program STAMP (Parks et al. 2014) to identify differentially abundant modules across temperature groups, colonization groups, and temperature groups within colonization groups (warm vs. cool in colonized or depleted tadpoles separately). Within STAMP, we used the two-group analysis option and compared KEGG module abundances between groups using a Welch's t-test and BH FDR corrected p-values. For all comparisons we chose the option to use unclassified reads only when calculating frequency profiles.

5.3 Results

Overall, we observed differences in the host gut transcriptome and gut microbial metagenome of tadpoles based on both temperature and microbial colonization treatment (Figure 5.1a-b). Specifically, temperature and the interaction between temperature and microbial colonization treatment impacted the host transcriptome (Figure 5.1a, PERMANOVA, temperature $F= 5.37$, temperature x colonization $F= 3.30$, $p<0.01$ for both). Temperature, microbial colonization, and their interaction impacted the microbial metagenome (Figure 5.1b, PERMANOVA, temperature $F= 3.70$, colonization $F=2.61$, temperature x colonization $F= 2.26$, $p<0.05$ for all). We explore the more detailed analyses were performed for the host transcriptome and microbial metagenome separately below.

5.3.1 Host transcriptome

Considering the effects of temperature alone, we identified 1,733 genes that were significantly upregulated or downregulated in response to heat stress across all tadpoles (Table 5.1, full gene lists and statistics in Supplemental File 1). Within genes upregulated in response to heat, there were five GO terms that were significantly enriched compared to the rest of the dataset (Supplemental File 1). These included rRNA processing, RNA modification, tRNA processing, negative regulation of mRNA splicing, and mitochondrial translation. Within genes downregulated in response to heat, there were 108 GO terms that were significantly enriched compared to the rest of the dataset (Supplemental File 1). The five most highly significant of these terms were positive regulation of transcription by RNA polymerase II, negative regulation of transcription by RNA polymerase II, positive regulation of NF-KappaB transcription factor activity, protein ubiquitination, and positive regulation of protein catabolic process.

In response to microbial colonization alone, we identified only six genes (three upregulated and three downregulated) that were differentially expressed in depleted tadpoles compared to colonized tadpoles (Table 5.1, full gene lists and statistics in Supplemental File 1). Notably, the three downregulated genes were cytochrome P450 genes associated with lipid hydroxylation and/or xenobiotic metabolism.

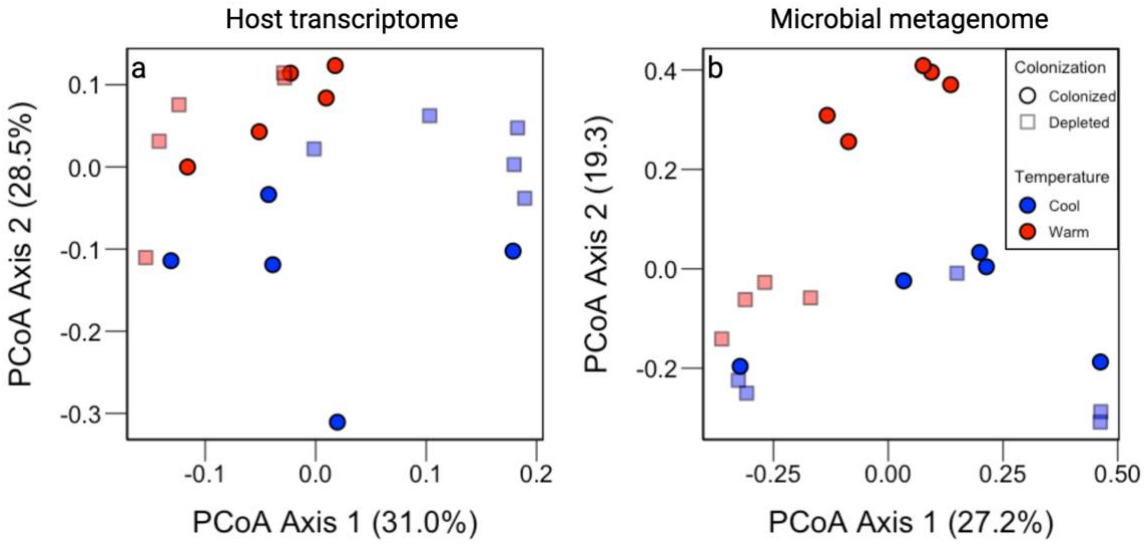


Figure 5.1 Impacts of temperature and microbial colonization on the tadpole host transcriptome and microbial metagenome.

Principal coordinate analyses plots created using Bray-Curtis distance matrices to compare (a) the host gut transcriptome of all expressed transcripts and (b) the gut bacterial metagenome of all identified functional KEGG orthologs across colonized and depleted tadpoles in cool and warm treatment groups. Within graphs, colonization treatment is denoted by shape, while temperature treatment is denoted by color. Percentages represent the proportion of variation explained by each axis.

Table 5.1 The number of differentially expressed host genes from the tadpole gut transcriptome and the number of differentially abundant KEGG modules from the tadpole gut metagenome across four treatment comparisons.

Comparison	No. of differentially expressed host genes	No. of differentially abundant bacterial KEGG modules
Cool vs. warm (all tadpoles)	1,733	8
Colonized vs. depleted (all tadpoles)	6	0
Cool vs. warm (colonized tadpoles)	287	31
Cool vs. warm (depleted tadpoles)	1,421	0

When considering the responses to heat of colonized and depleted tadpoles separately, depleted tadpoles exhibited more differentially expressed host genes (1,421 genes) than colonized tadpoles did (287 genes), and this trend was true for both upregulated and downregulated genes (Table 5.1, Figure 5.2a,b, full gene lists and statistics in Supplemental File 1). We created Venn diagrams to depict the number genes upregulated and downregulated in response to heat that were shared between colonized and depleted tadpoles. We found that only 10.5% and 8.3% of upregulated and downregulated genes respectively were shared between both colonization groups (Figure 5.2c,d). The majority of upregulated genes (79.9%) and downregulated genes (83.3%) were unique to depleted tadpoles, however, we still observed several upregulated genes (9.6%) and downregulated genes (8.3%) that were unique to colonized tadpoles (Figure 5.2c,d), indicating substantial differences in host responses to heat depending on microbial colonization. In downregulated genes, there were no differences in functional enrichment between colonized and depleted tadpoles in response to heat. However, we identified two GO terms that were significantly enriched in upregulated genes in colonized tadpoles exposed to heat as compared to depleted tadpoles (Figure 5.2e, Fisher's exact test, FDR $p < 0.05$ for both). Specifically, genes related to cellular amino acid catabolic processes and alpha-amino acid biosynthetic processes accounted for 8.2% and 6.8% of upregulated genes respectively in colonized tadpoles in response to heat. Of the genes that were assigned to these categories within colonized tadpoles, 57% were present in both categories. In depleted tadpoles, only 0.29% of genes upregulated in response to heat were related to cellular amino acid catabolic processes, and none were related to alpha-amino acid biosynthetic processes (Figure 5.2e).

5.3.2 Microbial metagenome

In response to temperature alone, we identified eight microbial KEGG modules that were differentially abundant between cool and warm temperatures (Table 5.1, full module list and statistics in Appendix Table 11). Of these eight KEGG modules, six were found within the higher order KEGG pathway of metabolism, and four of these were more specifically related to carbohydrate metabolism and tended to be present in greater abundance in the cool temperature group. When considering microbial colonization treatment alone, we did not identify any KEGG modules that were differentially abundant between colonized and depleted tadpoles (Table 5.1).

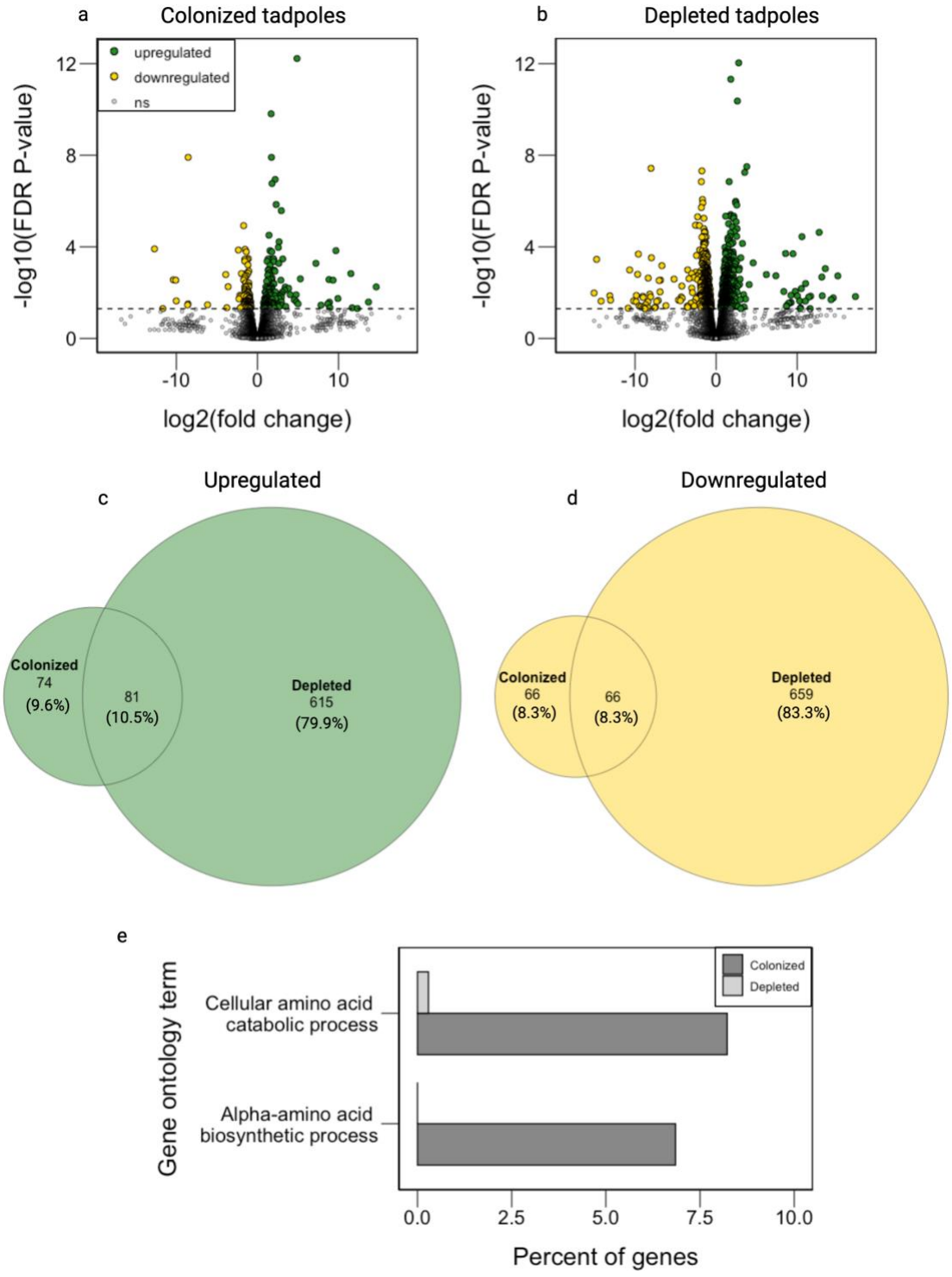


Figure 5.2 Comparison of colonized and depleted tadpole gut transcriptome responses to heat.

Volcano plots for (a) colonized and (b) depleted tadpoles showing genes upregulated (green), downregulated (yellow), or not affected (gray) in response to increased temperature. The y-axes demonstrate the significance (p-value) threshold at the dotted line and the x-axes show the direction and magnitude of each gene's change in

expression. For all genes that were either (c) upregulated or (d) downregulated in response to heat, Venn diagrams show the number of genes that were differentially expressed in colonized tadpoles only, depleted tadpoles only, or in both groups. The areas of each section in the Venn diagrams are weighted by the number of genes in those sections. Panel (e) shows gene ontology terms, and the percentage of genes identified under those terms, that were significantly differentially enriched among genes upregulated in colonized tadpoles upon exposure to heat compared to those upregulated in depleted tadpoles upon exposure to heat.

However, when comparing the response of colonized and depleted tadpole microbiome functions to heat, we observed that the colonized tadpole microbiome was more responsive to heat than the microbiome of depleted tadpoles. Specifically, we identified 31 KEGG modules in colonized tadpole microbiomes that were differentially abundant between cool and warm groups (Table 5.1, Figure 5.3, full module list and statistics in Appendix Table 11), while no KEGG modules were differentially abundant between temperature groups in depleted tadpoles. Of the heat sensitive pathways in colonized tadpoles, 29 of 31 were classified within the higher order KEGG pathway of metabolism which included amino acid metabolism, carbohydrate metabolism, energy metabolism, glycan metabolism, metabolism of cofactors and vitamins, metabolism of terpenoids and polyketides, and nucleotide metabolism. The most differentially abundant modules (12) were found within carbohydrate metabolism.

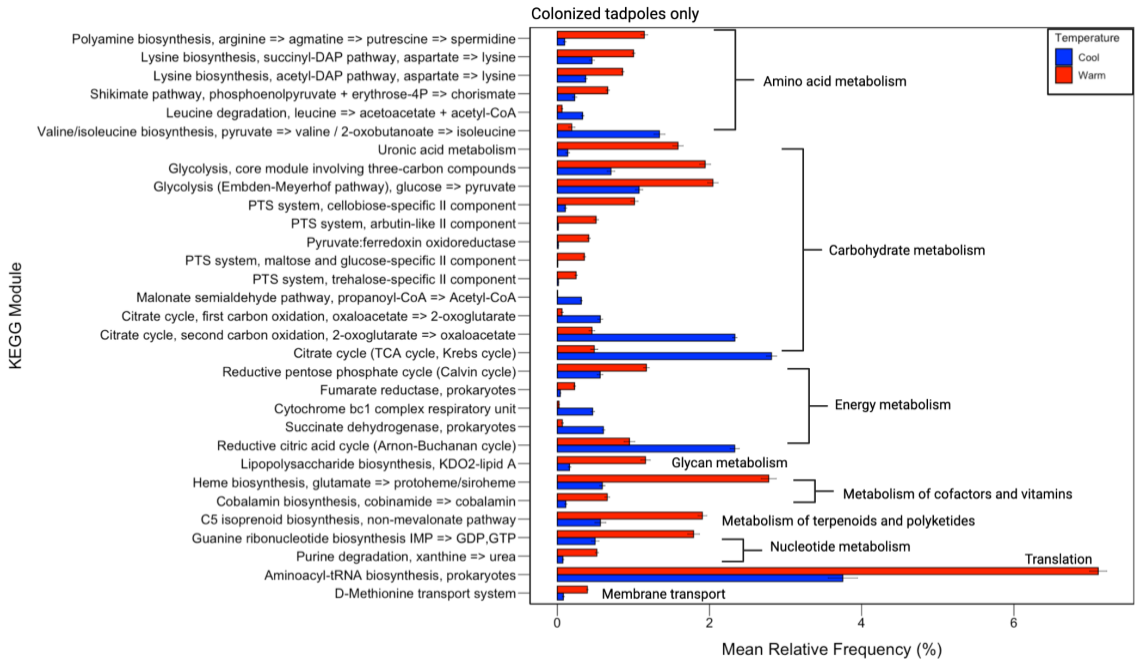


Figure 5.3 KEGG modules in the tadpole bacterial gut metagenome identified as differentially abundant between cool (blue) and warm (red) temperature groups in colonized tadpoles only.

Modules are grouped by their higher order KEGG pathways, and subsequently by the temperature at which they were most abundant. Bars and error bars represent the mean relative frequency (percentage of all modules) of each module ± s.e.m.

5.4 Discussion

The main goal of our study was to identify differences in host and microbial responses to heat stress between colonized (more diverse, natural microbiome) and depleted (less diverse, artificially disrupted microbiome) tadpoles which may underlie our previous observation that depleted tadpoles are less tolerant to heat than colonized tadpoles (Fontaine et al. 2022). We additionally wanted to understand the impacts of both heat and microbial colonization treatments on tadpole host and microbial function independently of one another.

The largest number of differentially expressed host genes we observed across any comparison was between cool and warm temperature groups among all tadpoles (Table 5.1), consistent with the consensus that heat induces large physiological changes in ectothermic animals (Huey and Kingsolver 1989). Similar to studies in other ectothermic animal groups, we found that heat-sensitive genes in the amphibians studied here were enriched for functions related to transcription and translation, as well as protein processing (Logan and Cox 2020) (Supplemental File 1). Interestingly, however, we did not observe differential expression in any host HSP genes in response to temperature, a response which is extremely common across ectotherms in general (Logan and Cox 2020) and among amphibians in particular (Ali et al. 1997). We also did not observe any changes in microbial HSPs upon exposure to heat, which are known to influence host heat tolerance (Dunbar et al. 2007, Burke et al. 2010), but did observe several changes to microbial metabolism, and specifically carbohydrate metabolism (Appendix Table 11), under heat stress, which is consistent with other studies predicting the functional effects of heat on the microbiome (Ziegler et al. 2017, Fontaine and Kohl 2020a).

We observed relatively few host genes whose expression were impacted by microbial colonization treatment alone (Table 5.1). Studies in lab mice show extensive differences in host gene expression between germ-free and conventionalized states, however, host gene expression profiles are relatively similar across mice colonized with microbiota from different sources (mice vs. zebrafish) (Rawls et al. 2006). In our system, all tadpoles are colonized with a microbiome, albeit microbiomes of different composition, and thus, if microbial colonization alone, and not microbial identity, impacts host gene expression, it is not surprising that we observed few differences across this comparison. A trend we did observe was a consistent downregulation of cytochrome P450 genes in depleted tadpoles compared to colonized tadpoles (Supplemental File

1). Each of these genes were within the cytochrome P450 CYP2 enzyme family, which are important in xenobiotic detoxification and metabolism (Kubota et al. 2011). Tadpoles readily ingest high concentrations of toxic plant secondary compounds through their herbivorous foraging strategy (Radanovic et al. 2017), and the cytochrome P450 CYP2 family is known to be important in metabolizing toxins from herbivorous diets in other animals (Greenhalgh et al. 2021). These enzymes also play an important role in lipid hydroxylation and aid in the breakdown of dietary fatty acids (Oliw 1994), and interestingly, we did observe some differences between the lipid profiles of colonized and depleted tadpoles previously (Fontaine et al. 2022). It would be interesting to manipulate the diets of colonized and depleted tadpoles with varying levels of dietary toxins and/or lipids to understand the functional consequences of these gene expression changes.

When considering the joint impacts of temperature and microbial colonization treatment, one major trend we observed was a difference in the degree of both host and microbiome plasticity in response to heat stress across tadpole colonization groups. In response to heat, depleted tadpoles exhibited more plastic host gene expression than colonized tadpoles. Specifically, in their gut, depleted tadpoles upregulated and downregulated 4.5x and 5.5x more genes, respectively, than colonized tadpoles (Table 5.1, Figure 5.2a,b), and very few of these genes were shared with those up or downregulated by colonized tadpoles in response to heat (Figure 5.2c,d). Although phenotypic plasticity in response to environmental change is often assumed to be beneficial, large numbers of genes that are actually maladaptive for heat tolerance have been shown to be differentially expressed in response to heat stress in other ectothermic vertebrates (Campbell-Staton et al. 2021). In fact, it is possible that the greater host transcriptomic response we observe in deleted tadpoles is indicative of greater stress caused by the heat treatment, as more dampened

gene expression responses are often observed in environmental stress-tolerant populations as compared to environmental-stress sensitive populations (Rivera et al. 2021).

In contrast to host gene expression responses, we observed greater plasticity in microbiome function in response to heat in colonized tadpoles as compared to depleted tadpoles via shotgun metagenomics. Specifically, we observed 31 functional KEGG modules that responded to heat in colonized tadpole microbiomes (Table 5.1, Figure 5.3), while there were zero KEGG modules that were differentially abundant between temperature treatments in depleted tadpole microbiomes. These results are consistent with a previous study demonstrating greater microbiome plasticity in response to heat in an invasive species as opposed to a non-invasive conspecific, suggesting animals that deal better with novel environmental stressors also tend to have more plastic microbiomes (Fontaine and Kohl 2020a). Our results are also congruent with the idea that the microbiome can buffer hosts from environmental stress, such that beneficial changes in microbiome function enable dampened host responses to stressful conditions (Timm et al. 2018).

Despite responding differently to heat, we did not observe any differences in microbial functional profiles between colonized and depleted tadpoles when analyzing for the effects of microbial colonization alone (Table 5.1). We previously observed that although depleted tadpoles do still host microbes, their gut microbiomes are less diverse and differ in composition compared to colonized tadpoles based on bacterial taxonomic sequencing of the 16S rRNA gene (Fontaine et al. 2022). Functional redundancy in host-associated microbial communities is common, and thus microbial communities that are disparate taxonomically may appear similar functionally (Moya and Ferrer 2016). However, taxonomically different microbes with the same metabolic potential may still respond differently (e.g., enzyme efficiencies, growth rates) when faced with changing conditions, such as temperature (Louca et al. 2018). Thus, we hypothesize that microbes in

depleted tadpole microbiomes may be more heat-sensitive in terms of their growth or survival than those of colonized tadpoles, explaining why they could appear functionally similar to colonized microbiomes but be unable to respond to heat in the same way.

In addition to quantifying plasticity in terms of the number of genes or bacterial KEGG modules that changed in response to heat across tadpoles, we sought to understand some functional implications of these changes. We identified two GO term pathways related to amino acid catabolism and anabolism (Figure 5.2e) that were enriched in the host transcriptomic response to heat within colonized tadpoles as compared to depleted tadpoles. Genes related to protein synthesis are commonly upregulated among ectotherms in response to heat (Logan and Cox 2020), and free amino acids may be important energy sources under warming and in other stressful conditions (Tripp-Valdez et al. 2017). Gut microbes have been shown to be important to their host's overall amino acid metabolism (Hooper et al. 2002, Matsumoto et al. 2012) and hosts often utilize bacterially-derived amino acids (Metges 2000). For example, up to 20% of host body lysine can be derived from gut microbial sources (Metges 2000). Of the 31 microbial functions that differed between warm and cool conditions in colonized tadpoles, six were related to amino acid metabolism, and two of these showed increases in microbial lysine biosynthetic pathways under warm conditions (Figure 5.3). It is possible that crosstalk between hosts and the gut microbiome related to amino acid metabolism facilitated increased host gene expression of amino acid anabolic and catabolic pathways in colonized tadpoles, ultimately increasing the host's tolerance to increased temperature. In contrast, depleted tadpoles may lack the necessary microbes to facilitate this process, ultimately resulting in their lowered heat tolerance.

Additionally, the gut microbiome of colonized tadpoles exhibited some specific responses to heat that could impact the thermal tolerance of tadpole hosts. For example, in the colonized

tadpole microbiome, sequences related to biosynthesis of cobalamin, or vitamin B_{12}, were increased in abundance under warm conditions (Figure 5.3). In the algal species, *Chlamydomonas reinhardtii*, cobalamin-producing bacteria are highly important in maintaining the host's thermal tolerance under warming temperatures due to the need for cobalamin for host methionine production (Xie et al. 2013). Methionine is an amino acid which modulates algal thermal tolerance through protein synthesis and maintenance of growth rate under high temperatures, and activation of heat shock responses (Xie et al. 2013). Interestingly, we also observed an increase in sequences related to the bacterial D-Methionine transport system in colonized tadpoles under warming (Figure 5.3), and expression of these bacterial transport proteins has been previously related to the level of methionine present (Gál et al. 2002).

We also observed increases in microbial sequences related to the enzyme pyruvate: ferredoxin oxidoreductase in colonized tadpoles under warm conditions (Figure 5.3). This enzyme reduces ferredoxin, an important mediator of reactive oxygen species (ROS) scavenging pathways, overexpression of which ultimately increases survival under heat stress in *C. reinhardti* (Lin et al. 2013). Interestingly, we previously observed greater activity of mitochondrial enzymes in colonized tadpoles at high temperatures compared to depleted tadpoles (Fontaine et al. 2022), and this activity could increase production of ROS and the need for ROS scavengers (Freeman and Crapo 1981). Increased abundance of these example bacterial functions under warm conditions may contribute to the greater heat tolerance of colonized tadpoles compared to depleted tadpoles, which lack the enrichment of these pathways under heat stress. However, it is currently unclear if similar pathways govern heat tolerance in our study tadpoles as would in organisms like algae. To test some of these described hypotheses, future experiments could focus on manipulating suspected

pathways (e.g., dietary-supplemented amino acids or vitamins) and observing how the treatments impact host heat tolerance.

In summary, depleted tadpoles, which are less heat tolerant, exhibited high plasticity in host responses to heat, while colonized tadpoles, which are more heat tolerant, exhibited greater microbiome plasticity in response to heat. Several host and microbial pathways (and potentially their interactions) may be responsible for these effects including amino acid metabolism, vitamin biosynthesis, and ROS scavenging pathways. Our findings suggest that hosting a microbial community that is functionally responsive to heat can help buffer hosts from deleterious effects of heat stress. Thus, rather than strictly a host response to environmental conditions, heat tolerance may represent an emergent phenotype that is governed in some way by interactions between hosts and their microbes (Lynch and Hsiao 2019). As global temperatures continue to rise, it will be important to incorporate host-microbe interactions into our understanding of host responses to climate change, as these interactions may ultimately alter evolutionary responses to warming conditions (Henry et al. 2021).

5.5 Acknowledgments

We thank Cory Duckworth for help with animal husbandry and dissections and Nick Barts for technical assistance. This project used the University of Pittsburgh Genomics Research Core, RRID: SCR_018301 for RNA sequencing library prep, the University of Pittsburgh Medical Center Genome Center for RNA sequencing, and Diversigen for metagenomic sequencing. This work was funded by the National Science Foundation (NSF GRFP to S.S.F and IOS-1942587 to K.D.K.). Permission to collect animals was obtained from Pennsylvania Fish and Boat

Commission under scientific collector's permit 2020-01-0131 and Pymatuning Laboratory of Ecology. Animal research was approved by the University of Pittsburgh IACUC under protocol ID 18062782.

6.0 Significance

6.1 Advancing the microbiome field

A recent surge in research has demonstrated the importance of host-associated microbial communities to many aspects of animal physiological function. However, several questions still remain regarding the ecological and evolutionary implications of these relationships for hosts and their microbes. It is thought that functions performed by the microbiome, and its high degree of plasticity, may confer benefits to animal hosts facing changing environments, thereby allowing them to better acclimate and adapt to novel conditions than they could alone (Alberdi et al. 2016). When hosts gain new physiological abilities due to associations with microbial partners, it is known as an "emergent phenotype" (Lynch and Hsiao 2019). Because emergent phenotypes can change the mean and variance of host phenotypic traits, it is possible that microbes may also significantly impact their host's evolutionary potential (Henry et al. 2021). Further, some believe that natural selection acts collectively on host and microbial genomes as a single unit, known as the hologenomic theory of evolution (Zilber-Rosenberg and Rosenberg 2008, Bordenstein and Theis 2015). However, this topic is hotly debated (Moran and Sloan 2015, Douglas and Werren 2016).

Testing these ideas, and demonstrating impacts of the microbiota on host ecology and evolution, has been challenging mainly because the majority of microbiome studies in natural systems are observational (Pascoe et al. 2017). Many studies describe how environmental variables impact the microbiome and can correlate aspects of the microbiome with host phenotypes but are unable to definitively link the microbiome to host traits. Indeed, a priority in the microbiome field

is to move past correlational studies and utilize microbiome manipulations to further understand the links between the microbiota and host biology (Hanage 2014).

With my dissertation research, I aimed to undercover direct links between the gut microbiota and host physiology that would increase our understanding of host-microbe interactions in an ecological and evolutionary context. To this end, I began studying relationships between amphibian microbiomes and temperature in a correlational context to initially demonstrate that environmental temperature significantly alters gut microbiome composition and diversity, and these changes could have implications for host physiology (Chapters 2 and 3). Following these studies, I sought to push past correlation, and in subsequent work (Chapters 4 and 5), I manipulated the tadpole microbiome to uncover causal links between host-associated microbiota and amphibian thermal tolerance. Through this manipulation, I discovered that tadpoles with a disrupted microbiome were less tolerant to heat than tadpoles with a more natural microbiome in terms of their acute heat tolerance, physiological performance at high temperatures, and ultimately their survival under heat stress (Chapter 4). Finally, to explore the potential mechanistic underpinnings of these findings, I utilized both host transcriptomics and microbial metagenomics to identify host and microbial pathways that differed under heat stress across tadpoles with unique histories of environmental microbial community exposure (Chapter 5). Ultimately, I uncovered several host and microbial pathways, and their interactions, that could underlie my previous results.

A substantial body of previous work has shown that microbes are integral to host heat tolerance in invertebrate ectotherms (Hector et al. 2022). However, until my work, no studies had causally linked host-associated microbiota and host thermal tolerance in a vertebrate ectotherm. My studies have shown that the relationship between microbes and host heat tolerance is likely widespread across ectothermic animals. Thus, heat tolerance could be considered an emergent

phenotype which depends on interactions between hosts and their microbes. This trait is of keen interest to biologists currently due to threat of global climate change, and it will be important to consider the role of microbes in shaping the ecological and evolutionary relationships between ectotherms and environmental temperature.

6.2 Conservation implications for amphibians

Amphibians are considered the most threatened class of vertebrates, with current rates of extinction that well exceed the background, and may be even higher than those during previous mass extinction events (Catenazzi 2015). Climate change is one of the most significant threats to amphibian populations due to their reliance on water and limited ability to regulate their body temperature (Catenazzi 2015). Although most studies that focus on amphibian responses to climate change study strictly host traits, amphibians do host diverse communities of gut microbes that influence several aspects of their physiology, including digestion (Pryor and Bjorndal 2005) and immune function (Knutie et al. 2017). Thus, warming-induced changes to host-associated microbial communities could be another indirect impact of climate change on amphibian physiology. While prior to my work it was known that changes in environmental temperature could significantly alter the amphibian microbiome (Kohl and Yahn 2016), little attention was paid to how these changes might in turn affect the host's physiology. A major focus of my dissertation was to understand these effects, and therefore increase our understanding of how climate change may ultimately impact amphibians.

To accomplish this goal, in Chapter 2, I demonstrated that increasing environmental temperature changed the composition and reduced the diversity of the gut microbiome in terrestrial

salamanders. Further, I identified several taxa in the gut of the salamanders that changed in abundance with temperature, were associated with salamander digestive performance, and were most abundant at the environmental temperature that salamander digestive performance was also the greatest. These results indicated that in addition to the direct effect of temperature on amphibian digestive performance, the impacts of temperature on microbes that aid in digestion may also indirectly impact this host trait. Importantly, many of these microbes were lost under the higher experimental temperatures, and thus climate change could negatively impact amphibians due to impacts on their symbiotic microbiota.

While informative, this study was limited in that animals were held at constant temperatures for relatively prolonged time periods before sampling (~two weeks). However, rather than maintaining a prolonged increase in environmental temperature, climate change is likely to increase the occurrence of short-term extreme weather events (e.g., heat waves, cold snaps) (Gao et al. 2012). To understand if such short-term events may also be capable of changing amphibian microbial communities (and potentially physiology), in Chapter 3, I exposed tadpoles to increased temperature and longitudinally sampled their gut microbiome to understand at what timepoint the communities significantly shifted. Ultimately, I found that gut microbial communities are capable of responding to temperature within just 12 hours, suggesting that even short-term exposures to increased temperature could have implications for amphibian physiology under climate change. However, these effects were host species-specific and thus, not all amphibians may be affected equally in response to changing temperatures.

Although climate change may impact amphibian microbial communities with potential impacts on host physiology, on the other hand, it is also possible that the function of microbial communities themselves could influence the way amphibians respond to climate change. To test

these ideas, in Chapter 4, I artificially manipulated the gut microbiome of tadpoles to change the microbial composition and reduce its diversity and found that this manipulation ultimately reduced the ability of tadpoles to tolerate and survive under heat stress. Importantly, during these experiments, tadpoles were also acclimated to three different environmental temperatures, and at the warmest environmental temperature, microbial community composition was changed and significantly reduced of its diversity. This result indicates that in nature a feedback loop could ensue where increasing temperatures alter the microbiome, and these disrupted microbiomes result in lowered host tolerance to heat, compounding the negative impacts of climate change for amphibian survival. Overall, the amphibian microbiome will likely play an important role in shaping the response of their hosts to global climate change, and efforts to maintain robust and diverse host-associated microbial communities should be of conservation concern.

Appendix A Supplementary Information for Chapter 3

Appendix A.1 Supplementary Methods

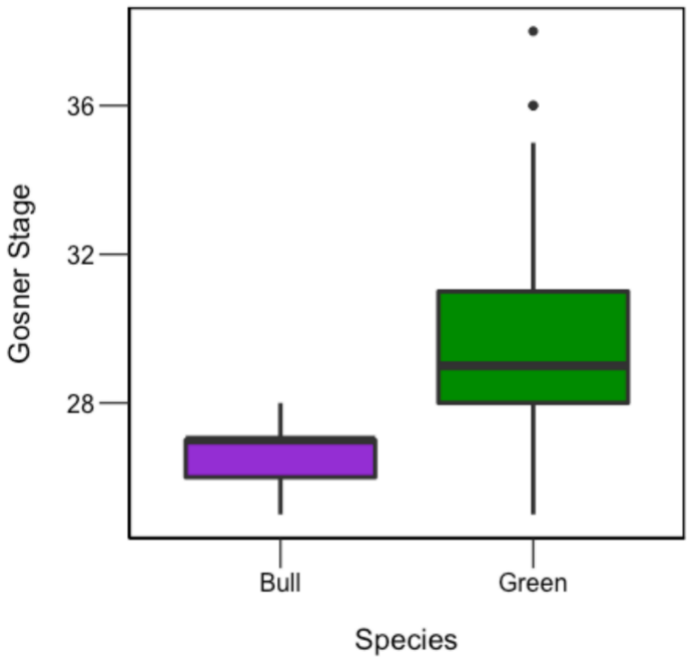
Appendix A.1.1 Detailed amplicon sequencing methods

All library preparation, PCR, and sequencing was performed by the DNA Services Facility at the University of Illinois at Chicago.

At the laboratory, PCR was used to amplify the V4 region of the 16S rRNA gene using the primers 515F and 806R (Caporaso et al. 2011) (GTGCCAGCMGCCGCGGTAA and GGACTACNVGGGTWTCTAAT). Forward primers contained common sequence linker tag 1 CS1 (ACACTGACGACATGGTTCTACA) and reverse primers contained common sequence linker tag 2 CS2 (TACGGTAGCAGA-GACTTGGTCT), as described in Moonsamy et al. (2013). Amplicons were generated using a two-stage targeted amplicon sequencing approach (Bybee et al. 2011). The first stage of amplifications was performed in 96-well plates (10μL reactions) using the MyTaq HS 2X mas-termix (Bioline, London, UK) and the following thermal cycling conditions: 95ºC for 5 minutes, 28 cycles of 95ºC for 30 seconds, 55ºC for 45 seconds and 72ºC for 30 seconds. The second stage of amplification was performed in 96-well plates (20μL reactions), again using the MyTaq HS 2X mas-termix. In each well, a unique primer pair was used with a 10-base barcode, obtained from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA, USA), and the following thermal cycling conditions were used: 95ºC for 5 minutes, 8 cycles of 95ºC for 30 seconds, 60ºC for 30 seconds, and 72ºC for 30 seconds. A final 7-minute elongation step was performed at 72ºC.

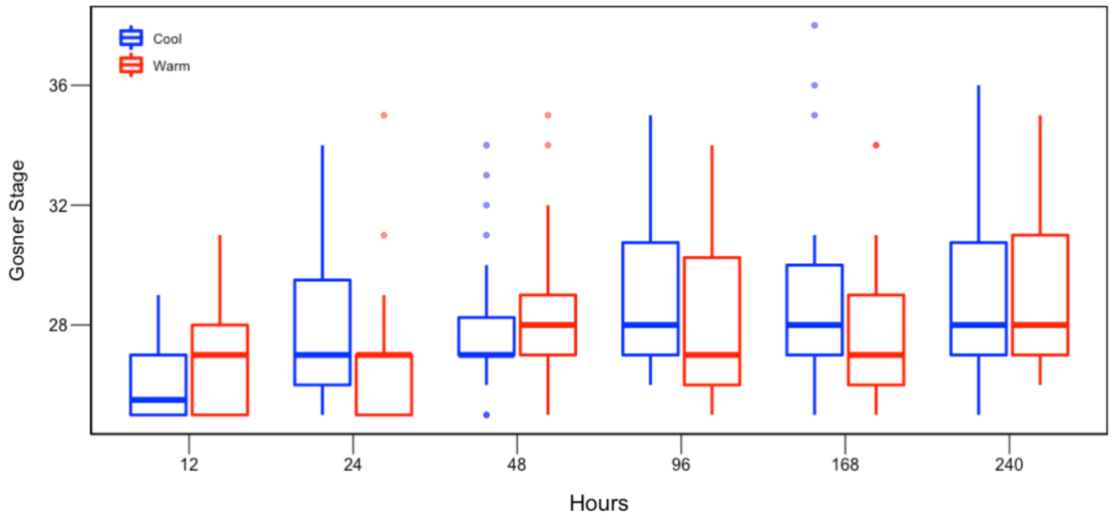
Amplified products were pooled in equal volume using an EpMotion 5075 liquid handling robot (Eppendorf, Hamburg, Germany), and the library was purified using an AMPure XP clean up protocol (0.6X, vol/vol; Agencourt, Beckmann-Coulter, Brea, CA, USA) removing fragments smaller than 300 base pairs. The pooled libraries, along with 20% phiX, were loaded onto an Illumina MiSeq flow cell (2×250 base paired-end reads). Sequencing primers targeting the CS1 and CS2 linker regions (Fluidigm, South San Francisco, CA, USA) were used to initiate sequencing.

Appendix A.2 Supplementary Figures



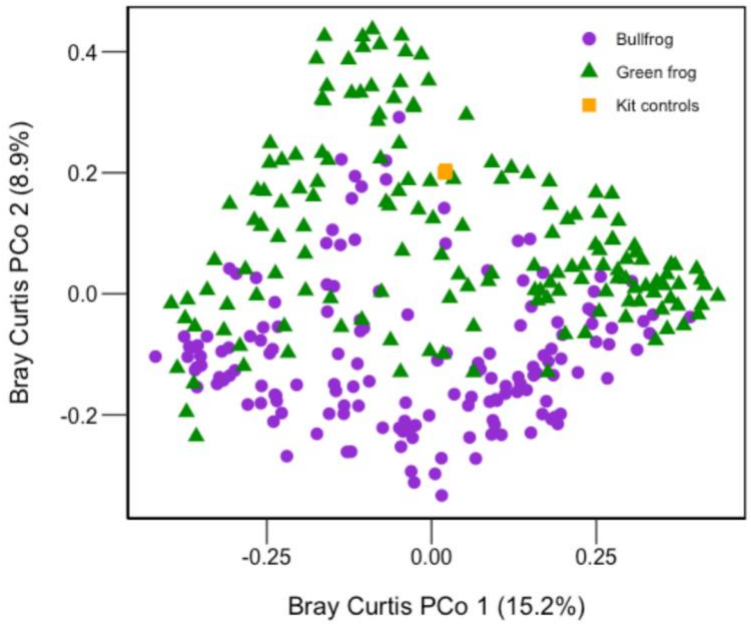
Appendix Figure 1 Green frog and bullfrog Gosner stage after removal from the experiment.

The center bolded line represents the median, the length of the box represents the IQR, and the whiskers extend to 1.5xIQR. Points beyond this value are plotted individually.



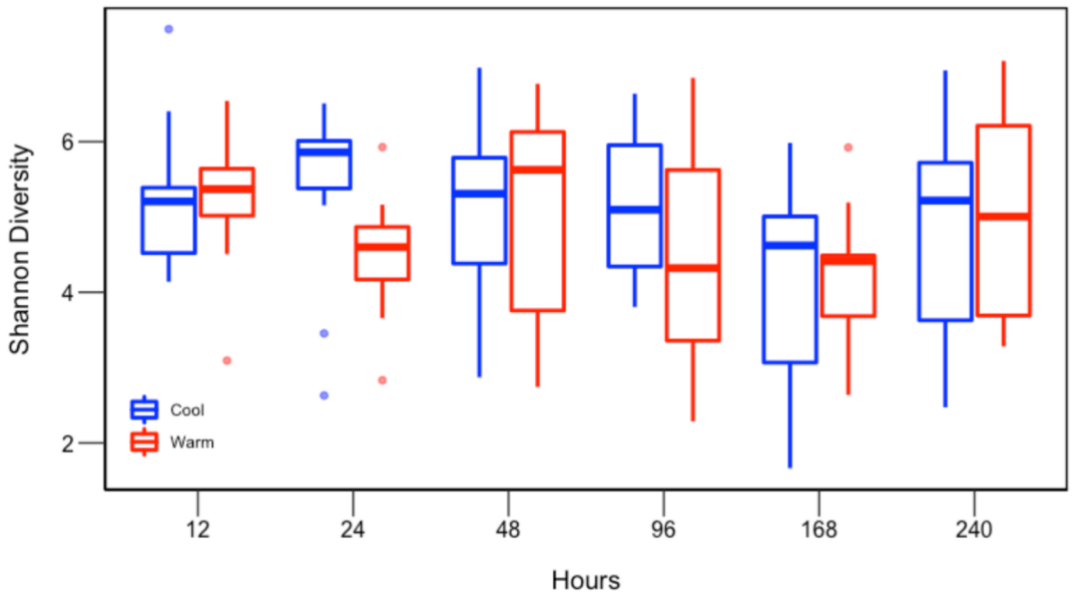
Appendix Figure 2 Gosner stage of all tadpoles (green frogs and bullfrogs) in two temperature treatments across six experimental time points.

The center bolded line represents the median, the length of the box represents the IQR, and the whiskers extend to 1.5xIQR. Points beyond this value are plotted individually.



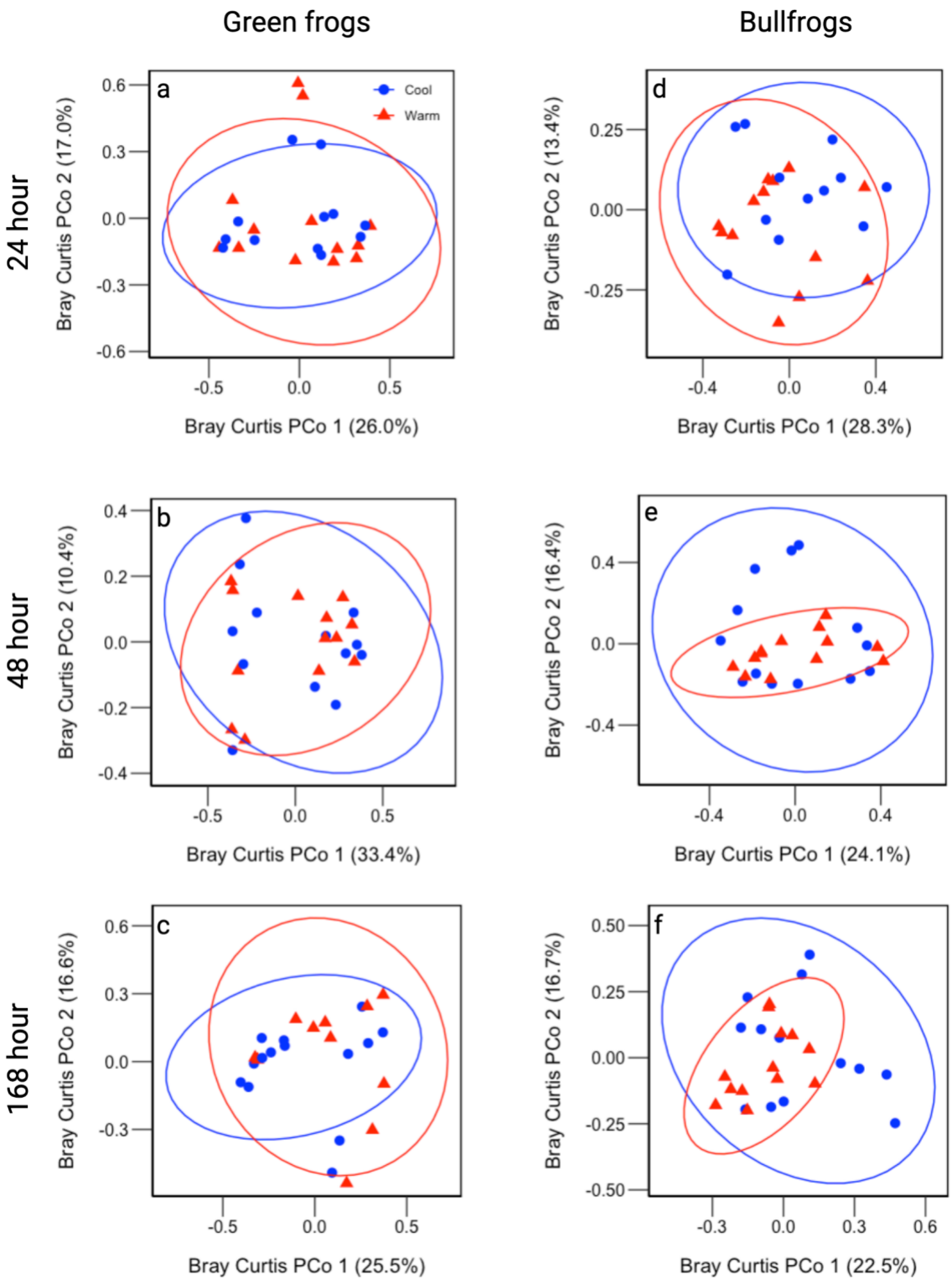
Appendix Figure 3 Microbial community composition of green frog tadpoles, bullfrog tadpoles, and kit control samples.

The plot is based on Bray-Curtis distance between samples and contains tadpole samples from all temperature and time point groups. The percentages represent the proportion of variation explained by each axis.



Appendix Figure 4 Shannon diversity of the bullfrog tadpole gut bacterial community in two temperature treatments at six time points.

The center bolded line represents the median, the length of the box represents the IQR, and the whiskers extend to 1.5xIQR. Points beyond this value are plotted individually.



Appendix Figure 5 Gut microbial community composition of green frogs and bullfrogs across two temperature treatments at three experimental timepoints.

Plots are based on Bray-Curtis distance between samples. (a),(b), and (c) show green frog tadpole communities and (d),(e), and (f) show bullfrog tadpole communities. Percentages represent the proportion of variation explained by each axis. Ellipses represent 95% confidence intervals.

Appendix A.3 Supplementary Tables

Appendix Table 1 Metrics of green frog and bullfrog tadpole development.

Tadpole Gosner stage, body mass (g), and body length (mm). Values are displayed as means ± SD. P-value columns indicate if these factors had a significant effect on the developmental metric using GLMMs. Significant p-values are in bold. N.S.=non-significant. Temp= temperature.

	Green frogs	Bullfrogs	Species p-value	Temp p-value	Time p-value
Gosner Stage	29.6 ± 2.82	26.5 ± 0.99	**<0.01**	N.S.	**<0.01**
Mass (g)	0.59 ± 0.26	0.56 ± 0.20	N.S.	N.S.	N.S.
Length (mm)	36.71 ± 5.81	35.72 ± 4.43	N.S.	N.S.	N.S.

Appendix Table 2 Relative abundance of gut bacterial genera that were differentially abundant between green frogs and bullfrogs.

Relative abundance values are displayed as mean percentages ± SD. Test statistics and FDR corrected p-values (q-values) are provided from t-tests. Genera are ordered based on in which species they were more abundant (higher value in bold) and subsequently by their effect size in decreasing order.

	Relative Abundance (%)			
Genus	**Green frogs**	**Bullfrogs**	**F statistic**	**q-value**
Epulopiscium	**0.64 ± 0.98**	0.10 ± 0.23	83.38	<0.01
Parabacteroides	**1.13 ± 3.01**	<0.01	46.25	<0.01
Clostridium sensu stricto 13	**0.25 ± 0.73**	N.O.	40.92	<0.01
Methylocystis	**0.08 ± 0.13**	0.02 ± 0.05	30.59	<0.01
Laribacter	**0.95 ± 3.84**	<0.01	22.38	<0.01
Crenobacter	**1.24 ± 4.35**	0.03 ± 0.08	21.97	<0.01
C39	**2.83 ± 4.17**	1.25 ± 1.93	21.46	<0.01
Exiguobacterium	**0.01 ± 0.03**	<0.01	17.94	<0.01
Cla-4 termite group	**0.28 ± 0.64**	0.07 ± 0.18	17.15	<0.01
Gemmobacter	**0.08 ± 0.12**	0.01 ± 0.06	17.05	<0.01
Chitinophagaceae bacterium lam-3	**0.08 ± 0.31**	<0.01	16.93	<0.01
Mycobacterium	**0.44 ± 0.74**	0.23 ± 0.73	16.30	<0.01
Clostridium sensu stricto 1	**0.73 ± 1.47**	0.45 ± 1.66	14.48	<0.01

Appendix Table 2 (Continued)

Bacteroides	**1.98 ± 2.88**	0.96 ± 1.43	14.13	<0.01
Ruminiclostridium 6	**0.03 ± 0.10**	<0.01	12.39	<0.01
Cellulosilyticum	**0.04 ± 0.23**	<0.01	12.17	<0.01
Edwardsiella	**0.88 ± 6.09**	<0.01	11.36	<0.01
Macellibacteroides	**4.65 ± 5.57**	2.99 ± 3.47	10.23	<0.01
Pseudomonas	**0.24 ± 0.83**	0.05 ± 0.14	7.08	0.02
Gordonibacter	**0.07 ± 0.16**	0.04 ± 0.10	6.63	0.03
Termite plantomycete cluster	**0.62 ± 0.95**	0.31 ± 0.48	5.88	0.04
Cetobacterium	13.05 ± 10.22	**25.43 ± 13.21**	88.61	<0.01
Candidatus *Amphibiichlamydia*	<0.01	**0.07 ± 0.22**	70.10	<0.01
Anaerorhabdus furcosa group	0.16 ± 0.33	**0.84 ± 1.34**	67.24	<0.01
Anaerofilum	0.01 ± 0.03	**0.05 ± 0.09**	38.52	<0.01
Endosymbiont TC1	0.05 ± 0.10	**0.21 ± 0.37**	34.66	<0.01
Ruminiclostridium 5	0.03 ± 0.05	**0.07 ± 0.10**	32.48	<0.01
Brachyspira	0.06 ± 0.14	**0.21 ± 0.38**	23.95	<0.01
Erysipelatoclostridium	0.02 ± 0.08	**0.12 ± 0.46**	20.98	<0.01
Treponema 2	0.12 ± 0.30	**0.34 ± 0.58**	20.32	<0.01
Ruminococcaceae UCG-014	0.50 ± 0.71	**0.86 ± 0.10**	14.06	<0.01
Anaerotruncus	<0.01	**0.02 ± 0.07**	13.70	<0.01
Angelakisella	<0.01	**0.04 ± 0.10**	13.69	<0.01
Succinispira	0.05 ± 0.26	**0.15 ± 0.52**	12.33	<0.01
Hydrogenoanaerobacterium	0.08 ± 0.18	**0.17 ± 0.33**	11.77	<0.01
Ruminococcus 1	0.03 ± 0.09	**0.09 ± 0.24**	10.00	<0.01
Dysgonomonas	<0.01	**0.02 ± 0.07**	9.55	<0.01
ZOR0006	0.33 ± 0.61	**0.61 ± 1.07**	9.40	<0.01
Erysipelotrichaceae UCG-004	<0.01	**0.02 ± 0.04**	8.68	0.01
Coprobacillus	0.06 ± 0.15	**0.12 ± 0.27**	8.53	0.01
Pygmaiobacter	0.10 ± 0.17	**0.17 ± 0.25**	8.24	0.01
SH-PL14	<0.01	**0.01 ± 0.08**	7.46	0.02
Acetobacterium	0.15 ± 0.47	**0.19 ± 0.29**	7.20	0.02
GKS98 freshwater group	0.03 ± 0.09	**0.12 ± 0.41**	6.49	0.03
Reyranella	0.18 ± 0.68	**0.51 ± 1.70**	6.10	0.04

Appendix Table 3 PERMANOVA results assessing the effects of temperature, time, and their interaction on green frog and bullfrog tadpole gut microbial community composition.

Three dissimilarity measures were tested for each species. Tank identity nested within temperatures was included as an additional factor in each model. The test statistic and FDR corrected p-value (q-value) is given for each test. Significant effects are shown in bold.

	Metric	Factor	F statistic	q-value
Green frogs	**Bray-Curtis**			
		Temp	4.9959	**0.001**
		Time	3.6058	**0.001**
		Tank (Temp)	2.2569	**0.001**

Appendix Table 3 (Continued)

		Temp x Time	1.7066	**0.003**
	Unweighted UniFrac			
		Temp	2.1036	**0.025**
		Time	2.3599	**0.001**
		Tank (Temp)	1.4486	**0.005**
		Temp x Time	0.99707	0.426
	Weighted UniFrac			
		Temp	1.8922	0.073
		Time	3.7191	**0.001**
		Tank (Temp)	3.5852	**0.001**
		Temp x Time	0.94236	0.518
Bullfrogs	**Bray-Curtis**			
		Temp	8.135	**0.001**
		Time	4.008	**0.001**
		Tank (Temp)	3.2197	**0.001**
		Temp x Time	2.1562	**0.001**
	Unweighted UniFrac			
		Temp	2.9036	**0.002**
		Time	2.7394	**0.001**
		Tank (Temp)	1.6458	**0.001**
		Temp x Time	1.3706	**0.028**
	Weighted UniFrac			
		Temp	12.239	**0.001**
		Time	3.8402	**0.001**
		Tank (Temp)	2.5093	**0.001**
		Temp x Time	1.5394	**0.043**

Appendix Table 4 Pairwise PERMANOVA results assessing the effect of temperature (warm vs. cool) on green frog and bullfrog gut microbial communities at six experimental timepoints.

Three dissimilarity measures were tested for each species. The test statistic and FDR corrected p-value (q-value) is given for each test. Significant effects are shown in bold.

Green frogs	**Metric**	**Time Point (Hours)**	**t statistic**	**q-value**
	Bray-Curtis			
		12	1.207	0.1995
		24	1.0387	0.36
		48	1.0619	0.3528
		96	1.8316	**0.009**
		168	1.7053	**0.016**
		240	2.0353	**0.006**
	Unweighted UniFrac			
		12	1.0891	0.4788
		24	1.009	0.4788
		48	0.90941	0.559
		96	1.3724	0.204
		168	1.0274	0.4788
		240	1.2982	0.300
	Weighted UniFrac			

Appendix Table 4 (Continued

		12	1.1113	0.408
		24	0.837	0.631
		48	0.79566	0.631
		96	1.413	0.204
		168	1.1477	0.408
		240	1.1755	0.408
Bullfrogs	**Bray-Curtis**			
		12	1.7171	**0.0036**
		24	1.7532	**0.003**
		48	1.8628	**0.003**
		96	1.7882	**0.003**
		168	1.8297	**0.004**
		240	2.0793	**0.003**
	Unweighted UniFrac			
		12	1.2604	0.123
		24	1.2836	0.123
		48	1.1822	0.132
		96	1.1973	0.132
		168	1.3793	0.108
		240	1.4164	0.108
	Weighted UniFrac			
		12	1.7668	**0.02**
		24	2.2766	**0.0075**
		48	1.9323	**0.0075**
		96	1.9958	**0.0075**
		168	1.7888	**0.0156**
		240	1.9148	**0.0075**

Appendix Table 5 Bacterial phyla and genera in green frog and bullfrog tadpole gut microbial communities which had relative abundances that were significantly affected by time.

The arrow in the time column indicates the direction of the relationship of that taxa's abundance with time (increasing or decreasing). Significance was determined with ANCOVA models and the F statistic for each variable in the model is shown, as well as FDR corrected p-values (q-values). Taxa are ordered by their effect size.

		Time	F statistic	q-value
Green frogs	**Phyla**			
	Bacteroidetes	↑	33.56	<0.01
	Chlamydiae	↑	33.13	<0.01
	Dependentiae	↓	22.32	<0.01
	Cyanobacteria	↑	10.16	0.03
	Patescibacteria	↓	9.23	<0.01
	Genera			
	Polynucleobacter	↓	13.93	<0.01
	Pirellula	↑	13.04	<0.01
	Acetobacterium	↓	11.64	<0.01
	Rikenella	↑	11.41	<0.01

Appendix Table 5 (Continued)

	Ruminiclostridium 6	↓	11.01	<0.01
	Terrimicrobium	↓	10.85	<0.01
	Macellibacteroides	↑	10.46	<0.01
	Luteolibacter	↓	10.10	<0.01
	Termite plantomycete cluster	↓	9.93	<0.01
	Clostridium sensu stricto 1	↑	9.85	<0.01
	Endosymbiont TC1	↓	9.31	<0.01
	Sphaerotilus	↓	9.10	<0.01
	Desulfovibrio	↓	8.73	<0.01
	Gordonibacter	↓	8.51	<0.01
	C39	↑	8.13	<0.01
	Erysipelothrix	↓	8.12	<0.01
	Tyzzerella	↓	7.85	<0.01
	Methyloparacoccus	↓	7.68	<0.01
	Ruminococcaceae UCG-014	↓	7.56	<0.01
	CPla-4 termite group	↓	7.49	<0.01
	GCA-900066225	↓	7.13	<0.01
	Novosphingobium	↓	7.10	<0.01
Bullfrogs	**Phyla**			
	Synergistetes	↓	14.9	<0.01
	Chlamydiae	↑	11.11	<0.01
	Patescibacteria	↓	8.23	<0.01
	Acidobacteria	↑	8.17	<0.01
	Fusobacteria	↓	7.15	<0.01
	Genera			
	Aurantimicrobium	↓	31.04	<0.01
	SH-PL14	↑	22.94	<0.01
	Mycobacterium	↑	21.45	<0.01
	Methylocystis	↓	19.81	<0.01
	Erysipelothrix	↓	19.36	<0.01
	Epulopiscium	↑	14.10	<0.01
	Anaerofustis	↓	13.79	<0.01
	dgA-11 gut group	↑	13.23	<0.01
	Phreatobacter	↓	11.46	<0.01
	Pirellula	↑	11.41	<0.01
	Bacteroides	↑	10.60	<0.01
	Anaerorhabdus furcosa group	↑	10.18	<0.01
	Anaerovorax	↓	10.02	<0.01
	Desulfovibrio	↓	9.20	<0.01
	Ruminococcaceae UCG-013	↓	8.45	<0.01
	Crenobacter	↑	8.18	<0.01
	ZOR0006	↓	8.18	<0.01
	Allorhizobium	↓	7.32	<0.01

Appendix B Supplementary Information for Chapter 4

Appendix B.1 Supplementary Discussion

Appendix B.1.1 Comparison of fresh pond water to pond water stored in the laboratory

In our first experiment, using 16S rRNA gene amplicon sequencing, we compared bacterial communities between pond water samples collected fresh from the pond (and immediately frozen) to pond water samples that were stored in the laboratory at 4ºC and used for our colonized water treatment. We found that bacterial community composition differed significantly at an ASV-level between fresh pond water and stored pond water samples (Appendix Figure 7a; PERMANOVA, pseudo-F= 1.92, p=0.03). While this result suggests there may be differences in microbes available to colonize tadpoles in the laboratory as opposed to the wild, we observed marked similarities between the two water sample types that suggest laboratory-colonized tadpoles will retain many common pond microbes. First, both water sample types differed significantly in bacterial community composition from tadpole gut communities (PERMANOVA, pseudo-F= 3.069 for gut to fresh pond water comparison, pseudo-F= 7.00 for gut to stored pond water comparison, $p<0.01$), and were much more similar to one another than either were to gut samples (Appendix Figure 7b). Additionally, both fresh and stored pond water sample microbial communities were dominated by Proteobacteria, Acidobacteria, Bacteroidetes, and Actinobacteria at a phylum level (Appendix Figure 7c). Further, four bacterial genera, *Polynucleobacter*, *Ferrovum*, Hgcl-clade, and *Candidatus Solibacter*, were among the top ten most abundant genera in both stored and fresh pond water, however the majority of bacteria in both groups were unable to be assigned to this

level of taxonomy (Appendix Figure 7d). Our findings are consistent with other studies which demonstrate that microbial communities of pond water stored at 4°C in the laboratory are largely representative of microbial communities in the pond of origin (Jani and Briggs 2018). Any small differences in water communities due to storage should not affect our conclusions, as our main comparison of interest is between a colonized and depleted environmental microbial community regardless of the composition of the initial colonized community. However, studies wishing to replicate natural pond water communities more accurately could consider microbial culturing techniques to retain specific microbes.

Appendix B.1.2 Comparison of colonized and depleted tadpole gut bacterial communities, body size and development, and heat tolerance in experiment 2

After development of tadpoles from experiment 2 in colonized or depleted water conditions at 22ºC, we remeasured several metrics from experiment 1 including composition and diversity of gut bacterial communities, body size and development, and acute heat tolerance to verify consistency between our two populations of tadpoles. With 16S rRNA gene amplicon sequencing, we demonstrated that compared to colonized tadpoles, the gut microbiota of depleted tadpoles was different in community composition (Appendix Figure 11d; Appendix Table 6; PERMANOVA, $p<0.001$), exhibited a greater degree of intraindividual variability (Appendix Table 6; PERMDISP, $p<0.05$) and was less diverse (Appendix Figure 11a-b; Appendix Table 6; Kruskal- Wallis, $p<0.001$), although in this set of animals, depleted tadpoles exhibited greater gut microbial community evenness than colonized tadpoles (Appendix Figure 11c; Appendix Table 6; Kruskal-Wallis, $p<0.01$). Additionally, in these tadpoles, six bacteria phyla and 15 bacterial genera had relative abundances that differed significantly between the two microbial colonization treatments

(Appendix Table 7; MaAsLin2, corrected $p<0.05$). Only two taxa, the phylum Dependentiae and the genus *Rikenella*, were differentially abundant between colonized and depleted animals in both populations of tadpoles. In both populations, Dependentiae was more abundant in colonized tadpoles, however, *Rickenella* was more abundant in depleted tadpoles from LA and colonized tadpoles from PA (Appendix Table 7). The lack of consistency in differentially abundant bacterial taxa across populations could be due to differences in environmental microbial communities in the original two pond water sources.

We additionally compared morphometrics and acute heat tolerance (CT_{max}) in this second population of tadpoles. We found that again, the depleted tadpoles were larger and more developed (Appendix Figure 12a-c; Appendix Table 8; GLM, $p<0.05$), and had lower CT_{max} values than colonized tadpoles (Appendix Figure 12d; GLM, $t= 2.23$, $p=0.03$). The consistency of our results demonstrates the impacts of host-associated microbiota on these phenotypes may be widespread across populations.

Appendix B.1.3 Muscle tissue phospholipid composition between colonized and depleted tadpoles

We compared the composition of phospholipid species in tail muscle tissue between colonized and depleted tadpoles from experiment 1 raised at 22ºC using LC-MS analysis. We detected twelve phospholipid species which had molar percentages that were significantly different between colonized and depleted tadpoles (Appendix Table 9; ANOVA, corrected $p<0.05$). However, in multivariate analyses using Bray-Curtis distance between samples, we did not detect significant changes in the overall phospholipid species community composition across groups (PERMANOVA, $p>0.05$). We also did not detect significant differences in the molar percentages

of any specific phospholipid classes (Phosphatidylcholines (PC), Phosphatidylethanolamines (PE), Phosphatidylglycerols (PG), Phosphatidylinositols (PI), and Phosphatidylserines (PS)) across groups (ANOVA, corrected $p>0.05$). Lastly, there were no significant differences in the proportions of saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids, or overall unsaturation indices, peroxidation indices, and average carbon chain length between colonized and depleted tadpoles (two-sided t-tests, $p>0.05$). In tadpoles and newts, increases or decreases in the latter three metrics have been associated with enhanced acclimation to cold or heat, respectively (Gray et al. 2016, Mineo et al. 2019).

Appendix B.2 Supplementary Methods

Appendix B.2.1 Frog and water collections

Adult green frogs (*Lithobates clamitans*) used in experiment 1 were collected from a single ephemeral pond in May 2019 from Kisatchie National Forest (LA, USA, 31.484564N -93.174226W) and shipped overnight to the University of Pittsburgh. Adult green frogs used in experiment 2 were collected in July 2020 from a single pond at Pymatuning Laboratory of Ecology (PA, USA, 41.672083N -80.513067W) and transported via automobile to the University of Pittsburgh. Permission to collect animals was obtained from LA Department of Wildlife and Fisheries (Scientific Collecting Permit WDP-19-010) and PA Fish and Boat Commission (Scientific Collector's Permit 2020-01-0131). In the laboratory, frogs were housed individually in a climate-controlled animal room maintained at 24°C, 65% humidity, and on a 14hr: 10hr light: dark cycle prior to experimentation. In June 2019, we collected 100L (divided into 10L carboys),

and two 1mL samples, of pond water from same location in Louisiana where frogs were collected. In 2020, 100L of pond water was collected monthly from July-October from the same location where frogs were collected in Pennsylvania. The carboys were kept cold on ice, and the 1mL samples frozen on dry ice, and driven back to the University of Pittsburgh. Upon return to the lab, the 1mL samples were frozen at -80°C, and the carboys of pond water were filtered through a 500μm sieve to remove macroorganisms and stored at 4°C until use.

Appendix B.2.2 Adult spawning, environmental microbial colonization manipulation, and tadpole husbandry

To obtain tadpoles for experimentation, in September 2019 (for experiment 1) and July 2020 (for experiment 2), we induced spawning in a single pair of adult frogs (one male, one female) using a hormonal injection method as described by Trudeau et al. (2010). Specifically, frogs were injected intraperitoneally with a hormonal cocktail containing [des-Gly^{10}, D-Ala^{6}]-LH-RH (0.4 μg/g body weight, Sigma Aldrich), metoclopramide (10 μg/g body weight, Sigma Aldrich), 0.7% saline (4 μL/g body weight) and DMSO (1 μL/g body weight) at a concentration of 7.5μL x body weight for females, and 10μL x body weight for males. Mating pairs were then placed in 55-gallon polypropylene tubs filled with ~4 inches of non-autoclaved laboratory water for spawning. Tubs contained pieces of floating styrofoam and PVC half pipes to be used as retreats. Spawning tubs were checked daily for up to one week for egg production. After egg mass production, viable embryos were separated from unfertilized eggs and placed in a 16-quart polypropylene container filled halfway with autoclaved laboratory water, which was changed daily to provide sufficient oxygenation to embryos.

After hatching and development to Gosner stage 25, tadpoles were divided into two treatment groups which differed in environmental microbial exposure. Tadpoles assigned to a "colonized" treatment group were placed in a water treatment consisting of 75% autoclaved laboratory water and 25% unmanipulated pond water from their parent's site of capture to provide a natural source of gut microbiota. Tadpoles assigned to a "depleted" treatment group were placed in a water treatment consisting of 75% autoclaved laboratory water and 25% autoclaved pond water from the same source to remove environmental sources of gut microbiota (Knutie et al. 2017). Pond and laboratory water were autoclaved in 10L carboys for 150 minutes at 121ºC. Throughout development, we fed tadpoles *ad libitum* a diet of 0.5g blocks of autoclaved ground rabbit chow suspended in autoclaved agar and supplemented with a small amount (~1 tsp) of commercial pet vitamins (Reptivite, Zoo Med Laboratories). Rabbit chow is high in plant material and fiber and thus should be similar nutritionally to the natural diet of these herbivorous tadpoles (Pryor and Bjorndal 2005). This diet has been used to successfully feed tadpoles in experimental settings previously (Gleason et al. 2016). Tadpoles were transferred weekly to fresh containers filled with the appropriate water treatment for the duration of all experiments. The sex distribution of our groups could not be determined because tadpole sex is not identifiable by observing external morphology. During experiment 1, we collected a 1mL sample of stored pond water weekly during water changes to monitor pond water microbial communities during laboratory storage. These samples were frozen at -80°C after collection.

Appendix B.2.3 Flow cytometry sample prep and protocol

To prepare tadpole gut samples for flow cytometry, we first removed gut contents from gut tissue. Previously minced gut samples were placed on a weigh boat and within each small section

of gut, the contents were lightly scraped out of the tissue using a sterile spatula and inoculating loop. After all contents were removed, the tissue was discarded, and the weigh boat containing the gut contents was flushed with 1mL sterile PBS. The gut contents and the PBS were carefully transferred to a pre-weighed microcentrifuge tube which was spun at 12,000 rpm for 10 minutes. The PBS supernatant was then removed, and the contents weighed. The gut content samples were then diluted in sterile PBS at a ratio of 100mL PBS to 1g of gut contents, and the resulting suspension was filtered through a 5μm cell strainer. To create a stock solution, 949μL of the cell suspension was mixed with 50μl CountBright Absolute Counting Beads (Thermo Fisher Scientific) as an internal reference, and 3μL of a 1:100 dilution of SYBR Green I dye (Sigma Aldrich). We then diluted this stock solution 1:4 with PBS.

We counted bacterial cells in each sample on the Attune NxT Flow Cytometer (Thermo Fisher Scientific). We initially adjusted the gating of the machine to count populations of both counting beads and stained bacterial cells. Specifically, plots of fluorescein isothiocyanate (FITC) were used to distinguish stained cells from all other events, and forward scatter (FSC) vs. side scatter (SSC) plots were used to distinguish counting beads from all other events and identify single prokaryotic cell events (Appendix Figure 14). To establish initial gates for counting beads, blank samples spiked only with beads were used. We ran 100μL of each sample on the machine and counted beads and bacterial cells until 500,000 events were reached, with a flow rate of 12.5 μL/min, and a threshold of 0.3. Instrument and gating parameters were kept constant across all samples. Based on the number of beads and bacterial cells counted, we then calculated the number of cells per gram of gut contents in each sample following the manufacturer's protocol.

Appendix B.2.4 Phospholipid analysis sample prep and protocol

Tadpole tail muscle samples were prepared for metabolic quenching, lysis, and lipid extraction by adding 500µL ice cold PBS and were homogenized using MP Bio Matrix A tubes at 60hz for 1 minute. 400µL of uncleared supernatant was transferred to a clean glass tube containing 10µL each PI, PC, PS, PE, and PG UltimateSplash deuterated phospholipid internal standards (Avanti Polar Lipids) and subjected to a Folch extraction. Samples were rested on ice for 10 minutes before phase separation via centrifugation at 2500 x g for 15 minutes. 700µL of organic phase was dried under nitrogen gas and resuspended in 1:1 acetonitrile:isopropanol and 3µL of sample was subjected to online LC-MS analysis.

Analyses were performed by untargeted LC-HRMS. Briefly, samples were injected via a Thermo Vanquish UHPLC and separated over a reversed phase Thermo Accucore C-18 column (2.1×100mm, 5µm particle size) maintained at 55°C. For the 30 minute LC gradient, the mobile phase consisted of the following: solvent A (50:50 H2O:ACN 10mM ammonium acetate / 0.1% acetic acid) and solvent B (90:10 IPA:ACN 10mM ammonium acetate / 0.1% acetic acid). Initial loading condition is 30% B. The gradient was the following: over 2 minutes, increase to 43%B, continue increasing to 55%B over 0.1 minutes, continue increasing to 65%B over 10 minutes, continue increasing to 85%B over 6 minutes, and finally increasing to 100% over 2 minutes. Hold at 100% for 5 minutes, followed by equilibration at 30%B for 5 minutes. The Thermo IDX tribrid mass spectrometer was operated in both positive and negative ESI mode. A data-dependent MS2 method scanning in Full MS mode from 200 to 1500 m/z at 120,000 resolution with an AGC target of 5e4 for triggering ms2 fragmentation using stepped HCD collision energies at 20,40, and 60% in the orbitrap at 15,000 resolution. Source ionization settings were 3.5 kV and 2.4kV spray voltage respectively for positive and negative mode. Source gas parameters were 35 sheath gas, 5 auxiliary

gas at 300°C, and 1 sweep gas. Calibration was performed prior to analysis using the PierceTM FlexMix Ion Calibration Solutions (Thermo Fisher Scientific). Internal standard peak areas were then extracted manually using Quan Browser (Thermo Fisher Xcalibur ver. 2.7) and normalized to weight and internal standard peak area.

Appendix B.2.5 Mitochondrial enzyme assay sample prep and protocol

To prepare homogenates for enzyme activity assays, frozen tail muscle was homogenized in 9 volumes of homogenization buffer (50 mM imidazole, 2 mM MgCL2, 5 mM EDTA, 1 mM glutathione, and 0.1% Triton X 100, pH=7.5) and centrifuged at 300 x g for 10 minutes at 4˚C. The supernatant that resulted from centrifugation was used for enzyme assays. The activities of CS and CCO were calculated from the linear change in absorbance with a temperature-controlled spectrophotometer (Evolution 201, Thermo Fisher Scientific) outfitted with an 8-cell changer that was connected to a circulating water bath (1160S, VWR). The activity of CS was determined from the reduction of DTNB (5,5' dithiobis-2-nitrobenzoic acid) at 412 nm in assay medium containing 100 mM Tris-HCL (pH=8.0), 0.1 mM DTNB, 0.15 mM acetyl-CoA, 0.15 mM oxaloacetate. The activity of CCO was determined from the oxidation of reduced cytochrome c at 550 nm against a reference of 0.075 mM cytochrome C oxidized with 0.33% potassium ferricyanide in assay medium containing 100 mM potassium phosphate (pH=7.5) and 0.075 mM reduced cytochrome c. Cytochrome c was reduced with sodium hydrosulfite, and excess sodium hydrosulfite was removed by bubbling with air for two hours (Spinazzi et al. 2012). All assays were performed in duplicate, and enzyme activity is expressed as units per g of tissue, where one unit is equal to 1 µmol of substrate modified per minute.

Appendix B.2.6 Resting metabolic rate protocol

Prior to the metabolic rate assay, tadpoles were weighed in grams, and their volume calculated via water displacement in a 10mL graduated cylinder. Tadpoles were then placed in a 9mL respirometry chamber filled with autoclaved laboratory water and rested for five minutes. We then measured eight cycles of oxygen consumption per animal, each of which consisted of an 87s flush phase, a 15s rest phase, and a 360s closed circulation phase. We exited the room during data collection to minimize stress to tadpoles, though small movements of tadpoles could not be prevented. Immediately following data collection, tadpoles were euthanized via immersion in buffered MS-222 (10 g/L), and their Gosner stage was recorded. For each of the eight cycles of respirometry per animal, mass-specific VO_2 was calculated in ng O_2/ mg body mass/hour using the formula:

*O_2 slope (ng/L/sec) * (respirometer volume (L)-animal volume (L)) * 3600s / body mass (mg).*

Because we could not account for small movements of animals, to best approximate resting metabolic rate, the minimum value of VO_2 of the eight measurements per animal was used for analyses.

Appendix B.2.7 Locomotor performance assay protocol

We measured locomotion of each tadpole in a water-jacketed 70 cm x 10 cm x 5 cm plexiglass track containing 3 cm of laboratory water. The bottom of the track was marked in 1 cm increments and the water jacket of the track was connected to a circulating water bath (1160S, VWR) that controlled the temperature of the water in the track. To measure locomotor

performance, we prompted tadpoles to swim by touching their tail with a blunt probe and filmed their escape response (frame rate = 60 fps) with a video camera (HC-V180, Panasonic) positioned 90˚ above the track. Three escape responses were recorded for each tadpole at a particular assay temperature, and each escape response was analyzed frame-by-frame using Tracker Video Motion Analysis and Modeling Tool software v5.1.5 (Open Source Physics, www.opensourcephysics.org) to calculate the maximum velocity (cm/s) reached during the first 10 cm for each escape response. We used the highest velocity measure calculated from the three escape responses for our analysis, and standardized these measures to body length (including tail) so maximum velocity was expressed in units of body lengths/s.

Appendix B.2.8 Microbiome DNA extraction, sequencing, and data processing

Total DNA was extracted from tadpole gut and water samples, along with blank extraction controls (one from experiment 1 and two from experiment 2), with the QIAamp PowerFecal Pro DNA Isolation Kit (QAIGEN) following the manufacturer's protocol. Water samples were vortexed after thawing and 200μL of water was used to begin the extraction. Extracted DNA was stored at -20°C prior to sequencing.

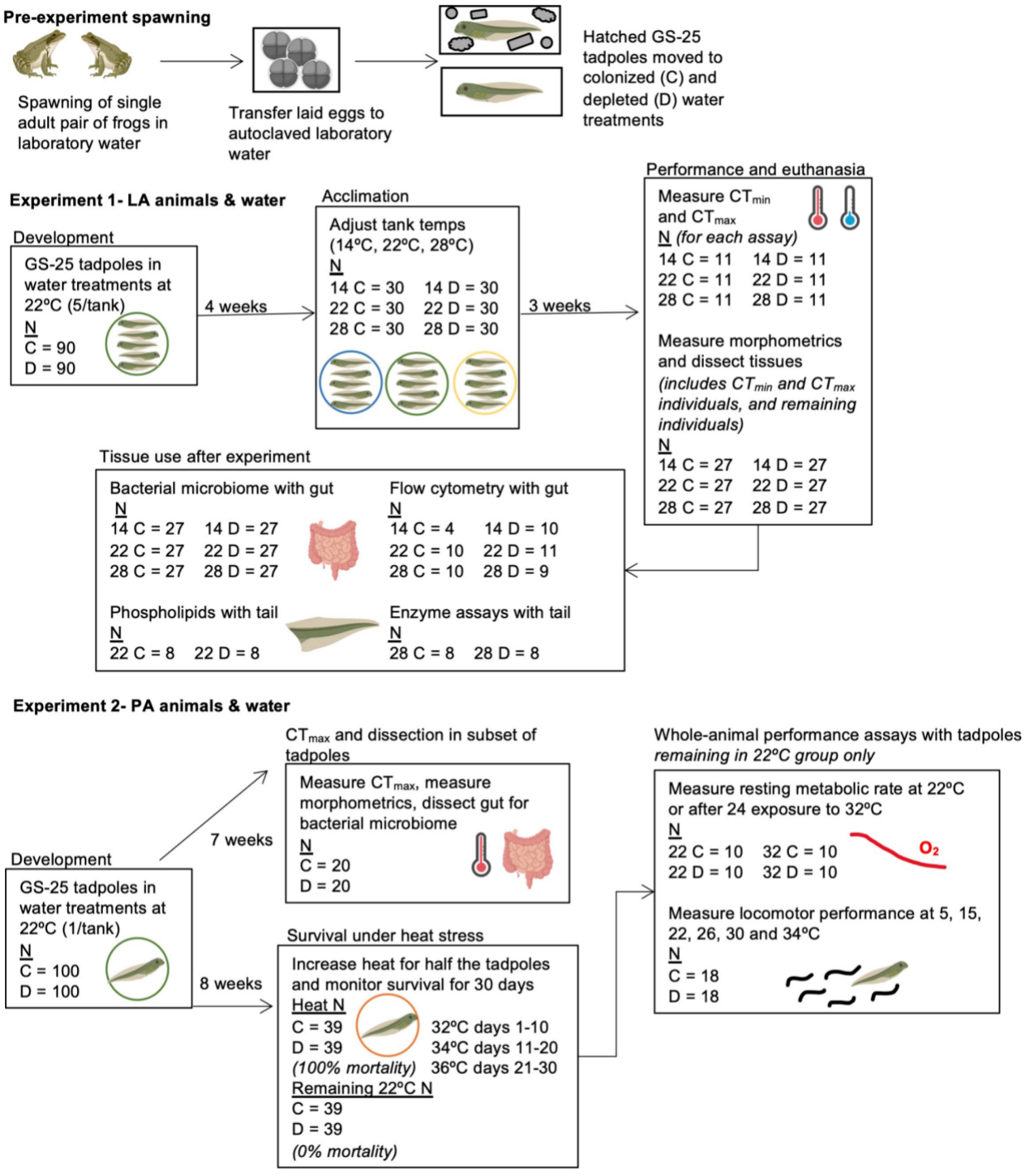
Extracted DNA from experiment 1 was sent to the University of Illinois at Chicago's DNA Services Facility and extracted DNA from experiment 2 was sent to the University of Connecticut's Microbial Analysis, Resources, and Services facility for library preparation, PCR, and sequencing. At the facilities, the bacterial 16S rRNA gene was amplified using primers 515F and 806R (Caporaso et al. 2012), libraries were pooled together and purified, and amplicons were then sequenced on the Illumina Miseq platform, resulting in 2x250 paired-end reads. Raw sequence data was processed using QIIME2 v2019.7 (Bolyen et al. 2019), and DADA2 was used

to trim reads to a length of 180 base pairs, filter reads for quality, merge forward and reverse reads, remove chimeric and singleton reads, and assign reads to amplicon sequence variants (ASVs) (Callahan et al. 2016). Subsequently, sequences were aligned, a phylogenetic tree was built using FastTree (Price et al. 2010), and ASVs were assigned taxonomy using the SILVA database classifier v132 (Quast et al. 2012). We removed any sequences identified as mitochondria, chloroplast, or archaea. Because kit control samples returned very low read counts compared to experimental samples (<1000 reads in experiment 1, <10 reads in experiment 2), and ASVs found in kit controls can often be found in the gastrointestinal tract (Glassing et al. 2016), we did not filter out kit control ASVs from experimental samples. However, we extracted samples in mixed batches, so any minor influences of contamination should be distributed equally across treatment groups (Eisenhofer et al. 2019).

We created several rarefied ASV tables to compare microbial diversity metrics across experimental groups. While the practice of rarefaction is debated, it is still relatively common and is beneficial for community-level comparisons of alpha and beta diversity metrics (McKnight et al. 2019) . For experiment 1, we created an ASV table which contained both gut and water samples and was rarefied to 1,567 sequences per sample (the number in the sample with the fewest reads). We then created an ASV table which was also rarefied to 1,567 sequences per sample and contained only water samples. Lastly, we created a table that contained only gut samples and was rarefied to 14,232 sequences per sample (the number in the sample with the fewest reads). For experiment 2 samples, we created an ASV table containing only gut samples that was rarefied to 13,216 sequences per sample which retained 13 colonized samples and 15 depleted samples in the analysis (other samples were removed due to low read counts). For each table, we calculated metrics of alpha diversity within each sample (Shannon diversity (Shannon 1948), Faith's

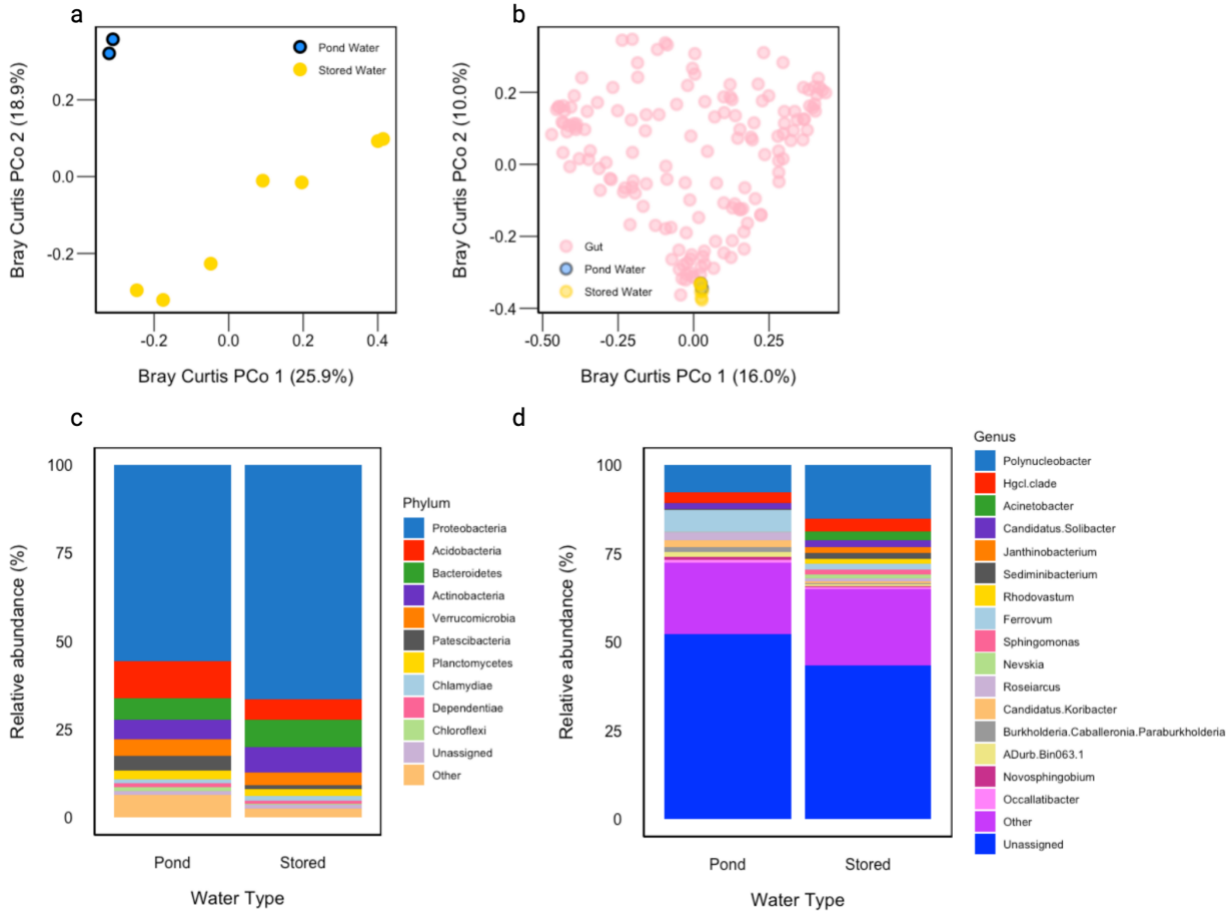
phylogenetic diversity (Faith 1992), Pielou's evenness (Pielou 1966), and the number of bacterial ASVs), and beta diversity between each pair of samples (Bray-Curtis dissimilarity (Bray and Curtis 1957), and unweighted and weighted UniFrac distance (Lozupone and Knight 2005)) within QIIME2.

Appendix B.3 Supplementary Figures



Appendix Figure 6 Experimental designs and sample sizes for chapter 4 experiments 1 and 2.

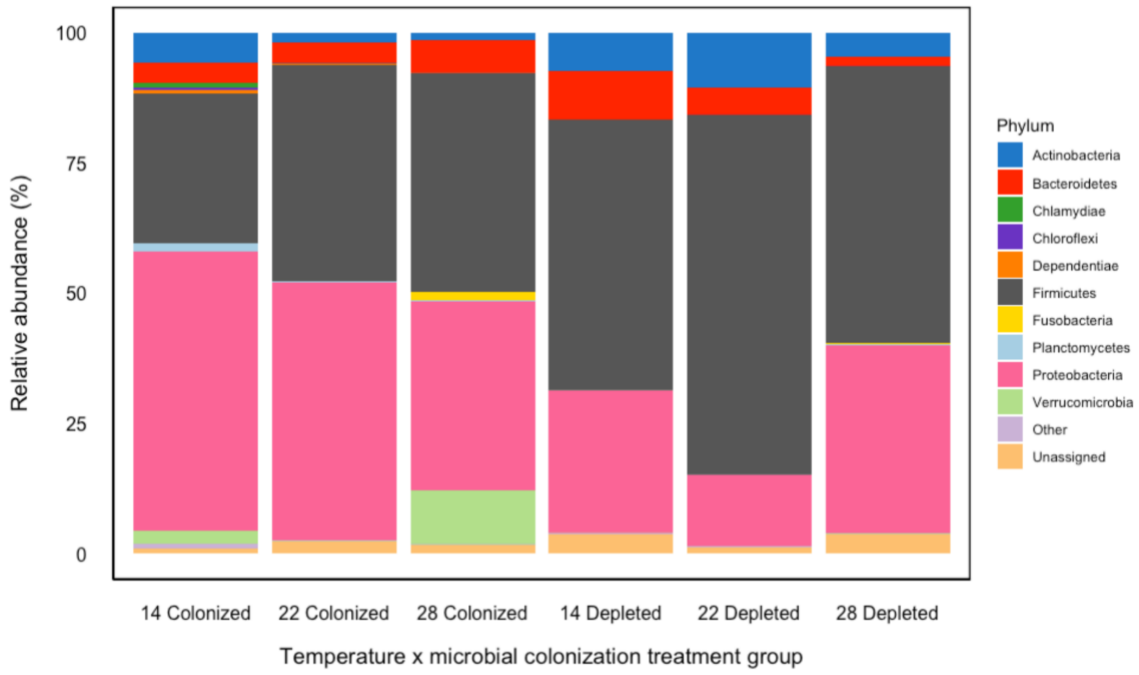
For both experiments, tadpoles were reared from pairs of adult frogs in the laboratory and divided into colonized (C) and depleted (D) water treatments at Gosner stage (GS) 25. For experiment 1, green frog tadpoles and pond water from Louisiana (LA, USA) were used and for experiment 2, green frog tadpoles and pond water from Pennsylvania (PA, USA) were used.



Appendix Figure 7 Environmental microbial communities of pond water used for the colonized microbial treatment

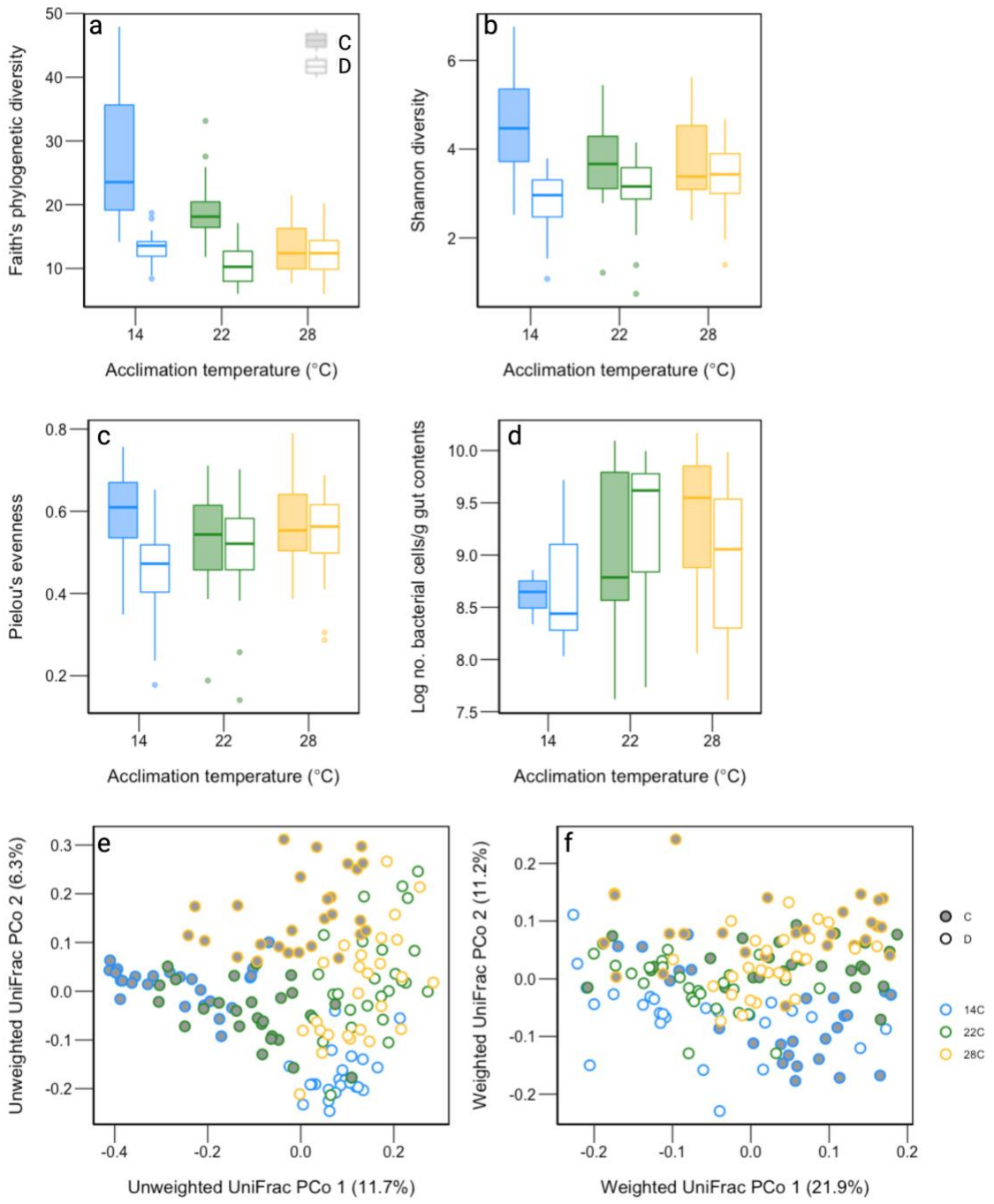
Principal Coordinate (PCo) analysis plots based on Bray-Curtis dissimilarity between (a) microbial communities of water samples collected fresh from the pond or after storage in the laboratory at 4ºC and (b) tadpole gut microbial communities and microbial communities of pond water collected fresh from the pond or after storage in the laboratory. Due to significant overlap between water types on the plot, fresh pond water samples are outlined in black. For both PCoA plots, percentages represent the proportion of variation explained by each axis. Mean relative

abundances of (a) bacterial phyla and (b) bacterial genera found in pond water samples fresh from the pond or after storage in the laboratory. The top ten most abundant taxa are shown individually, and the remainder are grouped together as “other”. Any bacteria that were unable to be assigned to a phylum or genus are grouped together as “unassigned”.



Appendix Figure 8 Mean relative abundances of bacterial phyla in gut microbial communities of tadpoles from experiment 1 across microbial colonization and acclimation temperature treatment groups.

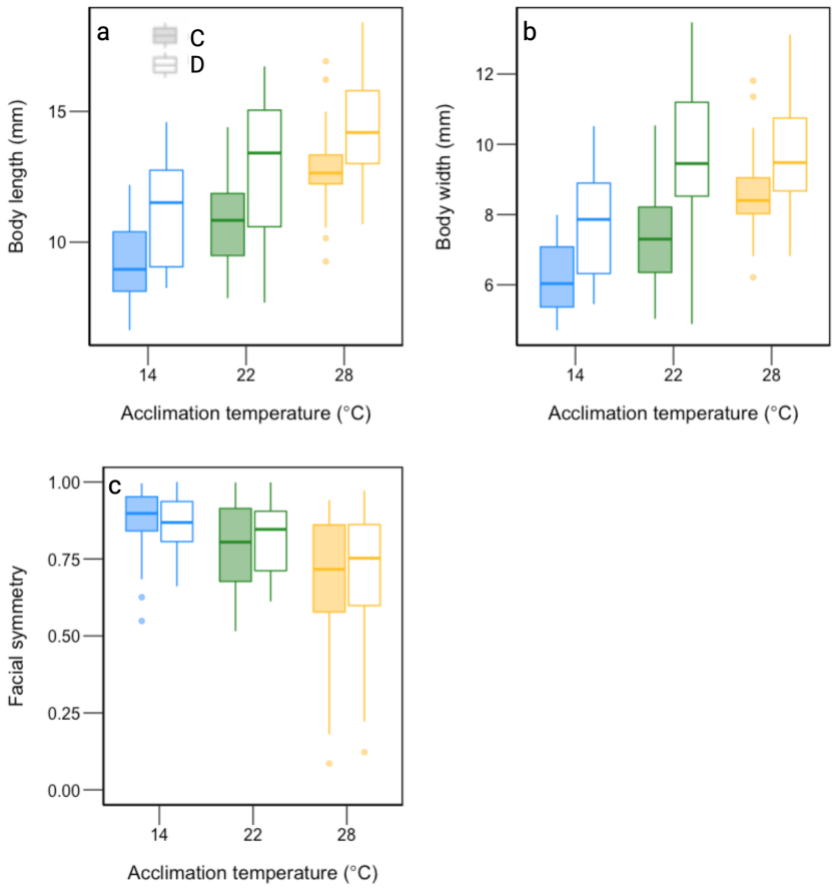
The top ten most abundant phyla are shown individually, and the remainder are grouped together as “other”. Any bacteria that were unable to be assigned to a phylum are grouped together as “unassigned”. N = 27 animals per group.



Appendix Figure 9 Impacts of microbial colonization treatment and acclimation temperature on tadpole gut microbial communities in experiment 1.

(a) Faith's phylogenetic diversity of the gut bacterial community (b) Shannon diversity of the gut bacterial community (c) Pielou's evenness within the gut bacterial community (d) Number of bacterial cells in tadpole gut contents measured using flow cytometry and shown on a log scale. Principal Coordinate (PCo)

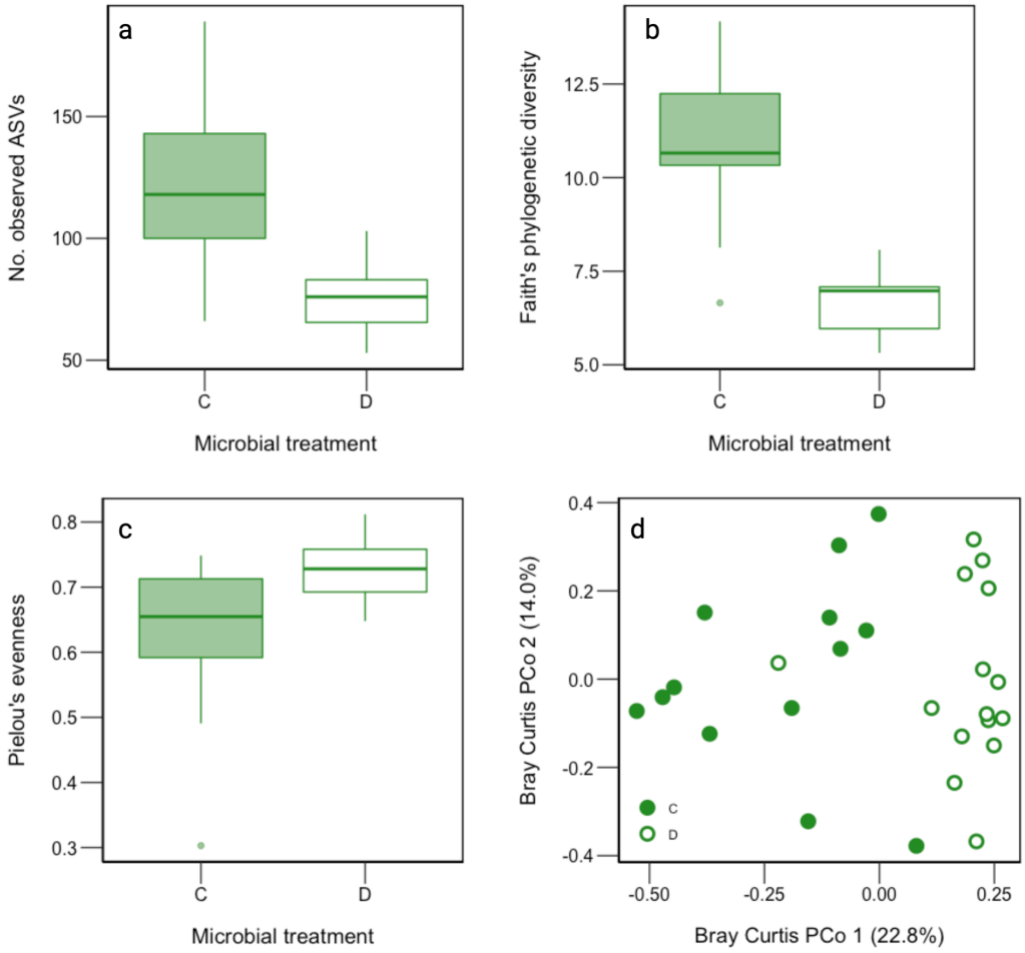
analysis plot based on (e) Unweighted UniFrac distance and (f) Weighted Unifrac between gut bacterial community samples. For all boxplots, the center line represents the median, the length of the box extends through the IQR, and whiskers extend to 1.5x IQR. All points outside this range are plotted individually. For all principal coordinate analysis plots, percentages represent the proportion of variation explained by each axis. C= colonized tadpoles and D= depleted tadpoles. Colors represent tadpole acclimation temperature.



Appendix Figure 10 Tadpole morphometrics across microbial colonization and acclimation temperature treatment groups from experiment 1.

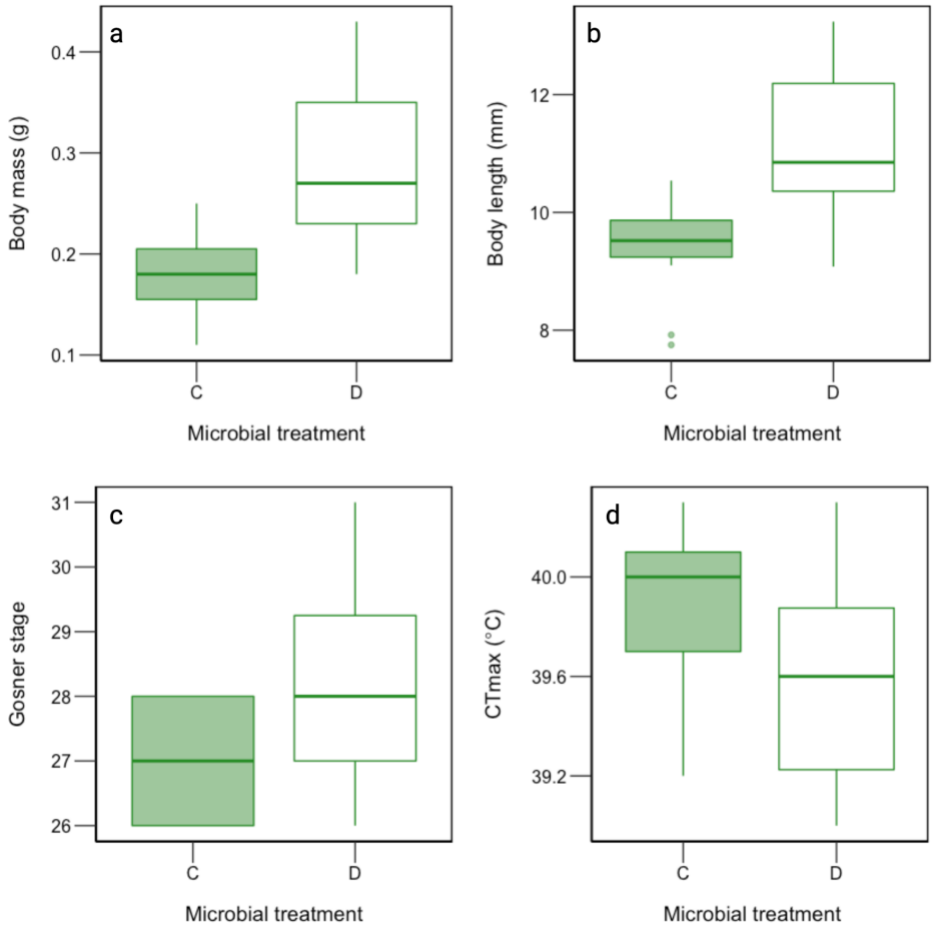
(a) tadpole body length (b) tadpole body width (c) tadpole facial symmetry, calculated as the absolute value, subtracted from 1, of the difference between the distance from the center of each eye to the tip of the nose. The center line of each boxplot represents the median, the length of the box extends through the IQR, and whiskers extend to 1.5x IQR. All

points outside this range are plotted individually. C= colonized tadpoles and D= depleted tadpoles. Colors represent tadpole acclimation temperature.



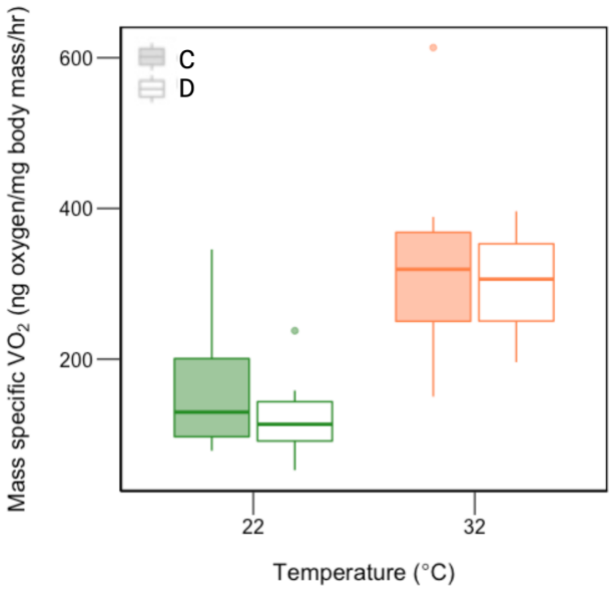
Appendix Figure 11 Impact of microbial colonization treatment on tadpole gut microbial communities in experiment 2.

(a) Number of bacterial ASVs in the tadpole gut bacterial community, (b) Faith's phylogenetic diversity of the tadpole gut bacterial community, (c) Pielou's evenness of the tadpole gut bacterial community, and (d) Principal Coordinate (PCo) analysis plot based on Bray-Curtis dissimilarity between gut bacterial community samples. Percentages represent the proportion of variation explained by each axis. The center line of each boxplot represents the median, the length of the box extends through the IQR, and whiskers extend to 1.5x IQR. All points outside this range are plotted individually. C= colonized tadpoles and D= depleted tadpoles.



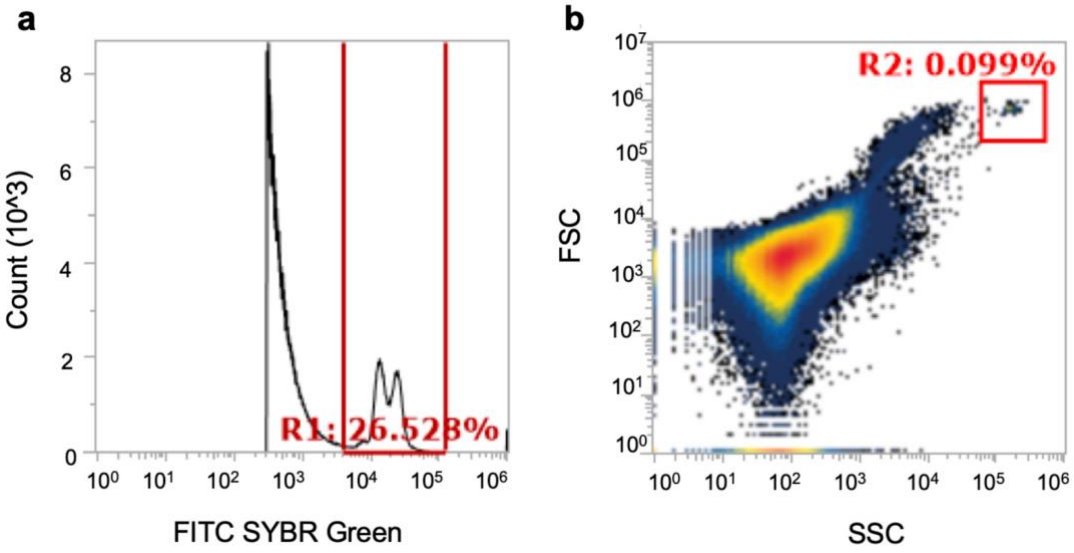
Appendix Figure 12 The impact of microbial colonization treatment on tadpole morphometrics and heat tolerance in experiment 2.

(a) tadpole body mass (b) tadpole body length (c) tadpole developmental stage based on the Gosner system (d) tadpole critical thermal maximum (CT_{max}). The center line of each boxplot represents the median, the length of the box extends through the IQR, and whiskers extend to 1.5x IQR. All points outside this range are plotted individually. C= colonized tadpoles and D= depleted tadpoles.



Appendix Figure 13 Impacts of microbial colonization treatment and assay temperature on tadpole mass-specific resting metabolic rate.

The center line of each boxplot represents the median, the length of the box extends through the IQR, and whiskers extend to 1.5x IQR. All points outside this range are plotted individually. C= colonized tadpoles and D= depleted tadpoles. On the y-axis VO_2 refers to oxygen consumption.



Appendix Figure 14 Plots used to determine gating parameters for flow cytometry from one representative sample.

(a) A plot of fluorescein isothiocyanate (FITC) vs. cell counts was used to distinguish cells stained with SYBR Green dye from all other cells (b) A plot of forward scatter (FSC) vs. side scatter (SSC) was used to distinguish populations of counting beads from all other events. To establish initial gates for counting beads, blank samples spiked only with beads were used. On both plots, red rectangles represent the gates and surround the events counted. Percentages indicate the proportion of events within the gate out of total events. R1= stained bacterial cells and R2=counting beads.

Appendix B.4 Supplementary Tables

Appendix Table 6 Results of statistical tests showing effects of acclimation temperature, microbial colonization treatment, and their interaction on tadpole gut microbial community alpha and beta diversity in experiments 1 and 2.

NS= non-significant. N/A= non-applicable, an interactive effect could not be measured for this test. For experiment 1 models, tadpole tank was included as a random effect. A single acclimation temperature and individual housing was used in experiment 2 so no effects of temperature were tested, and tank was not included in models. Significant effects of statistical tests (alpha diversity= GLMMs or Kruskal-Wallis, beta diversity= PERMANOVA, beta dispersion= PERMDISP) are in bold. For PERMANOVA and PERMDISP tests p-values are corrected using the BH FDR method.

Diversity metric	Temperature effect (P, test statistic)	Colonization effect (P, test statistic)	T x C interactive effect (P, test statistic)
Experiment 1			
Alpha diversity (GLMMs)			
No. observed ASVs	**<0.001, 33.8**	**<0.001, 67.0**	**<0.001, 38.2**
Faith's phylogenetic diversity	**<0.001, 54.0**	**<0.001, 71.4**	**<0.001, 40.5**
Shannon diversity	NS	**<0.001, 34.3**	**<0.001, 16.4**
Pielou's evenness	NS	**<0.001, 12.0**	**0.02, 5.9**
Beta diversity (PERMANOVAs)			
Bray-Curtis	**<0.001, 10.1**	**<0.001, 20.0**	**<0.001, 4.1**
Unweighted UniFrac	**<0.001, 5.3**	**<0.001, 13.3**	**<0.001, 3.9**
Weighted UniFrac	**<0.001, 8.8**	**<0.001, 15.4**	**<0.001, 5.9**

Appendix Table 6 (Continued)

Beta dispersion (PERMDISPs)			
Bray-Curtis	**<0.01, 6.1**	**<0.001, 20.8**	N/A
Unweighted UniFrac	**<0.001, 19.2**	**<0.001, 37.4**	N/A
Weighted UniFrac	**<0.01, 7.1**	**<0.01, 8.0**	N/A
Experiment 2			
Alpha diversity (Kruskal-Wallis)			
No. observed ASVs	N/A	**<0.001, 15.0**	N/A
Faith's phylogenetic diversity	N/A	**<0.001, 16.2**	N/A
Shannon diversity	N/A	NS	N/A
Pielou's evenness	N/A	**<0.01, 7.8**	N/A
Beta diversity (PERMANOVAs)			
Bray-Curtis	N/A	**<0.001, 5.2**	N/A
Unweighted UniFrac	N/A	**<0.001, 8.0**	N/A
Weighted UniFrac	N/A	**<0.001, 4.5**	N/A
Beta dispersion (PERMDISPs)			
Bray-Curtis	N/A	NS	N/A
Unweighted UniFrac	N/A	**<0.05, 5.7**	N/A
Weighted UniFrac	N/A	NS	N/A

Appendix Table 7 Relative abundances of bacterial phyla and genera in tadpole gut microbial communities that were significantly impacted by microbial colonization treatment or acclimation temperature in experiments 1 and 2.

A single acclimation temperature was used in experiment 2 so no effects of temperature were tested. Relative abundances are displayed as means ± s.e.m. The group in which the specific taxa was most abundant is in bold. Statistical testing was conducted using MaAsLin2. N.O=not observed. P-values were corrected using the BH FDR method.

Experiment 1					
	Relative abundance (%)			**FDR P-value**	**Coefficient**
Temperature effects	**14°C**	**22°C**	**28°C**		
Phyla					
Acidobacteria	**0.10 ± 0.03**	0.01 ± <0.01	<0.01	0.03	-0.04

Appendix Table 7 (Continued)

Colonization effects	Relative abundance (%)		FDR P-value	Coefficient
	Colonized	Depleted		
Phyla				
Chlamydiae	**0.33 ± 0.07**	<0.01	<0.01	-0.04
Chloroflexi	**0.20 ± 0.05**	0.02 ± 0.01	0.03	-0.02
Dependentiae	**0.32 ± 0.07**	<0.01	<0.01	-0.04
Plantomycetes	**0.62 ± 0.13**	0.11 ± 0.03	0.01	-0.04
Proteobacteria	**46.76 ± 2.77**	26.13 ± 2.68	<0.01	-0.27
WPS-2	**0.05 ± 0.01**	N.O.	0.02	-0.01
Firmicutes	37.83 ± 2.69	**59.13 ± 2.63**	<0.01	0.25
Genera				
Aquicella	**0.52 ± 0.13**	N.O.	<0.01	-0.05
Aquisphaera	**0.17 ± 0.04**	<0.01	0.01	-0.03
Coxiella	**0.08 ± 0.02**	<0.01	0.01	-0.02
Roseiarcus	**0.56 ± 0.13**	N.O.	<0.01	-0.05
Singulisphaera	**0.13 ± 0.33**	N.O.	<0.01	-0.02
Uncultured Diplorickettsiaceae	**0.21 ± 0.05**	<0.01	0.01	-0.03
Uncultured Simkaniaceae	**0.12 ± 0.02**	<0.01	0.01	-0.02
Uncultured Vermiphilaceae	**0.09 ± 0.03**	<0.01	0.01	-0.02
Xanthobacter	**8.49 ± 1.21**	1.46 ± 0.45	<0.01	-0.17
Bacillus	6.43 ± 1.35	**31.81 ± 2.85**	<0.01	0.43
Rikenella	1.59 ± 0.35	**5.45 ± 1.02**	0.03	0.13

Experiment 2

Colonization effects	Relative abundance (%)		FDR P-value	Coefficient
	Colonized	Depleted		
Phyla				
Cyanobacteria	**0.15 ± 0.08**	<0.01	0.03	-0.02
Deferribacteres	**0.11 ± 0.06**	N.O.	0.03	-0.02
Dependentiae	**<0.01**	N.O.	0.03	-0.004
Fusobacteria	**23.56 ± 7.07**	2.18 ± 2.17	<0.01	-0.39
Lentisphaerae	**0.03 ± 0.02**	N.O.	0.04	-0.01
Verrucomicrobia	**3.50 ± 1.16**	0.52 ± 0.25	0.01	-0.11
Genera				
Akkermansia	**1.47 ± 0.53**	N.O.	<0.01	-0.12
Cetobacterium	**23.59 ± 7.07**	2.18 ± 2.17	<0.01	-0.47
Desulfovibrio	**3.51 ± 1.14**	N.O.	<0.01	-0.19
Dysgonomonas	**0.34 ± 0.16**	N.O.	0.02	-0.05
Erysipelatoclostridium	**0.60 ± 0.16**	0.08 ± 0.04	0.01	-0.06
Mycobacterium	**0.27 ± 0.11**	0.02 ± 0.01	0.01	-0.04
Parabacteroides	**1.42 ± 0.33**	N.O.	<0.01	-0.12
Rikenella	**0.34 ± 0.12**	N.O.	<0.01	-0.05
Robinsoniella	**0.60 ± 0.20**	0.01 ± 0.01	<0.01	-0.07
Thiodictyon	**0.02 ± 0.01**	N.O.	0.04	-0.01
Uncultured Burkholderiaceae	**0.97 ± 0.26**	0.12 ± 0.07	<0.01	-0.08
Aeromonas	0.18 ± 0.12	**2.68 ± 0.94**	0.02	0.14

Colstridium sensu stricto 1	1.31 ± 0.64	**6.13 ± 1.28**	<0.01	0.22
Pseudoxanthobacter	0.17 ± 0.11	**0.96 ± 0.21**	<0.01	0.08
Tyzzerella	0.32 ± 0.13	**2.67 ± 0.73**	0.04	0.14

Appendix Table 8 Results of statistical tests examining the effects of acclimation temperature, microbial colonization treatment, and their interaction on tadpole morphometrics in experiments 1 and 2.

For experiment 1 models, tadpole tank was included as a random effect in all models, and body length was included as a covariate in the facial symmetry model. NS= non-significant. N/A= non-applicable. A single acclimation temperature and individual housing was used in experiment 2 so no effects of temperature were tested, and tank was not included in models. Significant effects of statistical tests (experiment 1= GLMMs, experiment 2= GLMs) are shown in bold.

Metric	Temperature effect (P, test statistic)	Colonization effect (P, test statistic)	T x C interactive effect (P, test statistic)
Experiment 1 (GLMMs)			
Body mass (g)	**<0.001, 48.0**	**<0.001, 44.8**	NS
Body length (mm)	**<0.001, 72.4**	**<0.001, 27.6**	NS
Body width (mm)	**<0.001, 53.9**	**<0.001, 40.7**	NS
Gosner stage	**<0.001, 85.7**	**<0.001, 36.6**	**<0.01, 12.1**
Facial symmetry	**<0.001, 8.5**	NS	NS
Experiment 2 (GLMs)			
Body mass (g)	N/A	**<0.001, 5.1**	N/A
Body length (mm)	N/A	**<0.001, 5.3**	N/A
Gosner Stage	N/A	**<0.001, 3.6**	N/A

Appendix Table 9 Relative abundances of phospholipid species that differed between colonized and depleted tadpoles.

Relative abundances are displayed as means ± s.e.m. The group in which a species was most abundant is shown in bold. Significance was determined using one-way ANOVAs and the response screening function in JMP. P-values were corrected using the BH FDR method. Phospholipid species nomenclature is depicted as "phospholipid class (no. carbons: no. double bonds)". PC= phosphatidylcholine, PE= phosphatidylethanolamine, PS= phosphatidylserine, PI= phosphatidylinositol.

	Relative abundance (%)		FDR P-value	F statistic
	Colonized	**Depleted**		
Phospholipid Species				
PC(35:2)	**0.71 ± 0.06**	0.48 ± 0.04	0.01	11.41
PC(36:1)	**1.01 ± 0.06**	0.68 ± 0.05	<0.01	18.03
PC(37:2)	**0.15 ± 0.02**	0.09 ± 0.01	0.02	10.48
PC(37:6)	**0.12 ± 0.01**	0.06 ± 0.01	<0.01	19.32
PC(38:6)	**0.55 ± 0.08**	0.43 ± 0.03	0.01	11.11
PE(29:1)	**1.50 ± 0.11**	1.00 ± 0.15	0.04	8.26
PS(42:9)	**0.12 ± 0.02**	0.05 ± 0.01	0.01	11.98
PS(44:12)	**0.11 ± 0.02**	0.05 ± 0.01	0.03	8.97
PC(34:3)	1.54 ± 0.06	**2.12 ± 0.15**	0.02	10.24
PE(32:0)	0.22 ± 0.01	**0.31 ± 0.02**	0.01	10.89
PI(36:4)	0.08 ± 0.01	**0.12 ± 0.01**	<0.01	12.90
PI(38:5)	0.37 ± 0.04	**0.61 ± 0.05**	<0.01	15.51

Appendix C Supplementary Information for Chapter 5

Appendix C.1 Supplementary Tables

Appendix Table 10 Individual tadpole identifiers and developmental data for samples used for transcriptomics and metagenomics.

Individuals from which samples were used for both analyses are shown in bold.

Transcriptomics				**Metagenomics**			
Sample ID	Body mass (g)	Body length (mm)	Gosner stage	Sample ID	Body mass (g)	Body length (mm)	Gosner stage
C2201	0.24	10.51	28	C2203	0.31	11.84	29
C2204	0.31	11.65	28	C2205	0.2	10.66	28
C2206	0.23	11.12	28	**C2207**	0.21	10.57	28
C2207	0.21	10.57	28	**C2208**	0.22	9.97	28
C2208	0.22	9.97	28	C2209	0.3	11.31	29
C3204	0.23	10.25	28	C3201	0.29	11.04	30
C3205	0.16	9.51	28	C3203	0.13	8.85	26
C3207	0.19	10.42	28	**C3204**	0.23	10.25	28
C3208	0.18	9.64	28	C3209	0.22	10.51	29
C3210	0.18	10.02	28	**C3210**	0.18	10.02	28
D2201	0.24	10.48	28	**D2202**	0.28	11.16	28
D2202	0.28	11.16	28	D2204	0.26	11.11	27
D2203	0.25	10.86	28	**D2206**	0.27	10.12	28
D2206	0.27	10.12	28	D2207	0.38	13.1	31
D2209	0.3	11.72	29	**D2209**	0.3	11.72	29
D3202	0.25	10.84	28	D3204	0.32	10.77	29
D3203	0.17	9.36	29	**D3205**	0.23	10.09	28
D3205	0.23	10.09	28	**D3206**	0.26	10.8	28
D3206	0.26	10.8	28	D3209	0.46	12.39	31
D3207	0.16	9.71	28	D3210	0.17	9.3	27

Appendix Table 11 KEGG module functions in tadpole gut microbiomes that differed in abundance between warm and cool conditions in all tadpoles and colonized tadpoles only.

Unique KEGG module identifiers and names are listed for each function, along with the first and second higher order pathways those modules are found in. Frequency categories represent the mean frequency (%) of that module ± the s.d. of the frequency.

Cool vs. Warm (all tadpoles)						
KEGG module ID	**KEGG module name**	**KEGG pathway 1**	**KEGG pathway 2**	**Cool frequency (%)**	**Warm frequency (%)**	**FDR P-value**
M00237	Branched-chain amino acid transport system	Environmental Information Processing	Phosphate and amino acid transport system	**1.5 ± 0.56**	0.31 ± 0.61	0.04
M00360	Aminoacyl-tRNA biosynthesis, prokaryotes	Genetic Information Processing	Translation	3.54 ± 0.91	**6.01 ± 1.41**	0.04
M00009	Citrate cycle (TCA cycle, Krebs cycle)	Metabolism	Carbohydrate metabolism	**2.77 ± 0.32**	1.04 ± 0.7	0.01
M00011	Citrate cycle, second carbon oxidation, 2-oxoglutarate => oxaloacetate	Metabolism	Carbohydrate metabolism	**2.22 ± 0.32**	1.02 ± 0.68	0.04
M00012	Glyoxylate cycle	Metabolism	Carbohydrate metabolism	**1.13 ± 0.42**	0.25 ± 0.25	0.01
M00060	Lipopolysaccharide biosynthesis, KDO2-lipid A	Metabolism	Carbohydrate metabolism	0.17 ± 0.09	**0.89 ± 0.4**	0.04
M00149	Succinate dehydrogenase, prokaryotes	Metabolism	Energy metabolism	**0.49 ± 0.15**	0.09 ± 0.11	0.01
M00115	NAD biosynthesis, aspartate => NAD	Metabolism	Metabolism of cofactors and vitamins	0.2 ± 0.12	**0.68 ± 0.23**	0.02
Cool vs. Warm (colonized tadpoles)						
KEGG module ID	**KEGG module name**	**KEGG pathway 1**	**KEGG pathway 2**	**Cool frequency (%)**	**Warm frequency (%)**	**FDR P-value**
M00238	D-Methionine transport system	Environmental Information Processing	Membrane transport	0.08 ± 0.07	**0.39 ± 0.05**	0.01
M00360	Aminoacyl-tRNA biosynthesis, prokaryotes	Genetic Information Processing	Translation	3.75 ± 1.07	**7.1 ± 0.62**	0.03
M00019	Valine/isoleucine biosynthesis, pyruvate => valine / 2-oxobutanoate => isoleucine	Metabolism	Amino acid metabolism	**1.34 ± 0.39**	0.19 ± 0.23	0.03
M00036	Leucine degradation, leucine => acetoacetate + acetyl-CoA	Metabolism	Amino acid metabolism	**0.33 ± 0.1**	0.06 ± 0.06	0.05

Appendix Table 11 (Continued)

M00016	Lysine biosynthesis, succinyl-DAP pathway, aspartate => lysine	Metabolism	Amino acid metabolism	0.45 ± 0.18	**1 ± 0.1**	0.03
M00525	Lysine biosynthesis, acetyl-DAP pathway, aspartate => lysine	Metabolism	Amino acid metabolism	0.37 ± 0.1	**0.85 ± 0.09**	0.01
M00022	Shikimate pathway, phosphoenolpyruvate + erythrose-4P => chorismate	Metabolism	Amino acid metabolism	0.22 ± 0.16	**0.66 ± 0.12**	0.05
M00133	Polyamine biosynthesis, arginine => agmatine => putrescine => spermidine	Metabolism	Amino acid metabolism	0.09 ± 0.1	**1.14 ± 0.24**	0.02
M00009	Citrate cycle (TCA cycle, Krebs cycle)	Metabolism	Carbohydrate metabolism	**2.81 ± 0.37**	0.48 ± 0.26	<0.01
M00011	Citrate cycle, second carbon oxidation, 2-oxoglutarate => oxaloacetate	Metabolism	Carbohydrate metabolism	**2.33 ± 0.15**	0.45 ± 0.21	<0.01
M00010	Citrate cycle, first carbon oxidation, oxaloacetate => 2-oxoglutarate	Metabolism	Carbohydrate metabolism	**0.56 ± 0.18**	0.06 ± 0.09	0.04
M00013	Malonate semialdehyde pathway, propanoyl-CoA => Acetyl-CoA	Metabolism	Carbohydrate metabolism	**0.31 ± 0.06**	0 ± 0	0.02
M00001	Glycolysis (Embden-Meyerhof pathway), glucose => pyruvate	Metabolism	Carbohydrate metabolism	1.07 ± 0.27	**2.04 ± 0.38**	0.05
M00002	Glycolysis, core module involving three-carbon compounds	Metabolism	Carbohydrate metabolism	0.7 ± 0.28	**1.94 ± 0.39**	0.03
M00061	Uronic acid metabolism	Metabolism	Carbohydrate metabolism	0.13 ± 0.14	**1.58 ± 0.37**	0.03
M00275	PTS system, cellobiose-specific II component	Metabolism	Carbohydrate metabolism	0.1 ± 0.1	**1.01 ± 0.26**	0.03
M00270	PTS system, trehalose-specific II component	Metabolism	Carbohydrate metabolism	0.01 ± 0.02	**0.24 ± 0.08**	0.05
M00310	Pyruvate:ferredoxin oxidoreductase	Metabolism	Carbohydrate metabolism	0.01 ± 0.02	**0.41 ± 0.1**	0.03
M00268	PTS system, arbutin-like II component	Metabolism	Carbohydrate metabolism	0.01 ± 0.01	**0.51 ± 0.17**	0.05
M00266	PTS system, maltose and glucose-specific II component	Metabolism	Carbohydrate metabolism	0 ± 0.01	**0.35 ± 0.09**	0.03
M00149	Succinate dehydrogenase, prokaryotes	Metabolism	Energy metabolism	**0.6 ± 0.1**	0.06 ± 0.07	0.01
M00151	Cytochrome bc1 complex respiratory unit	Metabolism	Energy metabolism	**0.46 ± 0.13**	0.02 ± 0.02	0.03
M00150	Fumarate reductase, prokaryotes	Metabolism	Energy metabolism	0.03 ± 0.03	**0.22 ± 0.07**	0.05
M00173	Reductive citric acid cycle (Arnon-Buchanan cycle)	Metabolism	Energy metabolism	**2.33 ± 0.33**	0.94 ± 0.39	0.03

M00165	Reductive pentose phosphate cycle (Calvin cycle)	Metabolism	Energy metabolism	0.56 ± 0.2	**1.17 ± 0.2**	0.04
M00060	Lipopolysaccharide biosynthesis, KDO2-lipid A	Metabolism	Glycan metabolism	0.16 ± 0.1	**1.15 ± 0.34**	0.04
M00121	Heme biosynthesis, glutamate => protoheme/siroheme	Metabolism	Metabolism of cofactors and vitamins	0.59 ± 0.19	**2.77 ± 0.55**	0.03
M00122	Cobalamin biosynthesis, cobinamide => cobalamin	Metabolism	Metabolism of cofactors and vitamins	0.11 ± 0.08	**0.65 ± 0.18**	0.03
M00096	C5 isoprenoid biosynthesis, non-mevalonate pathway	Metabolism	Metabolism of terpenoids and polyketides	0.56 ± 0.4	**1.9 ± 0.32**	0.03
M00050	Guanine ribonucleotide biosynthesis IMP => GDP,GTP	Metabolism	Nucleotide metabolism	0.49 ± 0.27	**1.79 ± 0.42**	0.03
M00546	Purine degradation, xanthine => urea	Metabolism	Nucleotide metabolism	0.07 ± 0.06	**0.52 ± 0.11**	0.02

Appendix D Investigating the use of fluid-preserved museum specimens for gut microbiome analyses

Appendix D.1 Background

As described throughout this dissertation, animal host-associated microbial communities play integral roles in shaping their host's physiology and responses to the external environment. Thus, understanding the composition and function of these communities should be of general interest to biologists working to understand the ecology and evolution of their study species. However, garnering this information ultimately relies on the ability to collect representative samples from the host species of interest. Currently, gut microbiomes can be analyzed from feces, cloacal/anal swabs of live animals or after euthanasia and dissection of the intestinal tract. These processes require location and/or sacrifice of the animal, which, depending on its rarity or protected status, may be difficult or impossible. Additionally, these methods only provide a snapshot of the gut microbial community at a single timepoint and therefore are limited in their ability to analyze changes over long time periods.

Museum specimens represent a potentially untapped resource for microbiome studies. With an estimated 3 billion specimens representing 2 million species in storage worldwide, natural history museums are rich repositories of biological diversity (Yeates et al. 2016). Specimens in which the whole animal is fluid preserved could be used to sample gut contents, circumventing some of the methodological challenges we face in cataloging gut microbiome diversity. However, it is unclear how museum storage changes the original gut microbial community, and thus, if this method is a reliable way to obtain samples. Previous studies have shown that storage length

(maximum investigated <2 months), and the medium of sample storage, such as ethanol or nucleic acid stabilizing reagents, affect aspects of sample integrity, including DNA concentration and microbial community composition (Vlckova et al. 2012, Hale et al. 2015). However, despite the effects of sample storage, individual and host species identity tend to be the most significant factors shaping the gut microbial community (Carroll et al. 2012, Song et al. 2016) .

A few studies have attempted to understand the specific effects of museum fluid preservation on host gut microbial communities (Heindler et al. 2018, Bodawatta et al. 2020, Greiman et al. 2020, Chalifour et al. 2022). However, there have been several drawbacks to these studies. First, most focus on a single host species, and thus, it is unclear whether or not the observed changes in the microbiome due to fluid preservation are minimal compared to differences in the microbiome observed across species, or if these changes override species-specific effects. Second, these studies compare only fresh-caught contemporary specimens to specimens preserved in museums that were captured during earlier time periods. Thus, it is impossible to know if differences in the microbiome between these groups are due to fluid preservation alone or due to real microbiome differences owing to changes over time or across populations.

Throughout my PhD, I have been working on a large side project to address these issues and more systematically investigate the use of fluid preserved museum specimens for microbiome analyses. Specifically, I am comparing gut microbial communities between fresh animals and those fluid preserved of the same species that I collected simultaneously from the same population, across a variety of vertebrate host taxa.

Appendix D.2 Methods in brief

During the summer of 2018, I collected 12-20 individuals each of 11 host species from the wild in western Pennsylvania (Appendix Table 12). Animals were collected from two field site locations: Pymatuning Laboratory of Ecology (Linesville, PA) or Powdermill Nature Reserve (Rector, PA). For each species, half of the individuals were dissected immediately upon capture to collect a fresh sample from the hindgut for gut microbiome analyses. These specimens are called "fresh". The other half of the individuals were fluid preserved in 10% formalin for 24 hours and then transferred to 70% EtOH for long term storage. The specimens remained fluid preserved for one year and then were dissected as described for fresh specimens to collect a gut microbiome sample. These specimens are called "preserved".

Appendix Table 12 List of study species and abbreviations used to denote them.

Fish	Bluegill, *Lepomis macrochirus* (LM) Yellow perch, *Perca flavescens* (PF)
Amphibians	American toad, *Anaxyrus americanus* (AA) Dusky salamander, *Desmognathus ochrophaeus* (DO) Green frog, *Lithobates clamitans* (LC) Eastern newt, *Notophthalmus viridescens* (NV) Red-backed salamander, *Plethodon cinereus* (PC) Slimy salamander, *Plethodon glutinosus* (PG)
Reptiles	Painted turtle, *Chrysemys picta* (CP) Garter snake, *Thamnophis sirtalis* (TS)
Mammals	Deer mouse, *Peromyscus maniculatus* (PE)

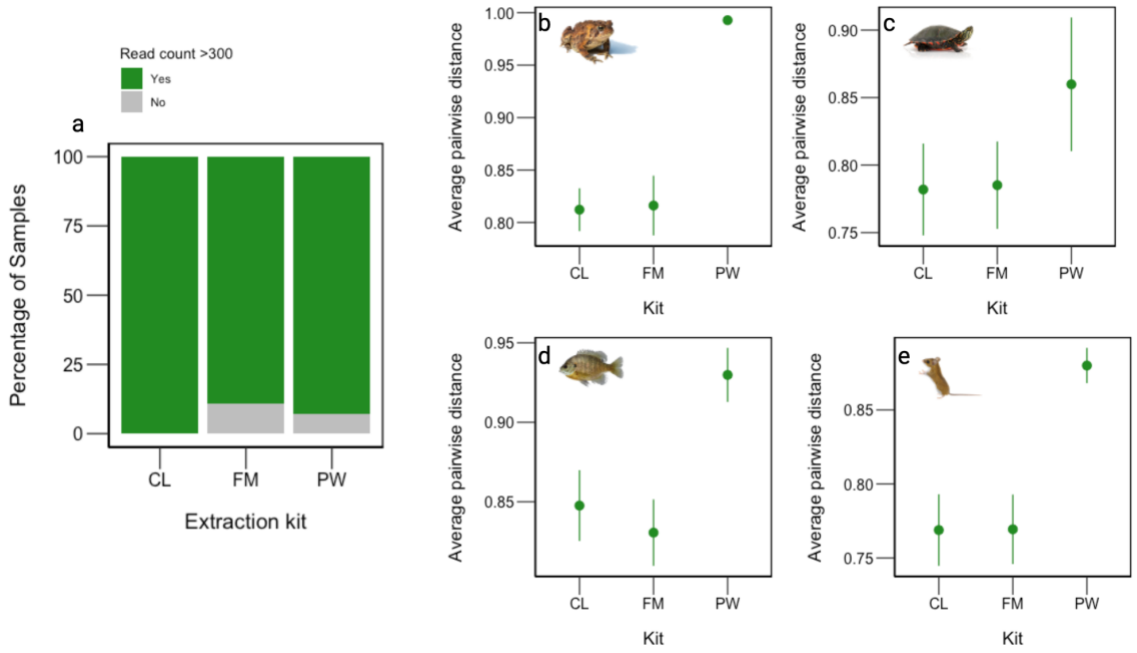
Because formalin can cause significant DNA damage, I conducted a preliminary analysis to determine an optimal method of DNA extraction for fluid preserved specimens. In a subset of species, one from each vertebrate class (bluegill, toad, turtle, and mouse), I split all samples (fresh and preserved) into three equal sections from which I then extracted DNA using three commercial DNA extraction kits from QIAGEN: PowerFecal DNA Kit (abbreviated PW, typically used to extract microbiome DNA from fecal samples), DNA FFPE Tissue Kit (abbreviated FM, designed to extract DNA from formalin-fixed paraffin-embedded medical tissue samples), and the DNA FFPE Tissue Kit + the DNeasy PowerClean Pro Cleanup Kit (abbreviated CL, removes PCR inhibitors from DNA samples). Bacterial 16S rRNA gene amplicons were then sequenced from DNA extracted from all samples with these three kits to determine which kit returned the most samples that were deemed usable (acceptable number of reads returned) and returned microbial communities that were most similar to fresh samples extracted with the PW kit (standard field practice).

After determining the most successful DNA extraction method (CL kit), I extracted DNA from all samples using this method and sequenced bacterial 16S rRNA gene amplicons to analyze gut microbial communities. The ultimate goal of the study is to determine the effects of both fluid preservation and host species identity on the microbiome.

Appendix D.3 Preliminary results

Appendix D.3.1 DNA extraction method

I determined that the CL DNA extraction method was the most reliable to extract DNA from fluid preserved specimens. This kit was the only kit that returned >300 sequences per sample, which we deemed adequate to use for gut microbiome analyses (Appendix Figure 15a, chi-square $p < 0.01$). Further, when comparing the resulting microbial communities, preserved samples extracted with the CL and FM kits returned communities that were significantly more similar to fresh samples extracted with the PW kit than preserved samples extracted with the PW kit in three species (toads, fish, and mice, Appendix Figure 15b,d,e, ANOVA $p < 0.05$ for all). In turtles, there was a similar trend, but no significant difference across the three kits (Appendix Figure C). Based on these data, the rest of the samples were extracted with the CL kit.

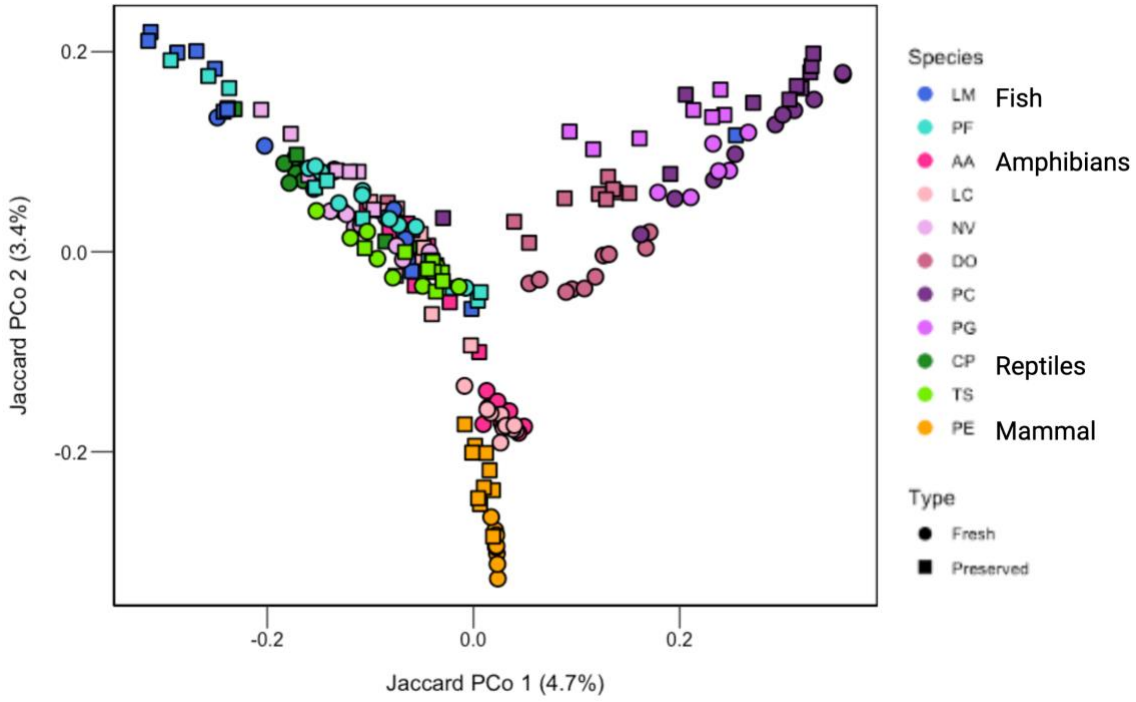


Appendix Figure 15 The CL DNA extraction kit is most reliable to extract DNA from preserved specimens. (a) The percent of samples that returned greater than 300 sequences across each extraction kit. The average pairwise distance between preserved samples extracted with each extraction kit to fresh samples of the same species extracted with the PW kit in (b) toads, (c) turtles, (d) fish, and (e) mice based on Bray-Curtis distance beween samples.

Appendix D.3.2 Comparison of gut microbial communities between fresh and preserved samples across all species

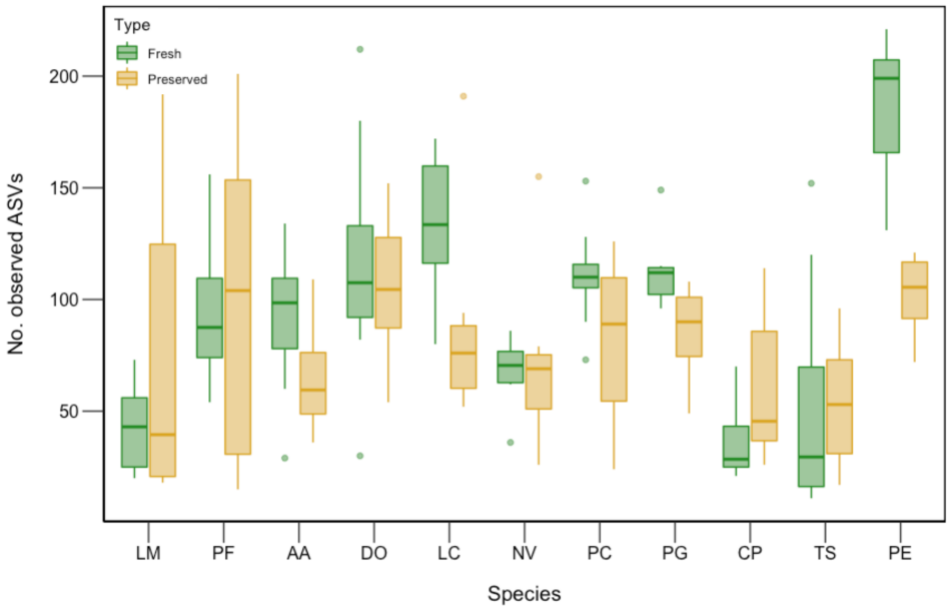
After extracting all samples using the CL kit and sequencing bacterial 16S rRNA gene amplicons, I have currently been analyzing the data to understand how both fluid preservation and host species impact the gut microbial community. I have found that each of these variables, and their interaction impacts microbial community composition (Appendix Figure 16, PERMANOVA $p = 0.001$ for each). However, based on effect sizes, the impact of species identity is stronger than the effect of preservation (F species = 3.94, F preservation = 2.31). When comparing alpha diversity based on the number of observed bacterial ASVs in samples, there was a significant

interaction between host species and preservation type (ANOVA, $p < 0.05$), such that only within mice was there a significant difference in diversity between fresh and preserved specimens (Appendix Figure 17). Thus far, these results suggest that fluid preservation of specimens does alter the gut microbial community, but species-specific differences in the microbiome are still retained. Analyses are currently ongoing and will further explore the impact of these variables on the microbiome using taxa relative abundance analyses, random forest analyses, and mantel tests.



Appendix Figure 16 Comparison of gut microbial communities based on host species identity and preservation.

Principal Coordinate analysis plot shows microbiome distance between samples based on a Jaccard distance matrix. Percentages represent the proporation of variation explained by each axis. Colors represent host species and shapes represent preservation type. Host species are identified by the abbreviated letters denoted in Appendix Table 12.



Appendix Figure 17 The impact of host species identity and preservation on alpha diversity of the gut microbial community.

Alpha diversity is measured as the number of observed bacterial ASVs in the gut microbiome. Host species are identified by the abbreviated letters denoted in Appendix Table 12.

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