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The Aquatic Microbial Environment Shapes the Gut Microbiota, Brain, and Behavior of Larval Amphibians

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THE AQUATIC MICROBIAL ENVIRONMENT SHAPES THE GUT MICROBIOTA, BRAIN, AND BEHAVIOR OF LARVAL AMPHIBIANS

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Kyle John Emerson

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ABSTRACT

THE AQUATIC MICROBIAL ENVIRONMENT SHAPES THE GUT MICROBIOTA, BRAIN, AND BEHAVIOR OF LARVAL AMPHIBIANS

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Dissertation supervised by Sarah K. Woodley

Microbial communities comprising bacteria, viruses, fungi, and protists live within and on the surfaces of animal hosts. These microbial communities exist in symbiosis with the host, and heavily influence host physiology, development, health, and fitness. Gut-dwelling microbes (i.e., gut microbiota) contribute to host neurodevelopment through a bidirectional Microbiota-Gut-Brain (MGB) axis. Evidence of the MGB axis has been primarily derived from studies that use germ-free (GF) models, which commonly display altered neurophysiology and behavior compared to conventionally raised counterparts. Almost all studies of the MGB axis have used mammalian models in a biomedical framework, leaving a knowledge gap regarding the role of the gut microbiota in neurodevelopment and behavior in non-mammalian animals and in more ecological contexts. The goal of my dissertation was to evaluate how the aquatic microbial environment influences the biodiversity of the amphibian gut microbiota, and how these shifts in the microbiota influence amphibian neurodevelopment and behavior. Findings of associations

between the gut microbiota and amphibian neurodevelopment and behavior are consistent with the presence of a MGB axis and can offer insight as to whether particular aspects of the gut microbiota (i.e., diversity and taxa abundance) were significantly associated with tadpole development and physiology. First, I tested whether manipulation of the gut microbiota affected the brain development of Green Frog (*Lithobates clamitans*) tadpoles. Tadpoles were raised in natural (unmanipulated) pond water or autoclaved pond water at three different water temperatures: 14, 22 and 28°C. Autoclaving reduced the number of aquatic microbes available to colonize the tadpole and thereby resulted in a gut microbiota with reduced microbial biodiversity and altered community composition compared to tadpoles raised in natural pond water. Both temperature and the aquatic microbial community during development affected tadpole brain shape, and the biodiversity of the tadpole gut microbiota was negatively associated with the size of the optic tectum. These results provide some of the first evidence of the MGB axis in amphibians. Next, I examined an additional species and incorporated behavioral assays to evaluate potential functional consequences of gut microbial manipulation. To do this, I raised Northern Leopard Frog (*Lithobates pipiens*) tadpoles in natural or autoclaved pond water. Compared to tadpoles raised in natural pond water, tadpoles raised in autoclaved pond water: (1) had altered gut microbial community composition and decreased biodiversity, (2) had decreased locomotory responses to visual stimuli, (3) had relatively heavier brains and altered brain shape, and (4) were larger and developed faster. Additionally, the composition and diversity of the gut microbiota was a significant predictor of tadpole brain size, brain shape, and locomotory behavior. Finally, I tested whether exposure to both a depleted aquatic microbial community and an ecological stressor had interactive effects on physiological and neurodevelopmental endpoints in tadpoles. In mammals, the composition of the gut microbiota, and subsequently the

development of the MGB axis, is shaped by stressors. Due to frequent exposure of wildlife to relevant ecological stressors such as predation, examining the role of stressors on the formation of the gut microbiota and subsequent physiological and neurodevelopmental endpoints is of interest. I exposed tadpoles to predation-derived chemical cues, exogenous corticosterone (CORT), or a vehicle control. I simultaneously exposed tadpoles to either natural or autoclaved pond water. I found no clear effects of predator cues, but tadpoles exposed to CORT had altered composition of their gut microbiota, altered brain shape, and altered tail shape compared to control. Additionally, I replicated previous results by finding that tadpoles raised in autoclaved pond water were larger, had altered brain development, and a dramatically altered gut microbiota that predicted several neurodevelopmental endpoints. Encouragingly, tadpoles raised in autoclaved pond water also displayed reduced ability to evade predators, which likely impacts fitness and survival. I found surprisingly few interactive effects of the aquatic microbial community and stressors. Overall, my dissertation work provides novel evidence of the MGB axis in larval amphibians. My work highlights the importance of the promotion and maintenance of freshwater ecosystem health, as aquatic microbial communities present in these waters are shown to have dramatic and consistent impacts on amphibian body size, neurodevelopment, and fitness-related behaviors.

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Chapter 1. Introduction

1. Overview

It has long been understood that all vertebrates live and develop in a microbially-dominated world. From birth, vertebrates are exposed to an innumerable number of colonizing microbes (i.e., bacteria, fungi, viruses, etc.) that form dense communities on them. Relatively recent advancements in genomic and metagenomic sequencing have continually improved our understanding of how these microbial communities participate in symbiotic relationships with vertebrates, which ultimately shape the evolution of both vertebrates and microbes in these symbiotic systems (see comprehensive review; (Perreau and Moran, 2022). The densest area of microbial colonization occurs in the gastrointestinal (GI) tract where these communities are comprised of around 10^{13} microbes in humans, and novel microbe species are continually being discovered (Leviatan et al., 2022; Sender et al., 2016). These communities, collectively referred to as the gut microbiota, are now recognized as integral pieces of vertebrate health, development, and fitness due to the various physiological roles they play within the host.

Host-microbe symbioses and their roles in animal physiology are becoming increasingly characterized and more well-defined (Gilbert et al., 2012; Ma et al., 2023; McFall-Ngai et al., 2013; McFall-Ngai, 2015; Peixoto et al., 2021). A primary function of the gut microbiota is to aid in digestion, by which microbes synthesize enzymes not encoded for within the host's genome and can ferment otherwise inaccessible carbohydrates (Fu et al., 2022). Enzymolysis of these dietary substances produces short-chain fatty acids (SCFAs) and other important metabolites (Fu et al., 2022; Koh et al., 2016) that influence numerous aspects of animal physiology, as many host cells express receptors and transporters that recognize and respond to these products (Guo et al., 2022; Kohl and Carey, 2016; Natarajan and Pluznick, 2014). As

discussed in a comprehensive review focusing on host-microbe symbiosis in both domesticated and wild animals (Peixoto et al., 2021), the microbiota has been implicated in influencing animal metabolism, digestive efficiency and food conversion, disease resistance and immune function, early life development and growth rates, adaptations to seasonal and environmental shifts, neuroendocrine function, and many others.

Recent and exciting work has begun to describe the imperative role of the gut microbiota in the establishment and function of the vertebrate nervous system. In addition to SCFA production, gut microbes also directly synthesize or coordinate the secretion of neuroactive compounds and metabolites, including neurotransmitters and neurotransmitter precursors, secondary bile acids, and other neuroactive compounds (Cowan et al., 2020; Cryan et al., 2019; Guo et al., 2022; Yano et al., 2015). These microbially-derived neuroactive compounds are able to communicate with the brain through many pathways, including via stimulation of nerves innervating the GI tract, the circulatory and immune systems, and various metabolic and neuroendocrine pathways (Cowan et al., 2020; Cryan et al., 2019; Guo et al., 2022); this bidirectional axis of communication is denoted as the microbiota-gut-brain (MGB) axis.

To better understand the role of the gut microbiota on neurodevelopment, many studies use germ-free (GF) animals, often times domesticated mammals. As discussed in recent reviews (Cowan et al., 2020; Cryan et al., 2019; Guo et al., 2022), GF animals commonly exhibit alterations in their neurophysiology, neuroimmunity, and behavior when compared to conventionally raised counterparts, including: alterations in neurogenesis in specific brain structures, altered dendritic growth, axon myelination, and synaptic connectivity, increased permeability of the Blood-Brain-Barrier, immature microglia cells, and alterations in several behavioral responses to various stimuli. Additionally, GF animals exhibit altered anxiety-like

behaviors and stress reactivity, likely due to a dysregulation of the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis (see comprehensive reviews; (Cowan et al., 2020; Cryan et al., 2019; Rusch et al., 2023). Specifically, GF mice exhibit hypersensitivity and altered behavioral responses to stressors, which have been associated with reductions in hippocampal NMDA and 5-HT_{1A} receptor mRNA expression, altered release rates and production of corticotropin releasing factor (CRF), adrenocorticotropic hormone (ACTH), and corticosteroids, and under expressed genes encoding for glucocorticoid receptors (Cowan et al., 2020; Cryan et al., 2019; Neufeld et al., 2011; Rusch et al., 2023; Sudo et al., 2004). Taken together, this seminal work mainly conducted in GF animals implicates gut microbes in coordinating the HPA/I axis and underscores how these microbes influence host neurodevelopment and the potential onset of neurological disorders (Cowan et al., 2020; Cryan et al., 2019; Rusch et al., 2023). While GF animals have advanced our understanding of host-microbe interactions, GF conditions are highly artificial and can be difficult to maintain (Kohl and Carey, 2016). Additionally, as the majority of microbiome studies take place in domesticated mammals, it has created a pertinent knowledge gap regarding host-microbe interactions in non-mammalian groups and also non-domesticated species (Colston and Jackson, 2016; Pascoe et al., 2017).

My dissertation explored host-microbe interactions relevant to the development of the MGB axis in understudied wildlife animals, specifically larval amphibians. To my knowledge, the amphibian MGB axis had not been examined prior to my work, and even the gut microbiota itself has been understudied in amphibians. Larval amphibians are useful models because they can be studied in controlled laboratory environments as well as in more ecologically relevant field experiments. Larval amphibians are commonly used to investigate vertebrate physiological

development, and findings in regards to their development have applicability to other vertebrates (Buchholz, 2015).

In most amphibians, the host-associated microbiota forms at hatching, a few days after fertilization, such that the vast majority of development is potentially modulated by host-microbe interactions. In mammals, the majority of development occurs in a sterile or microbially depauperate uterine environment. At birth, microbes are vertically transmitted from the mother to the newborn, whose microbiota resembles the maternal skin and vaginal microbiota (Arrieta et al., 2014; Perreau and Moran, 2022). Vertical transmission of microbes also occurs in amphibians, where females transmit diverse bacterial communities onto egg clutches (Hughey et al., 2017). However, amphibian development occurs completely externally in freshwater ecosystems that can drastically vary in aquatic microbial composition based on geography and local factors, including contaminants (Stocker et al., 2024). These aquatic microbial communities present in these waters represent a primary colonizing source and determinant of the amphibian microbiota (Correa et al., 2020), of which amphibians will be exposed to until metamorphosis or death.

Here, I focus on newly hatched larval amphibians because hatching represents a critical developmental window where the microbial colonization process at this time appears to shape the gut microbial community across ontogeny (Arrieta et al., 2014; Cox et al., 2014; Warne et al., 2017; Warne et al., 2019). At this developmental period, the gut microbiota is at its most susceptible to change, and significant hinderances to the colonization process or absence of critical symbiotic microbes can have potentially life-lasting effects on host immunity, disease resistance, development, and physiological and metabolic function (Arrieta et al., 2014; Cox et al., 2014; Warne et al., 2017; Warne et al., 2019).

By manipulating the aquatically available microbes at hatching, it is possible to non-invasively manipulate the composition of the larval amphibian microbiota and examine host physiology related to the brain and behavior. To manipulate the larval amphibian microbiota, I adapted methods used by recent studies whereby newly hatched tadpoles were raised in unmanipulated natural pond water or autoclaved pond water (Fontaine et al., 2022; Knutie et al., 2017). Development in autoclaved pond water serves as an experimental sterilization technique that manipulates the composition of the larval amphibian microbiota by reducing the abundance and diversity of colonizing aquatic microbes (Fontaine et al., 2022; Knutie et al., 2017). In these studies, tadpoles raised in autoclaved pond water displayed altered gut microbial community composition and reductions in community biodiversity compared to those raised in natural pond water. Additionally, these alterations in microbial community structure were associated with drastic shifts in tadpole phenotypes, including: increased body size, developmental rates, and reduced thermal tolerance under heat stress (Fontaine et al., 2022), as well as reduced resistance to parasitic worms (Knutie et al., 2017). Below, I discuss how I used a similar approach to investigate host-microbe relationships with an emphasis on the development of the MGB axis in larval amphibians.

Specific Aim 1: Temperature and the microbial environment alter brain morphology in a larval amphibian.

In Aim 1, I collaborated with Dr. Kevin Kohl and his PhD student Dr. Samantha Fontaine to study the aquatic microbial influence on neurodevelopment and potential evidence of the MGB axis. Fontaine et al. (2022) showed that newly hatched Green Frog (*Lithobates clamitans*) tadpole development in autoclaved pond water led to alterations to the composition of gut microbial communities and reductions in the biodiversity of these communities compared to

tadpoles raised in natural pond water (Fontaine et al., 2022). She also found that exposure to varying ambient temperatures that reflect ecologically relevant shifts in the global climate led to shifts and reduced biodiversity of gut microbial communities when compared to tadpoles raised in cooler temperatures (Fontaine et al., 2022). Tadpoles with reduced gut microbial biodiversity experienced reductions in acute thermal tolerance, altered metabolic rates, and reduced survivability under prolonged heat stress, demonstrating how host-microbe relationships can influence ectotherm physiology and fitness in response to climate change (Fontaine et al., 2022).

My role was to assess whether exposure to different aquatic microbial communities and ambient temperature, and the ensuing alterations to gut microbial communities, influenced other aspects of larval tadpole physiology, including the establishment of the MGB axis. I tested the hypothesis that the aquatic microbial environment and water temperature would influence relative brain size and morphology, potentially through the MGB axis. If the gut microbiota is influencing aspects of tadpole neurodevelopment, I also predicted that the composition and diversity of tadpole gut microbial communities would be significantly associated with changes in relative brain size and morphology.

Specific Aim 2: Something in the water: aquatic microbial communities influence the larval amphibian gut microbiota, neurodevelopment, and behaviour.

GF development has been associated with altered neurophysiology and neurogenesis of specific brain structures, but the functional consequences of these changes are not fully known (Cowan et al., 2020; Cryan et al., 2019). Studies investigating the MGB axis in GF models have evaluated vertebrate behavioral responses and found altered locomotory, exploratory, and anxiety-like behaviors compared to conventionally raised counterparts (Cryan et al., 2019; Davis et al., 2016; Heijtz et al., 2011). By evaluating behavior in addition to other parameters of

neurophysiology, researchers can gain a better understanding of how host-microbe interactions influence animal fitness and performance.

In this aim, I raised newly hatched Northern Leopard Frog (*L. pipiens*) in natural and autoclaved pond water to accomplish two primary goals: (1) test if the effects of pond water treatment on tadpole gut microbial communities, physiology, and neurodevelopment are reproducible in a different species, and (2) expand our evidence of the MGB axis by investigating tadpole performance during behavioral assays. I hypothesized that the aquatic microbial community would influence the developing tadpole gut microbiota, physiological development, neurodevelopment, and behavior. Compared to tadpoles raised in natural pond water, I predicted that tadpoles raised in autoclaved pond water would have altered brain size and shape and would exhibit differences in locomotory behavior when exposed to sensory stimuli. If the gut microbiota is influencing aspects of tadpole neurodevelopment and behavior, I also predicted that the composition and diversity of tadpole gut microbial communities would be significantly associated with changes in brain morphology and locomotory responses during behavioral assays.

Specific Aim 3: Effects of stress and the aquatic microbial environment on the microbiota, morphology, physiology, and behavior of larval amphibians.

The composition of the vertebrate microbiota, especially in early life, is likely heavily influenced by exposure to ecological stressors. In mammals, exposure to stressors triggers the activation of the HPA axis and increased circulation of glucocorticoids (GCs) that serves to mediate the effects of the stressor (Denver, 2009; Sapolsky et al., 2000). It is hypothesized that increased circulation of GCs exerts changes in physiology that can alter the habitability of a host for commensal microbes (Söderholm et al., 2002). Further, higher concentrations of GC

metabolites present within the body or feces is associated with the loss of gut microbial biodiversity in mammals (Huang et al., 2015; Petrullo et al., 2022), which is thought to be a fitness disadvantage (Stecher et al., 2010).

As described in Aim 1, increasing temperatures that fall within ecologically relevant ranges attributed to global climate change did alter the community composition and reduced the biodiversity of tadpole gut microbial communities, and reduction in the biodiversity of the microbiota was associated with reduced thermal tolerance and survival under heat stress (Fontaine et al., 2022). This novel work is some of the first to describe how ecological stressors influence the composition of the amphibian microbiota and subsequent physiology and neurodevelopment (Emerson et al., 2023; Fontaine et al., 2022). As whole-body CORT and other parameters of the vertebrate stress response were not evaluated in this study, it leaves questions as to the relationship between stressors, the HPA/I axis, and the host-associated microbiota in wildlife animals.

In this aim, I evaluated how prolonged exposure to a stressor influences host-microbe relationships in a tadpole model, serving as an ecologically relevant scenario that is well suited to test development, performance, fitness, and survival. To do this, I raised newly hatched Northern Leopard Frog (*L. pipiens*) tadpoles in pond water treatment conditions described above, and simultaneously exposed them to one of 3 stressor treatments: (1) predation-derived chemical cues; (2) exogenous CORT at physiologically relevant doses; (3) vehicle control. Predation-derived chemical cues have been used in previous experiments to simulate predator exposure which effect circulating levels of whole-body CORT as well as tadpole physiology and behavior (Fraker, 2008; Fraker et al., 2021; Middlemis Maher et al., 2013; Relyea, 2001). By maintaining pond water

treatments, I can evaluate if the effects of the aquatic microbial community on tadpoles persist during exposure to an ecologically relevant stressor.

I predicted that exposure to predation-derived chemical cues and exogenous CORT would alter the composition and diversity of the associated microbiota, as well as shape tadpole size, body and brain morphology, and behavior compared to tadpoles exposed to a vehicle control. Additionally, as the host-associated microbiota has been hypothesized to coordinate the HPA/I axis, circulating levels of GCs, and vertebrate stress responses (Cowan et al., 2020; Cryan et al., 2019; Davis et al., 2016; Heijtz et al., 2011), I predicted that simultaneous development in a depleted aquatic microbial community and exposure to stressors will have interactive effects on our endpoints of interest.

Chapter 2. Temperature and the microbial environment alter brain morphology in a larval amphibian

Reprinted from Journal of Experimental Biology Vol 226, Kyle J. Emerson, Samantha S. Fontaine, Kevin D. Kohl, and Sarah K. Woodley, Temperature and the microbial environment alter brain morphology in a larval amphibian., Copyright (2023), with permission.

ABSTRACT

Understanding how the global climate impacts the physiology of wildlife animals is of importance. Amphibians are particularly sensitive to climate change, and it is hypothesized that rising temperatures impair their neurodevelopment. Temperature influences the composition of the gut microbiota, which is critical to host neurodevelopment through the microbiota-gut-brain (MGB) axis. Most research investigating the link between the gut microbiota and neurodevelopment occurs in germ-free mammalian model systems, leaving the nature of the MGB axis in non-mammalian wildlife unclear. Here, we tested the hypothesis that the temperature and the microbial environment in which tadpoles were raised shapes neurodevelopment, possibly through the MGB axis. Newly hatched Green Frog tadpoles (*Lithobates clamitans*) were raised in natural pond water or autoclaved pond water, serving as an experimental manipulation of the microbiota by reducing colonizing microbes, at three different water temperatures: 14°C, 22°C, and 28°C. Neurodevelopment was analyzed through measures of relative brain mass and morphology of brain structures of interest. We found that tadpole development in warmer temperatures increased relative brain mass and optic tectum width and length. Further, tadpole development in autoclaved pond water increased relative optic tectum width and length. Additionally, the interaction of treatments altered relative diencephalon length. Lastly, we found that variation in brain morphology was associated with gut microbial diversity and the relative abundance of individual bacterial taxa. Our results indicate that both

environmental temperature and microbial communities influence relative brain mass and shape. Furthermore, we provide some of the first evidence for the MGB axis in amphibians.

INTRODUCTION

As the global climate continues to change, understanding how ecologically relevant shifts in temperature affect wildlife animal physiology remains pertinent. Perhaps no vertebrate group has been challenged by climate change more than ectotherms such as amphibians, as their biodiversity and global populations have been exponentially decreasing (Pounds et al., 2006). Temperature is particularly relevant for amphibians, as their body temperature largely tracks ambient temperatures. Fluctuating ambient temperatures modulate numerous aspects of amphibian physiology, including metabolic rate, developmental rate, locomotion, and digestive efficiency (Feder and Burggren, 1992; Fontaine et al., 2018; Gillooly et al., 2002; Huey and Stevenson, 1979). Interestingly, a literature review posited that rising temperatures stimulate neuronal activity in ectotherm brains up to a thermal threshold, beyond which it is detrimental to neuron development and nervous tissue (Beltrán et al., 2021). However, many of these temperature effects on neurodevelopment are likely species-dependent and we currently lack a complete understanding of how ecologically relevant increases in temperature influence brain development across ectotherms (Beltrán et al., 2021).

Environmental temperature impacts the diversity and composition of host-associated microbial communities harbored by both aquatic and terrestrial amphibians, which can impact their physiology (Fontaine and Kohl, 2020; Fontaine et al., 2018; Kohl and Yahn, 2016). A particularly large microbial community comprised of bacteria, archaea, fungi, and protists resides in the gastrointestinal (GI) tract. Called the gut microbiota, this host-associated microbial community participates in a symbiotic relationship with the host and impacts host performance,

health, and fitness (Kohl and Carey, 2016). In species with external fertilization, like most amphibians and fish, the gut microbiota is likely colonized by microorganisms present in their aquatic habitat immediately upon hatching, just a few days after fertilization (Correa et al., 2020). Although the composition of the gut microbiota is malleable throughout development, its trajectory throughout ontogeny is heavily dependent on these initial microbial colonizers (Litvak and Bäumler, 2019; Martínez et al., 2018; Warne et al., 2019). Manipulation of the gut microbiota in early life through exposure to antibiotics or sterilized environments impacts host growth and immunity (Knutie et al., 2017; Warne et al., 2019). Often, these impairments can persist throughout life, emphasizing the importance of the gut microbial colonization process in vertebrate development (Cox et al., 2014).

Gut microbiomes specifically contribute to the development of the central and peripheral nervous systems through the microbiota-gut-brain (MGB) axis (Foster and McVey Neufeld, 2013; Perry et al., 2016). Gut microbes produce molecules such as neurotransmitters, short chain fatty acids, secondary bile acids and peptidoglycans that can stimulate peripheral sensory neurons of the vagus nerve, the enteric nervous system, and the spinal cord (see comprehensive review, (Cryan et al., 2019). Further, the gut microbiota and its products can also modulate non-neural routes of communication within the MGB axis such as the hypothalamic-pituitary-adrenal (HPA) axis, which coordinates the vertebrate stress response (Cryan et al., 2019).

The importance of the MGB axis is most revealed in studies using germ-free animals that lack a gut microbiota. Germ-free mice exhibit altered locomotory behavior and long-term synaptic gene expression in brain regions involved in motor control and anxiety (Heijtz et al., 2011). In addition, germ-free mice had increased amygdalar and hippocampal volume, which are brain regions implicated in fear and anxiety (Luczynski et al., 2016; Tovote et al., 2015). Of

nonmammalian vertebrates, fish host-microbe interactions are relatively well studied due to their importance in aquaculture, and germ-free zebrafish are a common laboratory-based model. Zebrafish have *ex utero* development which allows investigators to manipulate microbial exposure more easily throughout all stages of development (Davis et al., 2016; Lee et al., 2021). Zebrafish raised in germ-free conditions exhibited altered locomotory and anxiety-like behavior as well as altered stress responses compared to conventionally raised zebrafish, demonstrating a potential link between the microbiota and neurodevelopment (Davis et al., 2016). Additionally, adult zebrafish exposed to beneficial intestinal microbes (i.e., probiotics) exhibited reduced anxiety-like behavior which was accompanied by altered neurotransmitter signaling in the brain (Davis et al., 2016). These effects of intestinal microbes on anxiety and locomotory behavior in fish could potentially regulate feeding behavior and energy homeostasis, which would have significant ramifications on host fitness (Butt and Volkoff, 2019; Xia et al., 2022).

While the use of germ-free mammals and fish has advanced our understanding of host-microbe interactions, study of other vertebrate groups has lagged, especially in more ecological contexts (Colston and Jackson, 2016; Pascoe et al., 2017; Woodhams et al., 2020). For example, correlational evidence for the MGB axis in songbirds was only recently reported (Slevin et al., 2020). Likewise, in amphibians, a few studies have found limited evidence for a link between the gut microbiota and the brain. In captive-bred Chinese Giant Salamanders, exposure to sterile soil from their natural habitat altered gene expression related to energy metabolism in the brain compared to exposure to non-sterile soil (Zhu et al., 2022). In Ranid frogs, exposure to an insecticide caused a shift in the composition of the gut microbiota and altered neurotransmitter production in the gut (Zhang et al., 2023a).

To better understand the role of host microbiota in brain development in amphibians, we tested the hypothesis that the temperature and the microbial environment in which larval amphibians were raised shapes neurodevelopment, possibly through the effects on the MGB axis. To do this, we took advantage of a previous study where newly hatched Green Frog tadpoles (*Lithobates clamitans*) were raised in varying water temperatures. Further, tadpoles were raised in natural pond water, or in pond water that was autoclaved to deplete the environmental microbial community available to colonize the gut (Fontaine et al., 2022). This disruption resulted in tadpole gut bacterial communities that were reduced in diversity and altered in composition, but similar in overall bacterial density, when compared with tadpoles colonized with microbes from natural pond water (Fontaine et al., 2022). Using autoclaved water to manipulate the host-associated microbiota is a less invasive method to reduce the diversity and abundance of colonizing microbes compared to using antimicrobials, which have undesirable effects on host physiology (Fontaine et al., 2022; Knutie et al., 2017; Morgun et al., 2015; Patangia et al., 2022). Additionally, tadpoles raised in autoclaved water still harbor a gut microbial community with indistinguishable bacterial densities compared to those raised in pond water. Thus, our experimental groups compare tadpoles with varying microbial communities, rather than using the highly artificial state of germ-free systems. Specifically, we used the specimens from "Experiment 1," of Fontaine et al., 2022, to examine the brains of tadpoles and test the predictions that 1) water temperature and the local microbial environment would alter relative brain size and morphology, and 2) the gut microbial diversity and composition would predict relative brain size and morphology.

MATERIALS AND METHODS

Here we present brief methodological details of animal husbandry and conditions. For detailed information on collection, rearing, treatment groups, and microbiome sequencing see Fontaine et al., 2022. All animal research was approved by the University of Pittsburgh IACUC (protocol #18062782) and animal collections were permitted by LA Department of Wildlife and Fisheries (Scientific Collecting Permit WDP-19-010).

Animal Information

In May 2019, a male and female adult Green Frog were collected from a pond in Kisatchie National Forest (LA, USA) and transported to the University of Pittsburgh. In September 2019, the frogs were injected with hormones to induce spawning following outlined methods in Trudeau et al. (Trudeau et al., 2010). Embryos were placed in a 16-quart polypropylene tank containing autoclaved laboratory-treated water that was changed daily to ensure proper oxygenation. Tadpoles were fed weekly. Food consisted of three 0.5g blocks of autoclaved rabbit chow suspended in autoclaved agar and supplemented with pet vitamins.

Treatments

Free-swimming tadpoles (Gosner stage 25, (Gosner, 1960)) were raised in either 25% unmanipulated pond water or 25% autoclaved pond water at one of three temperatures described below. The other 75% of water in each treatment consisted of autoclaved laboratory-treated water. Pond water was collected in June 2019 from the same pond from which the adult frogs were collected. Pond water was placed on ice and transported back to the University of Pittsburgh where it was filtered through a 500μm sieve and stored at 4°C until use. Details on the microbial community of stored pond water vs. pond water fresh from the pond are available in Fontaine et al. (2022). Tadpoles were raised in groups of 5 tadpoles in 900 ml of water in a 1 L polypropylene bin. Eighteen bins were filled with the natural microbial environment and 18 were

filled with the autoclaved microbial environment. Water treatments were re-administered during weekly water changes and were maintained for the duration of the experiment.

For the first four weeks of development, tadpoles were raised at 22°C by placing bins in water baths set to 22°C. There were three water baths, each with 12 bins, six each from the previously described microbial water treatments. After four weeks at 22°C, tadpoles were exposed to different acclimation temperatures. To do so, one of the three water baths was decreased to 14°C, and another water bath was increased to 28°C. The third water bath was maintained at 22°C. Tadpoles developed at these temperatures for an additional three weeks.

Each treatment combination (temperature treatment x microbial environment) consisted of six 1L polypropylene bins with five tadpoles per bin, for a total of 30 tadpoles per treatment combination. Final sample sizes were slightly lower than 30 tadpoles due to a small amount of mortality.

Dissections

Seven weeks after the start of the experiment (Nov. 2019), tadpoles were euthanized by immersion in buffered MS-222 (10g/L) and then were weighed and staged (Gosner, 1960). Using sterilized tools, the entire gastrointestinal tract was removed within ten minutes from euthanasia and immediately placed at -80°C. Carcasses were placed in 10% neutral buffered formalin.

In July-August 2020, brains were removed from carcasses using an Olympus SZ61 dissection scope with a camera attachment. Cranial nerves and the spinal cord were trimmed from the brains, brains were weighed, and the dorsal and ventral views of each brain were photographed three times for a total of six images per brain. In between taking each photograph, the brain was repositioned to produce three unique dorsal and ventral images. If damage to the

brain occurred during dissection that could alter brain mass or brain shape measurements, the brain was excluded from analysis. The same investigator conducted all brain dissections and photography.

Tissues are known to shrink with time in fixative, so we ensured that time in fixative was the same among our treatment groups by completing all the weighing and imaging of the brains within 12 days. Furthermore, we processed the samples in blocks that consisted of representatives from each treatment group. Within each block, samples were randomized so that the investigator remained blind to treatments.

Brain Morphometrics

We used geometric morphometrics to evaluate brain shape (Adams et al., 2004). Four linear brain dimensions on each dorsal image and three linear brain dimensions on each ventral image were measured using ImageJ software (US National Institutes of Health, Bethesda, MD) (**Fig. 2.1**). Each linear brain dimension was measured once from each of the three photographs, giving three measurements total, which were averaged to give a single estimate for each brain dimension for each tadpole. The same investigator conducted all measurements.

We measured the length and width of the telencephalon, diencephalon, and optic tectum, and we measured the width of the medulla. The telencephalon is involved in sensory processing, motor output, avoidance learning, and social behavior (Northcutt, 1981). The diencephalon is implicated in homeostasis and endocrine function, and contains sensory neurons vital to the vertebrate stress response (Charmandari et al., 2005; Denver, 2009). The optic tectum processes visually guided motor behaviors in amphibians (Bestman et al., 2012). The medulla is implicated in respiration and other autonomic functions (Gdovin et al., 1999).

Microbiome Sequencing

Total DNA was extracted from all gut samples using QIAamp PowerFecal Pro DNA isolation kit (QIAGEN) following kit directions. Extracted DNA was stored at -20°C until it was sent to the University of Illinois at Chicago's DNA Services Facility for library preparation, PCR, and sequencing. The bacterial 16S rRNA gene was amplified and amplicons were sequenced on the Illumina Miseq platform. Raw sequence data were processed and analyzed using QIIME2 v2019.7 (Bolyen et al., 2019). Full microbiome sequencing methods, statistical analyses, and results demonstrating relationships between temperature and microbial environment and bacterial community alpha diversity, beta diversity, bacterial taxa abundance, and bacterial load are available in Fontaine et al., 2022.

Statistical Analysis

Statistical analyses were performed in RStudio (v4.1.0) and using the lme4 package (Bates et al., 2015). All data analyzed with parametric statistics met assumptions of normal distribution and homogeneity of variance unless otherwise noted. Data were transformed to meet assumptions in some cases. Our criterion for statistical significance was $p = 0.05$.

Relative Brain Mass

Because tadpole body mass varied as a result of temperature and microbial environment (Fontaine et al., 2022), we adjusted brain mass measurements for differences in body mass. We used an analysis of covariance (ANCOVA) with temperature and microbial environment as main effects, and body mass as a covariate. We confirmed that the slopes of the lines for brain mass were parallel across treatment groups by demonstrating a nonsignificant interaction between treatment and body mass. To calculate a measure for brain mass that was adjusted for body mass, the unstandardized brain mass residual value for each animal (from the ANCOVA) was added to overall estimated marginal mean (EMM). To assess the effects of temperature, microbial

environment, and their interaction on our mass-adjusted brain mass measurements, we used generalized linear mixed models (GLMMs) including bin as a random effect.

Relative Brain Shape

Because linear brain dimensions covary with brain mass, we corrected brain dimensions for brain mass (McCoy et al., 2006). To do this, we used a multivariate analysis of covariance (MANCOVA) with temperature and microbial environment as main effects, and brain mass as a covariate. We confirmed that the slopes describing the relationship between brain mass and each brain dimension were parallel across each treatment group by demonstrating a nonsignificant interaction between treatments and brain mass. This was the case for all dimensions except optic tectum length and diencephalon length. Unstandardized residuals generated by the MANCOVA were added to the overall estimated marginal means to get a mass-adjusted value for each brain dimension for each tadpole.

Because mass-adjusted brain dimensions were highly correlated, we used a Principal Component Analysis (PCA) with a varimax rotation to obtain uncorrelated principal components (PCs). Assumptions of PCA were met, with KMO > 0.5 and Bartlett's tests ≤ 0.05. The PCA yielded three PCs with eigenvalues > 1. The effects of temperature, microbial environment, and their interaction on PCs, were assessed using GLMMs, with bin as a random effect.

Microbiome-Morphometric Associations

To further understand the effects of our treatments on the gut microbiota and brain development, we explored whether individual differences in the diversity of the bacterial gut microbiota predicted differences in brain mass and morphology. To do so, we used GLMMs that included bin as a random effect, with a measure of alpha diversity as a predictor variables and brain mass or morphometric values as the response variable. We examined 4 alpha diversity

metrics: the number of observed bacterial amplicon sequence variants (ASVs), Shannon diversity (Shannon, 2001), Faith's phylogenetic diversity (Faith, 1992), and Pielou's evenness (Pielou, 1966).

We were also interested in testing whether measurements of relative brain mass and shape were correlated with the relative abundances of specific gut bacterial taxa at the phylum and genus level. For this analysis, we used RStudio (v4.1.0) and the MaAsLin2 package (Microbiome Multivariable Association with Linear Models (Mallick et al., 2021)). P-values were corrected using the Benjamin Hochberg false discovery rate (BH FDR) method.

RESULTS

Relative Brain Mass

Tadpoles raised at warmer temperatures had relatively larger brains (adjusted for body mass) compared to tadpoles raised at cooler temperatures (**Fig. 2.2**; $\chi^2 = 22.8$, $p < 0.001$). For example, tadpoles raised at 28°C had relative brain masses that were roughly 20% larger than those raised at 14°C. There was no effect of microbial environment, and no interaction between temperature and microbial environment, on relative brain mass (**Fig. 2.2**).

Relative Brain Shape

The PCA of seven brain dimensions (adjusted for brain mass) yielded three PCs with eigenvalues greater than 1 (**Table 2.1**). PC-1 loaded strongly with telencephalon width, telencephalon length, diencephalon width and medulla width. PC-2 loaded strongly with optic tectum width and optic tectum length. PC-3 loaded strongly with diencephalon length.

Relative brain dimensions described by PC-1 were not affected by temperature or microbial environment, and there was no interaction between treatments (**Table 2.1; Fig. 2.3A**).

Relative brain dimensions described by PC-2 were affected by temperature and microbial environment, but there was no interaction between treatments (**Table 2.1**). Specifically, tadpoles raised in warmer temperatures and autoclaved pond water had increased relative optic tectum width and length compared to tadpoles raised in cooler temperatures and natural pond water (**Table 2.1; Fig. 2.3B)**. To better understand the change in PC-2, we analyzed optic tectum width and length separately, and found that both were altered by temperature and microbial environment (**Fig. 2.4).**

There was no main effect of temperature or microbial environment on relative brain dimensions described by PC-3, but there was a significant interaction between these variables (**Table 2.1**). Specifically, the effect of the microbial environment on diencephalon length depended on the temperature at which tadpoles were raised. At the intermediate temperature 22°C, the length of the diencephalon was shorter in tadpoles raised in autoclaved pond water compared to natural water, but not at the extreme temperatures (14°C or 28°C) (**Table 2.1; Fig. 2.3C**).

Microbiome-Morphometric Associations

Two measures of alpha diversity (observed ASVs and Faith's phylogenetic diversity) were predictors of the relative brain dimensions described by PC-2 (optic tectum width and length) (**Table 2.2**, **Fig. 2.5, Fig. 2.6, Table 2.3**). Specifically, tadpoles that harbored more diverse gut microbial communities in terms of the number of different bacterial taxa present had more narrow and shorter optic tecta. Additionally, Pielou's evenness was a predictor of relative brain dimensions described by PC-3 (diencephalon length) (**Table 2.2, Table 2.3**). There was no relationship between alpha diversity metrics and relative brain mass and relative brain dimensions described by PC-1 (**Table 2.2, Table 2.3**).

At the phylum taxonomic level, relative brain dimensions described by PC-2 (optic tectum width and length) were associated with the relative abundances of 13 bacterial phyla (**Table 2.4**). Of the 13 phyla, tadpoles with higher abundances of Firmicutes tended to have optic tecta with increased width and length, and this positive association is described in **Table 2.4**. Alternatively, the relative abundances of the other 12 phyla had negative associations with optic tectum width and length (**Table 2.4**). Relative brain mass and brain dimensions described by PC-1 and PC-3 were not associated with any relative abundances of bacterial phyla.

At the genus level, relative brain mass was associated with the relative abundances of five bacterial genera (**Table 2.5**). Additionally, relative brain dimensions described by PC-2 (optic tectum width and length) were associated with the relative abundances of 41 bacterial genera (**Table 2.5**). Relative brain dimensions described by PC-1 and PC-3 were not associated with any relative abundances of bacterial genera.

DISCUSSION

Here, we tested the effects of temperature and the microbial environment on neurodevelopment of Green Frog tadpoles, extending the results of a previous study (Fontaine et al., 2022). These tadpoles were raised in natural pond water or autoclaved pond water, as well as at three different temperatures. Tadpoles raised in warmer temperatures had a less diverse gut microbiota that varied in its composition compared to tadpoles raised in cooler temperatures (Fontaine et al., 2022). Similarly, tadpoles raised in autoclaved pond water had a less diverse and distinct gut microbiota compared tadpoles that developed in natural pond water (Fontaine et al., 2022). We extend the results of Fontaine et al., 2022 by showing that 1) water temperature, but not the microbial environment, affected relative brain mass, 2) both the water temperature and the microbial environment affected relative optic tectum length and width, 3) there was an

interactive effect between temperature and the microbial environment on relative diencephalon length, 4) metrics of alpha diversity predicted changes in relative brain shape, and 5) the relative abundances of several bacteria taxa, at the phylum and genus levels, were correlated with changes in relative brain mass and shape. Thus, water temperature and the microbial environment altered amphibian neurodevelopment, perhaps through changes to the gut microbiota and subsequently the microbiota-gut-brain (MGB) axis. Below, we discuss our results in more detail.

Effect of Temperature on Relative Brain Mass

Brain mass was influenced by water temperature, such that tadpoles raised in warmer water had heavier brains, relative to body mass, than tadpoles raised in cooler water. As reported in Fontaine et al., 2022, the tadpoles that were raised in warmer temperatures also attained a larger body mass than those exposed to cooler temperatures. Thus, in addition to a larger overall body mass, the brain was proportionally larger with exposure to warmer temperatures.

It is likely that temperature-dependent alterations to host metabolism contributed to larger brains seen in tadpoles raised at warmer temperatures. Increased sublethal temperatures enhance ectothermic growth and development (Goldstein et al., 2017; Marian and Pandian, 1985) due to increased metabolic rate (Feder and Burggren, 1992; Fontaine et al., 2022; Gillooly et al., 2001). Further, increased sublethal temperatures influence ectothermic digestive performance, energy assimilation, and increase appetite (Fontaine et al., 2018; McConnachie and Alexander, 2004). Interestingly, increasing temperatures have been shown to modulate tadpole feeding preferences such that they shift to a more herbivorous diet due to enhanced digestion of plant material at high temperatures compared to carnivorous diets that are high in lipids and proteins (Carreira et al., 2016). Although we did not measure food intake in our study and diet was consistent across all

treatment groups, investigating tadpole feeding preferences and appetite in the context of climate change would be an interesting future direction to better understand our results.

Increased metabolic rate and energy uptake as a result of higher, non-lethal temperatures could result in tadpoles developing and maintaining larger brains, as nervous tissue is energetically expensive and has been shown to account for 2-10% of vertebrate metabolic output, despite nervous tissue accounting for a much smaller percentage of total body weight (Aiello and Wheeler, 1995; Mink et al., 1981). Whether this increase in relative brain size has cognitive benefits for the host is beyond the scope of this study, but increased size of the brain and larger body-brain ratios are associated with increased cognition in numerous animal taxa (Roth and Dicke, 2005).

Effects of Temperature and Microbial Environment on the Optic Tectum and Diencephalon

In addition to increasing relative brain mass, we found that tadpoles raised in warmer water temperatures had relatively wider and longer optic tecta compared to tadpoles raised at cooler temperatures. It is important to note that these measurements of optic tectum size are corrected for relative brain mass, and changes in the size of this region are not driving the changes observed in overall brain size. A similar result to this has been seen in minnows, such that minnows raised in warmer water temperatures had larger overall brains and larger relative medullas compared to minnows raised in cooler water temperatures (Závorka et al., 2020). As with relative brain mass, we suggest that changes in the optic tectum size could be related to temperature-induced increases in metabolic rate (Fontaine et al., 2022). We also found that tadpoles raised in autoclaved pond water had relatively wider and longer optic tecta compared to tadpoles raised in natural pond water. These changes could also be attributed to host metabolism,

as other studies have found that newly-hatched tadpoles that developed in sterilized water exhibited altered metabolism and growth rates, although this result may vary across different species (Warne et al., 2019).

Due to the energetic cost to maintain and develop nervous tissue, any changes in brain morphology are expected to be adaptive responses to the external environment (Aiello and Wheeler, 1995; Gonda et al., 2013). In particular, we expect that the changes in brain mass and shape induced by our treatments contribute to changes in behavior. For example, pesticide-induced changes in brain shape altered behavioral responses to novel visual stimuli in Northern Leopard Frog tadpoles (McClelland and Woodley, 2022). Because the optic tectum processes visually guided motor behaviors in amphibians (Bestman et al., 2012; González et al., 2020), the changes in the optic tectum found in our study could alter the ability of tadpoles to evade predators, capture prey and affect their feeding behavior. For example, tadpoles raised in autoclaved pond water that had altered optic tectum width and length had slower escape responses when prodded by a blunt probe, although only after exposure to higher water temperatures (Fontaine et al., 2022). An intriguing possibility is that variation in the thermal and microbial environment in which tadpoles develop may contribute to natural behavioral variability of these aquatic animals through changes in the MGB axis. Future studies can incorporate behavioral assays to test if variations in the thermal and/or microbial environment influence tadpole anti-predator and foraging behaviors.

In addition to the effect on optic tectum shape, our microbial treatment altered diencephalon length, but only at the intermediate temperature of 22°C. While results are difficult to disentangle, alterations to diencephalon structure could alter the host stress response. Specifically, the vertebrate diencephalon contains neurons that produce corticotropin releasing

hormones, which activate the hypothalamic-pituitary-adrenal (HPA) axis so that glucocorticoids are released into the circulation (Charmandari et al., 2005; Denver, 2009). Increased circulation of glucocorticoids has been associated with changes in brain morphology in amphibians. Specifically, exposure of Northern Leopard Frog tadpoles (*Lithobates pipiens*) to glucocorticoids in the water resulted in wider diencephalons compared to control (Cha et al., 2021). Some studies have shown that gut microbial diversity is associated with physiological markers of stress such as glucocorticoids, and this relationship appears to be present across vertebrates (Stothart et al., 2016). For example, germ-free zebrafish had a blunted stress response compared to conventionally raised zebrafish (Davis et al., 2016). As glucocorticoids were not measured here, future studies can measure physiological markers of stress to further understand the relationship between the gut microbiota and the vertebrate stress response.

Microbiome-Morphometric Associations

To further understand the effects of our treatments on the gut microbiota and brain development, we explored whether individual differences in the diversity of the gut microbiota predicted differences in brain mass and morphology. We found that relative optic tectum width and length were negatively associated with the number of observed ASVs and Faith's phylogenetic diversity. Specifically, higher diversity of gut microbial communities in terms of the number of observed bacterial taxa tended to predict decreases in relative width and length of the tadpole optic tectum. Gut microbial diversity and composition have been associated with mammalian regional brain volume, as well as mammalian and avian cognitive ability (Carlson et al., 2018; Labus et al., 2017; Slevin et al., 2020), but the cellular causes driving these changes in brain morphology is unknown. Germ-free mice exhibit increased volume of brain regions such as the amygdala, hippocampus, and forebrain compared to control mice, which is hypothesized

to be due to neuronal remodeling that could alter their downstream stress responsivity and behavior (Cryan et al., 2019; Luczynski et al., 2016).

Alternatively, changes in brain morphology could be due to altered microglia morphology. The gut microbiota modulates the development of microglia, which are macrophages that serve as the main form of immune defense in the CNS to resolve threats to host health, such as neuroinflammation (Cryan et al., 2019; Erny et al., 2015). Germ-free mice exhibit increased proliferation of branched microglia with elongated processes, and their forebrains were 17% larger than control mice (Castillo-Ruiz et al., 2018). Future studies examining genetic, molecular, and cellular differences in brains from tadpoles raised in different microbial environments are warranted.

To further understand the effects of our treatments on brain shape and the gut microbiota, we explored whether individual differences in brain shape were correlated with the relative abundance of specific bacterial taxa. While these results are correlational, such results begin to give a picture of the wide-ranging interactions between hosts and the microbiome, especially in non-model systems. Making inferences as to the functional consequences of the microbiome (and specific microbial taxa within the microbiome) on host biology remain an important contribution and allows for downstream meta-analyses (Alberdi et al., 2021; Li et al., 2022) . Some of the bacterial phyla we found to be correlated with optic tectum shape have been previously linked to the nervous system. One example is the phylum Chlamydiae, which was more abundant in tadpoles that developed in natural pond water and was negatively correlated with optic tectum width and length (Fontaine et al., 2022). This phylum of bacteria has been shown to exploit host resources and elicit immune responses, and proliferations of this pathogenic bacteria have been seen in mammals experiencing neurodegenerative diseases such

as Alzheimer's (Balin et al., 1998; Collingro et al., 2020; Gitsels et al., 2019). Similarly, we found that the phylum Cyanobacteria was more abundant in tadpoles that developed in natural pond water and was also negatively correlated with optic tectum width and length (Fontaine et al., 2022). This phylum contains aquatic microbes commonly found in freshwater systems that can contribute to harmful algal blooms and have been implicated in neurodegenerative diseases due to their ability to synthesize neurotoxins (Sini et al., 2021; Zehr, 2011). It is important to note that not all taxa within these phylum are pathogenic, and no symptoms of disease or infection were seen in experimental animals (Fontaine et al., 2022).

Alternatively, we found the phylum Firmicutes was more abundant in tadpoles that developed in autoclaved pond water and was positively correlated with optic tectum width and length (Fontaine et al., 2022). Further, we found that the genus *Clostridium sensu stricto 5*, a member of the Firmicutes phylum, was positively correlated to the width and length of the optic tectum. Our results are supported by a previous study, that found positive correlations between the Firmicutes-associated *Clostridium* taxa and brain volume in regions involved in sensory integration in humans (Labus et al., 2017). While *Clostridium* has been implicated in modulation of peripheral serotonin levels and aspects of neurophysiology (Yano et al., 2015), the exact mechanisms behind these correlations that are driving changes in brain structure in this study are unknown at this time. It is also possible that the changes in relative brain morphology are purely a response to other environmental or physiological factors beyond our treatments that could still impact the composition of the gut microbiota. Future studies to establish causative roles of the gut microbiota in the MGB axis could directly manipulate the abundance of specific microbial taxa and observe changes in relative brain morphology.

CONCLUSION

We have shown that experimental manipulations of temperature and microbial environment alter tadpole relative brain mass and brain morphology. Further, we found these changes in neurodevelopment were significantly associated with metrics of gut microbial diversity and composition. Thus, our results provide some of the first evidence of the MGB axis in an amphibian model. Future experiments may include methods such as transcriptomics and/or isotropic fractionation to quantify neuronal and total cell counts in nervous tissue, which can help further investigate genetic, molecular, and cellular causes driving these changes in brain development (Herculano-Houzel, 2005). Additionally, functional consequences of these changes in brain development may be further tested through behavioral assays, which would strengthen support for the MGB axis in amphibians.

There is value in broadening our understanding of the vertebrate microbiota in more natural developmental settings, even though within this experiment we are unable to determine which specific aspects of the microbiome (i.e., individual bacterial taxa) are driving our observed changes in neurodevelopmental endpoints. Ectotherms such as amphibians represent an ecologically relevant model to investigate host-microbe relationships. Global increases in environmental temperature appears to affect the gut microbial composition and diversity in addition to the other aspects of host physiology modulated by temperature (i.e., metabolic demands, hydration, foraging behavior, digestive performance and brain development (Beltrán et al., 2021; Feder and Burggren, 1992; Fontaine et al., 2018; Rohr and Palmer, 2013)). Further, amphibians develop in freshwater systems that can be contaminated with environmental pollutants such as pesticides that alter host physiology as well as the community composition and diversity of colonizing microbes, and subsequently the host gut microbiota of inhabiting animals

(Gao et al., 2017; Kohl et al., 2015; Kolpin et al., 2002; McClelland et al., 2018; Woodley et al., 2015; Zhang et al., 2020; Zhu et al., 2022). With these challenges in mind, further investigating host-microbe relationships in amphibians, and other wildlife animals facing similar challenges, can help inform wildlife conservation studies and expand the microbiome field beyond mammalian biomedical applications (Trevelline et al., 2019).

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COMPETING INTERESTS

No competing interests declared.

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DATA AVAILABILITY

The raw microbiome sequencing data are available from NCBI's Sequence Read Archive under accession no. PRJNA732310.

Table 2.1: Effects of temperature and microbial environment on relative brain shape of Green Frog tadpoles. Principal components analysis (PCA) was used to reduce seven linear brain dimensions to three principal components (PCs). Principal components were analyzed with GLMMs with bin as a random effect. Significant results are bolded.

Results of PCA	**PC-1**	**PC-2**	**PC-3**
% of Variance	31.1%	24.4%	16.5%
Eigenvalue	2.68	1.35	1.01
Factor Loading*			
Telencephalon Width	0.753	-	0.335
Telencephalon Length	0.728	0.269	0.237
Optic Tectum Width	0.217	0.854	-
Optic Tectum Length	-	0.899	0.166
Diencephalon Width	0.797	0.278	-0.195
Diencephalon Length	-	-	0.924
Medulla Width	0.631	-	-0.254
Results of GLMM			
Temperature: ($\chi_{(2)}^2$, P)	4.44, 0.109	**116.2, < 0.001**	0.38, 0.827
Microbial Environment: ($\chi_{(1)}^2$, P)	0.88, 0.347	**36.7, < 0.001**	0.08, 0.772
Interaction: ($\chi_{(2)}^2$, P)	0.52, 0,771	0.18, 0.916	**7.69, 0.021**
*Factor loadings less than an absolute value of 0.1 are denoted as hyphens.			

Table 2.2. Results from GLMM with replicate as a random effect, testing whether alpha diversity metrics predict relative brain measurements in Green Frog tadpoles. Shown is a chi-square- distributed test statistic and p-value associated with a Type II Wald test. Significant effects are bolded.

Relative Brain Measurement	Shannon diversity (test statistic, P)	No. Observed ASVs (test statistic, P)	Faith's phylogenetic diversity (test statistic, P)	Pielou's evenness (test statistic, P)
Brain Mass	0.01, 0.99	1.34, 0.25	0.623, 0.43	0.18, 0.67
Telencephalon Width & Length, Diencephalon Width, Medulla Width (PC-1)	0.63, 0.43	2.38, 0.12	2.55, 0.11	0.03, 0.86
Optic Tectum Width & Length (PC-2)	0.54, 0.46	**12.2, < 0.001**	**16.9, < 0.001**	0.37, 0.847
Diencephalon Length (PC-3)	3.20, 0.07	0.45, 0.50	0.009, 0.93	**4.07, 0.044**

Table 2.3. Coefficients (beta) and their standard errors from the generalized linear mixed effects models (GLMMs).

Relative Brain Measurement	Shannon diversity (Beta, S.E.)	No. observed ASVs (Beta, S.E.)	Faith's phylogenetic diversity (Beta, S.E.)	Pielou's evenness (Beta, S.E.)
Brain Mass	-1.76e-05, 5.65e-03	-9.18e-05, 7.94e-05	-0.0007, 0.0009	0.019, 0.046
Telencephalon Width & Length, Diencephalon Width, Medulla Width (PC-1)	-0.065, 0.082	-0.002, 0.001	-0.019, 0.012	-0.12, 0.70
Optic Tectum Width & Length (PC-2)	-0.050, 0.068	-0.003, 0.001	-0.045, 0.011	0.11, 0.55
Diencephalon Length (PC-3)	0.14, 0.078	0.0007, 0.001	0.0012, 0.013	1.29, 0.64

Table 2.4: Relative abundances of gut bacterial phyla that were significantly associated with relative optic tectum size of Green Frog tadpoles. Prevalence is the percentage of tadpoles where the indicated bacterial phylum was found. Statistical testing was conducted using MaAsLin2. P-values were corrected using the BH FDR method. The sign of the coefficient indicates the direction of the correlation. Relative brain mass and the other brain PCs (PC-1 and PC-3) were not associated with relative abundances of any bacterial phyla (data not shown). N = 155 tadpoles.

Phyla	Prevalence	Coefficient	FDR P-value
Optic Tectum Width & Length (PC-2)			
Chlamydiae	49.7%	-0.015	<0.001
Acidobacteria	49.0%	-0.006	<0.001
Dependentiae	47.7%	-0.014	<0.001
WPS.2	16.1%	-0.006	<0.001
Chloroflexi	56.1%	-0.009	<0.001
Planctomycetes	87.1%	-0.014	<0.001
Patescibacteria	29.7%	-0.003	<0.001
Cyanobacteria	71.0%	-0.006	<0.01
Firmicutes	100%	0.067	<0.01
Halanaerobiaeota	10.3%	-0.002	<0.01
Dadabacteria	19.4%	-0.001	0.01
Gemmatimonadetes	18.7%	-0.002	0.01
Spirochaetes	16.8%	-0.001	0.03

Table 2.5: Relative abundances of gut bacterial genera that were significantly associated with relative brain morphology in Green Frog tadpoles. Prevalence represents the percentage of tadpoles where the indicated bacterial genera was found. Statistical testing was conducted usingMaAsLin2. The sign of the coefficient indicates the direction of the correlation. P-values were corrected using the BH FDR method. PC-1 and PC-3 were not associated with any relative abundances of bacterial genera. N = 155.

Genera	Prevalence	Coefficient	FDR P-value
Relative Brain Mass			
Reyrenalla	86.4%	1.03	< 0.01
Xanthobacter	81.2%	0.75	< 0.01
Polynucleobacter	54.8%	-0.43	< 0.01
Bacillus	98.7%	-1.20	0.02
Aurantimicrobium	48.4%	-0.38	0.02
Optic Tectum Width & Length (PC-2)			
Neochlamydia	25.2%	-0.006	< 0.001
Gemmata	11.6%	-0.003	< 0.001
Singulisphaera	26.5%	-0.009	< 0.001
Roseiarcus	27.7%	-0.021	< 0.001
Aquicella	41.9%	0.003	< 0.001
Rhodoblastus	17.4%	-0.012	< 0.001
uncultured.Syntrophobacteraceae.bacterium	18.7%	-0.008	< 0.001
Candidatus.Koribacter	11.6%	-0.005	< 0.001
Mycobacterium	60.0%	0.002	< 0.001
Aquisphaera	28.4%	-0.010	< 0.001
Methylocystis	16.1%	-0.008	< 0.001
Rhodovastum	11.6%	-0.005	< 0.001
Acidothermus	25.2%	-0.010	< 0.001
Candidatus.Xiphinematobacter	24.5%	-0.006	< 0.001
Schlesneria	18.1%	-0.005	< 0.001
Clostridium.sensu.stricto.5	87.1%	0.090	< 0.001
Rhodomicrobium	14.2%	-0.006	< 0.001
Candidatus.Berkiella	20.0%	-0.007	< 0.001
Candidatus.Protochlamydia	12.9%	-0.004	< 0.001
Coxiella	34.8%	-0.007	< 0.001
Desulfobacca	14.8%	-0.003	< 0.001
Polynucleobacter	54.8%	-0.034	< 0.001
Ancylobacter	52.9%	-0.016	< 0.001
uncultured.Planctomyces.sp.	10.3%	-0.004	< 0.001
Hydrogenispora	15.5%	-0.002	< 0.001
Epulopiscium	53.5%	0.031	< 0.001
uncultured.Chlamydia.sp.	10.3%	-0.002	< 0.001
Paenibacillus	41.9%	-0.010	< 0.001
uncultured.actinobacterium	12.9%	-0.003	< 0.01
Reyranella	86.5%	0.061	< 0.01
Conexibacter	11.0%	-0.003	< 0.01
Rhodococcus	10.3%	-0.002	< 0.01
Pajaroellobacter	11.0%	-0.002	< 0.01
Bdellovibrio	45.2%	0.016	< 0.01

Paramaledivibacter	10.3%	-0.004	< 0.01
Fulvivirga	11.6%	-0.001	0.02
Haliangium	13.5%	-0.001	0.02
Akkermansia	12.9%	-0.014	0.03
Hydrogenoanaerobacterium	57.4%	0.001	0.04
Brevundimonas	16.1%	-0.001	0.04
Vibrio	10.9%	-0.001	0.04

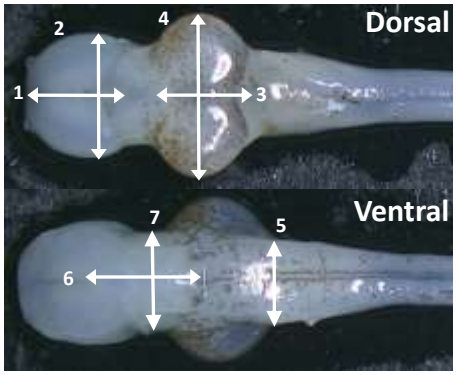

Figure 2.1: Dorsal and ventral view of a Green Frog tadpole brain. The anterior end of the brain is to the left. Arrows represent 7 linear dimensions used to describe brain morphology: 1) telencephalon length; 2) telencephalon width; 3) optic tectum length; 4) optic tectum width; 5) medulla width; 6) diencephalon length; 7) diencephalon width.

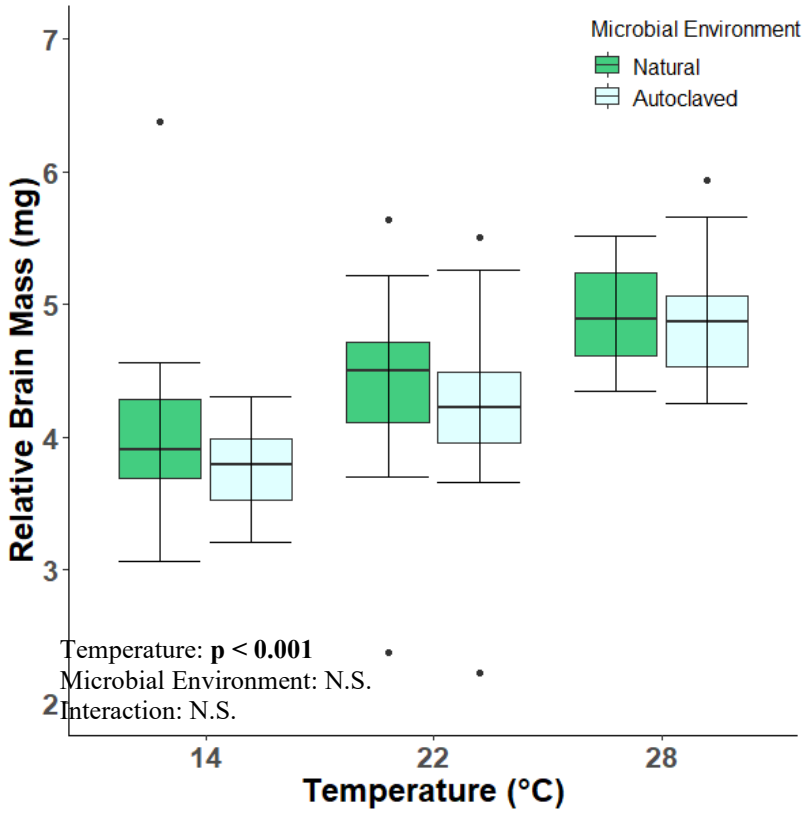

Figure 2.2: Effects of temperature and microbial environment on relative brain mass in Green Frog tadpoles. Tadpoles that developed in warmer temperatures had larger relative brains compared to development at cooler temperatures. Microbial environment did not alter relative brain mass. Brain mass was adjusted for body mass by using the unstandardized residuals of an ANCOVA (see main text). In each boxplot, the center line represents the median, the box length corresponds to the interquartile range (IQR), and whiskers extend to 1.5x IQR. Points outside this range are plotted individually. Significant effects of statistical tests (GLMMs with bin as a random effect) shown in bold. N.S. = nonsignificant. Sample sizes (N): 14°C Natural = 25; 22°C Natural = 27; 28°C Natural = 26; 14°C Autoclaved = 25; 22°C Autoclaved = 25; 28°C Autoclaved = 24.

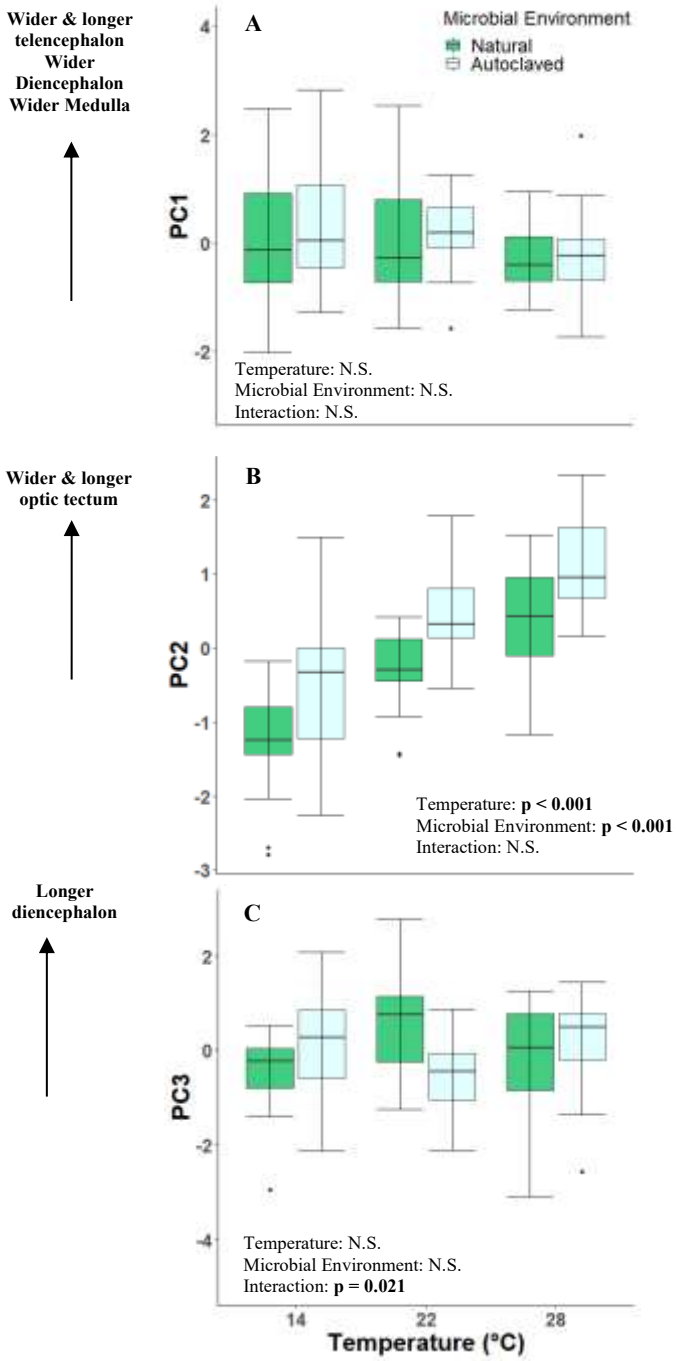
Wider & longer telencephalon
Wider Diencephalon
Wider Medulla
A
Microbial Environment
Natural
Autoclaved
PC1
Temperature: N.S.
Microbial Environment: N.S.
Interaction: N.S.
Wider & longer optic tectum
B
PC2
Temperature: p < 0.001
Microbial Environment: p < 0.001
Interaction: N.S.
Longer diencephalon
C
PC3
Temperature: N.S.
Microbial Environment: N.S.
Interaction: p = 0.021
14
22
28
Temperature (°C)

Figure 2.3: Effects of temperature and microbial environment on relative brain shape in Green Frog tadpoles. (A) Temperature and microbial environment had no effect on relative telencephalon width and length, diencephalon width, or medulla width. (B) Tadpoles that developed in warmer temperatures and in autoclaved pond water had increased relative optic tectum width and length. (C) Temperature and microbial environment had no effect on relative diencephalon length, but there was an interactive effect between treatments. Brain measurements were adjusted for brain mass (see text). 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. Points outside this range are plotted individually. Significant effects of statistical tests (GLMMs with bin as a random effect) shown in bold. N.S. = nonsignificant. Sample sizes (N): 14°C Natural = 24; 22°C Natural = 27; 28°C Natural = 25; 14°C Autoclaved = 24; 22°C Autoclaved = 24; 28°C Autoclaved = 24.

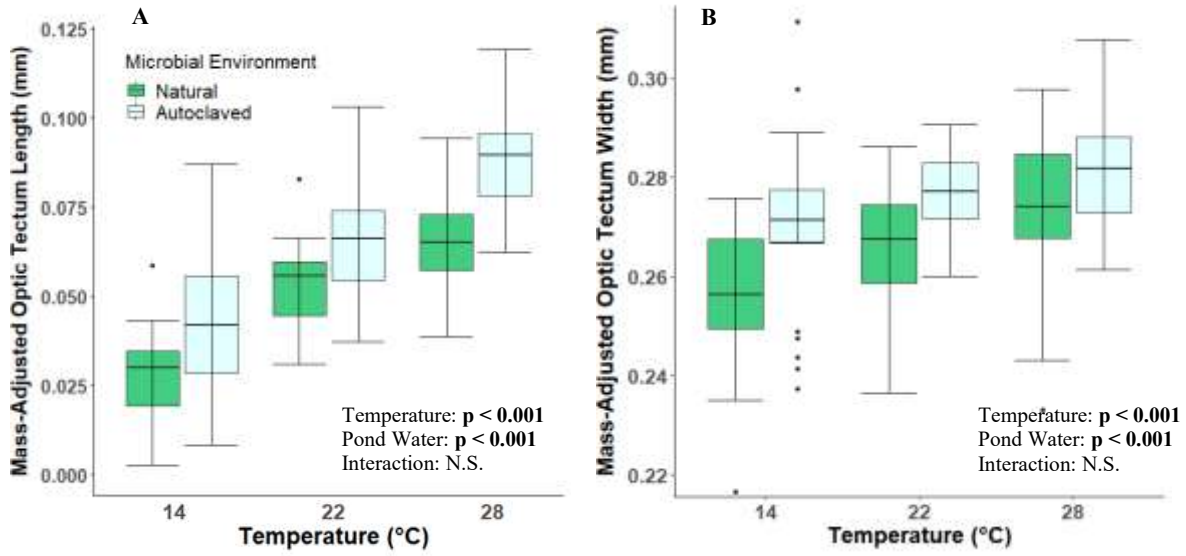

Figure 2.4: Effects of microbial environment and temperature on individual brain measurements describing optic tectum shape. These measurements were adjusted for brain mass and together were described by PC-2. (A) Development in warmer temperatures and in autoclaved pond water promoted tadpoles with increased relative optic tectum length. (B) Development in warmer temperatures and in autoclaved pond water promoted tadpoles with increased relative optic tectum width. 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. Points outside this range are plotted individually. Significant effects of statistical tests (GLMMs with bin as a random effect) shown in bold. N.S. = nonsignificant. Sample sizes (N): 14°C Natural = 24; 22°C Natural = 27; 28°C Natural = 25; 14°C Autoclaved = 24; 22°C Autoclaved = 24; 28°C Autoclaved = 24.

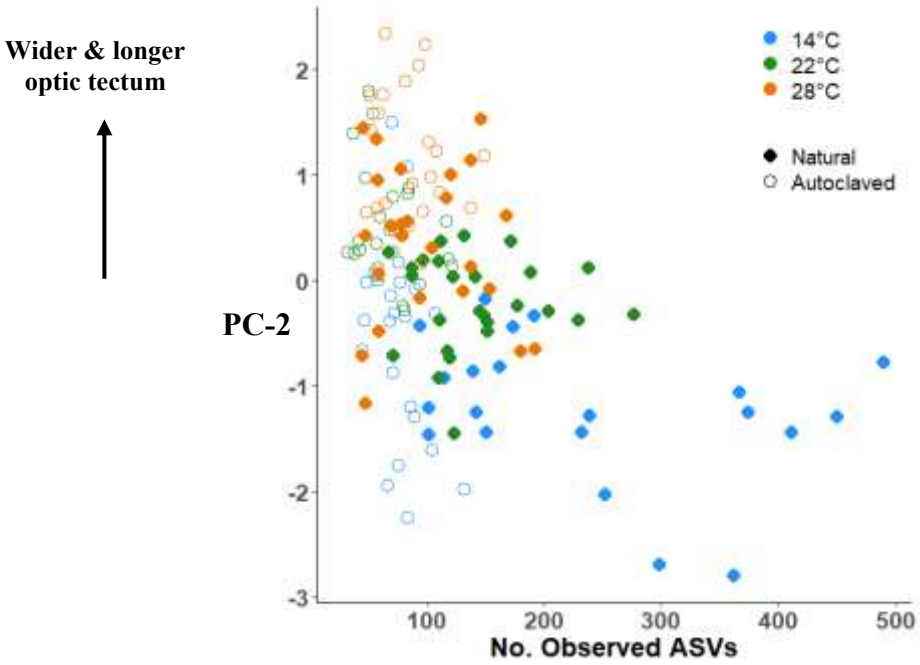

Figure 2.5. Association between relative optic tectum shape and number of ASVs in gut microbial communities of Green Frog tadpoles. Reduced optic tectum width and length was associated with increased number of observed ASVs. Brain measurements were adjusted for brain mass (see text). Colors represent temperature. Filled circles represent development in natural pond water. Open circles represent development in autoclaved pond water. Sample sizes (N): 14°C Natural = 24; 22°C Natural = 27; 28°C Natural = 25; 14°C Autoclaved = 24; 22°C Autoclaved = 24; 28°C Autoclaved = 24.

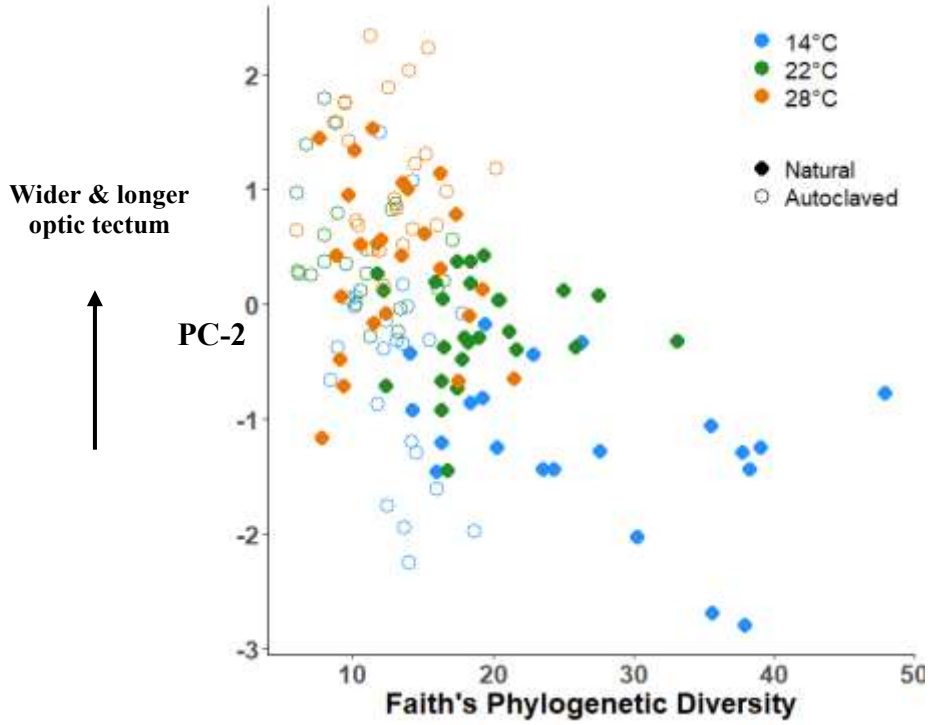

Figure 2.6. Associations between relative optic tectum shape and number of Faith's phylogenetic diversity in gut microbial communities of Green Frog tadpole. Reduced optic tectum width and length was associated with increases in Faith's phylogenetic diversity index. Brain measurements were adjusted for brain mass (see text). Colors represent temperature. Filled circles represent development in natural pond water. Open circles represent development in autoclaved pond water. Sample sizes (N): 14°C Natural = 24; 22°C Natural = 27; 28°C Natural = 25; 14°C Autoclaved = 24; 22°C Autoclaved = 24; 28°C Autoclaved = 24.

Chapter 3. Something in the water: aquatic microbial communities influence the larval amphibian gut microbiota, neurodevelopment, and behaviour

ABSTRACT

Microorganisms colonize the gastrointestinal tract of animals and establish symbiotic host-associated microbial communities that influence vertebrate physiology. More specifically, these gut microbial communities influence neurodevelopment through the microbiota-gut-brain (MGB) axis. We tested the hypothesis that larval amphibian neurodevelopment is affected by the aquatic microbial community present in their housing water. Newly hatched Northern Leopard Frog (*Lithobates pipiens*) tadpoles were raised in pond water that was unmanipulated (natural) or autoclaved. Tadpoles raised in autoclaved pond water had a gut microbiota with reduced bacterial diversity and altered community composition, had decreased behavioral responses to sensory stimuli, were larger in overall body mass, had relatively heavier brains, and had altered brain shape when compared with tadpoles raised in natural pond water. Further, the diversity and composition of the gut microbiota were associated with tadpole behavioral responses and brain measurements. Our results suggest that aquatic microbial communities shape tadpole behavior and brain development, providing strong support for the occurrence of the MGB axis in amphibians. Lastly, the dramatic role played by aquatic microbial communities on vertebrate neurodevelopment and behavior should be considered in future wildlife conservation efforts.

INTRODUCTION

Environmental microbes colonize the gastrointestinal (GI) tract of vertebrates, thereby forming the host-associated gut microbiota. Comprised of bacteria, viruses, archaea, and fungi, the gut microbiota participates in a bidirectional symbiotic relationship with the host by performing physiological functions that are essential for development (Kohl and Carey, 2016). The composition of the vertebrate gut microbiota is partly modulated by the biodiversity of microbes present in the local environment during early life (Correa et al., 2020), as initially colonizing microbes (i.e. "founder" microbes) have a greater chance to occupy a niche within the host compared to later arriving microbes (Litvak and Bäumler, 2019; Martínez et al., 2018).

In most animals, there is a relationship between neurodevelopment and the gut microbiota which is referred to as the microbiota-gut-brain (MGB) axis (Cryan et al., 2019). Gut microbes, primarily bacteria, synthesize neuroactive products that can communicate with the brain and aid in the development of the nervous system (Cryan et al., 2019; Foster and McVey Neufeld, 2013; Perry et al., 2016). Studies have found that germ-free (GF) mice have altered synaptic gene expression (Heijtz et al., 2011), locomotory activity (Cryan et al., 2019; Heijtz et al., 2011), and altered regional brain volume (Luczynski et al., 2016; Tovote et al., 2015) compared with conventionally raised animals. Support for the MGB axis in non-mammals has also been seen in GF zebrafish, which exhibited increased locomotory behavior and altered anxiety-like behavior compared to conventionally raised zebrafish (Davis et al., 2016). Further, a recent study using zebrafish found that the microbiota in early development was essential for neurodevelopment, as several bacterial taxa were implicated in stimulating microglial remodeling of neuronal circuits that modulate social behavior (Bruckner et al., 2022). As evidence continues to support the presence of the MGB axis and its influence on host

neurodevelopment, there is a need to evaluate host-microbe interactions in wildlife systems in more environmentally relevant contexts (Colston and Jackson, 2016; Pascoe et al., 2017).

Here, we investigated how manipulations of aquatic microbial communities influence the amphibian gut microbiota and subsequent physiology, with an emphasis on brain development and behavior. Amphibians are ideal for this work because they develop in freshwater environments and are recognized as bioindicators of ecosystem health and water quality (DeGarady and Halbrook, 2006; Schmeller et al., 2018; Welsh Jr. and Ollivier, 1998). Much is known about amphibian courtship behavior (Kelleher et al., 2018), foraging behavior (Placyk and Graves, 2001; Venesky et al., 2009), boldness (McClelland and Woodley, 2022), and antipredator responses (Fraker et al., 2009; Fraker et al., 2021), which provides a foundation to investigate microbial influence on amphibian behavior and fitness. We tested the hypothesis that larval amphibian neurodevelopment is affected by the aquatic microbial environment present in their housing water.

To test our hypothesis, we raised Northern Leopard Frog (*Lithobates pipiens*) tadpoles in natural pond water, or in natural pond water that was autoclaved. By autoclaving the pond water, the biodiversity of microbes available to colonize the gut was reduced. This methodology has been used in previous studies of larval amphibians (Emerson et al., 2023; Fontaine et al., 2022); specifically, green frog tadpoles (*Lithobates clamitans*) that developed in autoclaved pond water harbored distinct gut bacterial communities with decreased diversity compared with those that developed in natural pond water (Fontaine et al., 2022). Importantly, the density of gut bacteria in tadpoles raised in autoclaved pond water was similar to the density of gut bacteria of tadpoles raised in natural pond water (Fontaine et al., 2022). This contrasts with studies of GF animals, which are devoid of a gut microbiota, which is a highly artificial state.

We predicted that tadpoles raised in autoclaved pond water, compared with those raised in natural pond water, would (1) have reduced gut bacterial diversity and altered gut bacterial community composition, (2) exhibit altered behavioral responses to stimuli, and (3) have altered relative brain mass and morphology. We were also interested in whether our treatment affected body mass, development, and plasma corticosterone (CORT), an important stress and metabolic hormone. Finally, to better understand our treatment effects, we tested for associations between the gut bacterial communities and brain development/behavioral endpoints.

MATERIALS & METHODS

All protocols were approved by Duquesne University's Institutional Animal Care and Use Committee (protocol #2010-09). All experimental and analytical work was completed by the first author.

RStudio (v. 4.1.0) running the lme4 package (Bates et al., 2015) was used for statistical analysis. Unless otherwise noted, data analyzed with parametric statistics were normally distributed with homogeneity of variance. Data were transformed to meet assumptions in some cases.

General Information

We purchased three Northern Leopard Frog (*L. pipiens*) egg masses from Carolina Biological Supply Company (Burlington, NC). Upon arrival, embryos were placed into 15-L Sterilite plastic bins filled with 5 L of autoclaved laboratory tap water that had been passed through sediment and carbon filters, exposed to UV light, and treated with API Tap Water conditioner. Each bin had a bubbling air stone connected to an aquarium air pump to ensure proper oxygenation. Upon hatching, tadpoles were fed ad libitum two times per week or more as

needed until the experimental treatments began. Food consisted of Frog Brittle (Nasco) that was frozen (-20°C) until use.

Water temperature was tracked daily and maintained at 22°C. The photoperiod was confirmed at the start of the experiment and maintained at a 12 h light : 12 h dark photoperiod via a light timer. However, after the experiment was completed, we discovered that the light timer was providing 24 hours of light. It is unknown at what point during or after the experiment this malfunction occurred. However, the developmental rate (i.e., Gosner Stage (GS)/week) of tadpoles was 2.09, which is similar to the developmental rate of tadpoles (2.08) in a later, similar experiment that had a new timer system with a 12 h light; 12 h dark photoperiod (Emerson et al., in preparation). Additionally, many of the conclusions made in the current study were replicated and expanded on in our subsequent studies. Thus, we conclude that potential issues with the photoperiod did not obviously impact our conclusions.

Pond Water Collection and Treatments

Pond water was collected from the Great Outdoors Camping Ground (November 13th, 2020; 1525 Footville Richmond Rd, Linesville PA 16424; 41.69182, -80.50008) which is a pond where *L. pipiens* are found naturally. Pond water was filtered using an Advantech #35 500µm testing sieve on site to remove sediment. Filtered pond water was stored in 10-L HDPE carboys at 4°C until use. The natural pond water treatment consisted of 25% pond water and 75% autoclaved tap water. The autoclaved pond water treatment consisted of 25% autoclaved pond water and 75% autoclaved tap water. API Tap Water conditioner was added after autoclaving to dechlorinate the water.

Upon reaching GS 25 (Gosner, 1960), tadpoles were haphazardly distributed into plastic bins (Nasco Flex-Tanks: 5.7L, 27.9cm x 17.8cm x 12.7cm) filled with either pond water that was

unmanipulated (natural) or pond water that was autoclaved. Our pond water treatments were the same as our previous study (Emerson et al., 2023; Fontaine et al., 2022), and consisted of 75% autoclaved laboratory tap water that was combined with 25% pond water (i.e., natural pond water treatment) or 25% autoclaved pond water (i.e., autoclaved pond water treatment). API Tap Water conditioner was added after autoclaving to dechlorinate the water. There were 12 bins per treatment group filled with 2L of treatment water. Tadpoles were fed ad libitum, three times per week or more as needed. Water treatments were re-administered during weekly water changes for the duration of the experiment. During weekly water changes, tadpole bins were rotated clockwise along storage racks to distribute any slight room differences in environmental conditions. Additionally, we tested for ions that can be harmful if present in high abundance, such as nitrite, nitrate, and chlorine. We found no evidence that they were present in high abundance throughout the experiment.

Timeline and Sample Sizes

Tadpole egg masses were received on January 14th, 2021. The experiment started on January 20th, 2021, with eight tadpoles per bin and 12 bins per treatment group. On February 7th, 2021, three tadpoles from each bin were randomly selected and removed to prevent overcrowding, resulting in five tadpoles per bin for the remainder of the experiment. After four weeks of treatments (February 17th, 2021), we began collecting data. For logistical and financial reasons, we could not measure all the dependent variables from all tadpoles. **Table 3.1** shows how the data were collected from subsets of tadpoles within each bin.

One tadpole was randomly selected to be removed from each bin and had its locomotory behavior recorded (see below). When tadpoles finished their behavioral assays, they were euthanized via immersion in 0.2% buffered MS-222 solution that same day. After euthanasia,

tadpoles had their gastrointestinal (GI) tracts removed for assessment of the gut microbiota, had their body mass and Gosner Stage recorded, and had blood samples taken to analyze plasma CORT. These tadpole carcasses were then stored in 10% neutral buffered formalin.

The following day, another tadpole was randomly selected to be removed from a bin that previously had a tadpole removed the day prior for analysis of locomotory behavior. This tadpole was euthanized, had its body mass and Gosner Stage recorded, and had a blood sample taken to analyze plasma CORT. These tadpole carcasses were then stored in 10% neutral buffered formalin. Additionally, a third tadpole was randomly selected to be removed from the bin, euthanized, and had its body mass and Gosner Stage recorded. These tadpole carcasses were then stored in 10% neutral buffered formalin. Fixed carcasses later had their brains dissected for analysis of relative brain mass and shape

We did not collect any dependent variable information from the remaining two tadpoles in each bin, as they were kept in bins to maintain density and continued to develop when the experiment had concluded. Data collection took 7 days to complete, and the experiment concluded on Feb. 23, 2021.

Target sample sizes were: 12 tadpoles per treatment for assessment of behavior and the gut microbiota; 24 tadpoles per treatment for assessment of plasma CORT; and 36 tadpoles per treatment for assessment of body mass, GS, brain mass, and brain shape. Actual sample sizes were sometimes less than the target due to attrition and tissue damage. Survival rates were 97% of tadpoles in natural pond water and 78% of tadpoles in autoclaved pond water. The main source of attrition was the loss of all tadpoles from a single bin in autoclaved pond water for an unknown reason.

Behavioral Assays

One tadpole from each bin was randomly selected for behavioral assays (**Table 3.1**). Assays for all tadpoles took six days to complete, as we tested two tadpoles from each treatment group each day over those six days. Assays were completed daily from 12:00 to 14:30 hours. Tadpoles were removed from their bins using sterilized tea strainers and were rinsed with 100 ml of autoclaved laboratory tap water. After rinsing, tadpoles were placed individually into behavior arenas (Imagitarium Holding Bin, 10.5 in x 6.5 in x 7 in) filled with 2 L of autoclaved laboratory tap water. A webcam (360p, 16:9 30fps) was mounted above each behavior arena and attached to a computer to record tadpole movement. Average body mass and GS were 2.18 g and 35.2 (natural pond water treatment), and 2.7g and 35.7 GS (autoclaved pond water treatment).
We measured several parameters describing locomotory activity: average speed (mm/s), average acceleration (mm/s^2), mobility rate (%), total time frozen (m:s), exploration rate of the arena (%), total distance traveled (mm), time in center (m:s), and time near added stimulus (m:s; see below). Tadpoles were given 20 minutes to acclimate to the behavior arenas before recording behavior.

After the acclimation period, we recorded tadpole locomotory activity at baseline. Next, we recorded locomotory activity in response to the addition of an empty clear glass scintillation vial closed with a plastic cap, followed by locomotory activity in response to the replacement of the empty vial with an identical sealed vial filled with Nasco Frog Brittle. Finally, the vial was removed, and we then recorded locomotory activity after the addition of a frog brittle slurry (2-3 grams of Nasco Frog Brittle dissolved in autoclaved laboratory tap water and filtered through Whatman paper to remove large particles).

Locomotory activity at baseline and in response to the addition of the empty vial, full vial, and food slurry were recorded for 20 minutes each. Between each assay, tadpoles were

given five minutes to acclimate to the new stimulus where locomotory responses were not analyzed. The order of behavioral assays remained the same for all tadpoles.

The stimuli added to behavior arenas were selected as they are expected to be meaningful to a tadpole. The empty glass vial was used in previous studies to represent a novel visual cue (McClelland and Woodley, 2022), and the addition of food to the vial and in a slurry assumes the recognition of food as a potentially rewarding visual or olfactory stimulus. The stimuli were added to the left or right sides of the behavior arena and were alternated within treatments to avoid any bin biases.

Videos of tadpole behavioral assays were analyzed using ToxTrac software (Rodriguez et al., 2018). We analyzed locomotory responses at baseline using a PCA, with the following PC loadings: PC-1 (average speed, acceleration, mobility rate, and total time frozen), PC-2 (Exploration rate), and PC-3 (time in the center of the arena). We found that there was no significant treatment effect on baseline behaviors: PC-1 ($F = 0.03$, $P = 0.86$), PC-2 ($F = 0.33$, $P = 0.57$), and PC-3 ($F = 0.38$, $P = 0.55$). As there was no treatment effect on baseline behaviors, we analyzed relative changes in locomotory activity in response to sensory cues for each tadpole. For example, for each tadpole, we subtracted the average speed at baseline from the average speed in the presence of an empty vial to calculate the change in behavior in response to the empty vial.

Due to correlation between the behavior variables measured, we used a Principal Component Analysis (PCA) with a varimax rotation which yielded three uncorrelated Principal Components (PCs) with Eigenvalues $\sim \geq 1$. Assumptions of PCA were met for all behavioral assays and parameters (Kaiser Meyer Olkin Factor Adequacy > 0.5 and Bartlett's tests ≤ 0.05).

The effect of pond water treatment on behavior was analyzed using generalized linear models (GLMs). One tadpole's behavioral videos were corrupted and were not analyzed.

Gut Microbiota

After finishing the behavioral assays for a tadpole, it was euthanized by immersion in 0.2% buffered MS-222. Tadpoles were then rinsed in autoclaved laboratory tap water, blotted dry, weighed, and had their GS recorded. To assess the gut microbiota, GI tracts were dissected within nine minutes of euthanasia by removing the esophagus through the large intestine. Intestines were immediately placed into sterile 1.5 mL microcentrifuge tubes and frozen at -80°C. Equipment used for dissections was cleaned with 70% EtOH and heat sterilized in between dissections. A Qiagen QIAmp PowerFecal Pro DNA Isolation Kit was used to extract bacterial DNA from GI tissue by following kit instructions, which included kit controls.

Extracted DNA was placed into sterile microcentrifuge tubes and frozen at -80°C. Extractions were sent to the University of Illinois at Chicago's Genome Research Core for sequencing. Libraries were prepared through amplification of the V4 region of the 16S rRNA gene using primers 515F and 806R on an Illumina MiniSeq, resulting in 2x150 paired end reads (Caporaso et al., 2012). Raw sequence data was uploaded to an individual Illumina BaseSpace account when completed and was processed and analyzed by the author in RStudio (version 4.1.0) using the DADA2 package (Callahan et al., 2016). Specifically, primer sequences were removed by trimming reads to 131 (forward) and 130 (reverse) base pairs before denoising, filtration for quality, and merging of forward and reverse reads. When reads were properly processed, they were assigned to amplicon sequence variants (ASVs) (Callahan et al., 2016). Using the phangorn package (Schliep, 2011), sequences were aligned and a phylogenetic tree was rendered. Taxonomy was assigned to sequences using the SILVA database classifier v138.1

(Quast et al., 2013). Sequence variants identified as archaea were removed. DNA from all samples was extracted in one batch.

Raw sequence data from bacterial DNA extractions was analyzed in RStudio (version 4.1.0) using the DADA2 package (Callahan et al., 2016). To compare diversity metrics across experimental groups, a rarefied ASV table was created, and singleton reads were removed. Based on the experimental sample with the fewest reads, we rarefied the ASV table to 26,142 sequences per sample. Several alpha diversity metrics (i.e., no. of observed ASVs, Shannon Diversity Index (Shannon, 2001), and Faith's Phylogenetic Diversity (Faith, 1992)) were calculated and analyzed using RStudio and GLMs. To evaluate beta diversity and test if there were differences in gut bacterial composition across treatment groups, we calculated Bray Curtis distance matrices (Bray and Curtis, 1957) and performed a PERMANOVA (999 permutations) using the vegan package in RStudio (Oksanen et al., 2013). We also tested intraindividual variability in gut bacterial composition across treatment groups using the betadisper function to calculate PERMDISP. Bin was not included as a random effect because all tadpoles used for gut microbiota analyses came from different bins.

To test if the pond water treatment significantly altered the relative abundances of bacterial taxa at the phylum and genus levels, we used the MaAsLin2 (Microbiome Multivariable Association with Linear Models (Mallick et al., 2021)) package in RStudio. Pond water treatment was used as a fixed effect. To account for the number of tests being conducted and potential false positives, values of significance were corrected using the Benjamin Hochberg false discovery rate (BH FDR).

One tadpole in the natural pond water group had an abnormally high number of observed ASVs (~820). Other than being an outlier, there was nothing unusual about this sample. We

analyzed our data with and without this sample and found no difference in conclusions. Thus, we retained the sample in our analyses, although it was omitted from some figures to improve figure legibility (noted in figure captions).

Growth, Development, and Plasma CORT

After euthanasia (described above), all tadpoles were rinsed in autoclaved laboratory tap water, blotted dry, weighed, and had their GS recorded. Also, two tadpoles from each bin were randomly assigned to have their plasma CORT analyzed (**Table 3.1**). To do this, the tail vein was cut with sterilized scissors, and blood samples were collected in heparinized capillary tubes within four minutes of euthanasia, and then centrifuged. On average, we collected 16.7ul of plasma from each tadpole, which was frozen in heparinized microfuge tubes and stored at -20°C. Scissors were cleaned with 70% EtOH and heat sterilized between samples. Plasma samples were sent to the Endocrine Technologies core of the Oregon National Primate Research Center Endocrine Laboratory to measure plasma CORT following previously described methods (McClelland and Woodley, 2021). Samples were assayed in singlicate. The intra-assay coefficient of variation was 2.9%. All euthanized tadpole carcasses were stored in 10% neutral buffered formalin fixative.

The effects of pond water treatment on body mass, GS, and plasma CORT were analyzed using GLMMs (Generalized Linear Mixed Models) including bin as a random effect. CORT and GS values were not normally distributed, but variances were homogeneous. Thus, GLMMs were conducted for these variables because parametric statistics are generally robust to violations of normality (Glass et al., 1972; Harwell et al., 1992; Lix et al., 1996).

Brain Dissections

Methods regarding dissecting and processing brains are outlined in a previous study (Emerson et al., 2023). Brains were removed from fixed carcasses using an Olympus SZ61 dissection scope with a camera attachment. After trimming nerves and the spinal cord, brains were photographed six times: three dorsal and three ventral images, with repositioning between each photograph. If a brain was damaged during the dissection, it was excluded from the analysis. The same investigator conducted all dissections and photography. Because tissue can shrink over time in fixative, we dissected the brains within a month of completion of the study (March 19th, 2021 – March 21st, 2021). We ensured that all brain tissue spent an equal time in fixative across our treatment groups by finishing all weighing and photography within two days. Additionally, brains were processed in batches that included representatives from each treatment group.

Relative Brain Mass and Shape

Brain measurements followed the methods of a previous study (Emerson et al., 2023). To correct brain mass measurements for variations in body mass, we used an analysis of covariance (ANCOVA) with pond water treatment as a fixed effect and body mass as a covariate. We confirmed there was a nonsignificant interaction between treatment and body mass, signifying the slopes of the lines for brain mass were parallel across treatment groups. Unstandardized brain mass residual values for each tadpole were generated by the ANCOVA and added to the estimated marginal means (EMM) which yielded relative brain mass measurements that were corrected for any variation in tadpole body mass. A GLMM that included bin as a random effect was used to test the effect of our pond water treatment on relative brain mass values.

To assess changes in brain shape, we used geometric morphometrics (Adams et al., 2004) following the methods of a previous study (Emerson et al., 2023). Four brain structures on dorsal

images and three brain structures on ventral images were measured in triplicate using ImageJ software (US National Institutes of Health, Bethesda, MD). These three measurements for each structure were averaged to give a single value for each brain structure for each tadpole. In total, the same investigator solely measured telencephalon width and length, diencephalon width and length, optic tectum width and length, and medulla width for each tadpole (**Fig. 3.1**). We corrected the measurements of brain dimensions for brain mass (McCoy et al., 2006) as we did for brain mass using a multivariate analysis of covariance (MANCOVA) that found nonsignificant interactions between treatment and brain mass for all structures except medulla width.

Due to correlations between the corrected brain dimension measurements, we used a PCA with a varimax rotation to yield uncorrelated PCs (Emerson et al., 2023). Statistical assumptions of factor analysis were met with KMO > 0.5 and Bartlett's test ≤ 0.05, and the PCA yielded three PC's with Eigenvalues ~ ≥ 1. The effect of pond water treatment on relative brain shape was analyzed using GLMMs including bin as a random effect.

Microbiota – Neurodevelopment Associations

For tadpoles that had both their gut microbiota assessed and neurodevelopmental parameters measured, we investigated whether differences in the diversity of the gut microbiota were associated with changes in neurodevelopment (**Table 3.1**) . We used GLMs with our measurements of alpha diversity as predictor variables; our response variables included relative brain mass values and PCs from relative brain shape and behavioral analyses. Linear regressions were fitted to these data using the base statistics package in RStudio. Additionally, we tested whether the relative abundances of individual gut bacterial taxa (at the phylum and genus levels) were correlated with our neurodevelopmental parameters. We used the MaAsLin2 package

(Mallick et al., 2021) with relative brain mass values, PCs representing relative brain morphometric values, and/or PCs representing behavioral parameter values as the fixed effect, and values of significance were corrected using the BH FDR method.

RESULTS

Gut Microbiota

Pond water treatment significantly affected tadpole gut bacterial communities. Tadpoles raised in autoclaved pond water harbored communities with a distinct composition compared with tadpoles raised in natural pond water (**Fig. 3.2A**; PERMANOVA: $F = 3.2$, $p = 0.004$) but did not affect community dispersion (**Fig. 3.2A**; PERMDISP: $F = 0.043$, $p = 0.84$). Tadpoles raised in autoclaved pond water also had reduced gut bacterial diversity compared with tadpoles raised in natural pond water. Specifically, tadpoles raised in autoclaved pond water harbored fewer ASVs (**Fig. 3.2B**; $\chi_{(1)}^2$: 6.0, $p = 0.014$), had a lower Shannon Diversity Index (**Fig. 3.3A**; $\chi_{(1)}^2$: 10.2, $p = 0.0014$), and had a lower Faith's phylogenetic diversity score (**Fig. 3.3B**; $\chi_{(1)}^2$: 10.2, $p = 0.0014$).

Actinobacteriota, Bacteroidota, Firmicutes, Fusobacteriota, and Proteobacteria were the bacterial phyla with the highest relative abundances (**Fig. 3.4, Fig. 3.5**), with other phyla present in smaller quantities. Pond water treatment significantly altered the relative abundances of two bacterial phyla, Verruocomicrobiota and Planctomycetota (**Table 3.2**), and 24 bacterial genera (**Table 3.3**).

Behavioral Assays

Pond water treatment significantly affected tadpole behavior. In response to an empty vial, tadpoles raised in autoclaved pond water exhibited decreased swim speed, distance traveled, acceleration, and percentage of time mobile (described by PC-1) compared with tadpoles raised

in natural pond water (**Fig. 3.6A, Table 3.4**). Pond water treatment did not impact time spent near the empty vial (PC-2) or total time spent in the center of the arena (PC-3) in response to an empty vial (**Table 3.4; Fig. 3.7A-B**).

In response to a sealed vial containing food, tadpoles raised in autoclaved pond water again exhibited decreased swim speed, distance traveled, acceleration, and percentage of time mobile (PC-1) compared with tadpoles raised in natural pond water (**Fig. 3.6B, Table 3.5**). Pond water treatment did not impact the exploration of the arena (PC-2), or total time spent in the center of the arena (PC-3) in response to a sealed vial containing food (**Table 3.5, Fig. 3.8A-B**).

Pond water treatment had no impact on behavioral responses to the addition of a food-derived slurry (**Table 3.6, Fig. 3.9A-C**).

Growth, Development, and Plasma CORT

Tadpoles raised in autoclaved pond water were about 20-25% larger in body mass (**Fig. 3.10A**; $\chi_{(1)}^2$: 10.4, p = 0.0013). Further, tadpoles raised in autoclaved pond water were slightly more developed than tadpoles raised in natural pond water (**Fig. 3.11**; $\chi_{(1)}^2$: 5.8, p = 0.016). Specifically, tadpoles raised in autoclaved pond water had an average GS of 35.7 (SEM: 0.17) while tadpoles raised in natural pond water had an average GS of 35.2 (SEM: 0.13). There was no treatment effect on plasma CORT levels (**Fig. 3.12**; $\chi_{(1)}^2$: 0.016, p = 0.89).

Relative Brain Mass and Shape

Tadpoles raised in autoclaved pond water had relatively larger brains (12-13% heavier) compared with tadpoles raised in natural pond water (**Fig. 3.10B**; $\chi_{(1)}^2$: 4.1, p = 0.043). The PCA of seven linear brain dimensions yielded three PCs with eigenvalues greater than 1 (**Table 3.7**). Relative brain dimensions described by PC-1 (Optic tectum width and length, and Diencephalon width) and PC-2 (Telencephalon width and length) were not affected by pond water treatment

(**Table 3.7; Fig. 3.13A-B**). PC-3 (Medulla width) was smaller in tadpoles raised in autoclaved pond water compared to tadpoles raised in natural pond water (**Table 3.7**; Fig. 3C; $\chi_{(1)}^2$: 6.1, p = 0.013).

Microbiota – Neurodevelopment Associations

Metrics of alpha diversity were associated with neurodevelopmental endpoints (**Table 3.8; Table 3.9**). In response to a vial containing food, tadpoles with more ASVs and higher Faith's phylogenetic diversity scores had increased locomotory behaviors (PC-1: average swim speed, total distance traveled, average acceleration, percentage of time mobile, less time frozen) (**Table 3.8; Fig. 3.6C**). In response to a vial containing food, tadpoles with a higher Shannon Diversity Index exhibited increased exploration of the behavior arena (PC-2) (**Table 3.8**). In response to a food slurry, tadpoles with more ASVs and higher Faith's phylogenetic diversity scores had increased locomotory behaviors described by PC-2 (total time spent in the center of the arena and time spent near where the cue was administered) (**Table 3.8**). Additionally, tadpoles with a higher Shannon Diversity Index had relatively wider medullas (PC-3) (**Table 3.8; Fig. 3.10D**).

Several bacterial taxa found in the gut of tadpoles had relative abundances that were associated with locomotory responses during behavioral assays (**Table 3.10**). At the phylum level, tadpoles with higher relative abundances of Patescibacteria and Proteobacteria exhibited increased locomotory activity and less time frozen in response to an empty vial (PC-1: average swim speed, total distance traveled, average acceleration, percentage of time mobile) (**Table 3.10**). Additionally, tadpoles with higher abundances of Bdellovibrionota exhibited increased locomotory activity (PC-3: the amount tadpoles explored the behavior arena) in response to a food slurry, while tadpoles with higher abundances of Firmicutes exhibited a decreased

locomotory activity (PC3: exploration of the behavior arena). At the genus level, the relative abundance of *Clostridium.sensu.stricto.1* was negatively associated with tadpole locomotory responses to an empty vial or a food slurry. Lastly, the relative abundance of *Brevundimonas* was positively associated with locomotory responses to the food slurry (PC-3: exploration of the behavior arena).

DISCUSSION

Here, we tested how variation in the aquatic microbial community impacted Northern Leopard Frog tadpole physiological development, with an emphasis on the MGB Axis. Tadpoles raised in autoclaved pond water (1) had reduced gut bacterial diversity and altered bacterial community composition, (2) exhibited decreased locomotory activity in response to sensory stimuli (3) were 20-25% larger and had a higher average GS, and (4) had altered relative brain mass and shape compared with tadpoles raised in natural pond water. Additionally, we found that gut bacterial diversity and community composition were associated with changes in behavioral responses to sensory stimuli and relative brain shape. These results support our hypothesis that the aquatic microbial environment in which larval amphibians develop has dramatic effects on the amphibian gut microbiota, brain, and behavior. More importantly, our results indicate that changes in the microbial community of freshwater systems may have cascading effects on the animal species that rely on these microbial communities for their physiological development. Results are further described below.

Gut Microbiota

Tadpoles raised in autoclaved pond water harbored gut bacterial communities that were different in their diversity and composition, but not dispersion, compared with tadpoles raised in natural pond water. Comparable results were found in Green Frogs raised in similar conditions

(Fontaine et al., 2022). Additionally, the previous study using Green Frogs found that bacterial density did not differ between tadpoles raised in natural and autoclaved pond water (Fontaine et al., 2022). While density was not evaluated in this study, the previous result suggests that the differences in host phenotype in Northern Leopard Frogs are due to differences in the composition of the gut microbiota rather than bacterial deprivation. Supporting this, we found that raising tadpoles in autoclaved pond water for four weeks reduced the number of observed ASVs which accounts for species richness (Callahan et al., 2016), as well as Shannon Diversity Index and Faith's Phylogenetic Diversity, which account for relative abundances (Shannon, 2001) and phylogenetic differences (Faith, 1992) of bacterial taxa, respectively.

We compared the relative abundances of bacterial taxa among treatment groups to determine which taxa contributed to differences seen in diversity and overall composition. Regardless of treatment, five bacterial phyla commonly dominated the composition of the tadpole microbiota: Firmicutes, Proteobacteria, Actinobacteriota, Bacteriodota, and Fusobacteria. These bacterial phyla are ubiquitous and are commonly found on the skin and within the GI tracts of amphibians (Fontaine et al., 2022; Kohl et al., 2013; Zhou et al., 2023). Conversely, two bacterial phyla, Verrucomicrobiota and Planctomycetota, and several bacterial genera were more abundant in the guts of tadpoles raised in natural pond water. Because these taxa are found in relatively high abundances in aquatic vertebrates, we suspect they are commonly found in our regional soil and freshwater habitats and were eliminated from the pond water by autoclaving (Freitas et al., 2012; Hamilton et al., 2023; Orellana et al., 2022). Supporting this, a previous study showed that the microbial profile of autoclaved pond water is distinct and less diverse compared with the microbial profile of natural pond water, and this was reflected in the gut microbial profiles of tadpoles raised in those water treatments (Fontaine et al., 2022).

Behavioral Assays

We found multiple behavioral differences between tadpoles raised in natural versus autoclaved pond water. In response to an empty vial and a vial filled with food, tadpoles raised in autoclaved pond water had decreased locomotory responses compared with tadpoles raised in natural pond water. These findings are in line with a study that showed that exposure of Northern Leopard Frog tadpoles to pesticides altered exploratory behavior in response to an empty jar (McClelland and Woodley, 2022). Amphibian exploration of unfamiliar environments and stimuli is important for resource acquisition and dispersal in the larval and metamorphic life stages and can indicate personality traits such as boldness (Carlson and Langkilde, 2013; Dingemanse et al., 2003; Kelleher et al., 2018; Toms et al., 2010; Wilson and Krause, 2012), which can have major implications on amphibian fitness and survival (Araujo et al., 2016; Réale et al., 2007).

To better understand which bacterial taxa contributed to the differences in behavior, we tested for associations between diversity metrics and behavioral endpoints. In addition to overall treatment effects on behavioral endpoints, we found that changes in behavior were associated with the diversity and composition of the gut microbiota. Specifically, tadpoles with higher alpha diversity exhibited increased locomotory and explorative activity in response to a vial filled with food. Further, tadpoles with higher alpha diversity exhibited increased activity in response to the addition of a food slurry. Similar results have been seen in other aquatic ectotherms such as zebrafish, which when raised in sterilized conditions, exhibit drastically altered locomotory and anxiety-like behavior compared to conventionally raised zebrafish (Davis et al., 2016). A potential explanation for altered responses to the vial filled with food and the food slurry could be due to gut microbially mediated differences in host satiety and feeding behavior. Gut bacteria-

derived metabolites can stimulate the secretion of host-derived peptides that interface with enteric neurons and vagal afferents and stimulate or inhibit eating (see comprehensive reviews (Cryan et al., 2019; Warne and Dallas, 2022)). While the production of bacteria-derived metabolites was not evaluated in this study, this interpretation also assumes tadpole recognition of food in the vial and slurry. Future studies should focus on evaluating the link between the gut microbiota and feeding behavior by incorporating metabolomics and explicit tests of feeding behavior.

Additionally, several bacterial taxa were associated with locomotory activity during behavioral assays. At the phylum level, Patescibacteria and Proteobacteria were positively associated with increased locomotory activity. These phyla were both associated with neurotransmitter levels such as serotonin in the cerebral cortex and hippocampus in rats (Zhang et al., 2021). The phylum Bdellovibrionota was also positively associated with increased activity in our tadpole behavioral assays, and recent studies have described this phylum to contain natural antibiotics that positively influence gut microbial community composition through protection from pathogens (Zhang et al., 2023b), although to our knowledge it has yet to be associated with behavioral parameters in vertebrates. The phylum Firmicutes, one of the most abundant phyla across treatments, was negatively associated with tadpole activity. Interestingly, our previous study in Green Frog tadpoles found an association between Firmicutes and increased size of the optic tectum (Emerson et al., 2023). The Firmicutes-associated *Clostridium* genus was also negatively associated with tadpole activity during behavioral assays, marking our second consecutive study where this genus was associated with neurodevelopmental endpoints in a larval amphibian (Emerson et al., 2023). Additionally, the Clostridia class, which contains the *Clostridium* genus, have been associated with altered brain morphology (Labus et al., 2017),

potentially through modulation of neurotransmitter synthesis and circulation (Yano et al., 2015). While many of these findings are correlative, it is important to document these associations and infer how shifts in the composition of the gut microbiota impact host biology (Alberdi et al., 2021).

Growth, Development, and Plasma CORT

Tadpole growth was significantly affected by our pond water treatment, such that tadpoles raised in autoclaved pond water were 20-25% heavier than tadpoles raised in natural pond water. They were also advanced in developmental stage, although the average GS in both groups was 35, which is a prometamorphic developmental stage. Thus, the phenotypic effects of autoclaved pond water are unlikely to be due to differences in developmental rate. Taken together, these results are similar to a previous study in Green Frogs (Fontaine et al., 2022). While CORT is a metabolic hormone associated with growth, (Denver, 1997; Denver, 2009; Denver, 2021) circulating levels of plasma CORT were not affected by treatment.

Differences in body mass could be attributed to shifts in gut microbial community composition that could influence host digestive performance. Tadpoles raised in autoclaved pond water had higher relative abundances of *Enterococcus*, a bacterial genus containing numerous species that range from being pathogenic to acting as probiotics (Hanchi et al., 2018). In poultry, administration of bacterial strains within the *Enterococcus* genus increased size, growth rates, and feeding conversion (Franz et al., 2011). Increased body mass could result from increased digestive efficiency due to higher abundances of *Enterococcus* in the tadpole gut. Additionally, bacterial metabolites such as short-chain fatty acids (SCFAs) can interface with enteroendocrine cells via the vagus nerve to influence food intake and feeding behavior, which can contribute to differences seen in body mass (see comprehensive review: (Warne and Dallas, 2022)). Future

studies can investigate links between gut microbial community composition and metabolite production and how they influence host growth and development (Warne and Dallas, 2022).

Relative Brain Mass and Shape

Tadpoles that were raised in autoclaved pond water had relatively larger brains and altered brain shape compared with tadpoles that were raised in natural pond water. Nervous tissue is energetically costly to develop and maintain (Aiello and Wheeler, 1995; Franz et al., 2011; Mink et al., 1981), and the differences in relative brain mass could be due to differences in digestive efficiency, as described above. In addition to larger relative brains, tadpoles raised in autoclaved pond water had significantly narrower medullas compared with tadpoles raised in natural pond water. Furthermore, Shannon Diversity Index was positively associated with medulla width. Studies in mammals (primarily rodents) have demonstrated links between gut microbial diversity and regional brain morphology, such as the amygdala, hippocampus, and forebrain (Castillo-Ruiz et al., 2018; Cryan et al., 2019; Luczynski et al., 2016; Sharvin et al., 2023), potentially through neural remodeling, modulation of synaptic pathways, and the morphology of microglial cells (Cryan et al., 2019; Erny et al., 2015; Sharvin et al., 2023). Regardless of the mechanism, the medulla is implicated in vital autonomic functions, such as auditory function and respiration (Gdovin et al., 1999; Woldring and Dirken, 1951).

These results complement the results from our previous study showing that Green Frog tadpoles raised in autoclaved pond water had relatively larger brains and increased optic tecta size compared to tadpoles raised in natural pond water (Emerson et al., 2023). Additionally, we found that gut microbial alpha diversity was negatively associated with optic tectum size (Emerson et al., 2023). While the changes in brain architecture were not the same across these

studies, our work shows that the aquatic microbial environment influences brain development in multiple amphibian species.

Conclusion

We found that the nature of the aquatic microbial communities in which Northern Leopard Frog tadpoles develop affects the composition of their associated gut microbiota. Additionally, changes in neurodevelopment, measured through behavior and brain morphology, were associated with metrics of gut bacterial diversity and the composition of the gut microbiota. Collectively, our results suggest that the formation of the MGB axis can have important effects on amphibian biology related to health, performance, and fitness.

Future studies should evaluate gut bacterial influence on ecologically relevant behaviors such as anti-predatory and feeding behavior, as well as performing downstream meta-analyses of gut bacterial profiles (Alberdi et al., 2021; Li et al., 2022). Further, studies can continue to investigate the cellular and molecular mechanisms driving changes in neurodevelopmental phenotypes, such as exploring changes in microglia cell morphology and/or gene expression. In sum, our study used an experimental approach to demonstrate that aquatic microbial communities impact amphibian behavior and fitness through host-microbe interactions. Our work can help inform wildlife conservation efforts that focus on the health of aquatic ecosystems, especially as amphibians face numerous challenges due to anthropogenic influence (Trevelline et al., 2019).

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DATA + CODE AVAILABILITY

Raw sequence data for the tadpole gut microbiota are available at NCBI's Sequence Read Archive (SRA) under accession no. PRJNA1023542. Raw data sets and R code used for analyses are available from the Dryad repository ((Emerson and Woodley, (Forthcoming 2024)); https://doi.org/10.5061/dryad.wstqjq2t2) and Zenodo repository (https://doi.org/10.5281/zenodo.10070089), respectively.

Table 3.1: Sample sizes for each endpoint. In each bin, tadpoles were randomly selected for assessment of different endpoints. First, one tadpole in each bin was selected to assess behavior. After behavioral assays, the tadpole was euthanized, guts were removed for analysis of the gut microbiota, and a blood sample was collected for measurement of plasma CORT. Measurements of body mass, Gosner Stage (GS), and brain mass and shape were recorded. Second, another tadpole in each bin was selected to assess plasma CORT, body mass, GS, and brain mass and shape. A third tadpole in each bin was selected to assess body mass, GS, brain mass and shape. Tadpoles 4 and 5 were not used for data collection. + indicates that the endpoint was assessed in the tadpole.

Bin	Behavior	Gut Microbiota	Plasma CORT	Body Mass, Developmental Rate, Brain Mass and Shape
Tadpole 1	+	+	+	+
Tadpole 2	-	-	+	+
Tadpole 3	-	-	-	+
Tadpole 4	-	-	-	-
Tadpole 5	-	-	-	-

Table 3.2: Relative abundances of gut bacterial phyla that were significantly impacted by pond water treatment in Northern Leopard Frog tadpoles. Prevalence is the percentage of tadpoles where the phylum was found. Statistical testing was conducted using MaAsLin2. P-values were corrected using the BH FDR method. A negative coefficient signifies a higher abundance in the natural pond water; a positive coefficient signifies a higher abundance in the autoclaved pond water. N = 23 tadpoles.

Phyla	Prevalence	Coefficient	FDR P-value
Verrucomicrobiota	69.6	-0.05	<0.01
Planctomycetota	65.2	-0.05	0.013
Fusobacteriota	100.0	0.20	0.075

Table 3.3: Relative abundances of gut bacterial genera that were significantly impacted by pond water treatment in Northern Leopard Frog tadpoles. Prevalence is the percentage of tadpoles where the genus was found. Statistical testing was conducted using MaAsLin2. P-values were corrected using the BH FDR method. A negative coefficient signifies a higher abundance in the natural pond water; a positive coefficient signifies a higher abundance in the autoclaved pond water. N = 23 tadpoles.

Genus	Prevalence	Coefficient	FDR P-value
Mycobacterium	52.2	-0.08	<0.001
Pseudarthrobacter	52.2	-0.06	<0.001
Candidatus.Ovatusbacter	65.2	0.03	<0.01
Alsobacter	52.2	-0.03	<0.01
Iamia	43.5	-0.008	<0.01
Paracoccus	60.8	0.06	<0.05
Terrimicrobium	60.8	-0.04	<0.05
Nakamurella	39.1	-0.03	<0.05
Crenobacter	91.3	-0.06	<0.05
Leifsonia	30.4	-0.02	<0.05
Microvirga	78.3	0.03	<0.05
SH.PL14	43.5	-0.01	<0.05
Dysgonomonas	82.6	-0.2	<0.05
Enterococcus	73.9	0.05	<0.05
Rhodoblastus	34.8	-0.02	<0.05
Gemmobacter	30.4	-0.01	<0.05
Paludisphaera	30.4	-0.007	<0.05
Rhodomicrobium	39.1	-0.03	<0.05
Rhodovastum	34.8	-0.01	<0.05
Rosiearcus	39.1	-0.03	<0.05
Candidatus.Protochlamydia	39.1	-0.02	<0.05
Tundrisphaera	26.1	-0.008	<0.05
Microbacterium	87.0	0.03	<0.05
Longivirga	30.4	-0.05	<0.05

Table 3.4: Effects of pond water treatment on locomotory behavior in response to an empty vial in Northern Leopard Frog tadpoles. Principal components analysis (PCA) reduced eight behavior measurements into three principal components (PCs). PCs were analyzed with GLMMs. Significant results are bolded.

Results of PCA	**PC-1**	**PC-2**	**PC-3**
% of Variance	60.6	15.3	12.8
Eigenvalue	4.85	1.22	1.02
Factor Loading*			
Average Speed	0.976	-	-
Total Distance Travelled	0.975	-	-0.101
Average Acceleration	0.960	-	-0.136
Mobility Rate	0.936	-0.227	-
Exploration Rate	0.465	-0.517	0.273
Time in Center	-0.216	-	0.923
Total Time Frozen	-0.937	0.216	0.158
Time Near Object	-	0.912	0.161
Results of GLMM			
Pond Water: (F value, p)	**4.7, 0.043**	0.046, 0.83	1.9, 0.18

*Factor loadings less than an absolute value of 0.1 are denoted as hyphens.

Table 3.5: Effects of pond water treatment on locomotory behavior in response to a vial containing food in Northern Leopard Frog tadpoles. Principal components analysis (PCA) reduced eight behavior measurements in response to a vial containing frog brittle into three principal components (PCs). PCs were analyzed with GLMMs. Significant results are bolded.

Results of PCA	**PC-1**	**PC-2**	**PC-3**
% of Variance	59.9	14.8	12.9
Eigenvalue	4.79	1.19	1.03
Factor Loading*			
Average Speed	0.984	0.101	-
Total Distance Travelled	0.985	0.102	-
Average Acceleration	0.979	0.118	-
Mobility Rate	0.949	0.143	-0.104
Exploration Rate	-	0.874	-0.196
Time in Center	-0.135	-	0.876
Total Time Frozen	-0.953	-0.159	0.102
Time Near Object	-0.237	-0.576	-0.450
Results of GLMM			
Pond Water: (F value, P)	**4.12, 0.048**	0.14, 0.72	2.24, 0.15

*Factor loadings less than an absolute value of 0.1 are denoted as hyphens.

Table 3.6: Effects of pond water treatment on locomotory behavior in Northern Leopard Frog tadpoles in response to a food slurry. Principal components analysis (PCA) reduced eight behavior measurements into three principal components (PCs). Principal components were analyzed with GLMMs with bin as a random effect. Significant results are bolded.

Results of PCA	**PC-1**	**PC-2**	**PC-3**
% of Variance	60.1	16.9	14.3
Eigenvalue	4.81	1.35	1.14
Factor Loading*			
Average Speed	0.981	-0.104	-
Total Distance Travelled	0.981	-0.107	-
Average Acceleration	0.982	-	-
Mobility Rate	0.967	-	0.154
Exploration Rate	0.160	-	0.927
Time in Center	-0.104	0.786	0.362
Total Time Frozen	-0.974	-	-
Time Near Cue	-	0.838	-0.329
Results of GLMM			
Pond water: (F value, P)	1.6, 0.22	0.084, 0.78	0.19, 0.67

*Factor loadings less than an absolute value of 0.1 are denoted as hyphens.

Table 3.7: Effects of pond water treatment on relative brain shape of Northern Leopard Frog tadpoles. Principal components analysis (PCA) reduced seven linear brain dimensions to three principal components (PCs). Principal components were analyzed with GLMMs with bin as a random effect. Significant results are bolded.

Results of PCA	**PC-1**	**PC-2**	**PC-3**
% of Variance	32.1	26.4	16.8
Eigenvalue	2.25	1.85	1.18
Factor Loading*			
Telencephalon Width	0.426	0.750	0.173
Telencephalon Length	-	0.957	-
Optic Tectum Width	0.718	0.231	-
Optic Tectum Length	0.898	-	-
Diencephalon Width	0.661	0.407	0.383
Diencephalon Length	0.531	0.290	-0.500
Medulla Width	0.161	0.244	0.857
Results of GLMM			
Pond Water: ($\chi_{(1)}^2$, p)	0.83, 0.36	0.30, 0.59	**6.1, 0.013**

*Factor loadings less than an absolute value of 0.1 are denoted as hyphens.

Table 3.8. Results from GLMM testing whether alpha diversity metrics are associated with relative brain and behavioral endpoints in Northern Leopard Frog tadpoles. Shown is a chi-square distributed test statistic and p-value associated with a Type II Wald test. Significant effects are bolded.

Endpoint	Shannon Diversity Index (χ^2, P)	No. Observed ASVs (χ^2, P)	Faith's Phylogenetic Diversity (χ^2, P)
Relative Brain Mass	**4.4, 0.036**	0.05, 0.83	0.11, 0.75
Relative Brain Shape (PC-3)	**8.6, 0.003**	1.1, 0.29	0.69, 0.41
Response to vial containing food (PC-1)	1.7, 0.20	**4.0, 0.045**	**4.0, 0.046**
Response to vial containing food (PC-2)	**4.3, 0.039**	1.1, 0.30	0.56, 0.45
Response to food slurry (PC-2)	0.021, 0.88	**3.9, 0.049**	**5.8, 0.016**

Table 3.9. Complete results from GLMM testing whether alpha diversity metrics are associated with brain and behavioral endpoints in Northern Leopard Frog tadpoles. Shown is a chi-square distributed test statistic and p-value associated with a Type II Wald test. Significant effects are bolded.

Endpoint	Shannon Diversity Index (χ^2, P)	No. Observed ASVs (χ^2, P)	Faith's Phylogenetic Diversity (χ^2, P)
Relative Brain Mass	**4.4, 0.04**	0.05, 0.83	0.11, 0.75
Relative Brain Shape			
(PC-1)	0.36, 0.55	0.65, 0.42	0.58, 0.45
(PC-2)	3.5, 0.06	0.82, 0.37	0.52, 0.47
(PC-3)	**8.6, 0.003**	1.1, 0.29	0.69, 0.41
Response to Empty Vial			
(PC-1)	2.7, 0.10	0.99, 0.32	1.1, 0.29
(PC-2)	0.41, 0.52	0.92, 0.34	0.70, 0.41
(PC-3)	0.47, 0.49	1.1, 0.30	0.79, 0.37
Response to Vial containing Food			
(PC-1)	1.7, 0.20	**4.0, 0.045**	**4.0, 0.046**
(PC-2)	**4.3, 0.039**	1.1, 0.30	0.56, 0.45
(PC-3)	2.0, 0.16	0.014, 0.91	0.12, 0.73
Response to Food Slurry			
(PC-1)	1.9, 0.17	3.5, 0.063	3.5, 0.060

(PC-2)	0.02, 0.88	**3.9, 0.049**	**5.8, 0.016**
(PC-3)	3.6, 0.059	0.32, 0.57	0.020, 0.89

Table 3.10: Relative abundances of gut bacterial phyla and genera that were significantly associated with the locomotory behavior of Northern Leopard Frog tadpoles. Prevalence is the percentage of tadpoles where the taxon was found. Statistical testing was conducted using MaAsLin2. P-values were corrected using the BH FDR method. The sign of the coefficient indicates the direction of the correlation; positive coefficients indicate that the taxon is associated with increased locomotion. Relative brain mass and relative brain shape were not associated with relative abundances of any bacterial taxa (data not shown). N = 23 tadpoles.

Phylum	**Prevalence**	**Coefficient**	**FDR P-value**
Response to Empty Vial (PC-1)			
Patescibacteria	34.8	0.003	0.042
Proteobacteria	100	0.12	0.042
Response to Food Slurry (PC-3)			
Bdellovibrionota	82.6	0.03	0.05
Firmicutes	100	-0.08	0.05
Genus	**Prevalence**	**Coefficient**	**FDR P-value**
Response to Vial with Food (PC-2)			
Clostridium.sensu.stricto.1	69.6	-0.04	<0.01
Response to Food Slurry (PC-2)			
Clostridium.sensu.stricto.1	69.6	-0.04	0.05
Response to Food Slurry (PC-3)			
Brevundimonas	39.1	0.02	0.03

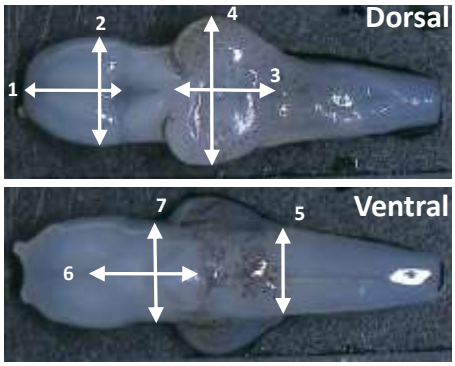

Figure 3.1: Dorsal and ventral view of a Northern Leopard Frog tadpole brain. The anterior end of the brain is on the left. Arrows represent the measurements of 7 linear brain dimensions that were analyzed to describe brain morphology: 1) telencephalon length; 2) telencephalon width; 3) optic tectum length; 4) optic tectum width; 5) medulla width; 6) diencephalon length; 7) diencephalon width.

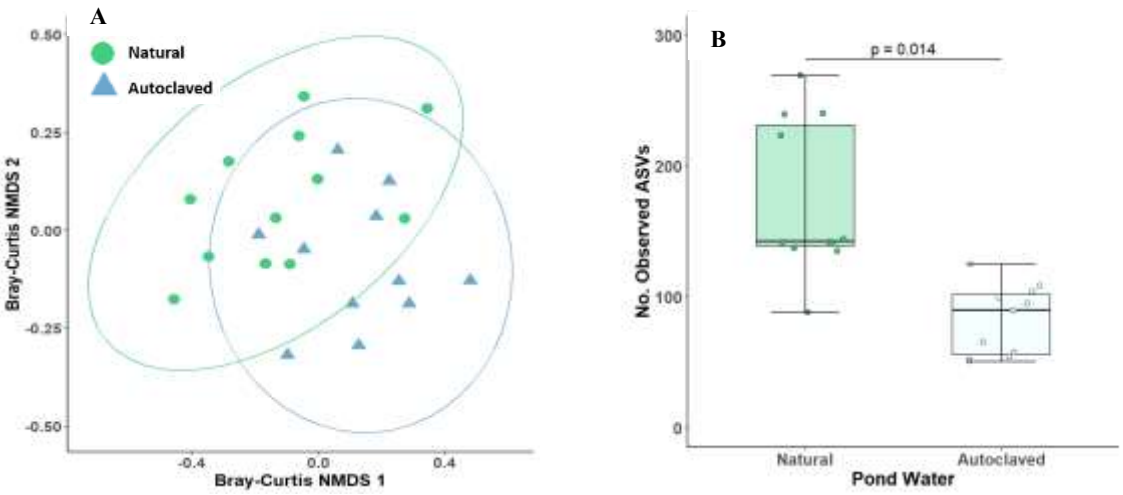

Figure 3.2: Effects of pond water treatment on the composition and diversity of the gut microbiota in Northern Leopard Frog tadpoles. (A) Non-metric multidimensional scaling plot based on Bray Curtis dissimilarity of gut microbial communities. Tadpoles raised in autoclaved pond water harbored distinct gut microbial communities compared to tadpoles raised in natural pond water ($p = 0.004$). N = 12 Natural, N = 11 Autoclaved. **(B)** Tadpoles raised in autoclaved pond water harbored fewer ASVs compared to tadpoles raised in natural pond water. In boxplots, the center line represents the median, the box length represents the interquartile range (IQR), and whiskers extend to 1.5x IQR. Points represent individual values. N = 12 Natural (outlier excluded), 11 Autoclaved.

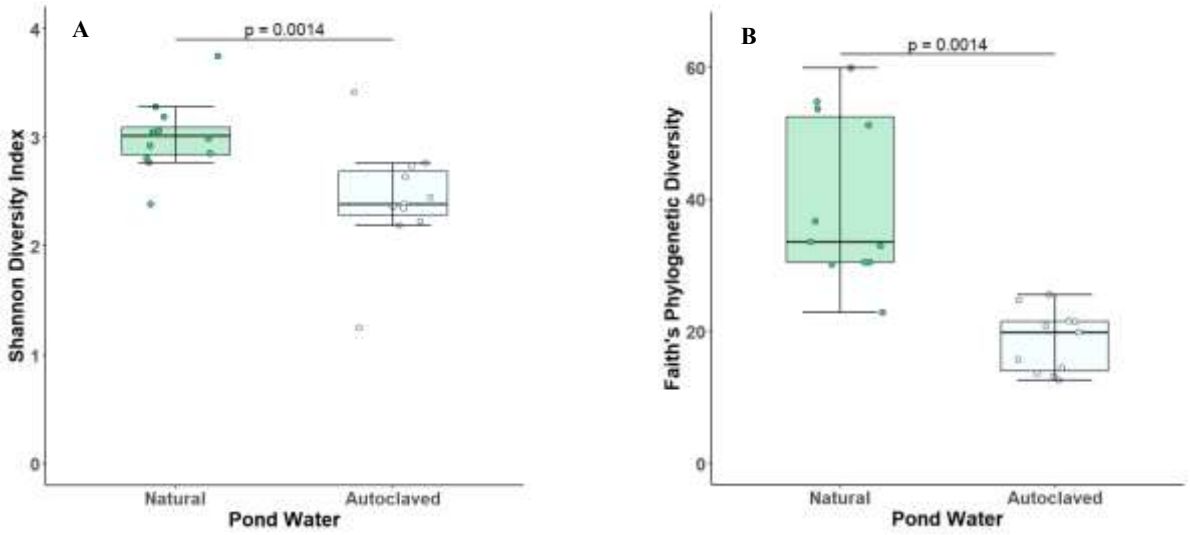

Figure 3.3: Effects of pond water treatment on Shannon Diversity Index and Faith's Phylogenetic Diversity in Northern Leopard Frog tadpoles. (A) Tadpoles raised in autoclaved pond water had a lower Shannon Diversity Index compared to tadpoles raised in natural pond water. **(B)** Tadpoles raised in autoclaved pond water had a lower Faith's Phylogenetic Diversity score compared to tadpoles raised in natural pond water. In boxplots, the center line represents the median, the box length represents the interquartile range (IQR), and whiskers extend to 1.5x IQR. Points represent individual values. Significant effects of statistical tests (GLMMs) are shown above box plots. N = 12 Natural (outlier excluded), 11 Autoclaved.

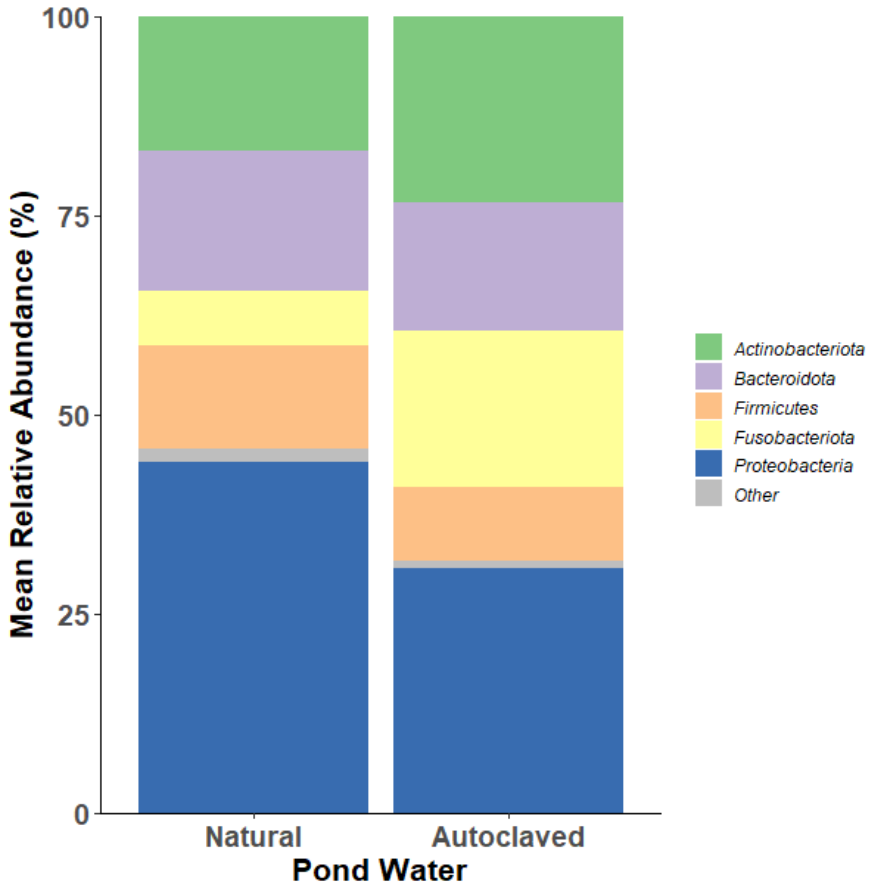

Figure 3.4: Effects of pond water treatment on the relative abundances of bacterial phyla in Northern Leopard Frog tadpole guts. The top five most abundant phyla are shown. Bacterial phyla with a mean relative abundance of less than 1% were pooled into the 'Other' category. N = 12 Natural, 11 Autoclaved.

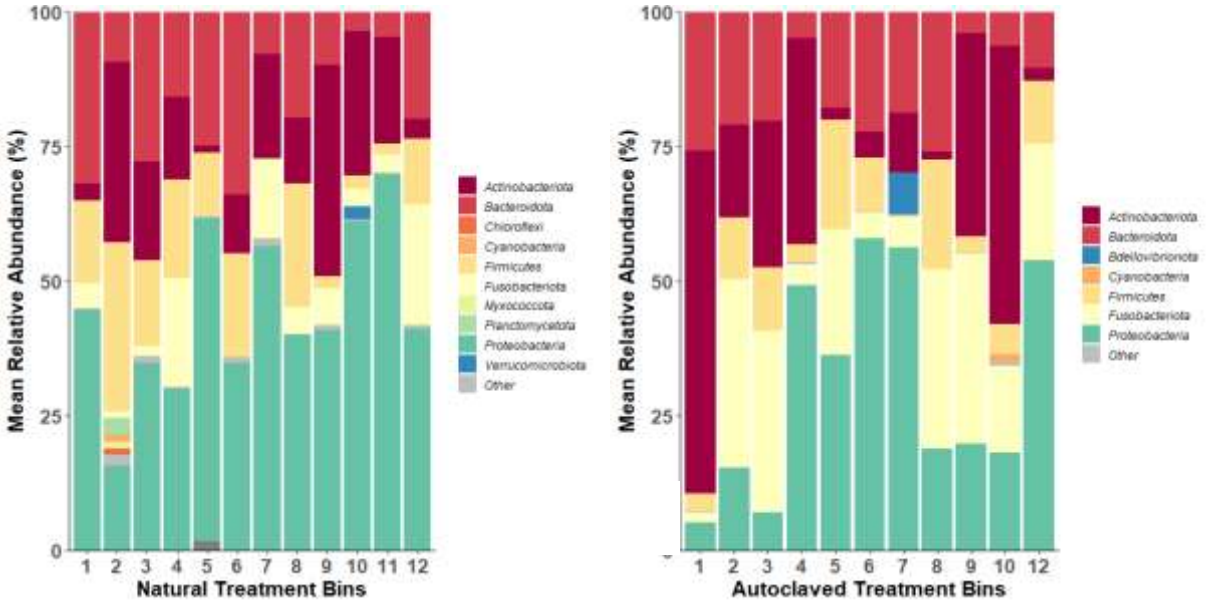

Figure 3.5: Relative abundances of bacterial phyla in Northern Leopard Frog tadpole guts. The gut microbiota was profiled for one tadpole per bin. The relative abundances of bacterial phyla with greater than or equal to 1% are shown. Bacterial phyla with less than 1% relative abundances were pooled into the 'Other' category. The gray bar in bin 5 in the natural pond water treatment is unassigned bacterial phyla.

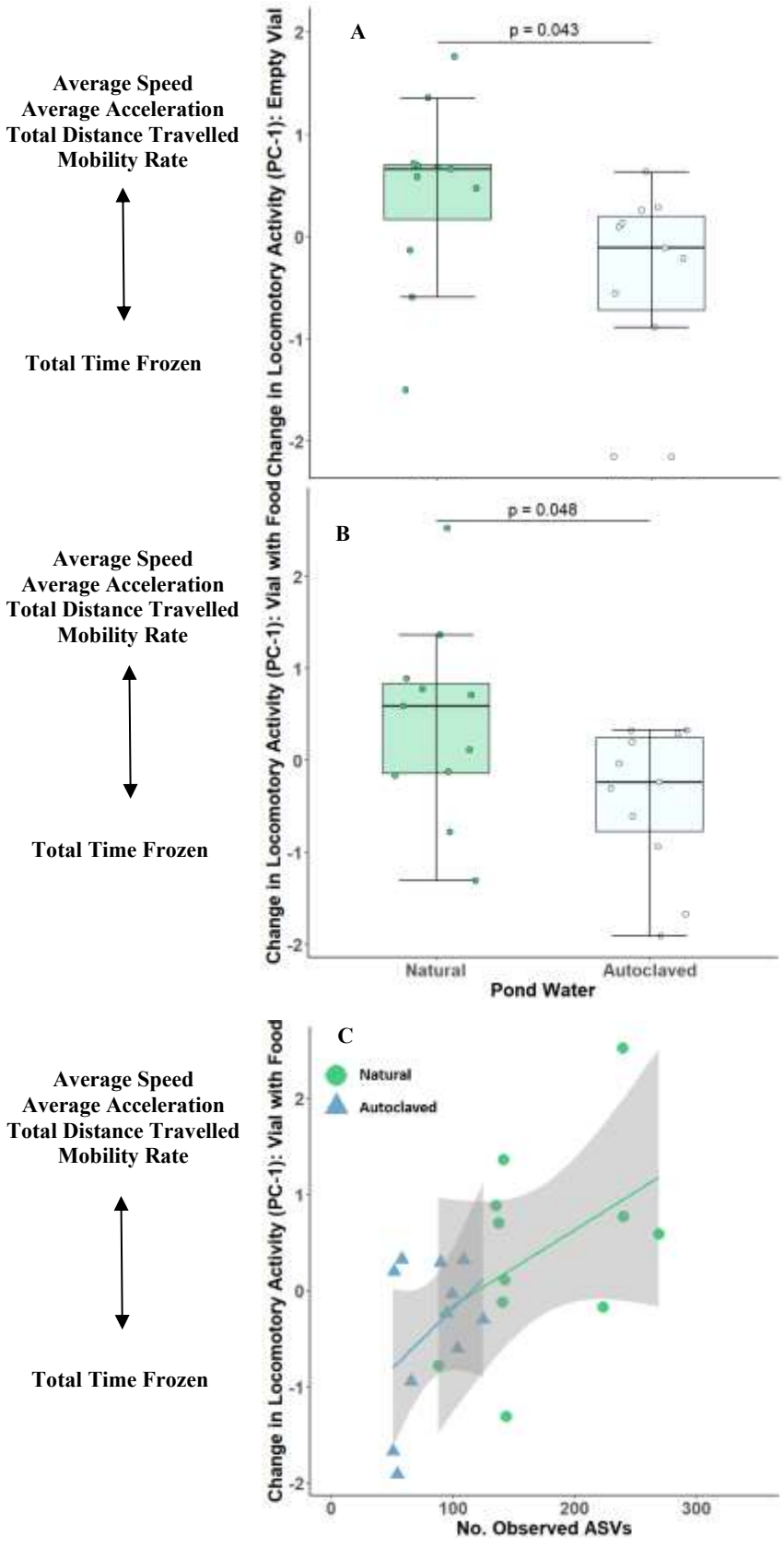
Average Speed
Average Acceleration
Total Distance Travelled
Mobility Rate
Total Time Frozen
A
p = 0.043
Change in Locomotory Activity (PC-1): Vial with Food
Change in Locomotory Activity (PC-1): Empty Vial
B
p = 0.048
Natural
Autoclaved
Pond Water
C
Natural
Autoclaved
No. Observed ASVs

Figure 3.6: Effects of pond water treatment on locomotory behavior response to sensory stimuli in Northern Leopard Frog tadpoles. (A) Empty vial: Tadpoles raised in natural pond water had a greater increase in locomotory activity compared with tadpoles raised in autoclaved pond water. **(B)** Vial containing food: Tadpoles raised in natural pond water had increased locomotory activity compared with tadpoles raised in autoclaved pond water. N = 11 Natural, 11 Autoclaved. Boxplots are as in **Fig. 3.3**. **(C)** The number of observed ASVs was associated with locomotory activity in response to a vial containing food ($F = 4.01$, $p = 0.045$). Trend lines were created using linear regression models, and grey shading indicates 95% confidence intervals. The variance in medulla width was modestly explained by the number of observed ASVs (Multiple $R^2 = 32.9$). N = 12 Natural (outlier excluded), 11 Autoclaved.

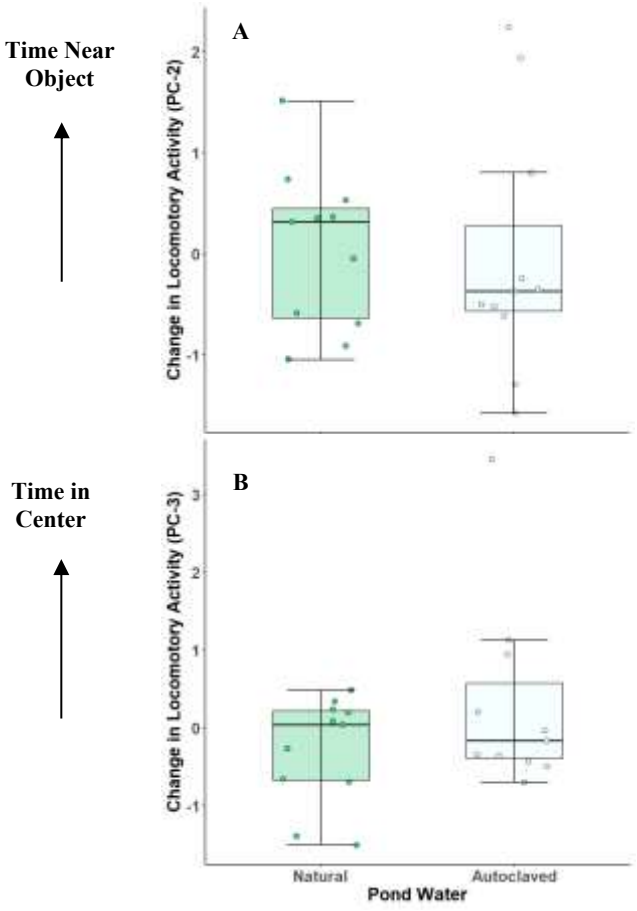

Figure 3.7: Effects of pond water treatment on locomotory behavior as described by PC-2 and PC-3 in Northern Leopard Frog tadpoles in response to an empty vial. In response to an empty vial, pond water treatment did not affect: **(A)** the duration of time that tadpoles spent near the empty vial, and **(B)** the duration of time that tadpoles spent in the center of the arena. Boxplots are as in **Figure 3.3**. N = 11 Natural, 11 Autoclaved.

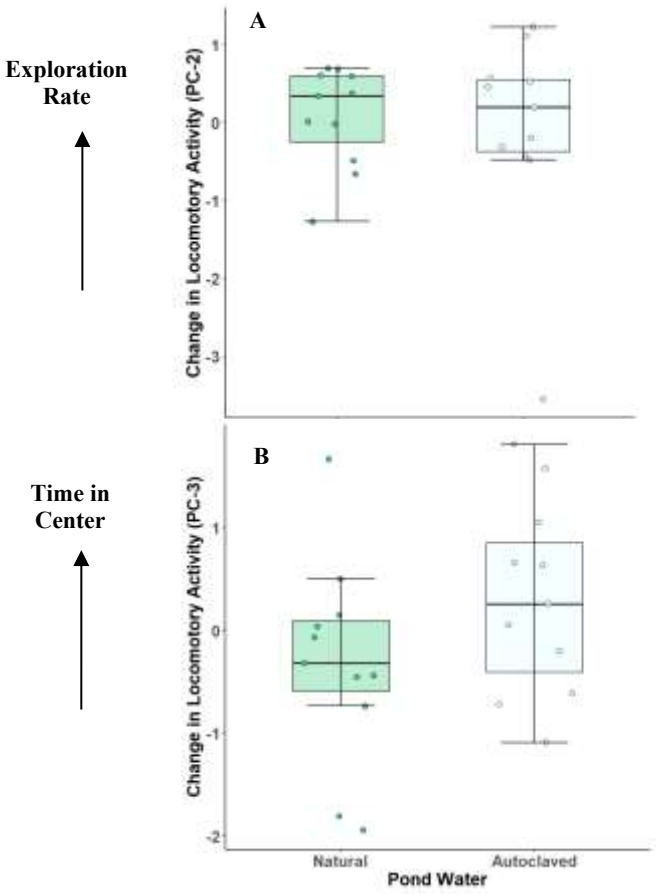

Figure 3.8: Effects of pond water treatment on locomotory behavior as described by PC-2 and PC-3 in Northern Leopard Frog tadpoles in response to a vial containing food brittle. In response to a vial containing food, pond water treatment had no effect on: **(A)** the exploration rate (PC-2), and **(B)** the time spent in the center of the arena (PC-3). Boxplots are as in **Figure 3.3.** N = 11 Natural, 11 Autoclaved.

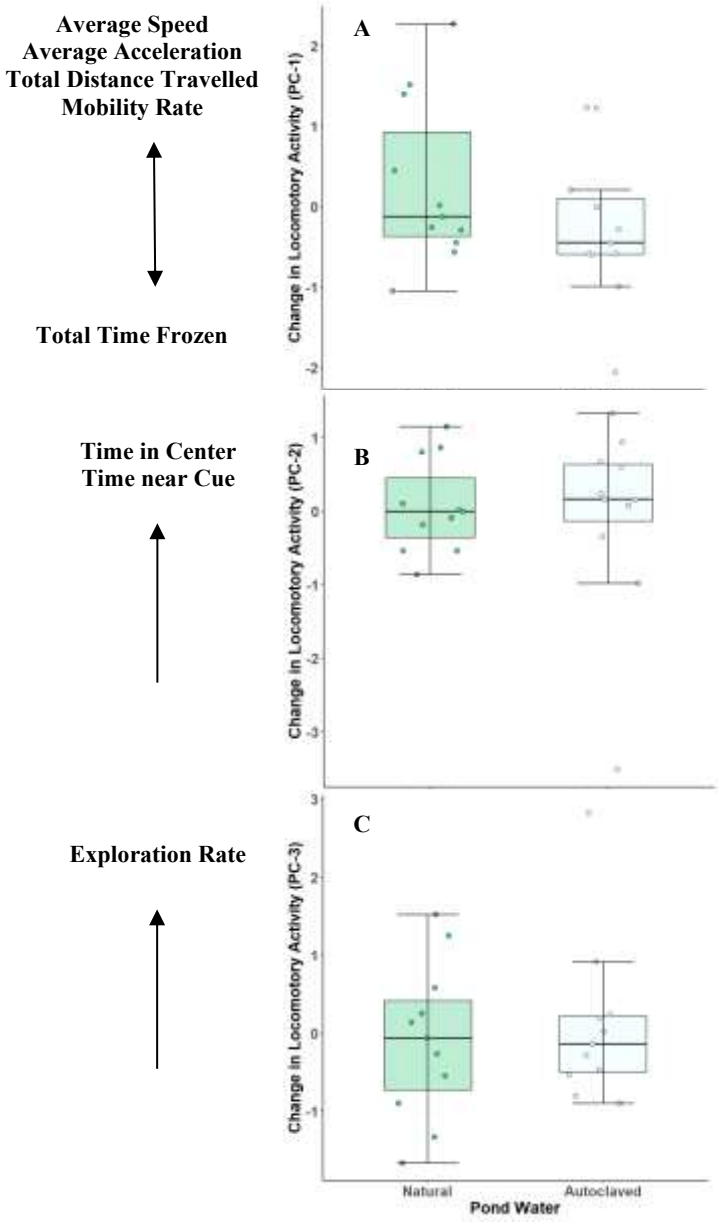

Figure 3.9: Effects of pond water on locomotory behavior in Northern Leopard Frog tadpoles in response to a food slurry. In response to a food slurry, the type of pond water had no effect on: **(A)** locomotory activity (PC-1), **(B)** the duration of time that tadpoles spent in the center of the behavior arena or time near the olfactory cue (PC-2), or **(C)** the exploration rate (PC-3). Boxplots are as in **Figure 3.3**. N = 11 Natural, 11 Autoclaved.

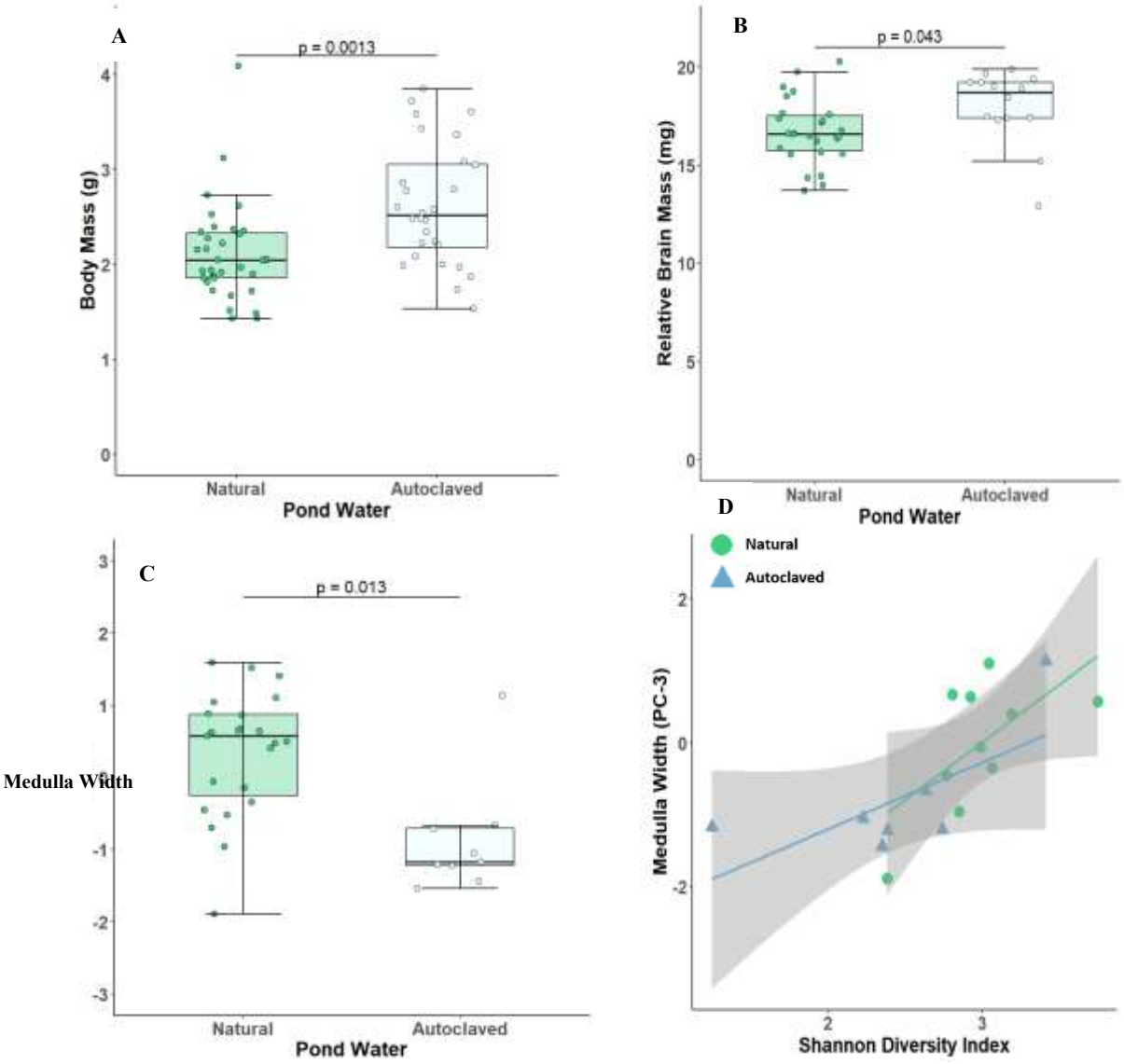

Figure 3.10: Effects of pond water treatment on growth and brain development in Northern Leopard Frog tadpoles. Compared with tadpoles raised in natural pond water, tadpoles raised in autoclaved pond water: **(A)** Were larger. N = 35 Natural, 28 Autoclaved. **(B)** Had relatively larger brains. N = 26 Natural, 14 Autoclaved. **(C)** Had relatively narrower medullas. N = 23 Natural, 9 Autoclaved. Boxplots are as in **Fig. 3.2B**. **(D)** Had a lower Shannon Diversity Index score, which was associated with medulla width ($F = 8.6$, $p = 0.003$). Trend lines are as in **Fig. 3.6C**. The variance in medulla width was modestly explained by Shannon Diversity Index (Multiple $R^2 = 48.5$). N = 10 Natural, 7 Autoclaved.

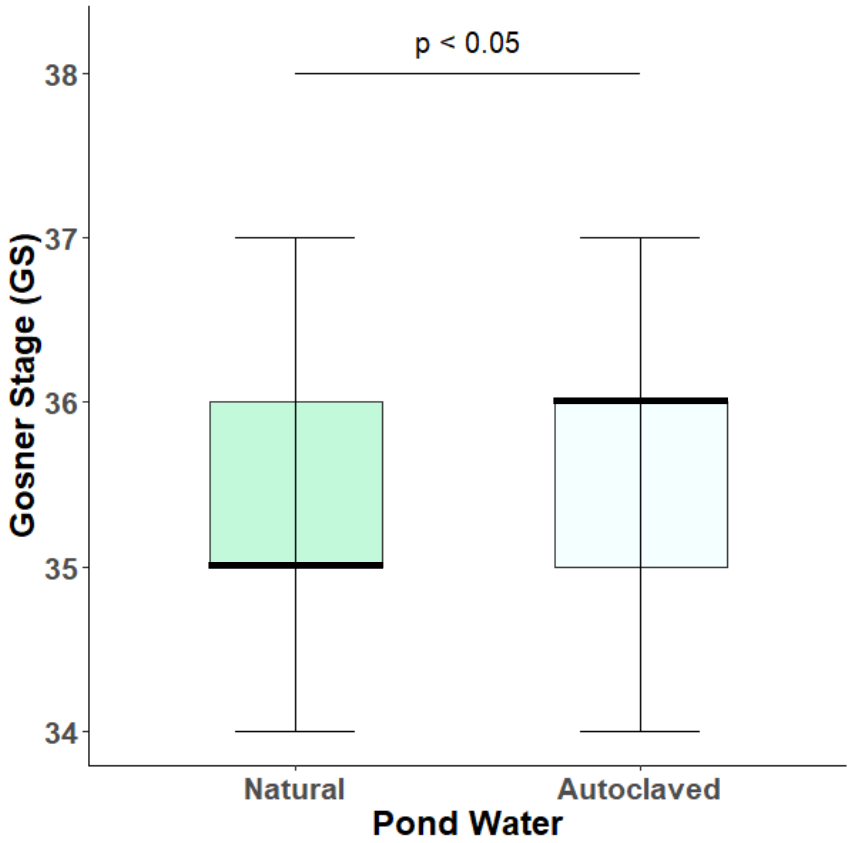

Figure 3.11: Effects of pond water treatment on development in Northern Leopard Frog tadpoles. Tadpoles raised in autoclaved pond water developed faster compared to tadpoles raised in natural pond water. Boxplots are as in **Figure 3.3**. Significant effects of statistical tests (GLMMs with bin as a random effect) are shown above box plots. N = 35 Natural, 28 Autoclaved.

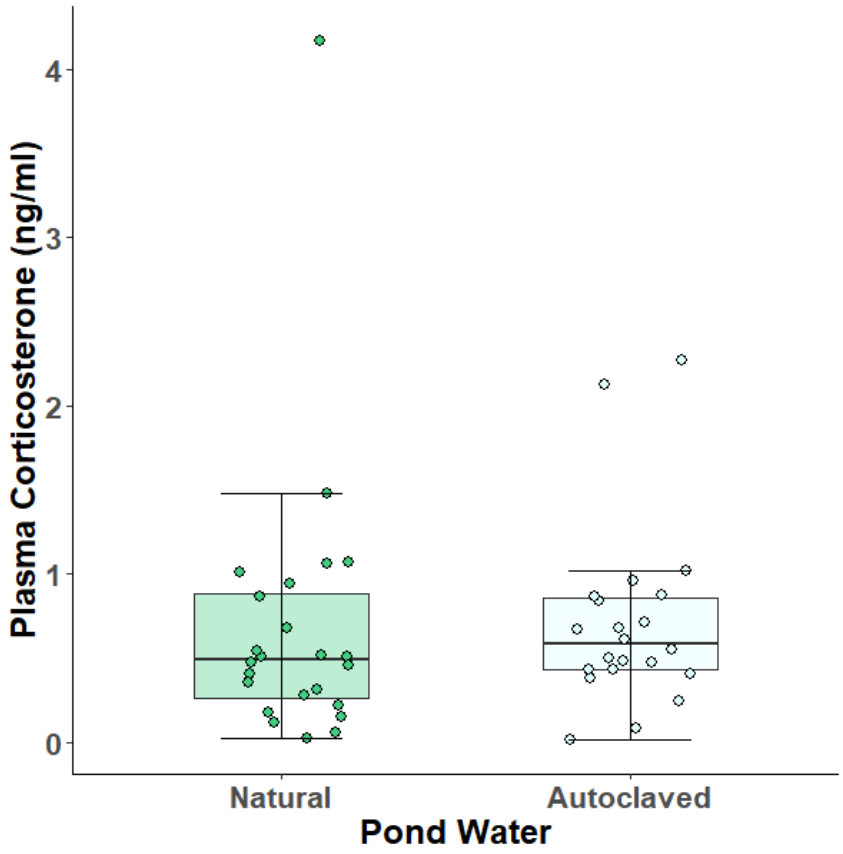

Figure 3.12: Effects of pond water treatment on plasma corticosterone production in Northern Leopard Frog tadpoles. Pond water did not affect plasma corticosterone production. Box plots are as in **Figure 3.3**. N = 24 Natural, 21 Autoclaved.

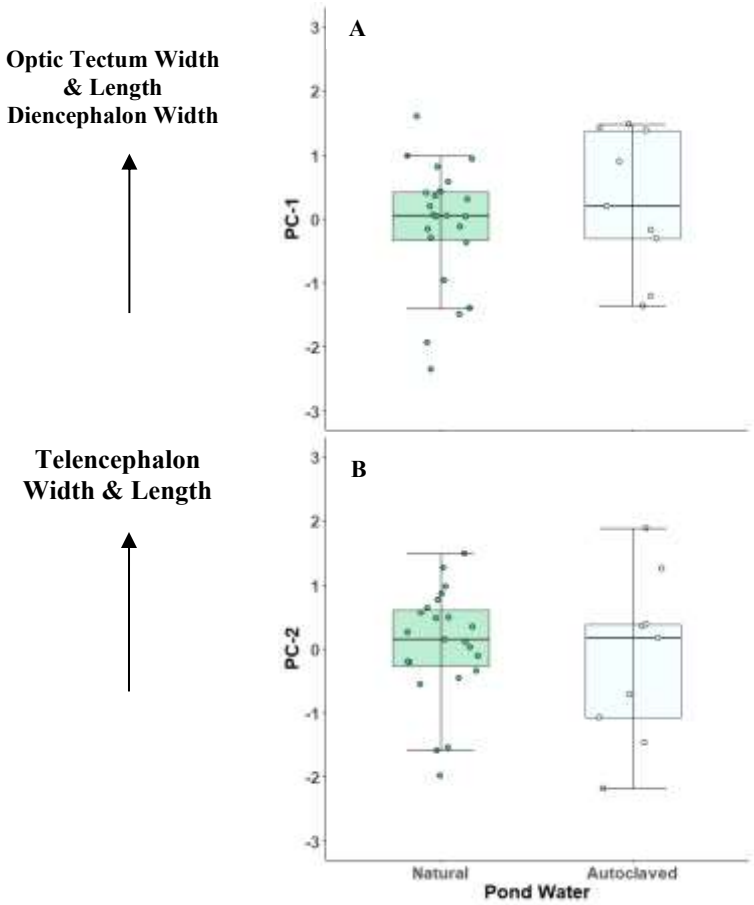

Figure 3.13: Effects of pond water treatment on PC-1 and PC-2 describing relative brain shape in Northern Leopard Frog tadpoles. (A) Pond water had no effect on optic tectum width, optic tectum length, or diencephalon width (PC-1). **(B)** Pond water had no effect on telencephalon width and length (PC-2). Boxplots are as in **Figure 3.3**. N = 23 Natural, 9 Autoclaved.

Chapter 4. Effects of stress and the aquatic microbial environment on the microbiota, morphology, physiology, and behavior of larval amphibians.

ABSTRACT

Animals participate in symbiotic relationships with microbes that live on and within them. Denoted as the host-associated microbiota, these communities contribute to physiological functions imperative for many aspects of development, including neurodevelopment through the microbiota-gut-brain (MGB) axis. The composition of the vertebrate microbiota, and subsequently the establishment of the MGB axis, has been shown to be influenced by stressors and stress hormones, such as glucocorticoids (GCs). Most investigations into the relationship between stress, GCs, and the microbiota use germ-free (GF) and/or mammalian models, while this relationship in wildlife animals is underexplored. Here, I raised newly hatched Northern Leopard Frog (*L. pipiens*) tadpoles in natural (unmanipulated) or autoclaved pond water to evaluate how aquatic microbial communities influence the host-associated microbiota, physiology, and behavior. At the same time, tadpoles were exposed to predation-derived cues or exogenous corticosterone (CORT, primary GC in amphibians) to evaluate if the impact of the aquatic microbial environment on tadpole development persisted when simultaneously exposed to ecologically relevant stressors. Development of tadpoles in autoclaved pond water influenced the gut and skin microbiota, body size, relative brain size and shape, and behavior compared to tadpoles raised in natural pond water. Interestingly, tadpoles raised in autoclaved pond water displayed increased locomotory activity when exposed to tadpole alarm pheromones, which is consistent with a reduced ability to avoid predation, compared to tadpoles raised in natural pond water. Additionally, stressor and pond water treatments had interactive effects on the composition of the gut microbiota, most likely due to CORT effects. CORT exposure also

increased tail muscle size, which has been found in previous studies. We found no clear effects of predator cues and surprisingly few interactive effects of the pond water treatment and stressors. Overall, our results replicated previous findings in support of an amphibian MGB axis but found minimal support for a role of stressors in shaping this axis.

INTRODUCTION

Animal health and development are strongly influenced by the microbial communities that colonize their external and internal surfaces (Kohl and Carey, 2016). These communities are known as the host-associated microbiota and are comprised of mostly bacteria, as well as fungi, protists, and viruses. The microbiota exists in a symbiotic relationship with the host that is rooted in co-evolution and interdependency (McFall-Ngai et al., 2013). In many cases, the microbiota provides services to the host (Gilbert et al., 2012; Kohl and Carey, 2016; Li et al., 2014; McFall-Ngai et al., 2013; McFall-Ngai, 2015) that impact development, performance, health, and fitness of the host. The initial composition of the host-associated microbiota is determined by the microbes present in the immediate environment at hatching or birth (Correa et al., 2020; Litvak and Bäumler, 2019; Martínez et al., 2018) and is relatively plastic in early life due to host-related and environmental factors (Cowan et al., 2020; Maritan et al., 2024; Warne et al., 2019).

Growing evidence supports the imperative role of the microbiota in the development of the vertebrate nervous system. Gut-dwelling microbes, denoted as the gut microbiota, can synthesize and/or stimulate the biosynthesis of neurotransmitters, short-chain fatty acids (SCFAs), and other neuroactive molecules that influence the enteric, peripheral, and central nervous systems (Cryan et al., 2019; Yano et al., 2015). The pathway of communication between the gut microbiota and brain is denoted as the microbiota-gut-brain (MGB) axis (Cryan et al., 2019). Much of our understanding of the MGB axis comes from studies of germ-free (GF)

animals, primarily domesticated mammals. Compared to conventionally raised counterparts, GF animals display differences in neurophysiology, such as altered neurogenesis, dendritic growth, myelination, synaptic plasticity, and immune parameters (see comprehensive review (Cryan et al., 2019)). Additionally, GF animals exhibit differences in their social, locomotory, and anxiety-like behaviors compared to control animals (Cryan et al., 2019; Davis et al., 2016; Heijtz et al., 2011). These alterations in neurophysiology and behavior are hypothesized to be detrimental to the host and could represent precursors to neurodevelopmental and neurodegenerative disorders (Cryan et al., 2019).

The composition of the gut microbiota, and subsequently the development of the MGB axis, is likely shaped by stressors. Exposure to stress is a normal part of the lives of animals and leads to the activation of the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis. The activation of the HPA/I axis leads to increased circulation of glucocorticoids (GCs) which have widespread effects that help an animal cope with the stressor (Denver, 2009; Martin, 2009; Sapolsky et al., 2000). Additionally, stressors and the associated GCs can influence the composition of the vertebrate gut microbiota. For example, chronic exposure to a stressor in rats led to impaired mucosal defenses against luminal bacteria (Söderholm et al., 2002), which can lead to shifts in the habitability of the host for certain microbes. Additionally, mice exposed to exogenous GCs exhibited drastic shifts in the composition of the gut microbiota (Huang et al., 2015), and increased concentrations of GC metabolites in squirrel feces was associated with decreased gut bacterial diversity (Petrullo et al., 2022). Interestingly, the majority of bacterial diversity loss was due to the loss of rare taxa with lower relative abundances (Petrullo et al., 2022), which is thought to be detrimental to host health (Stecher et al., 2010).

Despite the progress gained by studying GF and/or primarily mammalian animals, host-microbe relationships are understudied in many wildlife vertebrate species (Colston and Jackson, 2016; Pascoe et al., 2017; Trevelline et al., 2019). Larval amphibians are excellent models for furthering our understanding of these relationships as well as the development of the MGB axis. Larval amphibians develop in bodies of freshwater that contain microbial communities that drastically vary in their composition based on geography and local factors (Stocker et al., 2024). These aquatic microbial communities are a primary source of the host-associated microbiota in larval amphibians and likely contribute to population variation in the development of the gut microbiota (Correa et al., 2020). We and others have shown that raising tadpoles in autoclaved pond water is a non-invasive way to manipulate the composition and reduce the biodiversity of the gut microbiota by reducing the availability of colonizing aquatic microbes (Emerson and Woodley, 2024; Fontaine et al., 2022; Knutie et al., 2017). Importantly, these shifts in the tadpole gut microbiota are associated with changes in body size, developmental rates, and neurodevelopmental parameters such as brain size, brain architecture, and behavioral responses to visual stimuli (Emerson et al., 2023; Emerson and Woodley, 2024), providing some of the first evidence of the amphibian MGB axis.

Larval amphibians are also ideal models to study the impact of stress on the development of the MGB axis. Tadpole development and behavior is exquisitely sensitive to external stressors, including predator exposure (Fraker et al., 2009; Fraker et al., 2021; Middlemis Maher et al., 2013; Schoeppner and Relyea, 2005; Woodley et al., 2015). For example, tadpole exposure to predation-derived kairomones and alarm pheromones (i.e. chemical cues) affect circulating levels of whole body corticosterone (CORT, the primary GC in amphibians) and commonly results in reductions in locomotory behavior, which collectively help the tadpole avoid predation

(Fraker, 2008; Fraker et al., 2021; Middlemis Maher et al., 2013; Relyea, 2001). In some amphibians, exposure to natural stressors and/or exogenous CORT is associated with changes brain morphology (Cha et al., 2021; McClelland, 2020), body size and morphology (Crespi and Warne, 2013; Fraker et al., 2021; McClelland, 2020; Woodley et al., 2015), and anti-predator/escape behavior (Fraker, 2008; Fraker et al., 2009; Fraker et al., 2021; Middlemis Maher et al., 2013; Relyea, 2001; Woodley, 2017). However, little is known about the role of stress and CORT on the development of the amphibian gut microbiota, and subsequent changes in their neurodevelopment and fitness-related behaviors.

Here, we expanded on our recent findings that raising tadpoles in autoclaved pond water altered the gut microbiota as well as tadpole development, morphology, and behavior (Emerson et al., 2023; Emerson and Woodley, 2024). We sought to determine if these relationships persisted during exposure to ecologically relevant stressors, such as predation-derived chemical cues and the associated increases in CORT. Additionally, we explored treatment effects on the skin microbiota, which has been implicated in tadpole and frog disease resistance (Knutie et al., 2017). To achieve our objectives, newly hatched northern leopard frog tadpoles (*Lithobates pipiens*) were raised in natural pond water or autoclaved pond water. Within these pond water treatments, tadpoles were also exposed to one of three stressor treatments: (1) predation-derived chemical cues; (2) exogenous CORT at physiologically relevant doses; (3) vehicle control.

We expected that raising tadpoles in autoclaved pond water would alter the composition and diversity of gut and skin microbiota, tadpole size, body and brain morphology, and behavior compared to those raised in natural pond water. If the MGB axis is contributing to the brain and behavioral endpoints, we expected that the gut microbiota would be more closely associated with brain and behavioral endpoints compared to the skin microbiota. We also expected that exposure

to predation-derived chemical cues and CORT would alter the gut and skin microbiota, tadpole size, body and brain morphology, and behavior compared to those exposed to a vehicle control. Finally, we expected that simultaneous exposure to pond water treatments and stressors will have interactive effects on physiological, neurodevelopmental, and behavioral endpoints.

MATERIALS AND METHODS

Animal Information

Protocols were approved by the Duquesne University Institutional Animal Care and Use Committee (protocol #2021). We used laboratory tap water that was passed through sediment and carbon filters, exposed to UV light, and treated with API Tap Water Conditioner™. Three Northern Leopard Frog (*L. pipiens*) egg masses were obtained from Carolina Biological Supply Company (Burlington, NC) on 3/21/2022. Embryos from each clutch were evenly distributed into two 15-L Sterilite plastic bins (16.75" L, 11.9" W, 7" H) filled with 5 L of autoclaved laboratory tap water. These bins were placed into an incubator set to 16°C and 14h:10h light:dark photoperiod maintained by a light timer. Water was changed every other day to ensure proper oxygenation while embryos developed in the incubator. Hatching began in the incubator and gradually occurred over the course of several days. Upon hatching, tadpoles were offered food ad libitum two or three times per week. The tadpole diet throughout the experiment was Nasco Frog Brittle which was frozen at -20°C until use. Debris/fras was removed daily using sterilized transfer pipets and treatment-specific turkey basters. Complete water changes were made weekly.

On 4/26/2022, all tadpoles had reached Gosner Stage 25, by which time tadpoles are swimming and feeding (Gosner, 1960). Tadpoles were removed from the 16°C incubator and placed at room temperature (20°C) 24 hours prior to starting the experimental treatments. Water

was aerated using a bubbling airstone attached to an air pump. From then on, tadpoles experienced a 14h:10h light:dark photoperiod maintained by a light timer. Room temperature and water temperature were tracked daily and were 20°C-22°C and 18°C-20°C, respectively.

Treatments

Pond Water Treatment: On 4/27/2022, tadpoles were haphazardly assigned to 15-L Sterilite bins containing one of two pond water treatments: pond water that was not autoclaved (hereafter called natural pond water), or pond water that was autoclaved (autoclaved pond water). Each pond water treatment consisted of 12 bins filled with 4 L of water that each housed 6 tadpoles. To create the pond water treatments, each bin contained 75% autoclaved laboratory tap water (3 L) and either 25% (1 L) natural pond water or 25% autoclaved pond water, following the methodology of previous studies (Emerson et al., 2023; Emerson and Woodley, 2024; Fontaine et al., 2022). Like our previous study (Emerson and Woodley, 2024), pond water was collected near a commercial campground (1525 Footville Richmond Rd, Linesville PA 16424; 41.69182, -80.50008) which is a site where *L. pipiens* occur naturally. Pond water was collected on 11/23/2021, placed in 10-L HDPE carboys, and brought to Duquesne University, where it was filtered on 11/24/2021 using an Advantech #35 500μm testing sieve to remove sediment, leaves, and macroorganisms. Filtered pond water was stored in 10-L HDPE carboys at 4°C until use.

To ascertain the chemical composition and water quality, a few representative water samples were analyzed using inductively coupled plasma mass spectrometry prior to and after adding the API water conditioner.

At the same time (4/27/2022) that tadpoles were distributed into bins containing either autoclaved or natural pond water, bins were haphazardly assigned to receive one of three stressor

treatments: predation-derived chemical cues, exogenous CORT, or vehicle controls. Thus, there were eight treatment bins (four natural pond water and four autoclaved pond water) that received each of the stressor treatments. The investigator was blinded to stressor treatments throughout the duration of the experiment and statistical analysis. Each week, the water was completely changed in the bins are part of standard animal care. At this time, all pond water and stressor treatments were reapplied. In addition, tadpole bins were rotated clockwise around the storage racks on which they were placed to account for potential room biases.

To determine the microbial community of the natural and autoclaved pond water prior to the addition of tadpoles, we collected 3 mL samples of the natural pond water and autoclaved pond water that had been prepared for the water changes (and that did not contain tadpoles) each week (n = 6 per pond water treatment). The water samples were immediately frozen at -80°C until DNA extraction and microbial profiling.

Predation-derived chemical cues: To obtain predation-derived chemical cues, we collected *Anax junius* and *Aeshna umbrosa* dragonfly larvae from a Pennsylvania State Gameland (41°35'19.0"N 80°14'38.2"W) on 4/12/2022. The species of dragonfly were selected because they are natural predators of *L. pipiens* tadpoles. The dragonfly larvae were housed individually at Duquesne University in 1 L of autoclaved laboratory tap water and given mesh netting as a perch. We prepared predation-derived cues following methods described by Fraker, who demonstrated that tadpoles respond most strongly to cues derived from dragonfly larvae that are feeding on tadpoles. Such cues include kairomones from the predator and alarm pheromones from the tadpole (Fraker, 2008; Fraker et al., 2009; Fraker et al., 2021; Middlemis Maher et al., 2013). Thus, dragonfly larvae were initially fed 100-200mg of conspecific tadpoles (which were not experimental subjects) (Fraker et al., 2021). Because our supply of tadpole prey grew with

time, dragonfly larvae were fed 800mg–1.0g tadpoles in the later weeks. After dragonfly larvae finished feeding, the cue-containing water was immediately collected and pooled with cues from other dragonfly larvae that had been fed at the same time. Because these predation-derived cues also included dragonfly and tadpole-associated microbial communities, the pooled cue-containing water was filter-sterilized using a Sterivex Millipore 0.22 um sterile filter unit into a sterilized container. By filter-sterilizing, we ensured that we were not altering the aquatic microbial environment present in treatment bins. Bins assigned to be treated with predator-derived cues received 50mL of the filter-sterilized cue containing water weekly. The remaining treatment bins received a vehicle control, which consisted of 50mL of autoclaved laboratory tap water that did not contain predator-derived cues but was filter-sterilized as described above.

We also used predation-derived chemical cues in the behavioral tests, but we modified how they were prepared. This modification was required because by the time we conducted the behavioral tests, the tadpoles were too large for the dragonfly larvae to eat. Thus, we collected tadpole alarm pheromones by handling and poking the tadpoles in a simulated predator attack following previous methods (Fraker et al., 2009). Alarm pheromones produced in this way have been shown to elicit similar behavioral responses in tadpoles as predation-derived chemical cues from a feeding event. The solution of alarm pheromones was filter-sterilized as described above prior to use in order to remove any microbial contamination.

Exogenous CORT: To elevate whole-body CORT to concentrations that match the range observed during predator exposure, we added exogenous CORT to housing water using established methods (Cha et al., 2021; Glennemeier and Denver, 2002; McClelland, 2020). On 3/21/2022, a CORT stock solution was made by dissolving 11.7 mg of crystalline CORT (catalogue No. Q3919-000; Steraloids, Inc.) in 24.3 mL of 200-proof ethanol (Cha et al., 2021).

Eight treatment bins (four natural pond water and four autoclaved pond water) that were haphazardly assigned to receive exogenous CORT received 0.324 mL of the stock solution weekly. When combined with the 4 L present in the bin, the final concentration was 125nM (Glennemeier and Denver, 2002). We confirmed that we successfully elevated levels of water-borne CORT with CORT treatment, and that CORT was not detectable in the other treatment bins (see **Fig. 4.1, Table 4.1**).

Vehicle controls: To control for the addition of predation-derived cues, the other treatment bins received 50mL of autoclaved laboratory tap water that did not contain predation-derived cues but was filter-sterilized as described above. To control for the addition of CORT, the other treatment bins received 0.324 mL of 0.001% of 200-proof ethanol weekly.

Timeline and Sample Sizes

There were six treatment combinations, each comprising four bins containing six tadpoles each. Only two tadpoles died during the course of the experiment. Experimental treatments began on 4/27/2022. The final water change took place on 6/3/2022. After six weeks in treatments (6/6/2022), we began collecting data. For logistical and financial reasons, we could not measure every dependent variable from all six tadpoles in each bin (see **Table 4.2**). For each bin, we first measured behavior of one randomly selected tadpole. We then swabbed the tadpole to measure the skin microbiota and euthanized it to collect additional data. The remaining five tadpoles from the bin were euthanized and processed (**Table 4.2**). Each bin was processed within a single day. For logistical reasons, we could only process four bins on a given day, requiring six consecutive days to process all bins.

Behavioral Assays

We followed methods from a previous study to measure behavior (Emerson and Woodley, 2024). The tadpole selected for behavior was removed from its bin using sterilized tea strainers, rinsed with 100 mL of autoclaved laboratory tap water, and placed individually into a behavior arena (Imagitarium Holding Bin, 26.7 cm x 16.5 cm x 17.8 cm) containing 2 L of autoclaved laboratory tap water. A webcam (360p, 16:9 30fps) was mounted to a ring stand positioned above the arena and attached to a computer to track tadpole movements during assays. Assays were completed from 10:00 to 12:30 hours each day.

Tadpole behavior was measured in a series of assays: 1) baseline behavior in the absence of any stimulus, 20 minutes; 2) behavior in the presence of an empty, capped 20 mL glass vial, 20 minutes; 3) behavior in the presence of a capped glass vial filled with frog brittle, 20 minutes; 4) behavior after the addition of 100 mL of tadpole alarm pheromones, 10 minutes. The order of behavioral assays remained constant for all tadpoles. Each assay was preceded by an acclimation period of either 20 minutes (prior to the baseline assay) or 5 minutes (prior to the glass vial and alarm pheromone assays). To account for potential biases, stimuli were added to either the left or right sides of the arena and were alternated within treatments across all assays.

To quantify behavior, a single investigator analyzed the videos using ToxTrac (Rodriguez et al., 2018). ToxTrac measured average speed (mm/s), mobile average speed (mm/s), average acceleration (mm/s^2), mobility rate (%), total time frozen (m:s), exploration rate of the arena (%), total distance travelled (mm), time in center (m:s), and time near cue (m:s; not measured in baseline assays). Because many of the different behaviors were correlated, a Principal Component Analysis (PCA) was used to distill behavior into uncorrelated principal components (PCs) with eigenvalues ~ $\geq$ 1. In all cases, the PCs met the assumptions of PCA (Kaiser Meyer

Olkin Factor Adequacy > 0.5 and Bartlett's tests ≤ 0.05). PCs were analyzed as described in the statistics subsection.

Skin Swabs and GI Tract Dissections

After a tadpole was assayed for behavior, it was rinsed, and skin and gut samples were collected using sterilized materials for microbial profiling. To do so, tadpoles were transferred from the behavior arena into individual containers with 100 mL of autoclaved laboratory tap water for 5 minutes. After rinsing, tadpoles were held in sterile nitrile gloves and were swabbed over the entire surface of the body and tail for 30 seconds using a sterile swab (Hernández-Gómez et al., 2020). Two tadpoles during swabbing were dropped, which could contaminate skin microbe results, so they were excluded from this analysis. Upon completion, the swab was placed in sterile 1.5 mL microcentrifuge tubes and stored at -80°C. Upon completion, tadpoles were euthanized via immersion in 0.2% buffered MS-222 solution and weighed.

After euthanasia, the tadpole developmental stage was evaluated, and the gastrointestinal (GI) tracts were removed and stored at -80°C within eight minutes of euthanasia. The entire GI tract, from esophagus to the large intestine, was removed and cut into two matching halves. Each half was placed into a sterilized, 1.5 mL microcentrifuge tube and stored at -80°C. One half was used for DNA extractions to quantify the gut microbiota, and the other was stored as a backup. Surgical tools were autoclaved daily and were cleaned thoroughly with 70% EtOH and heat sterilization between each dissection. Carcasses were stored in 10% neutral buffered formalin.

Plasma CORT

After collecting behavioral data from a representative tadpole in a bin, on that same day two other tadpoles from the same housing bin were randomly selected for measurement of plasma CORT. To do so, two tadpoles were randomly selected, removed from bins via sterilized,

treatment-specific tea strainers, rinsed with autoclaved laboratory tap water, and euthanized via immersion in 0.2% buffered MS-222. Tadpoles were weighed and staged, rinsed again with autoclaved laboratory tap water, blotted dry, and tail fins were cut using sterilized scissors. Blood samples were collected from the tail vein using heparinized capillary tubes within six minutes of euthanasia and placed on ice until processing. Scissors were cleaned with 70% EtOH and heat sterilized between dissections. Samples were collected between 13:00 and 14:00 hours, at least 3 hours after the other tadpole had been removed for behavioral assays. Carcasses were stored in 10% neutral buffered formalin.

Blood samples were centrifuged to obtain plasma samples which were frozen in heparinized microcentrifuge tubes and stored at -80°C. Frozen plasma samples were shipped on dry ice to the Endocrine Technologies Core of the Oregon National Primate Research Center (ONPRC) Endocrine Laboratory to measure plasma CORT following previously described methods (Emerson and Woodley, 2024; McClelland and Woodley, 2021). The intra-assay CV was 6.5%, and the inter-assay CV was 5.0%.

Brain Transcriptome

For a future study examining changes in brain gene expression, we selected a tadpole from each bin for placement of the brain into RNALater. A tadpole was randomly selected, removed from the bin using sterilized, treatment-specific tea strainers and rinsed in autoclaved laboratory tap water. After rinsing, tadpoles were euthanized in 0.2% buffered MS-222, weighed and staged, rinsed again in autoclaved laboratory tap water, blotted dry, and was dissected to remove the brain. Brain dissections followed the methods of previous studies by trimming cranial nerves and the spinal cord using the aid of an Olympus SZ61 dissection scope (Emerson et al., 2023; Emerson and Woodley, 2024). Brains were placed into a 1.5 mL microcentrifuge

tube containing 0.5 mL of RNALater solution (Cat. R0901, Lot# MKCG5159) and stored overnight at 4°C. Carcasses were stored in 10% neutral buffered formalin. After 24 hours, the tissue submerged in RNALater solution was placed in -80°C for future RNA sequencing.

Microbial Profiling of Gut, Skin, and Pond Water

The Qiagen QIAamp PowerFecal Pro DNA Isolation Kit was used to extract bacterial DNA from GI tissue, skin swabs, and natural and autoclaved pond water samples, following manufacturer's instructions. DNA from GI tissue was extracted in a single batch on 3/16/2023, and DNA from skin and water samples was extracted in two batches on 4/20/2023. Extracted DNA was stored in sterile microcentrifuge tubes at -80°C. For sequencing services, frozen DNA was shipped on dry ice to the University of Illinois at Chicago's Genome Research Core (UIC GRC). At the UIC GRC, libraries were prepared via amplification of the V4 region of the 16S rRNA gene using 515F and 806R primers on an Illumina MiSeq platform (2x300 paired end reads) (Caporaso et al., 2012).

Raw sequence data was analyzed in RStudio (v 4.1.0) using the DADA2 package (Callahan et al., 2016). Primer sequences were removed, and sequences were trimmed to 270 (forward) and 220 (reverse) base pairs for gut, skin, and pond water samples. After trimming, forward and reverse sequences were denoised, filtered for quality, chimeric sequences were removed, and forward and reverse reads were merged. Processed reads were then assigned to Amplicon Sequence Variants (ASVs) and aligned for phylogenetic tree construction (Schliep, 2011). Using the SILVA database classifier v138.1 (Quast et al., 2013), taxonomy was assigned to ASVs, and any sequences identified as archaea or eukarya were removed. Samples of autoclaved pond water did not return enough sequence reads to quantify bacterial presence. Thus, future reference to pond water refers to the natural pond water samples.

To compare diversity metrics across treatment groups in gut, skin, and pond water samples, ASV tables were constructed, and singleton reads were removed. Two skin swab samples (in the autoclaved pond water – predator cue and natural pond water – predator cue treatment groups) returned an abnormally low amount of sequence reads compared to other samples and were excluded from the data set. Tables were rarefied to the fewest reads found in an experimental sample used in that dataset. Five ASV tables were constructed for analysis of alpha and beta diversity: (1) gut, skin, and pond water samples rarefied to 1,120 sequences per sample, (2) gut and pond water samples rarefied to 1,120 sequences, (3) skin and pond water samples rarefied to 1,120 sequences, (4) gut samples rarefied to 66,378 sequences, and (5) skin swab samples rarefied to 11,882 sequences.

Alpha diversity was assessed by calculating the number of observed ASVs, Shannon Diversity Index (Shannon, 2001), and Faith's Phylogenetic Diversity (Faith, 1992). Beta diversity was evaluated by calculating the Bray-Curtis Dissimilarity Index (Bray and Curtis, 1957).

Relative Brain Mass and Shape

In January 2023, brains were dissected from fixed carcasses using an Olympus SZ61 dissection scope with a camera attachment and processed following previously outlined methods (Emerson et al., 2023; Emerson and Woodley, 2024). To process brains uniformly, cranial nerves were trimmed, and the spinal cord was removed caudal to the medulla (**Fig. 4.2**). Brains were weighed and photographed three times on the dorsal and ventral side. Brains were repositioned after each photo to provide six unique images. If damage occurred to the brain, the brain was removed from the analysis. As tissues can shrink when stored over long periods of time in

fixative, brains were removed, weighed, and photographed within a three-week period, and brains were processed in batches that had equal representation of all treatment groups.

Because brain mass was correlated with overall body mass, we adjusted brain mass for differences in body mass. To do so, we used an Analysis of Covariance (ANCOVA) with pond water treatment and stressor treatment as fixed effects and body mass as a covariate. The ANCOVA indicated that there was no interaction between our treatments and tadpole body mass; also, the slopes of the lines for brain mass were parallel across our six treatment groups. The unstandardized residual brain mass values generated by the ANCOVA were added to estimated marginal means (EMMs), yielding brain mass values that were adjusted for body mass (hereafter called relative brain mass).

To evaluate differences in brain shape, geometric morphometrics were used (Adams et al., 2004) focusing on seven linear brain dimensions: telencephalon width and length, diencephalon width and length, optic tectum width and length, and medulla width (**Fig. 4.2**). We used ImageJ software (US National Institutes of Health, Bethesda, MD) to measure the linear dimensions on the images. Because each brain was imaged three times, the triplicate measurements were averaged to produce final values for each brain dimension for each tadpole.

Because the linear dimensions of brain shape were correlated with overall brain mass, we adjusted the brain dimensions for differences in brain mass. To do so, we used similar methods as described above for the correction of brain mass for differences in body mass. Because the mass-adjusted brain dimensions (hereafter called relative brain shape) were highly correlated, we reduced the brain dimensions to PCs as described above for behavior. PCs were analyzed as described in the statistics subsection.

Relative Body Shape

Tadpoles with intact carcasses (i.e., did not collect GI tracts, brains, or plasma) were photographed from the ventral position and right lateral position. To evaluate differences in body shape, geometric morphometrics were used, focusing on body length, body depth, tail muscle height, tail length, tail fin height, body width and tail fin width (**Fig. 4.3**). We used ImageJ software (US National Institutes of Health, Bethesda, MD) to measure the linear dimensions on the images.

Because the linear dimensions of body shape were correlated with overall body mass, we adjusted the body dimensions for differences in body mass. To do so, we used an ANCOVA following similar methods as described above measurement of brain shape. Because the mass-adjusted body dimensions (hereafter called relative body shape) were highly correlated, we reduced the body dimensions to PCs as described above for behavior. PCs were analyzed as described in the statistics subsection.

Relative Organ Mass

To evaluate differences in the mass of organs related to digestion, we weighed the liver, pancreas, and GI tracts in all tadpoles with intact carcasses. Organ masses were corrected for differences in body mass as described above for brain mass. The slopes of the relationship between liver mass and body mass were not similar across treatments, so liver values were not completely independent of body mass. Thus, results for relative liver mass should be interpreted cautiously.

Statistical Analyses

Tadpole Physiology and Behavior: Analyses were completed using RStudio (v. 4.1.0) running the lme4 package (Bates et al., 2015) unless otherwise noted. Most endpoints were analyzed with Generalized Linear Mixed Models (GLMMs). Data met assumptions of parametric

statistics unless otherwise noted; data were log-transformed in some instances. Values for developmental stage were not normally distributed nor homogeneous, but GLMMs are robust to violations of normality and homogeneity (Glass et al., 1972; Harwell et al., 1992; Lix et al., 1996). All statistical models included bin as a random effect when multiple tadpoles from a given bin were in the analysis. Tukey's HSD post hoc tests were used to analyze pairwise comparisons between stressor treatments using the emmeans package (Length, 2022).

Microbial Diversity & Composition: To determine if alpha diversity metrics differed between gut, skin, and natural pond water samples, these metrics were analyzed using Generalized Linear Mixed Models (GLMMs), followed by Tukey's pairwise comparison tests. Additionally, to determine if gut and skin alpha diversity metrics differed between treatment groups, GLMMs were used followed by Tukey's pairwise comparison tests for stressor treatments. Bin was not included as a random effect because all samples came from different bins and sources.

To determine if beta diversity metrics differed between gut, skin, and natural pond water samples, these metrics were analyzed using a PERMANOVA (999 permutations) using the vegan package in RStudio (Oksanen et al., 2013) followed by pairwise comparison tests using the pairwise Adonis package (Martinez, 2020). Further, the intraindividual variability in bacterial community composition across gut, skin, and pond water samples was evaluated by calculating PERMDISP by using the betadisper function. Additionally, to determine if gut and skin beta diversity metrics differed between treatment groups, a PERMANOVA (999 permutations) was used followed by pairwise comparison tests for stressor treatments using the pairwise Adonis package. Lastly, the intraindividual variability in gut and skin bacterial community composition within treatment groups was evaluated by calculating PERMDISP using the betadisper function.

To determine if our treatments significantly altered the relative abundances of bacterial taxa at the phylum and genus levels in gut and skin samples, we used the MaAsLin2 (Microbiome Multivariable Association with Linear Models (Mallick et al., 2021)) package in RStudio. To correct for the number of tests, P-values were adjusted using the Benjamin Hochberg false discovery rate (BH FDR).

Associations between microbial diversity and tadpole organismal biology: We sought to better understand whether differences in the diversity of the gut and skin microbiota could predict differences in tadpole development, morphology, and behavior. To do this, we used GLMMs with the metrics of alpha diversity as predictor variables, and the various phenotypic measurements were response variables. Lastly, to better understand whether the relative abundances of specific bacterial taxa were correlated with aspects of tadpole development, morphology, and behavior, we used the MaAsLin2 package and corrected P-values using BH FDR (Mallick et al., 2021).

RESULTS

Pond Water Microbial Communities, Chemical Composition, and Water Quality

Samples of autoclaved pond water used in the experiment did not contain detectable levels of bacteria. The microbial community of natural pond water was dominated by Proteobacteria, Actinobacteria, and Bacteroidota. These phyla were also part of the tadpole gut (**Fig. 4.4**) and skin microbiota (**Fig. 4.5**). Nonetheless, the microbial communities and biodiversity of the natural pond water significantly differed from the tadpole gut and skin microbiota, but community dispersion did not (**Fig. 4.6**; **Table 4.3**; **Fig. 4.7**).

The chemical composition and water quality was assessed in a few representative samples of natural and autoclaved pond water samples prior to the addition of API Tap Water

Conditioner. In unconditioned water samples, Arsenic and Aluminum were found in abundances higher than the Maximum Contaminant Level (MCL) set by the EPA (**Table 4.4**). Manganese also exceeded the MCL in the autoclaved pond water sample and the conditioned natural pond water sample but was undetectable in a different unconditioned natural pond water sample. In the conditioned natural pond water sample, all chemical and water quality components fell within MCL thresholds except Manganese (**Table 4.4**).

Gut Microbiota

Tadpole gut microbial communities were affected by both pond water and stressor treatments. The effects were strongest in response to the pond water treatment, where tadpoles raised in autoclaved pond water harbored gut microbial communities with altered composition (PERMANOVA; **Fig. 4.8; Table 4.5**) and dramatically reduced biodiversity (**Fig. 4.9; Table 4.5**) compared to tadpoles raised in natural pond water. Although stressor treatments did not affect gut microbial alpha diversity (**Fig. 4.9, Table 4.5**), gut bacterial communities were impacted by stressor treatment both directly as a main effect, and interactively with pond water treatment (PERMANOVA; **Fig. 4.8; Table 4.2**). Gut microbial community dispersion did not differ within pond water or stressor treatments (PERMDISP; **Table 4.5**).

Tadpole gut microbial communities were primarily composed of the following phyla: Proteobacteria, Bacteroidota, Fusobacteria, Firmicutes, and Actinobacteria (**Fig. 4.4**). Tadpoles raised in autoclaved pond water had higher relative abundances of Proteobacteria and Actinobacteria, but lower relative abundances of Bacteroidota and Desulfobacteria compared to tadpoles raised in natural pond water (**Table 4.6**). Additionally, pond water treatment significantly altered the relative abundances of 27 bacterial genera present in the tadpole gut

(**Table 4.6**). There was no effect of stressor treatment on the relative abundances of any gut bacterial taxa.

Skin Microbiota

Tadpole skin microbial communities were significantly altered by pond water treatment, but not stressor treatments. Specifically, tadpoles raised in autoclaved pond water harbored skin microbial communities that had altered composition (**Fig. 4.10; Table 4.5**) and lower measures of the number of observed ASVs and Faith's Phylogenetic Diversity (**Fig. 4.11; Table 4.5**) compared to tadpoles raised in natural pond water. There was no effect of stressor treatment on any measure of the skin microbiota, either as a main effect or interactively with pond water treatment. Skin microbial community dispersion did not differ within pond water or stressor treatments (PERMDISP**; Table 4.5**).

Tadpole skin microbial communities were primarily composed of the following phyla: Proteobacteria, Bacteroidota, Fusobacteria, Firmicutes, and Actinobacteria (**Fig. 4.5**). Tadpoles raised in autoclaved pond water had lower relative abundances of the phylum Verrucomicrobiota than tadpoles raised in natural pond water (**Table 4.7**). Additionally, tadpoles raised in autoclaved pond water had higher relative abundances of the bacterial genus *Taibaiella,* and lower relative abundances of *Mycobacterium* than those raised in natural pond water (**Table 4.7**). There was no effect of stressor treatment on the relative abundances of any skin bacterial taxa.

Tadpole gut and skin microbial communities were dominated by similar taxa (**Fig. 4.4; Fig. 4.5**) and had similar measurements of alpha diversity (**Table 4.3**). Nonetheless, tadpole gut and skin microbial communities differed in their community composition (**Fig. 4.6; Table 4.3**)

Behavioral Assays

Baseline

The eight behaviors that were measured under baseline conditions were distilled into two principal components (**Table 4.8**). Tadpole behavior measured under baseline conditions was not affected by either pond water or stressor treatments (**Table 4.8, Fig. 4.12**).

Visual Stimuli

The nine behaviors that were measured in the presence of visual stimuli (either an empty glass vial or a glass vial filled with frog brittle) were distilled into three PCs (**Table 4.9; Table 4.10**). Tadpole behavior in the presence of a glass jar, either empty or filled with frog brittle, was not affected by either pond water or stressor treatment (**Table 4.9 – 4.10; Fig. 4.13; Fig. 4.14**).

Alarm Pheromonal Stimuli

The nine behaviors measured in the presence of tadpole-derived alarm pheromones were distilled into two PCs (**Table 4.11**). PC-1, which loaded average speed, mobile average speed, average acceleration, distance travelled, mobility rate, and negatively loads total time frozen, was significantly affected by pond water treatment but not stressor treatment (**Table 4.11**). Specifically, tadpoles raised in autoclaved pond water exhibited increased locomotory activity when exposed to alarm pheromones, while tadpoles raised in natural pond water exhibited behavioral inhibition and increased time frozen (**Fig. 4.15**). PC-2 was not affected by either pond water or stressor treatment (**Table 4.11**, **Fig. 4.15**).

Body Mass, Developmental Stage, and Whole-Body CORT

Tadpoles raised in autoclaved pond water were significantly heavier (~16%) than tadpoles raised in natural pond water (**Fig. 4.16A**, χ^2: 9.5, P = 0.002). Additionally, tadpoles raised in autoclaved pond water were slightly more developed than tadpoles raised in natural pond water (median Gosner stage 37 versus 38) (**Fig. 4.16B**, χ^2: 6.1, P = 0.013). There was no effect of stressor treatment on tadpole body mass (**Fig. 4.16A** , χ^2: 5.9, P = 0.053) or

developmental stage (**Fig. 4.16B**, χ^2: 2.0, P = 0.37). Plasma CORT in tadpoles did not differ according to pond water (**Fig. 4.17**, χ^2: 0.005, P = 0.95) or stressor treatments (**Fig. 4.17**, χ^2: 0.72, P = 0.70).

Relative Brain Mass and Shape

Tadpoles that were raised in natural pond water had significantly heavier brains than tadpoles raised in autoclaved pond water (**Fig. 4.16C**, χ^2: 4.0, P = 0.045), while there was no effect of stressor treatment on relative brain mass (**Fig. 4.16C**, χ^2: 1.8, P = 0.41).

In evaluating tadpole brain shape, seven dimensions of brain morphology were distilled into three principal components (**Table 4.12**). Neither PC-1 nor PC-3 were significantly affected by either pond water or stressor treatment (**Table 4.12, Fig. 4.18**). PC-2, which loaded optic tectum width, medulla width, and diencephalon width was significantly affected pond water and stressor treatments. Specifically, tadpoles raised in autoclaved pond water had decreased optic tectum width, medulla width, and diencephalon width. (**Fig. 4.16D**; **Table 4.12**). Although the stressor treatment affected PC-2, pairwise comparisons with Tukey's HSD failed to distinguish differences between predator cues, CORT, and vehicle control, thus results must be interpreted carefully (**Fig. 4.16D**; **Table 4.12**).

Relative Body Shape

The seven measurements of tadpole body shape were best described by three PCs (**Table 4.13**). PC-3 was significantly affected by stressor treatment but not pond water treatment (**Table 4.13**). Specifically, tadpoles exposed to exogenous CORT had relatively wider and shorter tail fins compared to tadpoles exposed to predator cues or vehicle control, based on post hoc analysis with Tukey's HSD (**Table 4.13**, **Fig. 4.19**). Neither PC-1 nor PC-2 was significantly affected by pond water or stressor treatment (**Table 4.13, Fig. 4.20**).

Relative Intestine Mass

There was no effect of either pond water or stressor treatments on relative GI tract mass, relative liver mass, or relative pancreas mass (**Fig. 4.21**).

Microbiome Associations with Tadpole Behavior and Physiology

Several metrics of alpha diversity of the tadpole gut microbial communities were predictors of tadpole behavior. Specifically, tadpoles with a lower number of observed ASVs and a lower Faith's Phylogenetic Diversity score exhibited increased locomotory activity and more time in the center of the testing arena (PC-1 and PC-2, respectively) during baseline behavioral assays (**Table 4.14**). Additionally, tadpoles with lower Shannon Diversity Index scores spent more time near a glass vial containing frog brittle (PC-3) during the visual stimulus assay (**Table 4.14**). Lastly, tadpoles with lower Faith's Phylogenetic Diversity scores exhibited increased locomotory activity (PC-1) when exposed to alarm pheromones during behavioral assays (**Table 4.14, Fig. 4.22**). In addition, there were several gut bacterial phyla and genera that were significantly associated with changes in relative brain mass, relative brain shape, and locomotory activity during behavioral assays (**Table 4.15**).

There were no significant associations between skin bacterial taxa and physiological or behavioral endpoints (**Table 4.16**).

DISCUSSION

To test the effects of the aquatic microbial environment and stressors on the development of the tadpole-associated microbiota, morphology, and behavior, we designed a two-factor experiment to test each factor separately and in combination. We found widespread impacts of our pond water treatment on the tadpole gut and skin microbiota, body mass, brain morphology, and behavior. As found in previous experiments, tadpoles raised in autoclaved pond water

harbored distinct gut microbial communities that had reduced diversity compared to tadpoles raised in natural pond water (Emerson et al., 2023; Emerson and Woodley, 2024; Fontaine et al., 2022). In addition, tadpoles raised in autoclaved pond water were 16% larger and had relatively smaller brains and altered brain shape. Finally, we found behavioral differences suggesting reduced ability to evade predators in tadpoles raised in autoclaved pond water. We suspect that the effects of the aquatic microbial community on tadpole morphology and behavior are mediated, at least somewhat, by the gut microbiota and not the skin microbiota. This is because the gut microbiota, but not the skin microbiota, was a significant predictor of many physiological and behavioral endpoints, such as predator evasion behavior. These findings confirm and extend our previous studies demonstrating strong impacts of the aquatic microbial community on the development of the tadpole MGB axis (Emerson et al., 2023; Emerson and Woodley, 2024).

We also provide novel data on how predation stress and stress hormones (CORT) impact the development of the tadpole gut microbiota as well as body and brain morphology. It is important to note that we used methodology from previous studies to administer predation stress, and we administered ecologically relevant doses of exogenous CORT. We found that the stressor treatments significantly altered the composition, but not the biodiversity, of the gut microbiota. Surprisingly, there was no effect of stressor treatments on the skin microbiota, on body size and development, or behavior. However, exposure to CORT, but not predation cues, resulted in dramatic alterations in tail morphology. Interestingly, there was only one interactive effect between pond water and stress treatments on gut microbial community composition. Below, we discuss our results in greater detail.

Gut Microbiota

Across treatments, the composition of the gut microbiota was dominated by five bacterial phyla: Proteobacteria, Firmicutes, Fusobacteria, Bacteroidota, and Actinobacteria. These phyla are commonly found in the amphibian microbiota (Emerson and Woodley, 2024; Fontaine et al., 2022; Kohl et al., 2013; Zhou et al., 2023), and these phyla were also found in pond water that did not contain tadpoles. This supports previous work showing that a large portion of colonizing microbes are environmentally encountered (Correa et al., 2020; Litvak and Bäumler, 2019; Martínez et al., 2018). Despite this overlap, we found that the aquatic microbial communities found in pond water (that did not contain tadpoles) were more diverse and differed significantly from those found in tadpole guts. This is expected, as tadpole GI tracts have carrying capacities to maintain microbial populations and act as a filter to constrain population biodiversity (Contijoch et al., 2019). Interestingly, tadpoles raised in autoclaved pond water had significantly higher relative abundances of Actinobacteria and Proteobacteria than tadpoles that developed in natural pond water. This difference is likely driven by stochastic processes by which the increased diversity of microbes present in natural pond water can increase the abundance of rarer taxa present within the gut, therefore decreasing the abundance of dominant taxa (Petrullo et al., 2022; Shade et al., 2014).

The microbial communities available in the pond water could be influenced by the water composition. Freshwater systems commonly contain pollutants that may select for proliferation of opportunistic bacteria. An example of this is an increased abundance of Desulfobacterota in the guts of tadpoles raised in natural pond water. This increase in abundance is mainly driven by *Desulfovibrio*, which is a genus that contains opportunistic bacteria associated with human gastrointestinal diseases (Rowan et al., 2010; Zhang et al., 2016). Previous studies have found higher abundances of *Desulfovibrio* in amphibians that develop in ponds contaminated with

heavy metals (Chai et al., 2022; Zhang et al., 2016), suggesting that metals could potentially lead to pathogenic bacterial proliferation within the amphibian gut. Interestingly, in our representative water sample, we found that our autoclaved pond water sample and natural pond water sample that was conditioned with API Tap Water Conditioner had concentrations of Manganese that reached the Maximum Contaminant Level set by the EPA. Manganese is a neurotoxic, heavy metal contaminant that has displayed modulatory effects on the vertebrate microbiota. Specifically, manganese exposure influenced the composition of the mouse gut microbiota in a sex-dependent manner, which was associated with altered bacterial gene expression involved in neurotransmitter synthesis (Chi et al., 2017). We did not see any evidence of disease or neurotoxicity in tadpoles in this study. Nonetheless, it is important to recognize that environmental factors such as heavy metals could potentially affect the composition of the amphibian microbiota, which can subsequently influence the MGB axis through altered expression of bacterial genes involved in neurotransmitter synthesis (Chi et al., 2017).

Tadpoles harbored microbial communities that had significantly distinct compositions across stressor treatment groups. Despite these overall differences in composition, pairwise comparison tests failed to find differences between stressor treatment groups, precluding interpretation. Interestingly, there was a strong interaction between pond water treatment and stressor treatment on gut microbial composition. This interaction was most evident within the exogenous CORT treatment group, in which CORT-treated tadpoles clustered according to their pond water treatments.

The potential effect of CORT on community composition suggests that exposure to exogenous GCs does indeed modulate the composition of the amphibian gut microbiota. In other species, endogenous GC circulation has been associated with shifts in the composition of the gut

microbiota, although these changes are context dependent and often related to overall microbial biodiversity. Higher fecal GC concentration has been associated with decreased microbial diversity in fish and red squirrels (Petrullo et al., 2022; Stothart et al., 2016; Uren Webster et al., 2020), meanwhile baseline CORT release rate was positively associated with gut microbial diversity in amphibians (Gabor et al., 2022). Additionally, exposure to exogenous CORT altered the diversity and composition of the gut microbiome in lizards, but these changes were dependent on reproductive contexts (Macleod et al., 2022). Stressor-related shifts in gut microbial community structure are often hypothesized to be driven by GC-induced shifts in GI physiology and mucosal immunity (Söderholm et al., 2002). For example, mammalian exposure to GCs has been associated with decreased probiotic, lactic acid producing bacteria and increases in proinflammatory microbes (Huang et al., 2015; Wu et al., 2018), Similar results were found in fish (Uren Webster et al., 2020). However, our stressor treatments did not significantly affect the relative abundances of any bacterial taxa, making it difficult to compare our results to previous experiments. Nonetheless, these shifts in gut microbial community structure likely reflect alterations in GC-tolerant taxa that may proliferate due to decreased niche competition and altered energetic demands (Hibbing et al., 2010).

Skin Microbiota

Tadpoles raised in autoclaved pond water had distinct skin microbial communities with reduced biodiversity compared to tadpoles raised in natural pond water. Similarly to the gut microbiota, tadpole skin microbial communities across treatments were dominated by similar phyla to those found in pond water samples. These results are similar to that of a previous study that raised tadpoles in these pond water treatments (Knutie et al., 2017). Interestingly, this reduction in skin microbial diversity was associated with increased infection by parasitic worm

(Knutie et al., 2017). Early-life disruption of the skin microbiota appeared to reduce amphibians' ability to resist infection and supports studies that implicate the amphibian skin microbiota as a vital component of their disease resistance (Knutie et al., 2017; Longo et al., 2015; Martins et al., 2022; Neely et al., 2023; Rebollar et al., 2020; Thaiss et al., 2016; Walke and Belden, 2016; Woodhams et al., 2014).

We found that the relative abundance of one bacterial phyla, Verrucomicrobiota, differed between pond water treatments and was higher in tadpoles raised in natural pond water. This falls in line with our previous study that found higher abundances of Verrucomicrobiota in tadpole guts that were raised in natural pond water (Emerson and Woodley, 2024), likely due to the presence of this phyla in freshwater and soil habitats, which was eliminated during the sterilization process (Freitas et al., 2012; Hamilton et al., 2023; Orellana et al., 2022). Tadpoles raised in natural pond water also had higher relative abundances of the genus *Mycobacterium* which is thought to contain opportunistic pathogens, as it is associated with vertebrate skin diseases and abundantly found in metal-acid contaminated waters (Chai, 2011; Ross et al., 2019). However, as mentioned above, we evaluated the chemical composition and quality of pond water used in this experiment, and found that the water, especially after conditioning, was relatively free of contaminants.

The composition of microbial communities harbored on the tadpole skin were not affected by stressor treatments. Additionally, the diversity of these microbial communities was not affected by stressor treatments. This is similar to a study where newts were exposed to exogenous CORT; CORT had no effect on skin microbial alpha or beta diversity (Pereira et al., 2023). Additionally, a recent study found that exposure of salmon to GCs led to significant restructuring of the gut microbiota, but not the skin microbiota (Uren Webster et al., 2020).

Similar to tadpoles used in this study, the skin microbiota in salmon was heavily dominated by Proteobacteria, which may be less sensitive to GC exposure compared to more diverse, lactic acid producing bacteria present in the intestinal microbiota (Uren Webster et al., 2020). Additionally, fish had low concentrations of GCs in skin mucous compared to GC concentration found in fecal samples, which could explain the lack of an effect (Uren Webster et al., 2020). While we did not measure CORT concentrations present on the tadpole skin, this result could suggest why ectotherm skin microbial communities are more tolerant to GC exposure than gut microbial communities.

While tadpole exposure to predation cues did not influence the composition of the skin microbiota, some studies in amphibians support a link between ecological stressors and skin microbial community structure. A previous study in wild tropical tree frogs found that increases in GC circulation was strongly associated with the loss of transient skin bacteria, as well as skin bacterial community restructuring when frogs were exposed to habitat fragmentation and translocation (Neely et al., 2023). Thus, environmental stressors may reduce microbial community resilience (Capdevila et al., 2021; Neely et al., 2023). While our results do not support this, it is important to keep in mind that the relationship between the stress response, GCs, and associated microbes is very complex and likely context and species dependent. Additionally, habitat translocation could introduce environmental microbes that can influence the composition of the skin microbiota (Longo et al., 2015; Neely et al., 2023).

Behavioral Assays

Tadpoles that developed in autoclaved pond water exhibited increased locomotory activity during behavioral assays when exposed to tadpole-derived alarm pheromones compared to tadpoles that developed in natural pond water. This result expands on our previous work

showing that the aquatic microbial community in which tadpoles develop shape locomotory behaviors (Emerson and Woodley, 2024). Increased locomotory behavior in this case is thought to be detrimental to health and survival, because a characteristic tadpole antipredator behavior is inhibition of locomotory and exploratory activity, which makes the tadpole less conspicuous to a predator (Denver, 2021; Fraker, 2008; Fraker et al., 2009; Fraker et al., 2021; Kelleher et al., 2018; Middlemis Maher et al., 2013; Relyea, 2001; Wells, 2019). Interestingly, lower Faith's Phylogenetic Diversity was associated with higher locomotory behavior during this assay, suggesting that gut microbial diversity was a predictor of this uncharacteristic behavior.

Additionally, locomotion in the presence of alarm pheromones was associated with individual gut bacterial taxa. Tadpoles with a higher relative abundance of the Bacteroidota phyla exhibited decreased locomotory activity during this behavioral assay, which is to be expected, as Bacteroidota abundance was higher in tadpoles raised in natural pond water. Two gut bacterial genus, *Ancylobacter* and *Staphylococcus*, had relative abundances that were positively associated with increased activity during this behavioral assay, but to our knowledge there is no evidence of these taxa influencing vertebrate behavior.

Other studies in ectotherms have implicated gut microbial communities in coordinating stress responses (Cryan et al., 2019; Davis et al., 2016). GF zebrafish placed in a novel environment displayed increased locomotory behavior and decreased thigmotaxis compared to controls, which is an uncharacteristic behavioral response that represents alterations in anxiety-like behavior (Davis et al., 2016). Additionally, GF zebrafish that were exposed to an osmotic stress test displayed a blunted hormonal and behavioral stress response (Davis et al., 2016). Fascinatingly, upon colonization of GF zebrafish with commensal gut bacterial taxa, these uncharacteristic hormonal and behavioral stress responses returned to control levels (Davis et al.,

2016). Taken together, these results suggest that the host-associated microbiota is integral in coordinating the HPA/I axis and characteristic responses as evidenced through mediations in animal behavior when exposed to a stressor (Cryan et al., 2019). Additionally, it supports the efficacy of the use of alarm pheromones as recognizable stimuli to tadpoles (Fraker et al., 2009).

We found no effect of our stressor treatments on tadpole locomotory activity during behavioral assays. This is surprising, as exposure to exogenous CORT has been shown to alter amphibian behavior in other studies (Bliley and Woodley, 2012; Fraker et al., 2009; Fraker et al., 2021; Woodley, 2017). It has been shown that short-term exposure (3 hours) of tadpoles to 125nM of exogenous CORT was associated with increased locomotory activity, but this increased activity declined over long term exposure (8 days) (Middlemis Maher et al., 2013). As our behavioral assays did not evaluate tadpole behavior immediately after exogenous CORT was added to the behavior arena, our longer-term experimental design and less frequent administration of the hormone could explain the lack of CORT effects on behavior.

Additionally, there was no effect of predation-derived chemical cues on tadpole locomotory activity. This result was also surprising, as tadpoles exposed to these chemical cues exhibit reductions in locomotory activity (Fraker, 2008; Fraker et al., 2009; Fraker et al., 2021). It is important to note that our behavioral assays differed from these previous studies in that we evaluated locomotory activity at baseline and in response to visual stimuli in the absence of these cues in the behavior arena (Fraker, 2008; Fraker et al., 2009; Fraker et al., 2021). Additionally, previous studies have evaluated tadpole behavior in response to these cues that were derived from a caged predator that is feeding on conspecifics in the same holding water (Fraker et al., 2021; Relyea, 2001). It is possible that a combination of the lack of a visual, non-lethal predator

exposure and differences in behavioral assay structure could have contributed to our lack of treatment effect.

Plasma CORT

The pond water treatment, despite altering the gut microbiota, had no effect on plasma CORT levels, similar to our previous study (Emerson and Woodley, 2024). While the composition of the host-associated microbiota has been implicated in the coordination of the HPA/I axis and circulating levels of GCs (Cryan et al., 2019), our work suggests that neither the pond water treatments nor the ensuing changes in gut microbiota influences the HPA/I axis and circulating levels of GCs in larval amphibians. Our stressor treatments also did not affect plasma CORT levels. As our administration of stressor treatments followed similar methodology outlined in previous studies, the lack of stressor treatment effects on plasma CORT was unexpected.

Although we were unable to detect elevated plasma CORT in tadpoles raised in the presence of exogenous CORT, we are confident that our treatment of the water with CORT impacted the tadpoles. We used a concentration of CORT shown by others to raise whole-body CORT levels to within a physiological range seen during a stress response (Cha et al., 2021; Fraker et al., 2021; Glennemeier and Denver, 2002; McClelland, 2020). One difference between our study and others was the time frame. We applied our treatments only one time per week while other studies had more frequent application. Fraker et al. (2021) exposed tadpoles to exogenous CORT concentrations every four days. Cha et al. (2021) and McClelland (2020) applied CORT multiple times per week. Therefore, it is possible that our inability to detect elevated plasma CORT levels in tadpoles exposed to exogenous CORT could be attributed to less frequent tadpole exposure to the hormone over a longer experimental timeframe. Also, as

CORT circulation follows the diel cycle, it is possible that our time of plasma collection was unable to capture treatment differences (Buckingham, 2006; Woodley, 2017).

Note that we treated water with exogenous CORT (in bins that did not have tadpoles) and assayed that water, and we found that water-borne CORT was elevated for the 7-day duration between water changes. Finally, it is important to note that exposure to CORT altered the composition of the gut microbiota and drastically influenced tail morphology, confirming that our CORT treatments were effective.

Additionally, tadpoles exposed to predation-derived chemical cues throughout development did not have elevated plasma CORT. Other studies have found that exposure to predation-derived cues influenced levels of CORT, although this is often context dependent. Specifically, exposure to conspecific alarm pheromones suppressed tadpole whole-body CORT (Fraker et al., 2009). In another study, tadpole exposure to predation-derived chemical cues from a caged predator in the same housing water suppressed whole-body CORT in the short-term but elevated CORT in the long term (Middlemis Maher et al., 2013). Also, tadpole whole-body CORT was positively associated with predator biomass in natural ponds (Middlemis Maher et al., 2013).

Response to Predation-derived Chemical Cues

There was no observable effect of predation-derived chemical cues on endpoints measured in this study. This contrasts with previous studies that found changes in morphology and behavior (Fraker, 2008; Fraker et al., 2009; Fraker et al., 2021; Middlemis Maher et al., 2013). A potential explanation for this difference could be due to our filter sterilization of cue containing water prior to administration. It is possible that microbes or other components of the predator-prey interaction that are filtered out may be critical components of the predation-

derived chemical cues, necessary to elicit physiological reactions seen in previous studies (Fraker, 2008; Fraker et al., 2009; Fraker et al., 2021; Middlemis Maher et al., 2013). It is noteworthy that tadpoles in this experiment did respond to the tadpole-derived alarm pheromone stimulus during behavioral assays. This provides support for previous work showing that tadpoles can recognize conspecific alarm pheromones (Fraker et al., 2009), and shows that tadpoles can recognize these chemical cues after filter sterilization.

Additionally, some of these previous studies collected predation-derived chemical cues from a caged predator, which is fed a conspecific in the same water as tadpoles used in the experiment (Fraker et al., 2021; Middlemis Maher et al., 2013; Relyea, 2001). This absence of a visually caged predator could contribute to this absent treatment effect. It is also possible that tadpoles grew accustomed to the routine and infrequent administration of this cue and did not recognize it as a viable predation threat. For example, our timeline and frequency of exposure differed from previous work such as Fraker at al., in which tadpoles were exposed to predation derived cues daily over a 9-day experimental period (2021).

Body Mass and Developmental Stage

Tadpoles that developed in autoclaved pond water were about 16% larger and were on average one Gosner Stage further into their development than tadpoles that developed in natural pond water. These results are similar to results seen in our previous experiment using the same pond water treatments and tadpole species (Emerson and Woodley, 2024) and another study using Green Frogs in similar pond water conditions (Fontaine et al., 2022). This may be due to microbially mediated differences in host digestive efficiency and feeding behavior. Tadpoles that developed in autoclaved pond water had higher abundances of *Clostridium sensu stricto 1*, a genus within the Firmicutes phyla. This genus has highly conserved translational machinery that

is implicated in glucose and glycerol metabolism (Udaondo et al., 2017), the suppression of lipogenesis (Zhao et al., 2014), and is associated with increased obesity in developing mammals (Mei et al., 2022; Shang et al., 2016). Additionally, our previous study found that tadpoles that harbored higher abundances of *Clostridium sensu stricto* displayed altered locomotory activity during behavioral testing when glass jars filled with frog brittle and a food slurry were added to the behavior arena (Emerson and Woodley, 2024). Taken together, these results suggest that *Clostridium sensu stricto* may be a bacterial genus that is associated with amphibian size, growth rates, and behavioral responses to food-derived cues.

Tadpole exposure to predation-derived chemical cues and exogenous CORT had no effect on tadpole body size or developmental rates. This is not supported by previous work in amphibians that has found that exposure to a stressor (i.e. chronic handling) and exogenous CORT lead to decreased body weight (Bliley and Woodley, 2012; McClelland, 2020). Also, previous studies have found that exposure of tadpoles to stressors accelerates developmental rates, through hormonally-mediated developmental plasticity that allow tadpoles to reach metamorphosis and escape from the pond (Denver, 2021). While evidence of this developmental plasticity was not expressed through changes in body mass or developmental rate, below we discuss how stressor treatments influenced relative brain development and body morphology.

Relative Brain Mass and Shape

Tadpoles raised in autoclaved pond water had significant reductions in relative brain size and width of the optic tectum, diencephalon, and medulla. This falls in line with our previous work that found that pond water treatments significantly altered brain development, although specific changes to brain size and morphology appear to be context and species dependent (Emerson et al., 2023; Emerson and Woodley, 2024). Our results are also consistent with studies

in other organisms which have found associations between gut microbial diversity and brain development in brain regions such as the amygdala, hippocampus, and forebrain (Castillo-Ruiz et al., 2018; Luczynski et al., 2016; Sharvin et al., 2023). As the optic tectum (processes visually guided motor behaviors (Bestman et al., 2012)), diencephalon (homeostatic and endocrine function (Charmandari et al., 2005; Denver, 2009)) and medulla (autonomic function (Gdovin et al., 1999)) carry out functions vital to amphibian health, understanding mechanisms driving these changes in brain architecture requires further attention.

Stressor treatments elicited reductions in the width of the optic tectum, diencephalon, and medulla, but not overall brain size, although post-hoc analysis could not determine differences in relative brain shape between these treatments. This result partially supports previous work showing that tadpole exposure to predator cues reduces the width and length of the telencephalon, optic tectum, diencephalon, and the medulla (Woodley et al., 2015) and overall brain size (Gonda et al., 2013). Further, tadpole exposure to CORT led to reductions in overall brain mass and reduced medulla and olfactory bulb length (McClelland, 2020), as well as increases in diencephalon length (Cha et al., 2021). As stressor treatments did not alter the biodiversity of the associated microbiota, these results may reflect instances of amphibian phenotypic plasticity in response to GC and/or predator exposure (Buskirk and Relyea, 1998; Woodley et al., 2015).

The relative abundance of the Cyanobacteria phyla was positively associated with increased relative brain mass, marking our second study in which this phyla is associated with amphibian brain development (Emerson et al., 2023). This is noteworthy, as this phylum contains bacteria that are implicated in algal fungal blooms and neurotoxicity (Emerson et al., 2023; Sini et al., 2021; Zehr, 2011). Previous studies in ectotherms have shown that freshwater

pollutants such as pesticides, microplastics, and nanoplastics all exhibit neurotoxicity that alter relative brain mass and morphology (Mattsson et al., 2017; McClelland, 2020; McClelland and Woodley, 2022; Prüst et al., 2020). While we cannot confirm that tadpoles in this experiment were experiencing bacterially induced neurotoxicity, documenting these associations is important to potentially inform ecological conservation studies focused on the health of freshwater systems.

Relative Body Shape

Despite changes in body size and developmental rates due to pond water treatments, there was no effect of pond water treatments on body morphology. In contrast, tadpoles exposed to exogenous CORT had drastic increases in tail fin width and reduction in tail fin height. This supports work done in previous studies that have shown tadpole exposure to CORT increased tail muscle depth and tail fin width (Glennemeier and Denver, 2002; McClelland, 2020). These alterations to tadpole tail morphology are thought to represent phenotypic plasticity that aids escape and survivability when exposed to a predator (Fraker et al., 2021). Additionally, increased tadpole tail surface area could induce a 'lure effect' that can lead predator strikes to the tail and away from vital organs/areas of the body (Van Buskirk et al., 2003).

This result leads to a new question: why did predation-derived chemical cues not elicit these same morphological changes? In previous studies in which tadpoles were exposed to a simulated predation event, drastic changes to tail morphology were observed (Denver, 2021; Fraker et al., 2021; Middlemis Maher et al., 2013; Woodley et al., 2015). A potential explanation for our deviation from results could be attributed to differences in experimental design. For example, in Woodley et al., predator cues were accompanied by visual exposure to a caged predator (2015), and in Middlemis Maher et al., predator cues were readministered every other

day (2013). Additionally, in Fraker et al., these changes in tail morphology were only seen when the administration of chemically-derived predator cues were combined with exogenous CORT (Fraker et al., 2021), suggesting that CORT is the primary driving factor in this instance of phenotypic plasticity. Tadpole tail explants treated with exogenous CORT *in vitro* had increased weight compared to control, supporting the direct effect of CORT on the tail (Middlemis Maher et al., 2013). As there is no evidence of predation-derived chemical cues influencing whole body CORT in this experiment, this could explain the absence of tail morphological plasticity.

Another potential explanation for the lack of an effect of predation-derived chemical cues on tail morphology could be that tadpoles grew accustomed to addition of predation cues and didn't perceive them as a viable threat (Kruger and Morin, 2020; Schoeppner and Relyea, 2009). Alternatively, tadpoles could be perceiving these cues as a threat, but are instead exhibiting anti-predator plasticity in a non-morphological manner. For example, a previous study found that tadpoles exhibited differential phenotypic plasticity based on which predator they were exposed to (Innes-Gold et al., 2019). Examples could be through change in tadpole pigmentation instead of a more costly change to tadpole tail morphology, although this was not examined in this current study. Alternatively, as it was seen that alarm pheromones administered during behavioral assays elicited changes in tadpole locomotory activity, tadpoles could be exhibiting adaptive anti-predator responses through changes in their behavioral phenotype.

Relative Intestine Mass

We found that neither pond water nor stressor treatments influenced the relative mass of the gut, liver, or the pancreas. This result is surprising, as both the associated microbiota and stress hormones are heavily involved in host metabolism (Sommer and Bäckhed, 2013; Wack et al., 2012). For example, in addition to well characterized roles in host digestion and adiposity,

the gut microbiota is also integral in intestinal vascularization, tissue homeostasis, and organ morphogenesis (Sommer and Bäckhed, 2013). Additionally, elevations of circulating GCs in early life are necessary for the maturation of many vertebrate organs, including the small intestine and liver (Liggins, 1994; Taves and Ashwell, 2021; Wada, 2008). Despite the lack of any treatment effect, any induced changes to the development and physiology of these organs cannot be solely measured by their mass, and further investigation is likely required.

CONCLUSION

In sum, we found that in newly hatched tadpoles, exposure to predation stress and exogenous CORT changes the composition of the tadpole gut microbiota, as well as influences relative brain shape. Further, tadpole exposure to exogenous CORT drastically changes tail morphology. In addition, we were able to replicate and expand upon our previous work by showing that tadpole development in autoclaved pond water significantly influences the composition and biodiversity of the gut and skin microbiota, and subsequently influences body size, development, and brain architecture. Lastly, and perhaps most importantly, tadpoles raised in autoclaved pond water with reduced gut microbial biodiversity exhibited uncharacteristic locomotory responses to alarm pheromones. This provides additional support of the amphibian MGB axis, in that reductions in the biodiversity of the associated microbiota may influence characteristic tadpole behavioral responses to a simulated predation event.

Author contributions

Conceptualization: KJE, SKW

Methodology: KJE, DB-S, SS, SKW

Software: KJE

Formal Analyses: KJE

Resources: SKW

Data Curation: KJE

Writing-original draft: KJE

Writing-review and editing: SKW

Visualization: KJE

Supervision: SKW

Project Administration: KJE, SKW

Funding acquisition: SKW

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Table 4.1. Mean ± standard error (SEM) of water-borne corticosterone present in treatment bins over the duration of a week. Below, APW denotes Autoclaved Pond Water and NPW denotes Natural Pond Water. For stressor treatments, Pred. denoted Predation-derived chemical cues.

Treatment Group	WB CORT (pg/mL) Day 0 Mean ± SEM (n)	WB CORT (pg/mL) Day 7 Mean ± SEM (n)
NPW – Pred.	1.3 ± 0.3 (3)	2.7 ± 0.7 (3)
NPW – CORT	42730.7 ± 8868.2 (3)	4539.3 ± 2007.8 (3)
NPW – Vehicle	1.0 ± 0.0 (3)	1.3 ± 0.3 (3)
APW – Pred.	1.3 ±0.3 (3)	2.0 ± 0.0 (3)
APW – CORT	6092.0 ± 3067.4 (3)	14793.0 ± 7308.7 (3)
APW– Vehicle	1.3 ± 0.3 (3)	2.0 ± 0.0 (3)
Pred.	1.3 ± 0.2 (6)	2.3 ± 0.3 (6)
CORT	24411.3 ± 9204.9 (6)	9666.2 ± 4092.3 (6)
Vehicle	1.2 ± 0.2 (6)	1.7 ± 0.2 (6)

Table 4.2. Sample sizes for each endpoint. Tadpoles from each bin were randomly selected to be evaluated for certain endpoints. + indicates that the endpoint was assessed in that tadpole. – indicates that the endpoint was not assessed in that tadpole. N = 24 bins, each with 6 tadpoles. The brain of tadpole #4 was placed in RNALater for a future transcriptome study.

Bin	Gut & Skin Microbiota	Behavior	Plasma CORT	Relative Brain Mass & Shape	Relative Body Shape	Relative Organ Mass	Body Mass & Developmental Stage
Tadpole 1	+	+	-	+	-	-	+
Tadpole 2	-	-	+	+	-	+	+
Tadpole 3	-	-	+	+	-	+	+
Tadpole 4	-	-	-	-	-	+	+
Tadpole 5	-	-	-	+	+	+	+
Tadpole 6	-	-	-	+	+	+	+

Table 4.3. Statistical differences in microbial diversity among tadpole gut, tadpole skin, and natural pond water. Shown are the test statistic and P-values derived from GLMMs analyzing alpha diversity and the PERMANOVA analyzing beta diversity among samples from tadpole gut, tadpole skin, and natural pond water samples. Pairwise comparisons for alpha diversity were derived from Tukey's Honest Significant Difference (HSD). Pairwise comparisons of Bray-Curtis Dissimilarity were derived using the pairwise.adonis functions as part of the pairwiseAdonis package in RStudio. N = 24 (gut), 20 (skin), and 6 (water). For Bray-Curtis Dissimilarity pairwise comparisons, all significant differences are ≤ 0.003. Significant effects are bolded.

	Diversity Metric	**Results of GLMM or PERMANOVA** Test statistic, P	**Pairwise Comparisons** (Test statistic, P)
Alpha	No. Observed ASVs	**χ^2 = 22.4, < 0.001**	Gut ≠ Water (**-3.5, 0.003**); Skin ≠ Water (**-4.7, < 0.001**); Gut = Skin (2.0, 0.12)
	Shannon Diversity Index	**χ^2 = 39.8, < 0.001**	Gut ≠ Water (**-5.9, < 0.001**); Skin ≠ Water (**-6.0, < 0.001**); Gut = Skin (0.33, 0.94)
	Faith's Phylogenetic Diversity	**χ^2 = 49.9, < 0.001**	Gut ≠ Water (**-6.3, < 0.001**); Skin ≠ Water (**-7.0, < 0.001**); Gut = Skin (1.2, 0.48)
Beta	Bray-Curtis Dissimilarity	**F = 9.7, < 0.001**	Gut ≠ Skin ≠ Water (all **P ≤ 0.003**)
	PERMDISP	F = 1.2, 0.29	

Table 4.4. Presence of chemical elements and water quality measurements in pond water samples. Three samples of pond water were analyzed: Unconditioned APW (Autoclaved Pond Water) and Unconditioned NPW (Natural Pond Water) on 4/14/2022, and NPW that was conditioned with API Tap Water Conditioner on 5/31/2022. Note that the Conditioned NPW analyzed on 5/31/2022 came from the same source as samples evaluated on 4/14/2022 but was not the same exact sample that was previously analyzed. MCL, ppm represents the Maximum Contaminant Level concentration based on EPA water standards. Bdl denotes below detectable limit. Bolded values exceed the MCL. Samples were evaluated using Inductively Coupled Plasma Mass Spectrometry and a water quality photometer.

Chemical Components	MCL, ppm	Unconditioned APW (4/14/2022)	Unconditioned NPW (4/14/2022)	Conditioned NPW (5/31/2022)
Li	Not regulated	0.001	0.001	0.001
B	Not regulated	0.011	0.016	0.019
Na	Not regulated	11.58	11.78	24.43
Mg	Not regulated	1.71	1.62	1.05
Al	0.05-0.2	**0.05**	**0.05**	0.016
Si	Not regulated	1.20	1.31	0.84
P	Not regulated	0.53	0.34	0.117
K	Not regulated	5.82	5.36	1.41
Ca	Not regulated	8.92	9.78	6.12
Ti	Not regulated	bdl	bdl	0.001
V	Not regulated	bdl	bdl	bdl
Cr	0.1 (total)	0.002	0.002	0.003
Mn	0.050	**0.12**	bdl	**0.05**
Fe	0.30	0.167	0.132	0.27
Co	Not regulated	<0.001	<0.001	<0.001
Ni	Not regulated	bdl	bdl	bdl
Cu	<1.3	bdl	bdl	0.006
Zn	5.000	0.038	0.036	0.009
As	0.010	**0.077**	**0.078**	0.001

Se	0.050	0.004	0.001	0.001
Rb	Not regulated	0.012	0.011	0.003
Sr	Not regulated	0.04	0.04	0.03
Mo	Not regulated	bdl	bdl	<0.0001
Ag	0.1000	0.003	0.007	0.0032
Cd	0.0050	bdl	bdl	bdl
Sn	Not regulated	bdl	bdl	bdl
Sb	0.0060	0.0001	0.0001	bdl
Cs	Not regulated	0.00006	0.00004	0.00001
Ba	2.00	0.01	0.01	0.01
W	Not regulated	bdl	bdl	bdl
Hg	0.0020	NA	NA	NA
Pb	0.015	0.0003	0.0001	0.0005
Bi	Not regulated	bdl	bdl	bdl
U	0.03000	bdl	bdl	bdl
Fluoride	4 (2)	bdl	bdl	bdl
Chloride	250.00	14.9	12.7	15.7
Nitrite	3.30	bdl	bdl	bdl
Bromide	Not regulated	bdl	bdl	0.53
Nitrate	44.30	2.3	2.1	0.2
Phosphate	Not regulated	1.1	0.9	0.49
Sulfate	250.00	1.2	1.0	1.2
Bicarbonate	4 (2)	25.6	26.8	19.5
Carbonate	250.00	bdl	bdl	bdl
Specific Conductance	Not regulated	107	109.9	NA
Total Dissolved Solids	500	69.6	71.4	NA
pH	6.5 – 8.5	7.5	7.5	NA

Table 4.5. Statistical differences in microbial diversity in gut and skin samples of Northern Leopard Frog tadpoles. Shown are the test statistic and P values derived from GLMMs analyzing alpha diversity and PERMANOVAs analyzing beta diversity. Significant effects are bolded. N = 24 (gut) and 20 (skin).

Source	Diversity Metric	**Pond Water** test statistic, P	**Stressor** test statistic, P	**Interaction** test statistic, P
Gut	No. Observed ASVs	**χ^2 = 41, < 0.001**	χ^2 = 0.69, 0.71	χ^2 = 3.7, 0.16
	Shannon Diversity Index	**χ^2 = 32, < 0.001**	χ^2 = 4.4, 0.11	χ^2 = 4.0, 0.14
	Faith's Phylogenetic Diversity	**χ^2 = 17, < 0.001**	χ^2 = 1.3, 0.52	χ^2 = 3.0, 0.22
	Bray-Curtis Dissimilarity	**F = 9.8, < 0.001**	**F = 1.8, 0.03**	**F = 2.7, 0.002**
	PERMDISP	F = 1.1, 0.29	F = 0.08, 0.93	
Skin	No. Observed ASVs	**χ^2 = 21, < 0.001**	χ^2 = 5.4, 0.07	χ^2 = 1.2, 0.56
	Shannon Diversity Index	χ^2 = 1.5, 0.22	χ^2 = 1.4, 0.48	χ^2 = 0.71, 0.70
	Faith's Phylogenetic Diversity	**χ^2 = 14, < 0.001**	χ^2 = 4.2, 0.12	χ^2 = 1.2, 054
	Bray-Curtis Dissimilarity	**F = 2.5, 0.004**	F = 1.0, 0.46	F = 0.99, 0.48
	PERMDISP	F = 0.64, 0.43	F = 0.22, 0.81	

Table 4.6: Relative abundances of gut bacterial phyla and genera that were significantly different between Northern Leopard Frog tadpoles raised in natural versus autoclaved pond water. Prevalence is the percentage of tadpoles in which the taxon was found. Statistical testing was conducted using MaAsLin2. P-values represent the effects of pond water treatment and were corrected using the BH FDR method. A negative coefficient signifies a higher abundance in the natural pond water treatment; a positive coefficient signifies a higher abundance in the autoclaved pond water treatment. Stressor treatment did not significantly impact the relative abundance of any bacterial phylum or genus. N = 4 x 6 treatment groups.

Taxon		Prevalence (%)	Coefficient	FDR P
Phylum	Actinobacteriota	100	0.13	0.04
	Desulfobacterota	33.3	-0.03	0.04
	Bacteroidota	95.8	-0.15	0.04
	Proteobacteria	100	0.13	0.04
Genus	*Clostridium.sensu.stricto.1*	83.3	0.059	0.001
	Aurantimicrobium	87.5	0.213	0.002
	Roseomonas	87.5	0.046	0.002
	Oerskovia	41.7	-0.020	0.003
	Ancylobacter	83.3	0.189	0.005
	Xanthobacter	83.3	0.038	0.005
	Reyranella	87.5	0.037	0.005
	Dysgonomonas	45.8	-0.060	0.011
	Agromyces	33.3	0.012	0.020
	Anaerostignum	54.2	-0.069	0.021
	X.Anaerorhabdus (furcosa.group)	54.2	-0.035	0.023
	Bosea	100.0	0.110	0.026
	Microbacterium	33.3	0.013	0.026
	UCG.009	50.0	-0.043	0.026
	X.Eubacterium (brachy.group)	95.8	-0.046	0.030
	Ensifer	91.7	0.023	0.030
	Fonticella	33.3	-0.007	0.030
	Paeniglutamicibacter	29.2	-0.016	0.030
	Rhodoblastus	33.3	-0.015	0.030
	Desulfovibrio	33.3	-0.030	0.032
	Propionivibrio	37.5	-0.068	0.032
	Breznakia	33.3	0.018	0.035
	Candidatus.Soleaferrea	54.2	-0.070	0.039
	Parabacteroides	45.8	-0.086	0.039
	Pseudarthrobacter	37.5	-0.029	0.039
	Coprobacillus	79.2	-0.027	0.046
	Dielma	62.5	-0.059	0.046

Table 4.7: Relative abundances of skin bacterial phyla and genera that were significantly different between Northern Leopard Frog tadpoles raised in natural versus autoclaved pond water. Prevalence is the percentage of tadpoles in which the taxon was found. Statistical testing was conducted using MaAsLin2. P-values represent the effects of pond water treatment and were corrected using the BH FDR method. A negative coefficient signifies a higher abundance in the natural pond water treatment; a positive coefficient signifies a higher abundance in the autoclaved pond water treatment. Stressor treatment did not significantly impact the relative abundance of any bacterial phylum or genus. N = 2 – 4 x 6 treatment groups.

Taxon		**Prevalence (%)**	**Coefficient**	**FDR P**
Phylum	Verrucomicrobiota	70	-0.08	0.015
Genus	*Mycobacterium*	65	-0.06	0.002
	Taibaiella	60	0.16	0.02

Table 4.8: Effects of treatments on behavior of Northern Leopard Frog tadpoles under baseline conditions. Principal components analysis (PCA) was used to reduce 8 behaviors to 2 principal components (PCs). Principal components were analyzed with GLMMs. Values with less than an absolute value of 0.1 are denoted as hyphens.

Analysis		**PC-1**	**PC-2**
PCA	% of Variance	69.4	14.8
	Eigenvalue	5.6	1.2
Factor Loadings	Average Speed	0.98	-
	Mobile Average Speed	0.78	0.36
	Total Distance Travelled	0.98	-
	Average Acceleration	0.96	-
	Mobility Rate	0.95	-
	Exploration Rate	0.72	0.55
	Time in Center of Arena	-0.15	0.84
	Total Time Frozen	-0.81	0.20
GLMM	Pond Water: F, P	1.11, 0.31	3.77, 0.07
	Stressor: F, P	0.71, 0.51	0.32, 0.73
	Interaction: F, P	1.79, 0.20	0.16, 0.86

Table 4.9: Effects of pond water and stressor treatments on behavior of Northern Leopard Frog tadpoles in response to the presentation of an empty glass vial. Principal components analysis (PCA) was used to reduce 9 behaviors to 3 principal components (PCs). Principal components were analyzed with GLMMs. Factor loadings with an absolute value of < 0.1 are denoted as hyphens.

Analysis		**PC-1**	**PC-2**	**PC-3**
PCA	% of Variance	57.1	21.5	12.1
	Eigenvalue	5.1	1.9	1.1
Factor Loadings	Average Speed	0.98	0.17	-
	Mobile Average Speed	0.75	0.39	-0.26
	Total Distance Travelled	0.98	0.17	-
	Average Acceleration	0.97	0.13	-0.10
	Mobility Rate	0.92	0.27	-
	Exploration Rate	0.32	0.86	0.13
	Time in Center of Arena	-0.14	-0.93	0.10
	Total Time Frozen	-0.88	-0.21	-0.18
	Time Near Cue	-	-	0.96
GLMM	Pond Water: F, P	1.9, 0.19	2.2, 0.16	0.16, 0.69
	Stressor: F, P	0.19, 0.83	0.47, 0.64	1.0, 0.38
	Interaction: F, P	0.32, 0.73	0.39, 0.68	1.4, 0.28

Table 4.10: Effects of pond water and stressor treatments on behavior of Northern Leopard Frog tadpoles when presented with a glass vial filled with frog brittle. Principal components analysis (PCA) was used to reduce 9 behaviors to 3 principal components (PCs). Principal components were analyzed with GLMMs. Factor loadings with an absolute value of < 0.1 are denoted as hyphens.

Analysis		**PC-1**	**PC-2**	**PC-3**
PCA	Variance (%)	62.5	15.4	11.7
	Eigenvalue	5.6	1.4	1.1
Factor Loadings	Average Speed	0.99	-	-
	Mobile Average Speed	0.76	0.34	-
	Total Distance Travelled	0.99	-	-
	Average Acceleration	0.97	-	-
	Mobility Rate	0.96	-	-
	Exploration Rate	0.60	0.64	0.25
	Time in Center of Arena	-	-	0.99
	Total Time Frozen	-0.93	-	-
	Time Near Cue	-	0.92	-
GLMM	Pond Water: F, P	2.9, 0.11	1.6, 0.23	0.31, 0.59
	Stressor: F, P	1.8, 0.20	0.001, 0.99	0.27, 0.77
	Interaction: F, P	1.3, 0.29	0.042, 0.96	1.7, 0.21

Table 4.11. Effects of pond water and stressor treatments on behavior of Northern Leopard Frog tadpoles in response to the addition of tadpole-derived alarm pheromones. Principal components analysis (PCA) was used to reduce 9 behaviors to 2 principal components (PCs). Principal components were analyzed with GLMMs. Factor loadings with an absolute value of < 0.1 are denoted as hyphens. Significant results are bolded.

Analysis		**PC-1**	**PC-2**
PCA	% of Variance	59.0	14.3
	Eigenvalue	5.31	1.3
Factor Loadings	Average Speed	0.96	0.19
	Mobile Average Speed	0.69	-
	Total Distance Travelled	0.96	0.20
	Average Acceleration	0.95	0.18
	Mobility Rate	0.93	0.19
	Exploration Rate	0.75	-0.23
	Time in Center of Arena	-	-0.83
	Total Time Frozen	-0.81	-0.17
	Time Near Cue	0.19	0.60
GLMM	Pond Water: F, P	**12.4, 0.002**	0.08, 0.79
	Stressor: F, P	2.1, 0.15	0.13, 0.88
	Interaction: F, P	1.0, 0.37	1.8, 0.20

Table 4.12: Effects of treatments on the relative brain shape of Northern Leopard Frog tadpoles. Principal components analysis (PCA) was used to reduce 7 linear brain dimensions to 3 principal components (PCs). Principal components were analyzed with GLMMs with bin as a random effect. Factor loadings with an absolute value of < 0.1 are denoted as hyphens. Significant results are bolded.

Analysis		**PC-1**	**PC-2**	**PC-3**
PCA	Variance (%)	30.6	25.8	14.3
	Eigenvalue	2.14	1.81	1.00
Factor Loading	Telencephalon Width	0.840	0.114	-
	Telencephalon Length	0.844	-	-
	Optic Tectum Width	0.720	0.440	-
	Optic Tectum Length	0.158	0.721	-
	Diencephalon Width	0.408	0.685	-
	Diencephalon Length	-	-	0.984
	Medulla Width	-	0.781	0.131
GLMM	Pond Water: $\chi_{(1)}^2$, P	0.301, 0.58	**4.52, 0.033**	0.005, 0.94
	Stressor: $\chi_{(1)}^2$, P	3.72, 0.15	**9.08, 0.011**	0.999, 0.61
	Interaction: $\chi_{(1)}^2$, P	3.13, 0.20	3.54, 0.17	3.00, 0.22

Table 4.13: Effects of pond water and stressor treatments on the relative body shape of Northern Leopard Frog tadpoles. Principal components analysis (PCA) was used to reduce 7 linear body dimensions to 3 principal components (PCs). Principal components were analyzed with GLMMs with bin as a random effect. Factor loadings with an absolute value of < 0.1 are denoted as hyphens. Significant results are bolded.

Analysis		**PC-1**	**PC-2**	**PC-3**
PCA	% of Variance	26.4	21.1	20.3
	Eigenvalue	1.846	1.48	1.42
Factor Loading	Body Length	0.566	0.178	-
	Body Depth	0.829	-	0.196
	Tail Muscle Height	0.344	0.534	0.244
	Tail Length	-	0.882	-
	Tail Fin Height	0.125	0.582	-0.688
	Body Width	0.820	-	0.260
	Tail Fin Width	0.152	0.209	0.882
GLMM	Pond Water: $\chi_{(1)}^2$, P	0.644, 0.42	0.851, 0.356	0.142, 0.707
	Stressor: $\chi_{(1)}^2$, P	4.66, 0.097	0.905, 0.636	**45.9, < 0.001**
	Interaction: $\chi_{(1)}^2$, P	3.75, 0.154	1.17, 0.556	0.036, 0.982

Table 4.14. Results of tests of whether gut alpha diversity metrics predict physiological endpoints in Northern Leopard Frog tadpoles. Shown is a chi-square distributed test statistic and P-value associated with a Type II Wald test. Significant effects are bolded.

Endpoint		Shannon Diversity Index: χ^2, P	No. Observed ASVs: χ^2, P	Faith's Phylogenetic Diversity: χ^2, P
Body Mass		0.55, 0.46	0.40, 0.53	1.3, 0.25
Gosner Stage		1.1, 0.29	0.85, 0.36	0.91, 0.34
Relative Brain Mass		0.01, 0.92	3.6, 0.057	2.4, 0.12
Relative Brain Shape	PC-1	0.27, 0.60	0.11, 0.75	0.01, 0.91
	PC-2	0.16, 0.68	0.01, 0.98	0.10, 0.75
	PC-3	0.01, 0.92	0.06, 0.80	0.01, 0.93
Behavior: Baseline	PC-1	2.8, 0.10	**8.8, 0.003**	**4.1, 0.04**
	PC-2	0.06, 0.81	**6.95, 0.01**	**4.6, 0.03**
Behavior: Empty Glass Vial	PC-1	1.9, 0.16	0.77, 0.38	0.62, 0.43
	PC-2	0.96, 0.33	0.14, 0.71	0.36, 0.55
	PC-3	0.22, 0.64	2.1, 0.15	0.33, 0.57
Behavior: Food in Glass Vial	PC-1	1.1, 0.29	1.6, 0.20	1.4, 0.24
	PC-2	0.84, 0.36	0.09, 0.77	0.83, 0.36
	PC-3	**4.3, 0.038**	0.68, 0.41	1.0, 0.32
Behavior: Chemical Cues	PC-1	0.41, 0.52	3.4, 0.06	**6.2, 0.013**
	PC-2	0.30, 0.58	0.09, 0.76	0.06, 0.80

Table 4.15. Relative abundances of gut bacterial phyla and genera that were significantly associated with host physiology and behavior in Northern Leopard Frog tadpoles.

Prevalence is the percentage of tadpoles where the taxon was found. Statistical testing was conducted using MaAsLin2. P-values were corrected using the BH FDR method. The sign of the coefficient indicates the direction of the correlation: positive correlations indicate a positive relationship between the abundance of the taxa and the endpoint it describes. N = 24 tadpoles.

Phylum	Prevalence	Coefficient	FDR P-value
Relative Brain Mass			
Cyanobacteria	33.3	0.003	0.03
Relative Brain Shape (PC-3)			
Deinococcota	12.5	-0.004	0.05
Behavior – Baseline (PC-1)			
Bacteroidota	95.8	-0.09	0.03
Behavior – Baseline (PC-2)			
Patescibacteria	20.8	0.002	0.001
Behavior – Empty Glass Jar (PC-1)			
Bacteroidota	95.8	-0.08	0.01
Proteobacteria	100	0.07	0.01
Behavior – Glass Jar with Food (PC-1)			
Actinobacteriota	100	0.06	0.04
Behavior – Glass Jar with Food (PC-2)			
Dependentiae	12.5	-0.001	0.02
Behavior – Olfactory (PC-1)			
Bacteroidota	95.8	-0.08	0.04
Genus	**Prevalence**	**Coefficient**	**FDR P-value**
Behavior – Empty Glass Jar (PC-2)			
Thermoactinomyces	12.5	-0.01	0.001
Planococcus	12.5	-0.01	0.03
Roseiarcus	16.6	-0.003	0.03
Paenibacillus	20.8	-0.01	0.05
Behavior – Olfactory (PC-1)			
Staphylococcus	75	0.01	0.04
Ancylobacter	83.3	0.08	0.04

Table 4.16. Results of tests of whether skin alpha diversity metrics predict physiological endpoints in Northern Leopard Frog tadpoles. Shown is a chi-square distributed test statistic and P-value associated with a Type II Wald test.

Endpoint		Shannon Diversity Index: χ^2, P	No. Observed ASVs: χ^2, P	Faith's Phylogenetic Diversity: χ^2, P
Body Mass		0.44, 0.50	0.005, 0.94	0.08, 0.78
Gosner Stage		2.7, 0.10	0.11, 0.74	0.01, 0.94
Relative Brain Mass		2.5, 0.12	0.05, 0.83	0.26, 0.61
Relative Brain Shape	PC-1	0.37, 0.55	0.64, 0.42	0.74, 0.39
	PC-2	0.01, 0.98	0.31, 0.58	0.014, 0.91
	PC-3	1.98, 0.16	0.056, 0.81	0.58, 0.45
Behavior: Baseline	PC-1	2.1, 0.15	0.37, 0.54	1.8, 0.18
	PC-2	0.70, 0.40	3.7, 0.054	2.6, 0.11
Behavior: Empty Glass Vial	PC-1	0.0, 0.99	0.12, 0.73	0.11, 0.73
	PC-2	0.19, 0.66	0.19, 0.66	1.3, 0.25
	PC-3	1.5, 0.22	0.03, 0.87	0.36, 055
Behavior: Food in Glass Vial	PC-1	0.16, 0.68	0.02, 0.89	0.35, 0.55
	PC-2	0.42, 0.52	0.07, 0.79	0.002, 0.96
	PC-3	4.5, 0.34	0.03, 0.87	0.002, 0.97
Behavior: Chemical Cues	PC-1	0.04, 0.85	0.07, 0.80	0.002, 0.97
	PC-2	2.1, 0.14	1.2, 0.27	3.7, 0.056

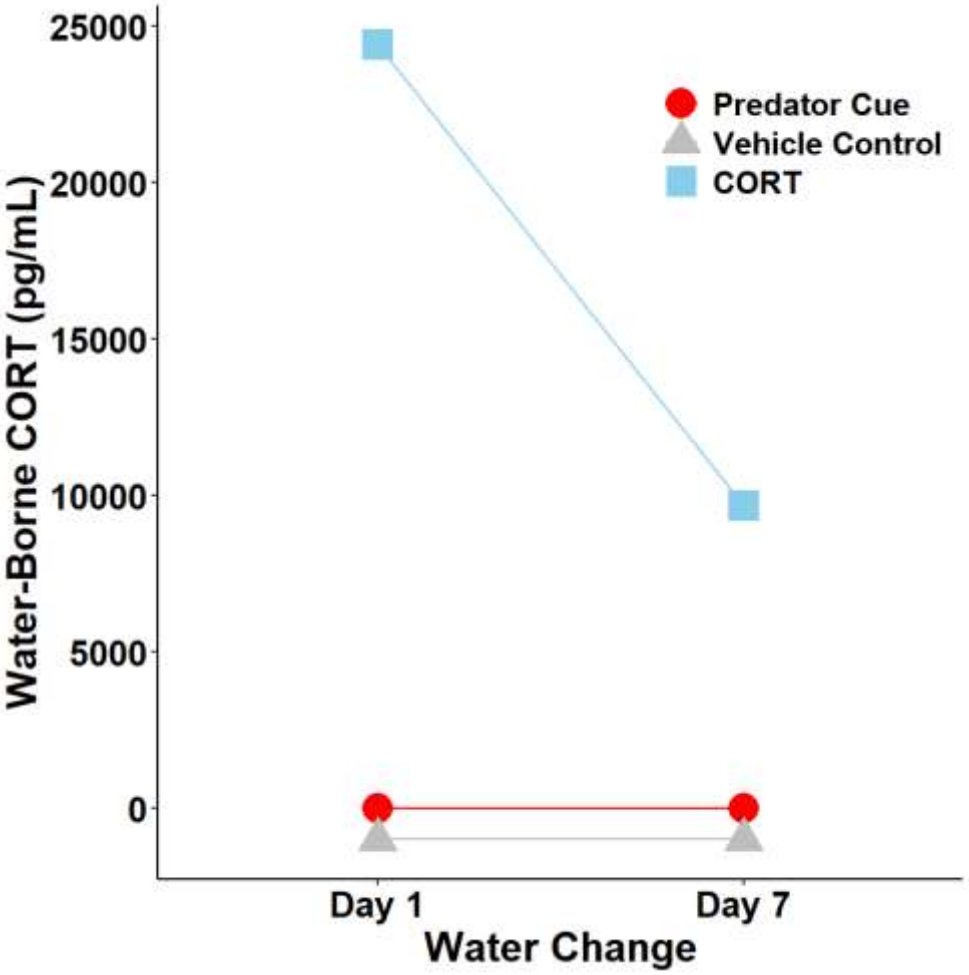


Figure 4.1: Validation of CORT treatments. To determine if the concentrations of CORT in the water changed over time in the absence of tadpoles, three bins per treatment combination (N = 3 x 6) were set up. At the time of set-up and after 7 days, a 40mL sample was taken from each bin and frozen at -80°C. CORT concentrations in the water were measured by the Endocrine Technologies Core of the Oregon National Primate Research Center (ONPRC). Bins treated with CORT had ~ 25,000 pg/mL of CORT immediately after adding CORT, and ~ 9,000 pg/mL 7 days after adding CORT.

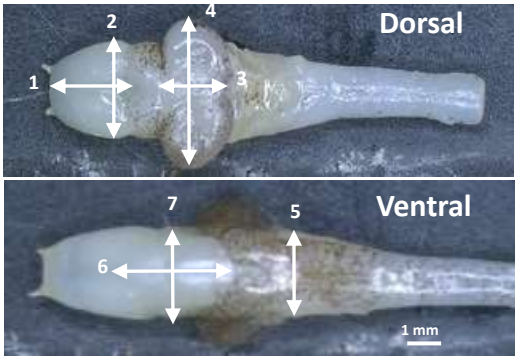


Figure 4.2. Dorsal and ventral view of a Northern Leopard Frog tadpole brain (GS 36). The anterior end of the brain is on the left. Arrows represent the measurements of 7 linear brain dimensions that were analyzed to describe brain morphology: 1) telencephalon length; 2) telencephalon width; 3) optic tectum length; 4) optic tectum width; 5) medulla width; 6) diencephalon length; 7) diencephalon width.

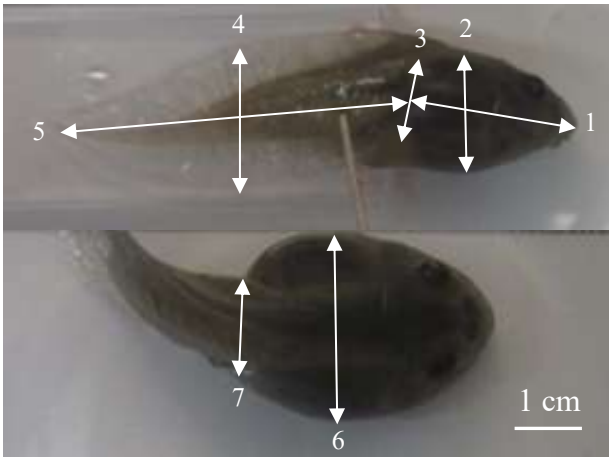


Figure 4.3. Lateral and dorsal view of a Northern Leopard Frog tadpole (GS 37). Arrows represent the measurements of 7 linear body dimensions that were analyzed to describe body morphology: 1) body length; 2) body depth; 3) tail muscle height; 4) tail fin height; 5) tail length; 6) body width; 7) tail fin width.

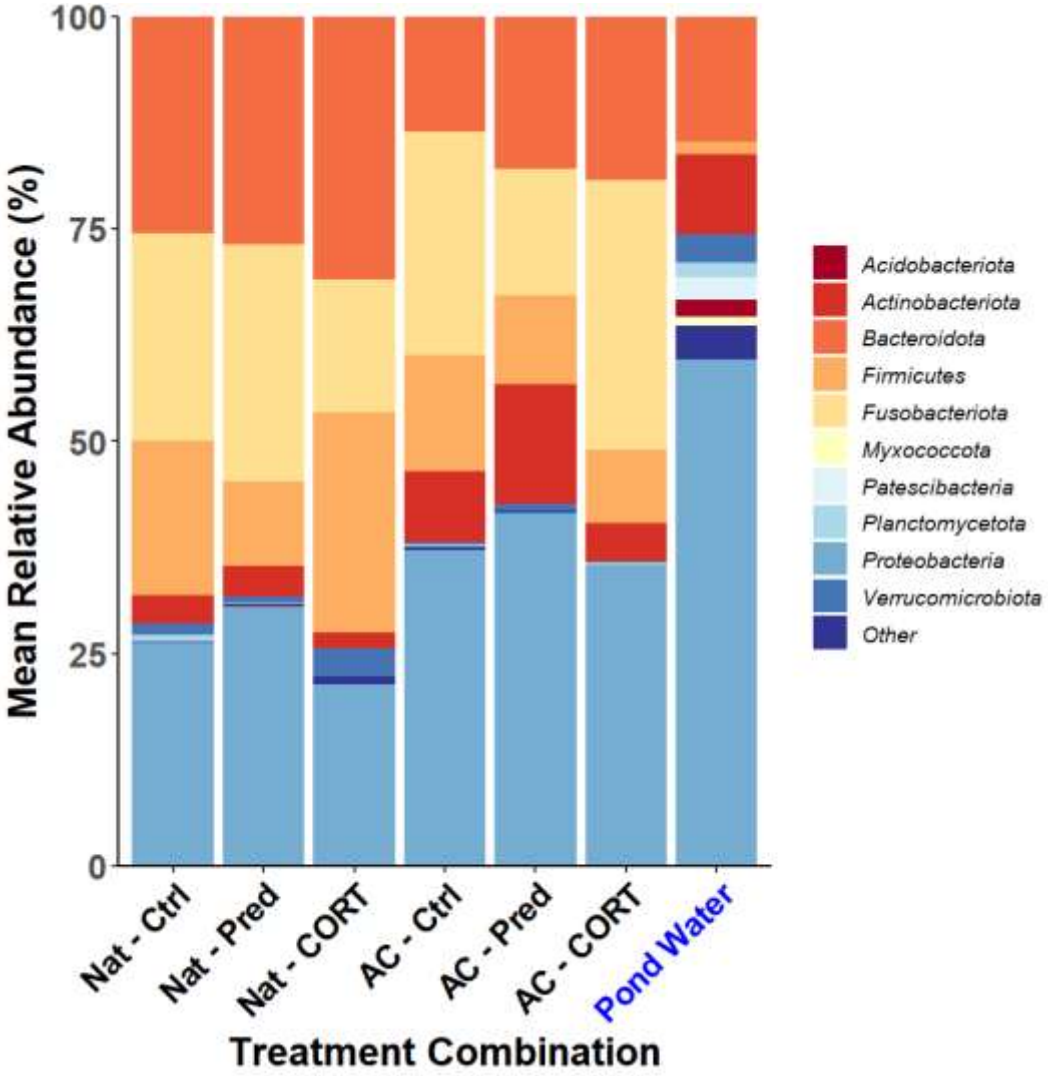


Figure 4.4. Bacterial phyla in guts of Northern Leopard Frog tadpoles and in natural pond water. Tadpoles were raised in either natural pond water (Nat) or autoclaved pond water (AC). In addition to the pond water treatments, tadpoles were raised in the presence of a vehicle control (Ctrl), predator cues (Pred), or CORT. Bacterial phyla with a mean relative abundance greater than 1% are shown; all other phyla were pooled into the 'Other' category. N = 4 tadpoles per treatment group and N = 6 pond water samples. No microbial DNA was detected in the 6 samples of autoclaved pond water.

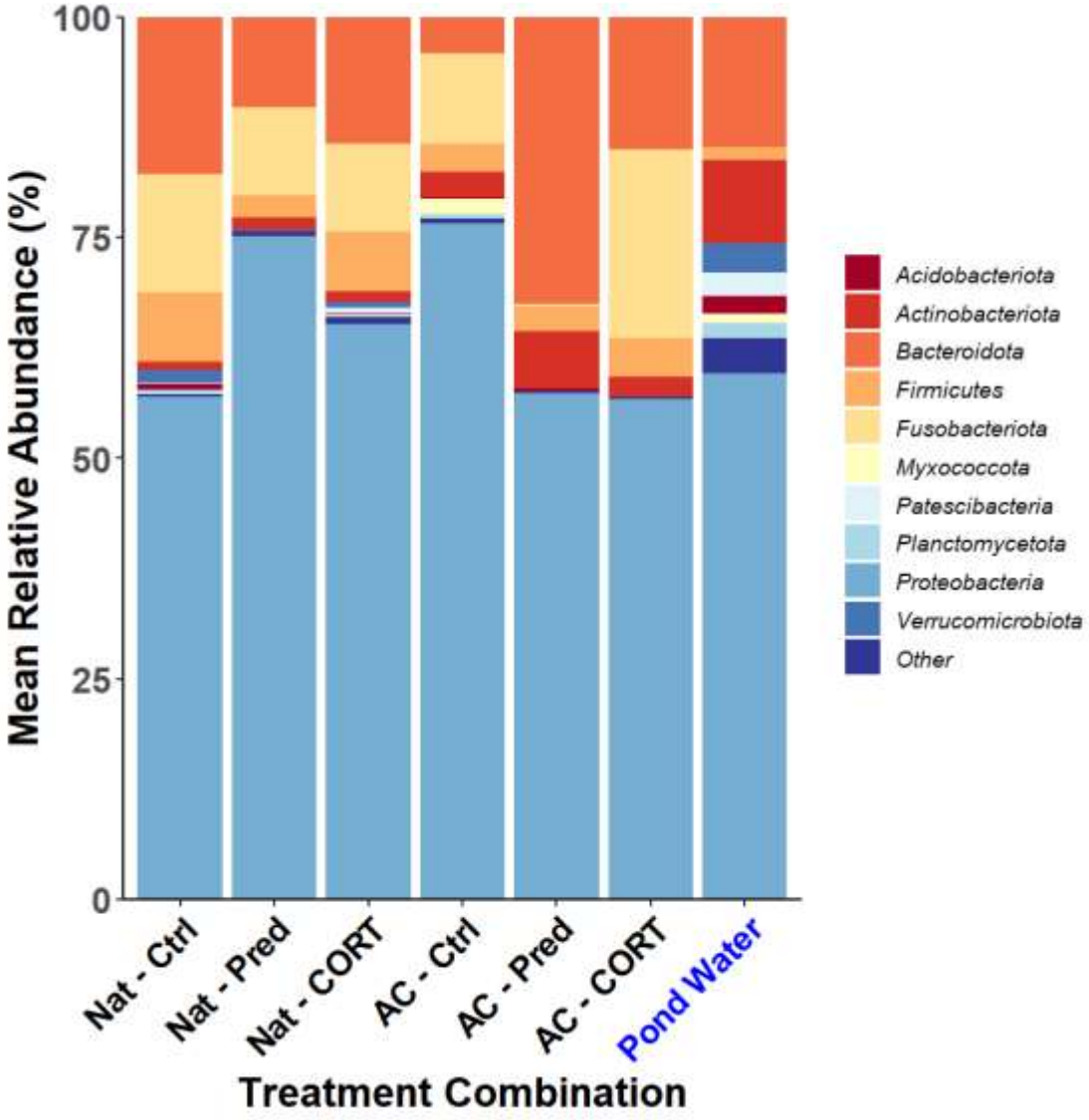


Figure 4.5. Bacterial phyla on skin of Northern Leopard Frog tadpoles and in natural pond water. Tadpoles were raised in either natural pond water (Nat) or autoclaved pond water (AC). In addition to the pond water treatments, tadpoles were raised in the presence of a vehicle control (Ctrl), predator cues (Pred), or CORT. Bacterial phyla with a mean relative abundance greater than 1% are shown; all other phyla were pooled into the 'Other' category. N = 2-4 tadpoles per treatment group and N = 6 pond water samples. No microbial DNA was detected in the 6 samples of autoclaved pond water.

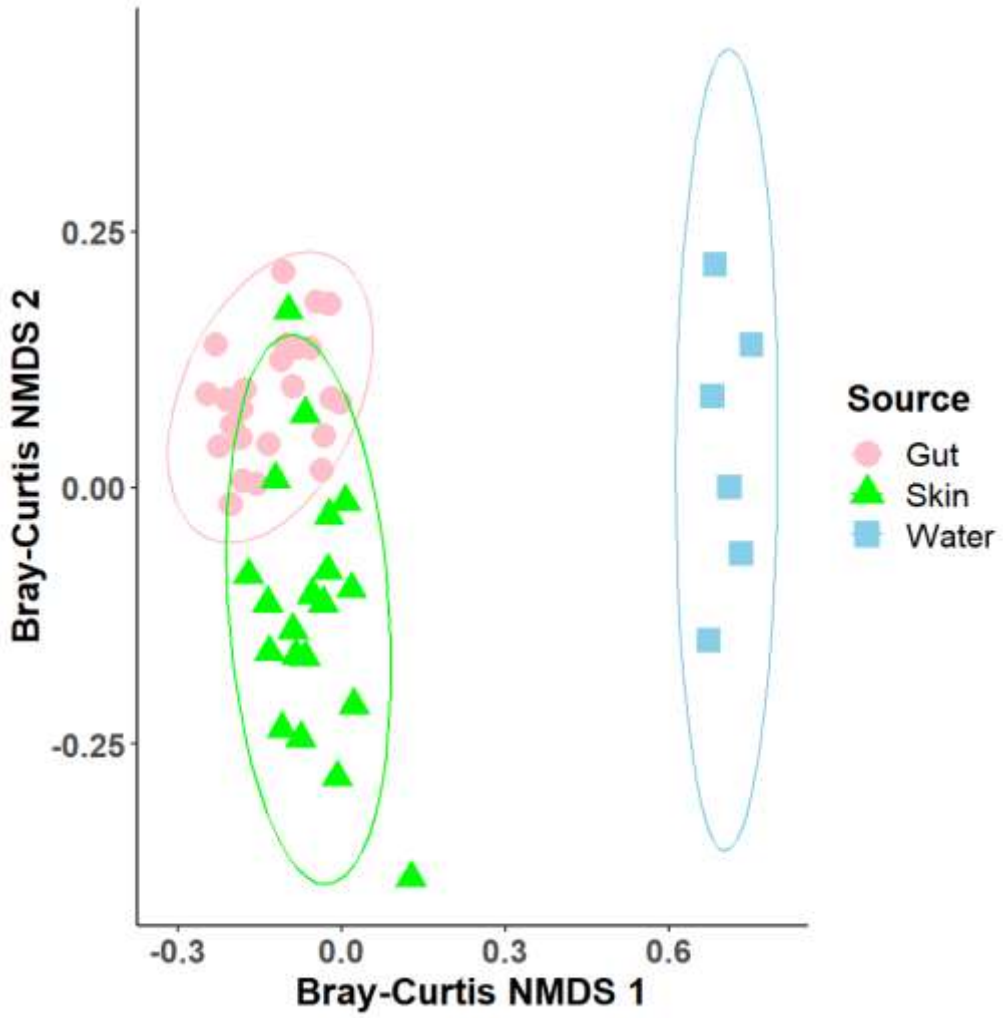
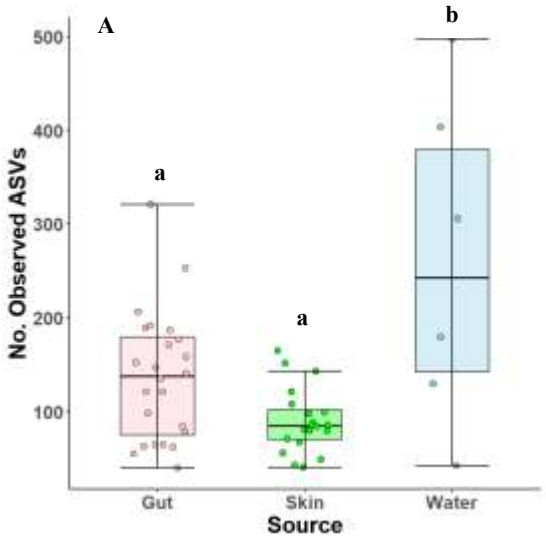
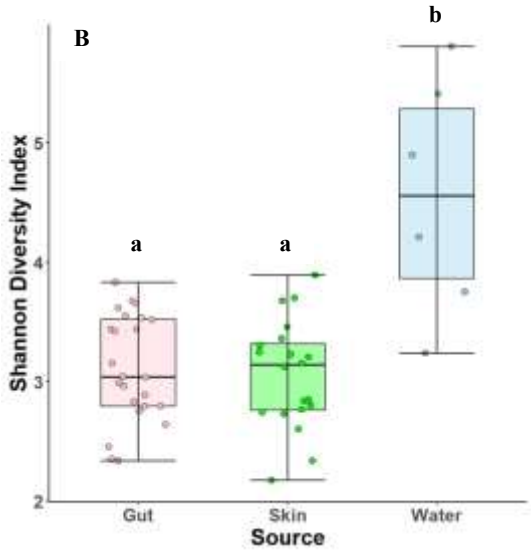


Figure 4.6. Microbial community composition present in the tadpole gut, on tadpole skin, and in tadpole-free natural pond water. Non-metric multidimensional scaling plot based on Bray Curtis dissimilarity showed that tadpole gut, tadpole skin, and natural pond water microbial communities differed significantly in composition (PERMANOVA: $F = 9.69$, $P < 0.001$). Tadpole gut and skin microbial communities were also significantly different (Adonis Pairwise Comparisons: $F = 10.3$, $P = 0.003$). See **Table 4.3** for full statistical output. Each point represents a sample: N = 24 (gut), 20 (skin), and 6 (water). Autoclaved pond water was not plotted because no microbial DNA was detected.


A
No. Observed ASVs
500
400
300
200
100
a
a
b
Gut
Skin
Water
Source


B
Shannon Diversity Index
5
4
3
2
a
a
b
Gut
Skin
Water
Source

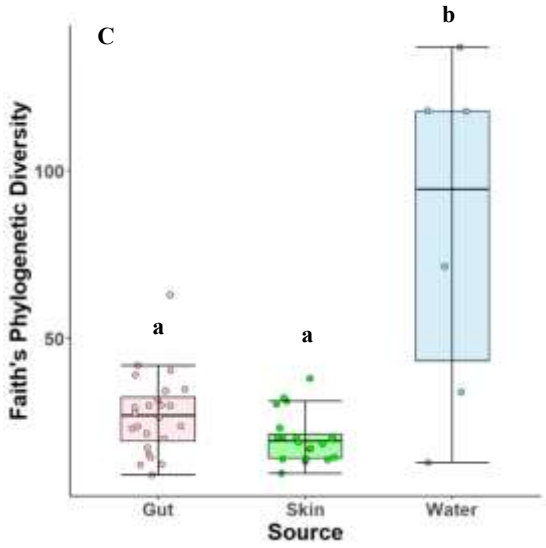

C
Faith's Phylogenetic Diversity
100
50
a
a
b
Gut
Skin
Water
Source

Figure 4.7: Diversity of microbial communities present in tadpole gut, tadpole skin, and natural pond water. Microbial communities differed significantly in: (**A)** the number of observed ASVs (χ^2 = 22.4, P < 0.001), **(B)** Shannon Diversity Index (χ^2 = 39.8, P < 0.001), and **(C)** Faith's Phylogenetic Diversity (χ^2 = 49.9, P < 0.001). In boxplots, the center line represents the median, the box length represents the interquartile range (IQR), and whiskers extend to 1.5x IQR. Points represent individual values. Box plots that do not share letter are statistically different based on Tukey's HSD. Full statistical analysis shown in **Table 4.3**. N = 24 (gut), 20 (skin), and 6 (natural pond water). No microbial DNA was detected in the 6 samples of autoclaved pond water.

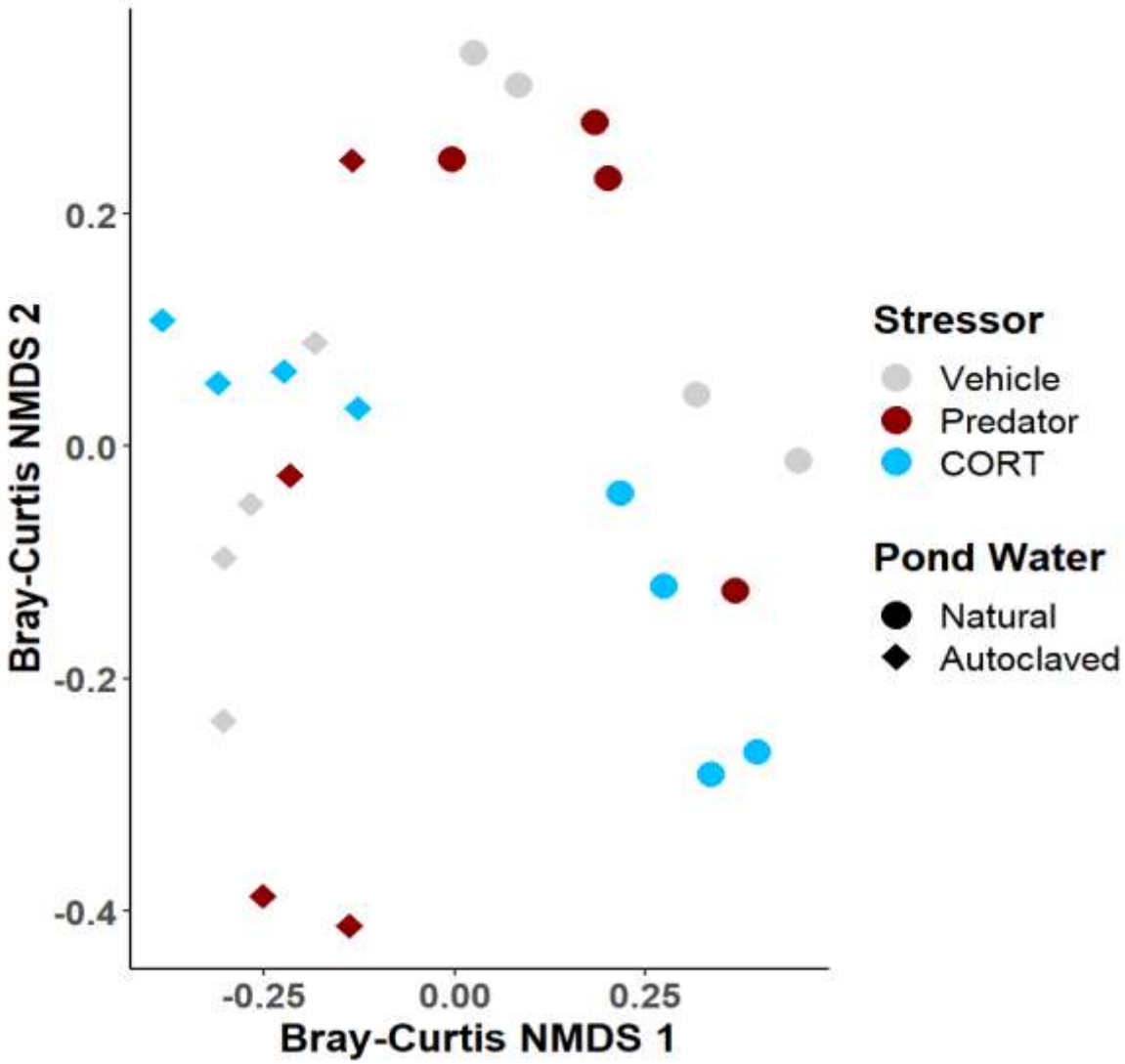


Figure 4.8. Microbial community composition present in the gut of Northern Leopard Frog tadpoles. Non-metric multidimensional scaling plot based on Bray Curtis dissimilarity showed that tadpoles raised in autoclaved pond water harbored gut microbial communities that differed from tadpoles raised in natural pond water (PERMANOVA: $F = 9.8$, $P < 0.001$). Gut bacterial communities also differed according to stressor (PERMANOVA: $F = 1.8$, $P = 0.033$). Lastly, there was a significant interaction between pond and stressor treatments (PERMANOVA: $F = 2.7$, $P = 0.002$). N = 4 per treatment group. See **Table 4.5** for full statistical output.

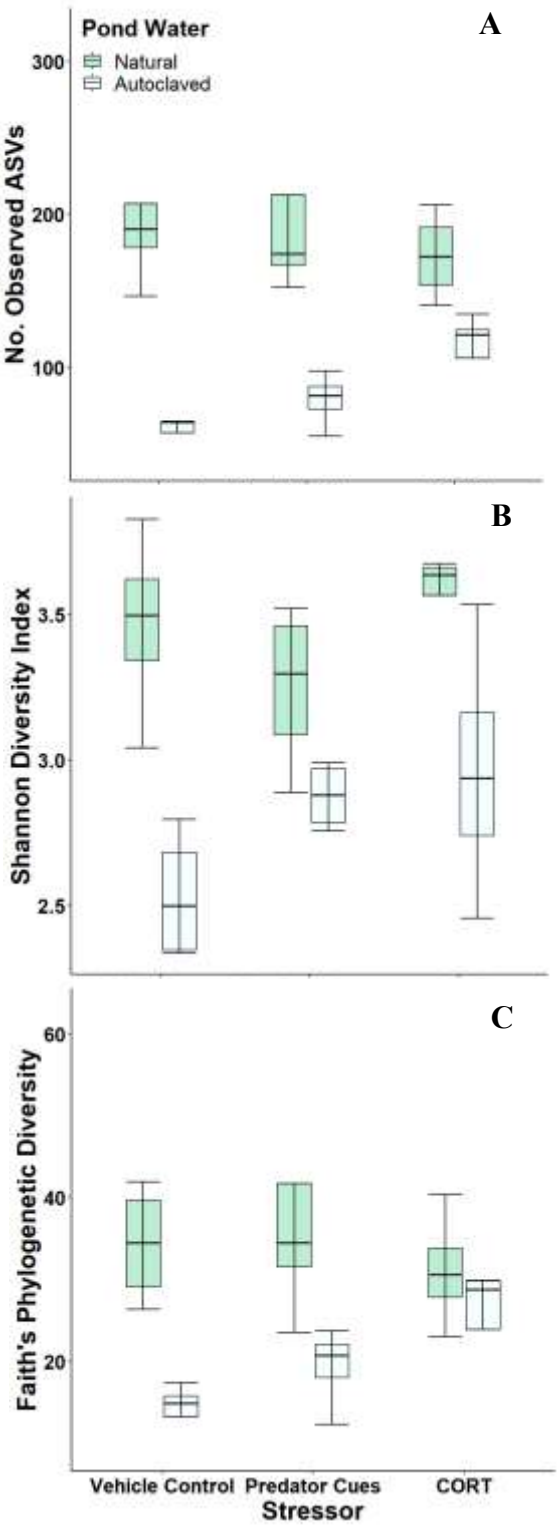

A
Pond Water
Natural
Autoclaved
No. Observed ASVs
300
200
100
B
Shannon Diversity Index
3.5
3.0
2.5
C
Faith's Phylogenetic Diversity
60
40
20
Vehicle Control
Predator Cues
CORT
Stressor

Figure 4.9. Effects of pond water treatment on the diversity of the gut microbiota in Northern Leopard Frog tadpoles. Compared with tadpoles raised in natural pond water, tadpoles raised in autoclaved pond water had: **(A)** a lower number of observed ASVs (χ^2= 41, P < 0.001), **(B)** a lower Shannon Diversity Index score (χ^2= 32, P < 0.001), and **(C)** a lower Faith's Phylogenetic Diversity score (χ^2= 17, < 0.001). There was no effect of stressor treatments on the diversity of the tadpole gut microbiota (**Table 4.5**). In boxplots, the center line represents the median, the box length represents the interquartile range (IQR), and whiskers extend to 1.5x IQR. N = 4 in each of the 6 treatment groups.

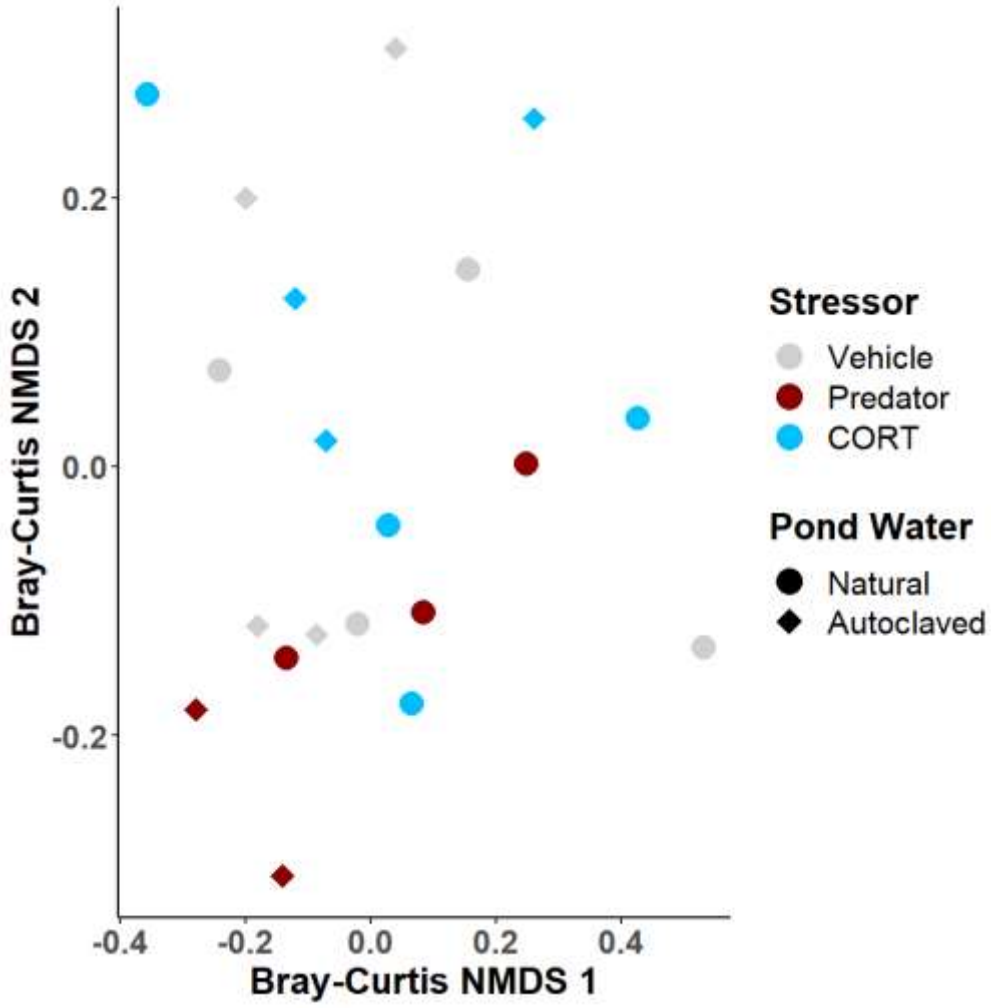


Figure 4.10: Microbial community composition present on the skin of Northern Leopard Frog tadpoles. Non-metric multidimensional scaling plot based on Bray-Curtis Dissimilarity showed that tadpoles raised in autoclaved pond water harbored skin microbial communities that differed from tadpoles raised in natural pond water (PERMANOVA: $F = 2.5$, $P < 0.004$). There was no effect of stressor treatments on skin microbial community composition, either alone (PERMANOVA: $F = 1.0$, $P = 0.46$) or in interaction with pond water treatments (PERMANOVA: $F = 0.99$, $P = 0.48$). Each point represents a sample; N = 2-4 in each of the 6 treatment groups. See **Table 4.5** for full statistical output.

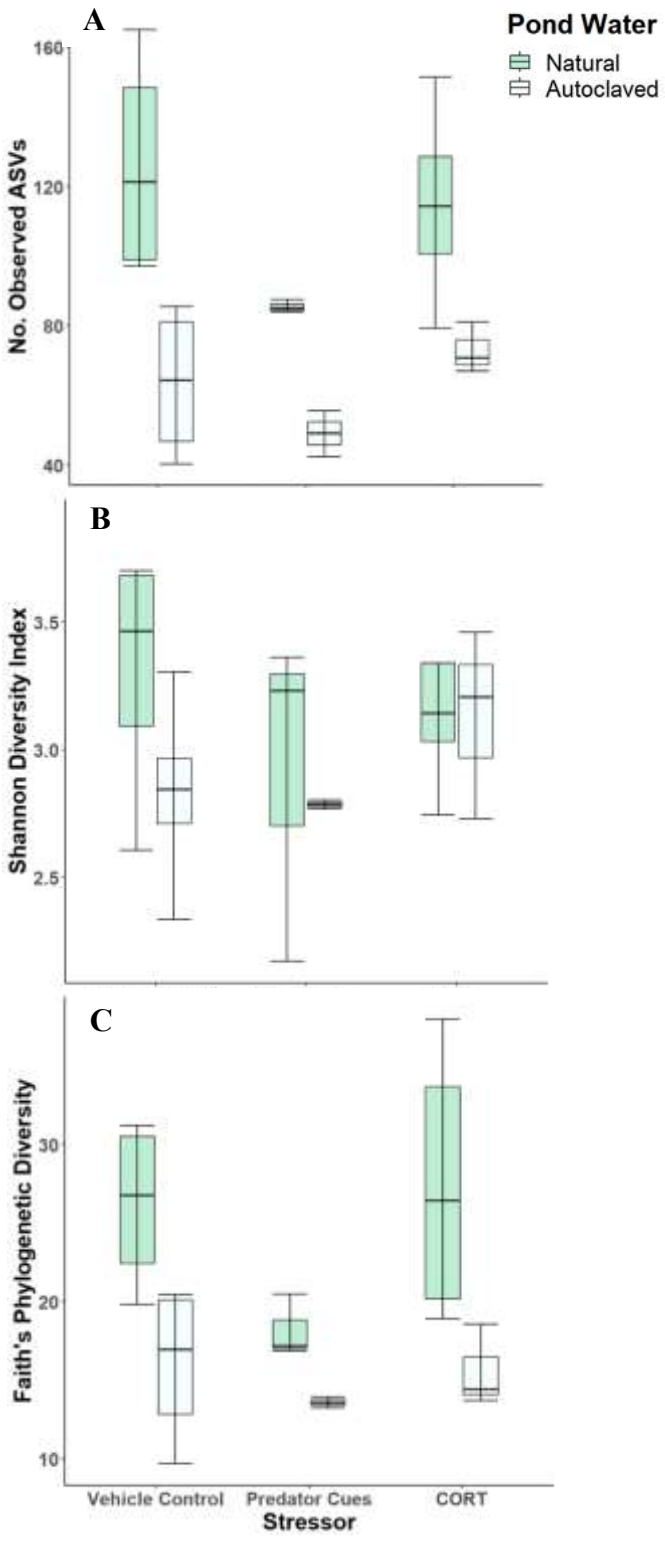

A
Pond Water
Natural
Autoclaved
No. Observed ASVs
160
120
80
40
B
Shannon Diversity Index
3.5
3.0
2.5
C
Faith's Phylogenetic Diversity
30
20
10
Vehicle Control
Predator Cues
CORT
Stressor

Figure 4.11: Effects of pond water treatment on the diversity of the skin microbiota on Northern Leopard Frog tadpoles. Compared to tadpoles raised in natural pond water, tadpoles raised in autoclaved pond water had: **(A)** a lower number of observed ASVs ($\chi^2 = 21$, $P < 0.001$), **(B)** no significant difference in Shannon Diversity Index ($\chi^2 = 1.5$, $P = 0.22$), and **(C)** lower Faith's Phylogenetic Diversity ($\chi^2 = 14$, $P < 0.001$). There was no effect of stressor treatments on the diversity of the tadpole skin microbiota. See **Figure 4.7** for explanation of boxplots. N = 2-4 for each of the 6 treatment groups. See **Table 4.5** for full statistical output.

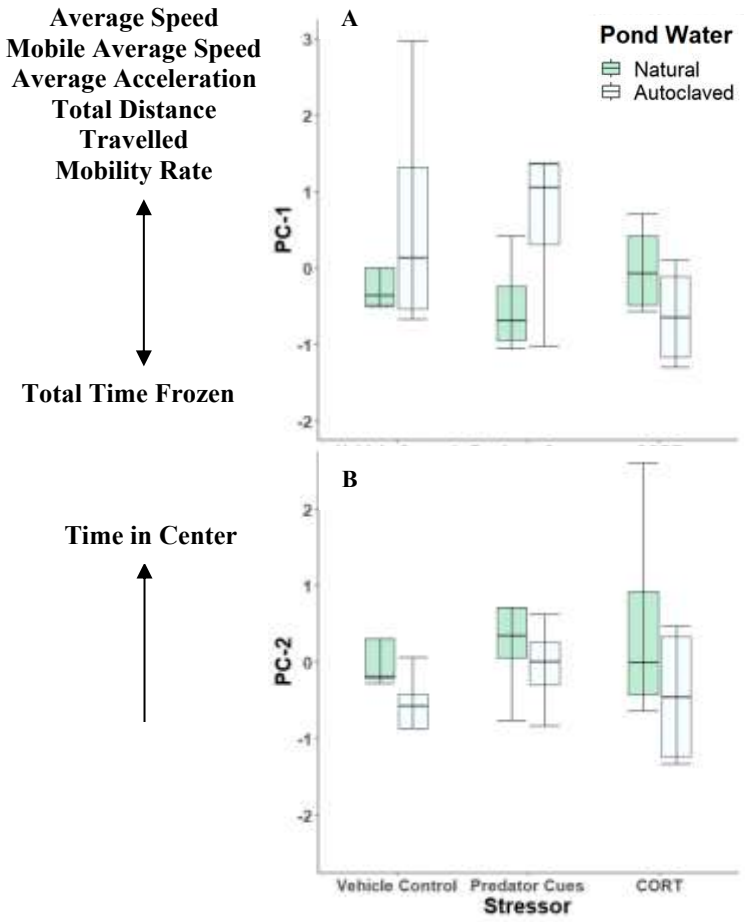


Figure 4.12: Effects of treatments on behavior of Northern Leopard Frog tadpoles under baseline conditions. Pond water and stressor treatment had no effect on baseline locomotory behavior. See **Figure 4.7** for explanation of boxplots. N = 4 for each treatment group.

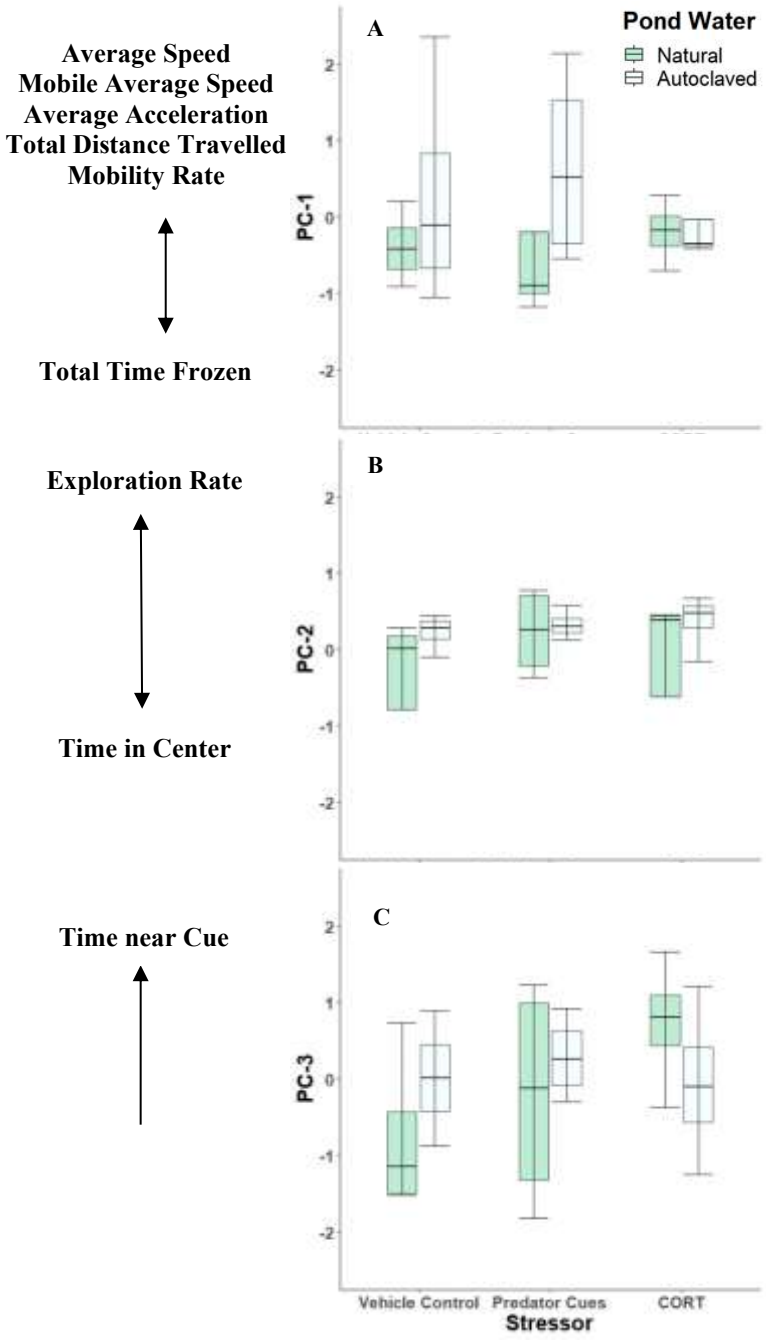


Figure 4.13: Effects of pond water and stressor treatments on behavior of Northern Leopard Frog tadpoles in response to the presentation of an empty glass vial. Pond water and stressor treatment had no effect on tadpole behavior when presented with an empty glass vial (see **Table 4.9**). **See Figure 4.7** for explanation of boxplots. N = 4 for each treatment group.

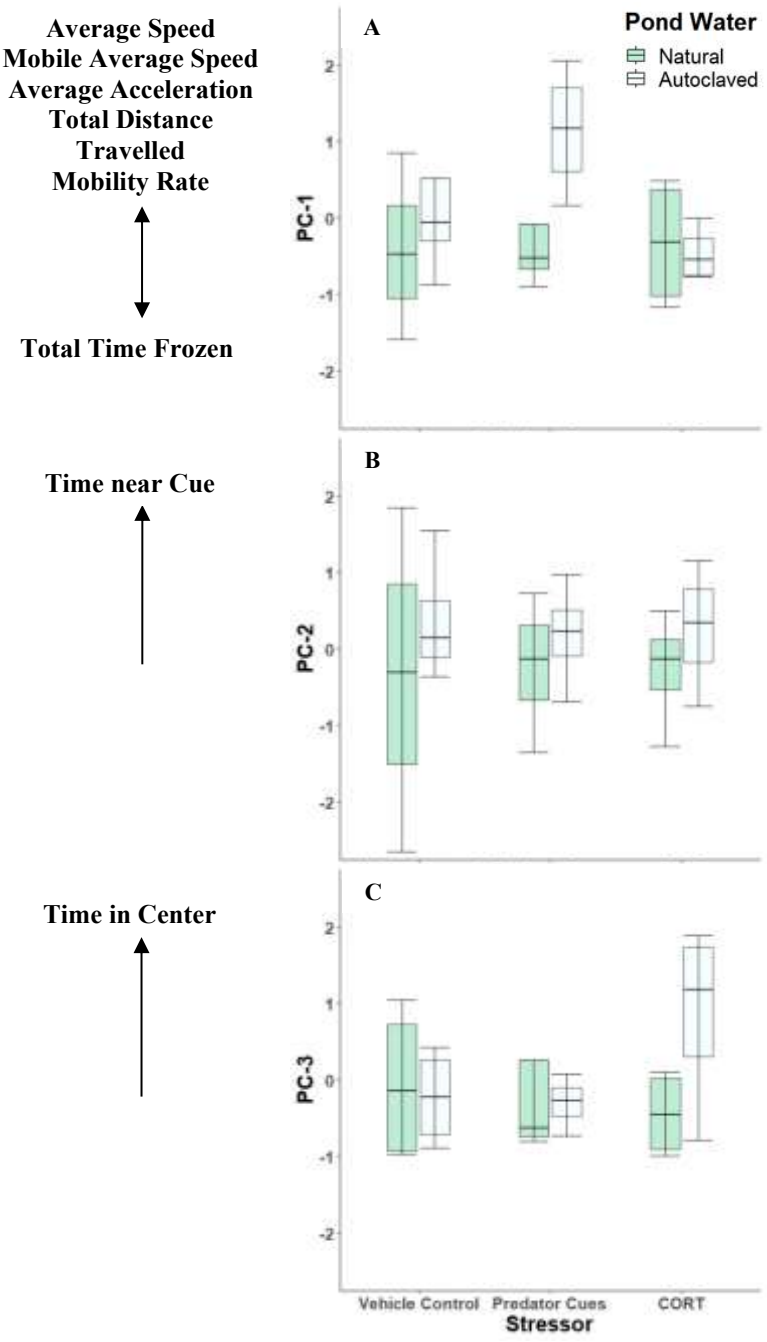


Figure 4.14: Effects of pond water and stressor treatments on behavior of Northern Leopard Frog tadpoles when presented with a glass vial filled with frog brittle. Pond water and stressor treatment had no effect on tadpole behavior when presented with a glass vial filled with frog brittle (see **Table 4.10**). See **Figure 4.7** for explanation of boxplots. N = 4 for each treatment group.

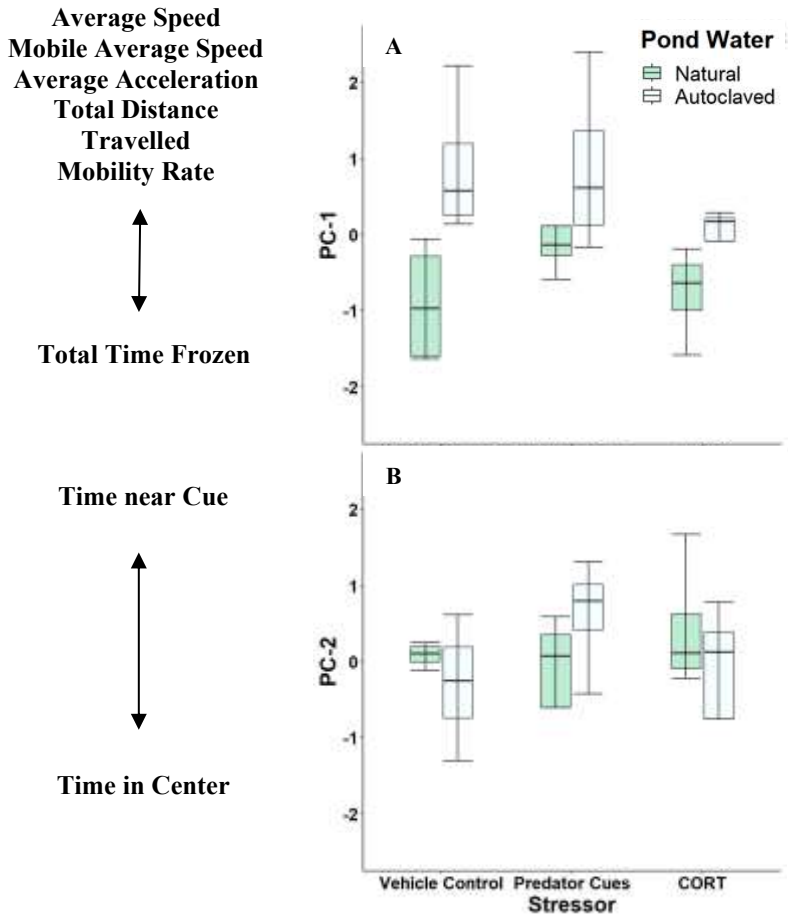


Figure 4.15. Effects of pond water and stressor treatments on behavior of Northern Leopard Frog tadpoles in response to the addition of tadpole-derived alarm pheromones. **(A)** After the addition of alarm pheromones, tadpoles that were raised in autoclaved pond water increased their overall locomotory activity compared to tadpoles that developed in natural pond water ($F = 12.4$, $P = 0.002$). Stressor treatment had no effect on tadpole locomotory behavior. **(B)** After the addition of alarm pheromones, there was no effect of treatment on location of tadpole relative to the cue or center of the arena (see **Table 4.11**). See **Figure 4.7** for explanation of boxplots. $N = 4$ for each of the 6 treatment groups.

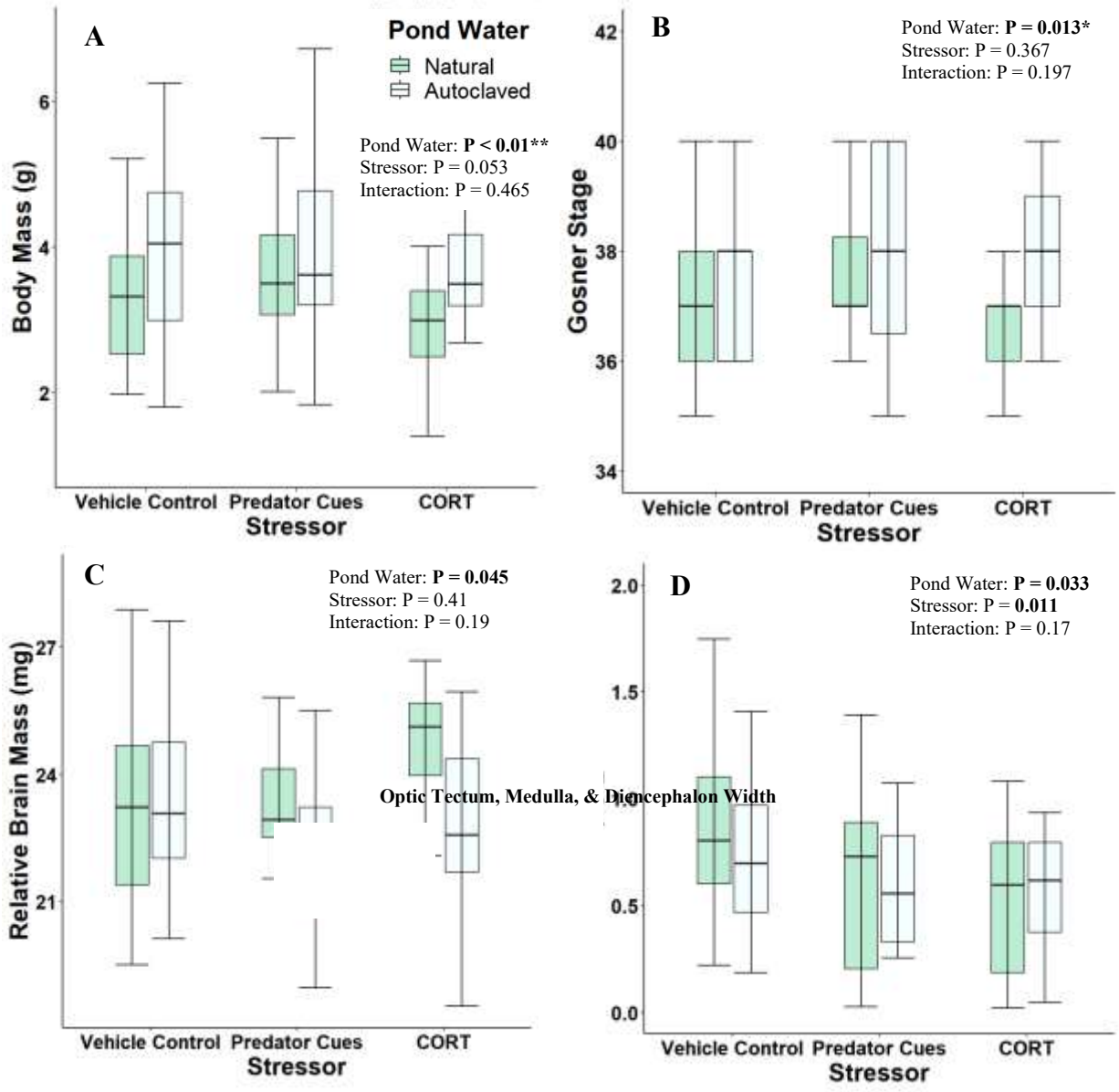


Figure 4.16. Effects of treatments on body mass, developmental stage, relative brain mass and shape in Northern Leopard Frog tadpoles. Compared to tadpoles raised in natural pond water, tadpoles raised in autoclaved pond water: **(A)** were larger (χ^2 = 9.5, P = 0.002), **(B)** developed faster (χ^2 = 6.1, P = 0.013), **(C)** had a smaller relative brain mass (corrected for body mass) (χ^2 = 4.0, P = 0.045) and **(D)** had relatively narrower optic tectum, medulla, and diencephalon. Stressor treatment impacted relative brain shape, although no pairwise differences were detected. See **Table 4.12** for statistical output. See **Figure 4.7** for explanation of boxplots. N= 17-24 for each of the 6 treatment groups.

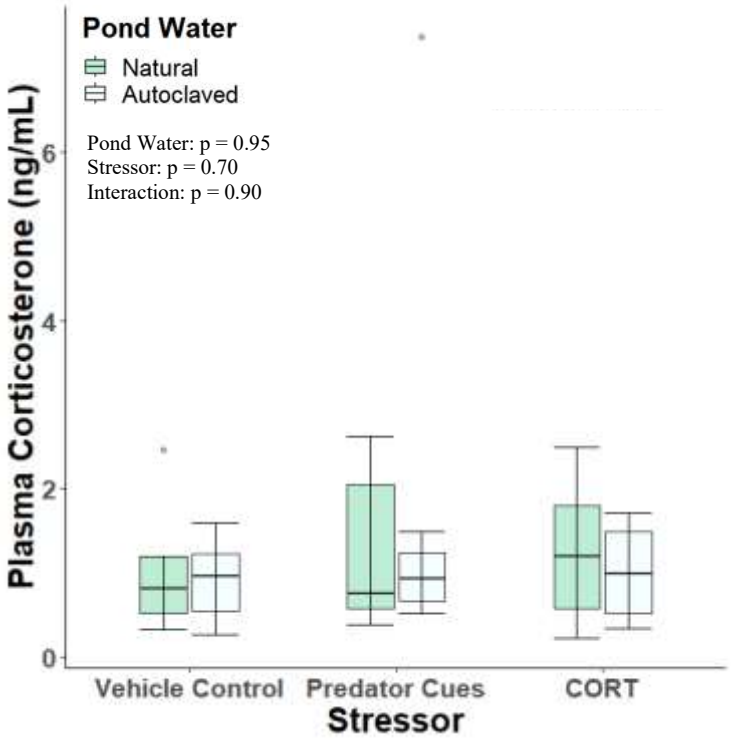


Figure 4.17: Effects of treatments on plasma corticosterone (CORT) levels in Northern Leopard Frog tadpoles. Neither Pond water (χ^2: 0.005, P = 0.95) and stressor (χ^2: 0.72, P = 0.70) treatment had no effect on the levels of plasma CORT. See **Figure 4.7** for explanation of boxplots. Points outside boxplot range are plotted individually. N= 7-8 for each treatment group.

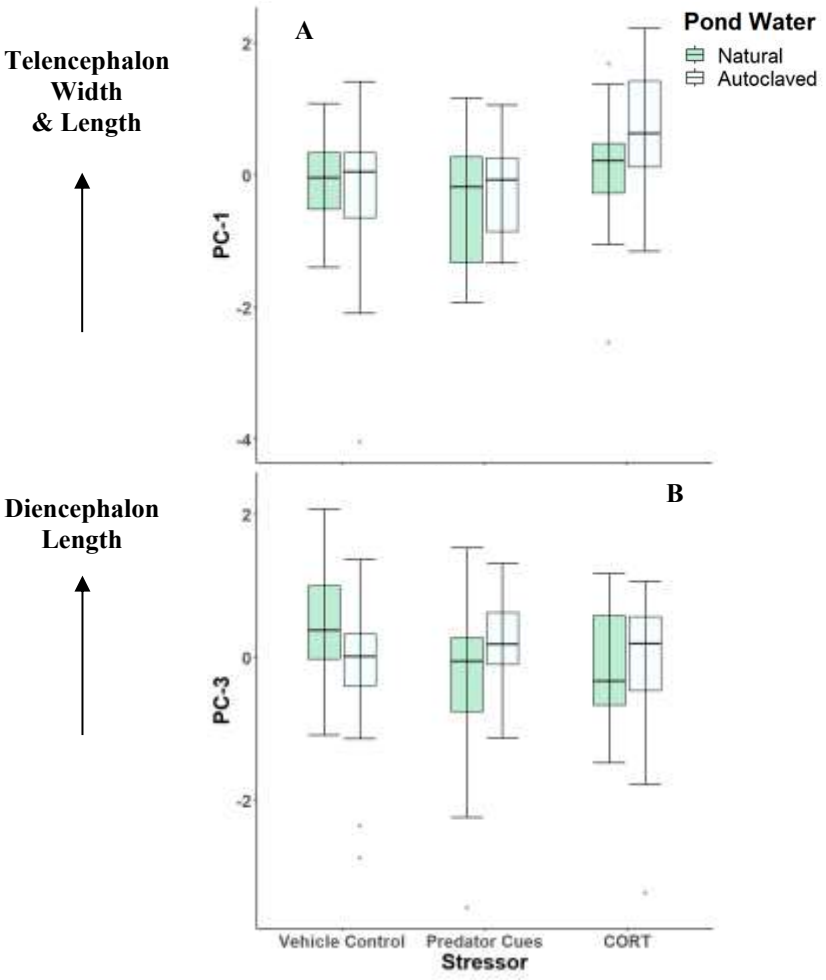


Figure 4.18: Effects of treatments on relative brain shape in Northern Leopard Frog tadpoles. (A) Neither pond water nor stressor treatment affected brain shape represented by PC-1. **(B)** Neither pond water nor stressor treatment affected brain shape represented by PC-3. Statistical analysis can be found in **Table 4.12**. See **Figure 4.7** for explanation of boxplots. Points outside boxplot range are plotted individually. N = 17-19 per treatment group.

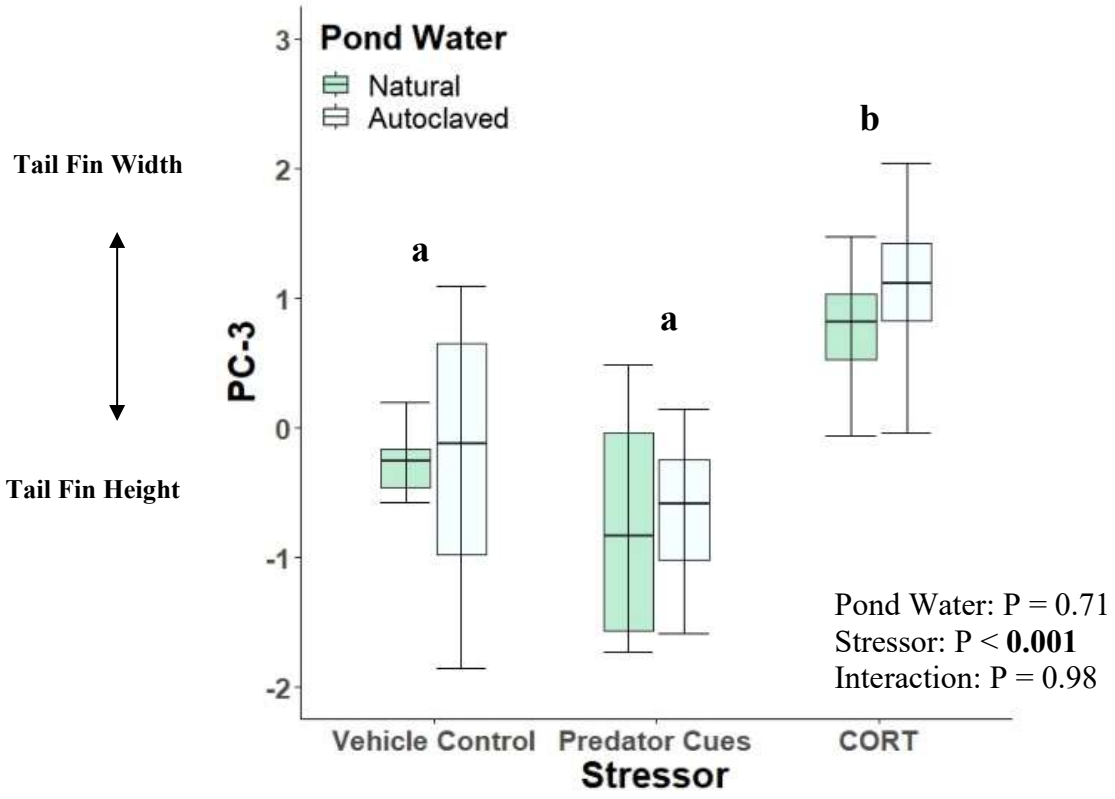


Figure 4.19. Effects of pond water and stressor treatments on body shape in Northern Leopard Frog tadpoles. Tadpoles exposed to exogenous CORT had relatively wider and shorter tail fins compared to the other stressors. Pond water had no effect on tadpole relative tail fin width and tail fin height. Statistical analysis can be found in **Table 4.13**. See **Figure 4.7** for explanation of boxplots. Box plots that do not share letter are statistically different based on Tukey's HSD. N= 6-8 for each of the 6 treatment groups.

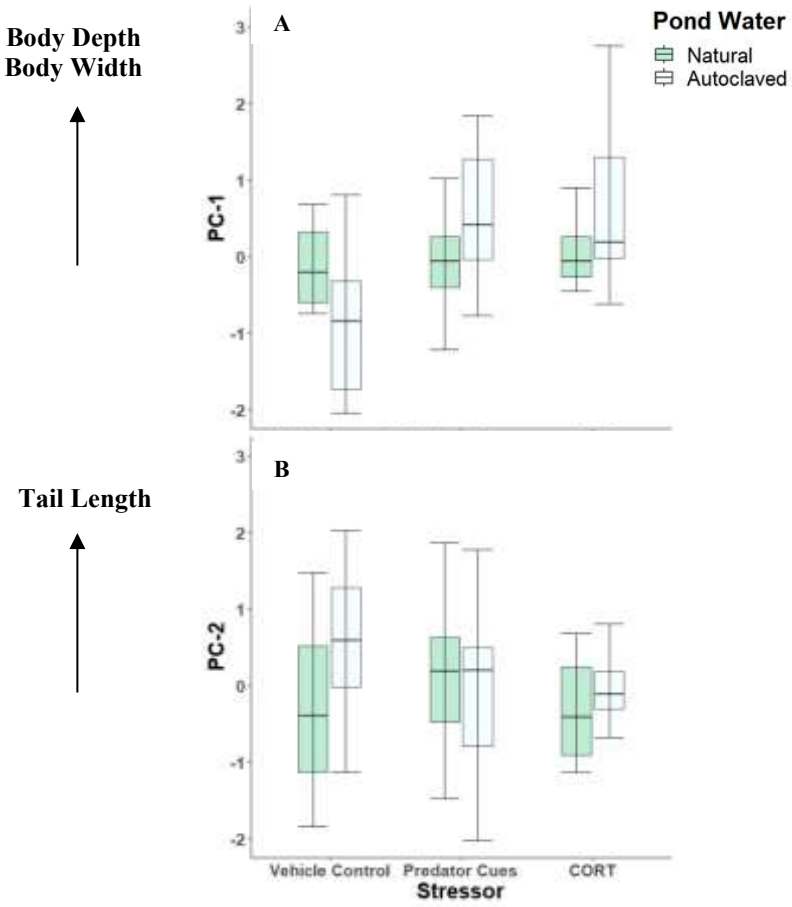


Figure 4.20: Effects of pond water and stressor treatments on relative body shape in Northern Leopard Frog tadpoles. (A) Pond water and stressor treatments had no effect on tadpole relative body depth and body length. **(B)** Pond water and stressor treatments had no effect on relative tail length. Statistical analysis can be found in **Table 4.13**. See **Figure 4.7** for explanation of boxplots. N= 6-8 for each treatment group.

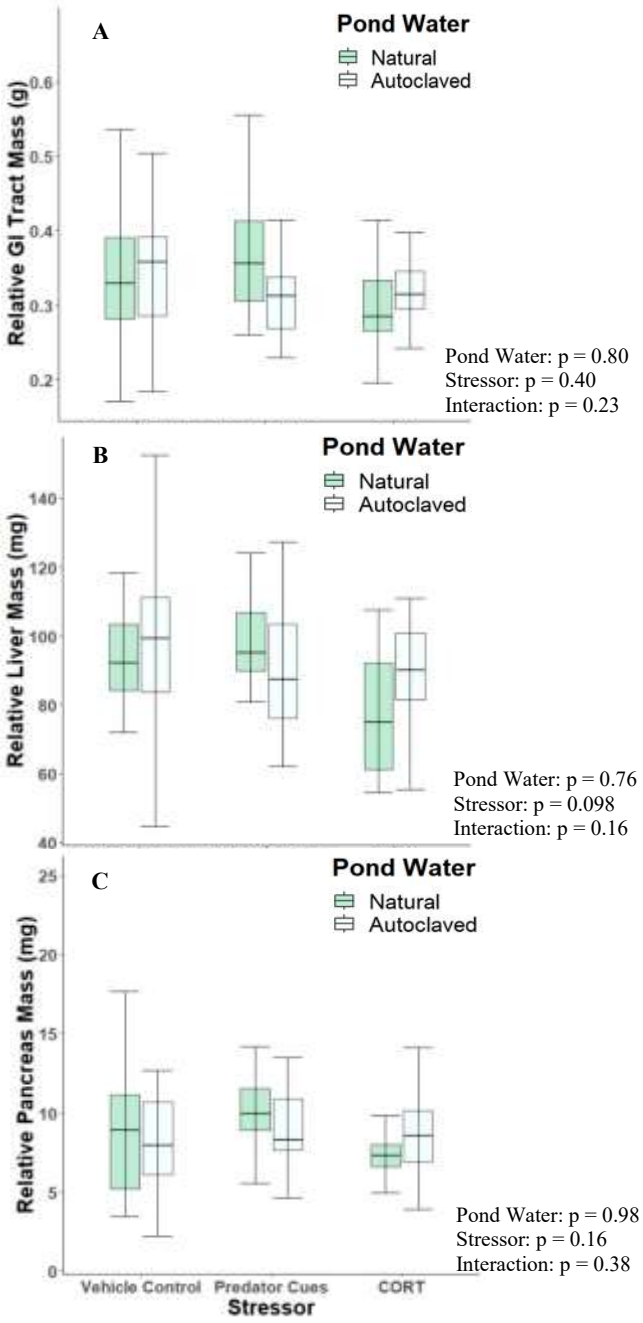


Figure 4.21: Pond water and stressor treatments had no effect on organ mass (corrected for body mass) in Northern Leopard Frog tadpoles. (A) Relative gastrointestinal tract mass. **(B)** Relative liver mass. **(C)** Relative pancreas mass. See **Figure 4.7** for explanation of boxplots.

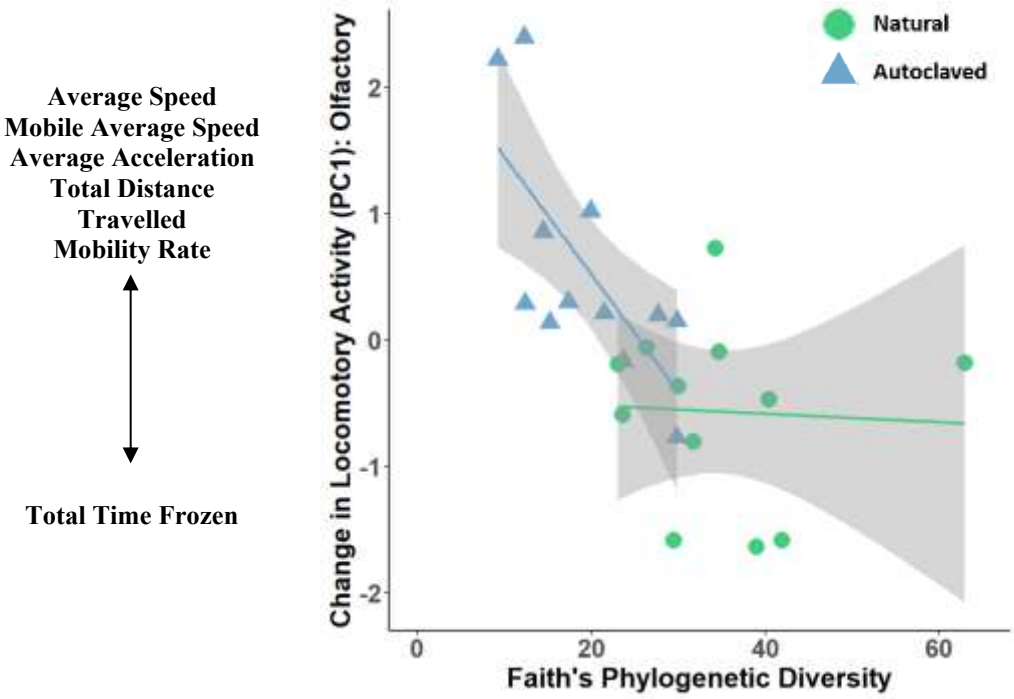


Figure 4.22. Association between gut microbial alpha diversity and tadpole locomotory behavior in response to alarm pheromones. Variance in locomotory behavior was modestly explained by Faith's Phylogenetic Diversity (R^2: 0.125). A higher Faith's Phylogenetic Diversity score predicted decreased tadpole locomotory activity in response to the addition of alarm pheromones ($\chi^2 = 6.2$, P = 0.013). Trend lines were created using linear regression models, and gray shading indicates 95% confidence intervals.

Chapter 5. Conclusions

Summary of Dissertation Goals and Results

The goal of my dissertation was to evaluate how the aquatic microbial environment influences the biodiversity of the amphibian gut microbiota, and how these shifts in the microbiota influence amphibian neurodevelopment and behavior. Findings of associations between the gut microbiota and amphibian neurodevelopment and behavior are consistent with the presence of a MGB axis and can offer insight as to whether particular aspects of the gut microbiota (i.e. diversity and taxa abundance) were significantly associated with tadpole development and physiology, including the MGB axis.

My first aim tested the hypothesis that the aquatic microbial environment and water temperature would influence tadpole neurodevelopment through changes in brain size and morphology. By taking advantage of a collaborative opportunity, I found evidence of the amphibian MGB axis. Fontaine et al. (2022) had shown that tadpoles that were raised in autoclaved pond water and at warmer water temperatures had a larger body size and altered composition and reduced biodiversity of gut microbial communities compared to tadpoles raised in natural pond water and at cooler temperatures. I extended her findings to show that tadpoles raised in warmer temperatures had increased relative brain size and increased width and length of the optic tectum compared to tadpoles raised in cooler temperatures (**Table 5.1**). Similarly, tadpoles raised in autoclaved pond water exhibited similar increases in optic tectum width and length compared to those raised in natural pond water. Interestingly, I also found significant associations between gut microbial communities and brain shape. Collectively, these results are novel and important, as they describe how relevant increases in global temperatures and how

aquatic microbial communities influence larval amphibian neurodevelopment, which provides some of the first evidence of the amphibian MGB axis.

My second aim tested the hypothesis that the aquatic microbial environment would influence the tadpole gut microbiota, neurodevelopment, and behavior (Emerson and Woodley, 2024). In this aim, I switched to Northern Leopard Frog tadpoles, which allowed me to test the generality of the findings in Aim 1 that used Green Frog tadpoles. Compared to tadpoles raised in natural pond water, tadpoles raised in autoclaved pond water had a gut microbiota that differed in its composition and had reduced bacterial diversity, had decreased behavioral responses to sensory stimuli, were larger, had relatively heavier brains, and had relatively narrower medullas (**Table 5.1 – 5.2**). Additionally, I found that the composition and diversity of the amphibian gut microbiota were significant predictors of brain development and behavior (**Table 5.3 – 5.4**). Collectively, these results supported the hypothesis and accomplished the primary goals of replicating and expanding upon our Aim 1 results.

My third aim sought to determine if these phenotypes induced by pond water treatments persisted during exposure to ecologically relevant stressors, such as predation-derived chemical cues and the associated increases in CORT. Stress is a normal part of animal development, and stressors and stress hormones have been linked to changes in the gut microbiota in mammals. Compared to tadpoles raised in natural pond water, tadpoles raised in autoclaved pond water exhibited similar phenotypes seen in our previous experiments (**Table 5.1 – 5.2**), such as: altered composition and reduced diversity of gut and skin microbial communities, increased body size, decreased relative brain mass, and decreased size of the optic tectum, medulla, and diencephalon. Novel findings in Aim 3 were that tadpoles raised in autoclaved pond water had impaired predator avoidance behavior and this result was significantly associated with the composition

and diversity of the gut microbiota. This result suggests that the tadpole gut microbiota influences behaviors directly linked to recognition of chemical cues and subsequently survival (Cowan et al., 2020; Cryan et al., 2019; Fraker et al., 2009; Schoeppner and Relyea, 2005). Also, the gut microbiota, but not the skin microbiota, was a significant predictor of several aspects of tadpole phenotype (**Table 5.3 – 5.4**).

In Aim 3, there were relatively few impacts of the stressor treatments, either in interaction with the pond water treatments or alone. It is surprising that no clear effects of tadpole exposure to predation-derived chemical cues were found, as previous studies using similar methodology found effects on body morphology, whole-body CORT, and locomotory and anti-predator behaviors (Fraker, 2008; Fraker et al., 2009; Fraker et al., 2021; Middlemis Maher et al., 2013; Relyea, 2001; Woodley et al., 2015). As expected, exogenous CORT exposure had dramatic impacts on tadpole tail morphology, as has been found in other studies (Fraker et al., 2021; Glennemeier and Denver, 2002; McClelland, 2020; Middlemis Maher et al., 2013), which confirms that our CORT treatments were effective. Exposure to CORT also altered community composition but not biodiversity of the gut microbiota. Surprisingly, CORT did not affect body mass or other endpoints. Overall, Aim 3 provided additional support for the presence of the amphibian MGB axis through evaluations of tadpole physiology, brain development, and behavior. Additionally, while the composition of the amphibian microbiota appears somewhat malleable in the face of ecological stressors, aquatic microbial communities appear to be the primary determining factor in the composition and diversity of the larval amphibian microbiota.

Evidence for a Microbiota-Gut-Brain Axis in Larval Amphibians

With my dissertation, I provide the first evidence that the MGB axis shapes brain development and behavior in an amphibian model. Specifically, I found that raising tadpoles in

autoclaved pond water resulted in tadpoles that harbored distinct gut microbial communities with reduced biodiversity compared to tadpoles raised in natural pond water. Further, development in these conditions had replicated effects on tadpole brain development and architecture, as well as behavioral responses to sensory stimuli. Lastly, aspects of the gut microbiota were significant predictors of tadpole brain and behavioral endpoints.

Although my results are consistent in their support of the presence of the amphibian MGB axis, it is very difficult to design experiments to discern direct causal relationships between the gut microbiota and host phenotype. For example, raising tadpoles in autoclaved water alters the gut microbiota, but also the microbiota of other surfaces such as skin. However, I found no associations between skin microbiota and tadpole phenotype. It is also possible that the process of autoclaving the water impacts the heavy metal content which in turn impacts tadpole development. However, initial results indicated that the chemical composition and water quality of both natural and autoclaved pond water samples are very similar. Perhaps the aquatic microbial environment somehow alters the palatability of the food, so that the tadpoles eat more, and the increased nutrition/energy can contribute to the growth of nervous tissue or can be allocated to immune parameters. Future studies could track food consumption in these pond water treatments.

Relationship between Host Phenotype and Specific Bacterial Taxa

A goal of my dissertation work was to evaluate if aspects of the gut microbiota were significantly associated with tadpole development and physiology. This is important, because describing associations between the gut microbiota and physiology can aid in downstream metanalyses that can better describe conserved relationships between a host and specific commensal taxa. Here, I describe patterns of associations between the microbiota and amphibian

neurodevelopment across my studies, which will contribute to future experiments interested in evaluating host-microbe relationships in wildlife species.

The relative abundances of several gut bacterial taxa were associated with neurodevelopmental phenotypes across multiple studies. For example, the phylum Patescibacteria was found to be negatively associated with the size of the optic tectum (Chapter 2), but positively associated with tadpole locomotion in response to sensory stimuli (Chapter 3). A previous study investigating the MGB axis found that this phyla was associated with upregulation of serotonin that was associated with the amelioration of depression-like symptoms in rats (Zhang et al., 2021). Additionally, the relative abundance of the *Clostridium.sensu.stricto* genus was also significantly associated with changes in the size of the optic tectum (Chapter 2) and locomotory behavior (Chapter 3). This is noteworthy, as this genus has also been implicated in the modulation of peripheral serotonin levels and aspects of neurophysiology (Yano et al., 2015). The mechanism behind this elevation in serotonin levels is hypothesized to be driven by commensal microbes that produce metabolites that stimulate the release of gut-derived serotonin from enterochromaffin cells present in the intestinal epithelia that reach the brain (Yano et al., 2015). Future studies investigating the MGB axis in wildlife can further explore this association by colonizing animals with bacterial taxa associated with neurotransmitter expression, such as *clostridium.sensu.stricto.*

I was also interested in evaluating potential associations between bacterial taxa and tadpole behavioral responses to alarm pheromones in Chapter 4. The relative abundances of the *Staphylococcus* genus and *Ancylobacter* genus were positively associated with tadpole locomotory activity when exposed to alarm pheromones. Interestingly, both bacterial genus are either aerobic (Firsova et al., 2009) or facultative anaerobes, and *Staphylococcus* contains

bacterial species commonly associated with mammalian pathogens (Foster, 1996). The association of aerobic bacteria with this uncharacteristic response is interesting, as within the vertebrate GI tract, higher abundances of obligate anaerobic bacteria are much preferred. Obligate anaerobes are the primary producers of SCFAs through carbohydrate enzymolysis (Litvak et al., 2018). This activity by obligate anaerobic bacteria is vital to vertebrate health, as it limits oxygen content emanating from the mucosal surface (Litvak et al., 2018) and increases the production of epithelium tight junction proteins that maintain gut barrier integrity (Fachi et al., 2019). Often, the increase in relative abundances of aerobic bacteria and loss of gut hypoxia is associated with decreased host health and increased instances of bacterial translocation into systemic circulation (Fachi et al., 2019), which can influence the MGB axis (Cryan et al., 2019). While the uncharacteristic behavioral response in Chapter 4 cannot be attributed to the presence of these bacterial taxa, it is interesting that this response was associated with aerobic microbes that have been associated with vertebrate pathogens and decreased host health.

Factors Shaping the Amphibian Gut Microbiota

My dissertation provided evidence that the aquatic microbial environment is a determining factor in the composition and diversity of the amphibian gut and skin microbiota (**Table 5.2**). As measured in Chapter 4, we also measured the composition and diversity of the microbial communities present in natural pond water. This allowed us to confirm that while natural pond water contained significantly more diverse bacterial communities with different compositions, this is likely due to differences in carrying capacity of water verses a tadpole GI tract (Contijoch et al., 2019). Additionally, there was significant overlap in the most abundant bacterial phyla present in the water and those found in and on the tadpole, providing support for

previous work suggesting these aquatic microbial communities are a primary determining factor in the larval amphibian microbiota (Correa et al., 2020).

Temperature is also an important factor shaping the amphibian gut microbiota. Elevated temperature is a relevant ecological stressor, as amphibian populations and biodiversity have been decreasing considerably due to climate change (Pounds et al., 2006). Previous work has demonstrated that amphibian exposure to varying or increasing temperatures has dramatic impacts on the composition of their microbiome and their physiology, such as metabolic rate, developmental rate, locomotion, thermal tolerance under heat stress, and digestive efficiency (Feder and Burggren, 1992; Fontaine and Kohl, 2020; Fontaine et al., 2022; Fontaine et al., 2018; Gillooly et al., 2001; Gillooly et al., 2002; Huey and Stevenson, 1979; Kohl and Carey, 2016; Kohl and Yahn, 2016).

Additional factors that can influence the composition of the amphibian-associated microbiota include vertical transmission of maternal microbes to egg clutches (Hughey et al., 2017), which can influence the initial amphibian gut and skin microbiota. A potential future study could raise amphibians in natural pond water after egg clutches were unmanipulated or after they were sterilized to remove vertically transmitted microbes to determine the sole influence of aquatic microbial communities. Further, while diet and feeding frequency was kept constant in my dissertation studies, the composition of an animal diet has a large determining factor on the intestinal microbiota (Kohl and Carey, 2016). Future studies can explore multi-factor experimental designs testing shifts in diet while also incorporating pond water treatments to better investigate the relationship between environmental microbes and diet.

The anatomy of the GI tract can also influence the composition of the gut microbiota (Maritan et al., 2024), but we found no effect of pond water treatment on the relative weight of

organs associated with digestion. Weight of the GI tract is not an all-encompassing measurement when it comes to evaluating treatment effect, so future studies can investigate GI histology and gut-associated lymphoid tissue, which can impact the habitability of certain gut-dwelling microbes. Lastly, it is important to note that the microbial community of pond water can vary greatly based on geography, season, and local factors (Stocker et al., 2024). The source of the pond water used in Aim 1 differed from the pond water used in Aims 2 and 3, nonetheless, I found similar outcomes.

Effects of the Aquatic Microbial Community on Amphibian Brain and Behavior

Tadpoles that developed in autoclaved pond water consistently experienced drastic alterations to their brain size and architecture compared to tadpoles raised in natural pond water (**Table 5.1**). Despite differences in morphological changes between experiments, it is important and novel that we have demonstrated consistent effects of a depleted microbial environment on neurodevelopment in an amphibian model. Further, these results fall in line with previous studies that demonstrate brain plasticity in an amphibian model when exposed to both abiotic and biotic factors (Cha et al., 2021; Gonda et al., 2013; Liao et al., 2016; McClelland, 2020; McClelland et al., 2018; McClelland and Woodley, 2022; Woodley et al., 2015). Numerous previous studies in GF mice have found that they exhibit significant differences in brain regions such as the amygdala, hippocampus, hypothalamus and others (Cowan et al., 2020; Cryan et al., 2019). The development and maintenance of larger brains is known to be energetically costly (Aiello and Wheeler, 1995; Mink et al., 1981) and likely impact animal cognition and behavior (Roth and Dicke, 2005).

Tadpoles that developed in autoclaved pond water consistently experienced changes in locomotory behavioral responses when exposed to sensory stimuli when compared to tadpoles

raised in natural pond water (**Table 5.1**). In Chapter 3, tadpoles that developed in autoclaved pond water displayed reduced locomotory and exploratory behavior in response to visual stimuli, which could indicate animal personality traits such as boldness (Dingemanse et al., 2003; Kelleher et al., 2018; Toms et al., 2010). In a previous study, tadpoles spent more time near a visual stimulus after exposure to trace concentrations of a pesticide compared to control (McClelland and Woodley, 2022). Tadpole responses in novel environments and/or to sensory stimuli have been described as factors that influence fitness and survivability (Carlson and Langkilde, 2013; Wilson and Krause, 2012).

In Chapter 4, tadpoles displayed increased locomotory activity compared to control when exposed to conspecific alarm pheromones, which is an uncharacteristic behavioral response based on previous work that likely makes tadpoles more detectable to a predator (Denver, 2021; Fraker et al., 2009; Fraker et al., 2021; Middlemis Maher et al., 2013). While we cannot say for certain that development in a depleted microbial environment is driving these changes in behavior and potential shifts in tadpole personality, this result does support a previous study using a GF zebrafish model that displayed differences in characteristic behavioral and stress responses compared to conventionally raised conspecifics, of which were obliterated upon microbial reconstitution (Davis et al., 2016). Future studies should expose tadpoles to live predators to firmly establish fitness consequences of these behaviors that appear to be mediated by through the MGB axis (Fraker et al., 2021; Mogali et al., 2012).

During our Aim 3 work, dissected brains were stored in RNALater for future assessments of brain transcriptomics, which will allow for future analysis of differential gene expression in the brain based on treatment. Future work can also incorporate different approaches such immunohistochemistry to evaluate target molecule localization in nervous tissue or isotropic

fractionation to quantify neuronal cell counts (Herculano-Houzel, 2005), which could help explain differences in brain size and morphology.

Body Mass

Development in autoclaved pond water elicited dramatic and repeatable changes in tadpole body mass (**Table 5.2**). Across all studies, tadpoles that developed in autoclaved pond water were significantly larger than tadpoles raised in natural pond water. Developmental rate was also higher in tadpoles raised in autoclaved pond water, but this rate only slightly increased. Additionally, there was no difference in plasma CORT levels between treatments in any study, eliminating the associated stress hormone as a potential explanation.

Interestingly, increased body mass is commonly found in livestock that are chronically treated with antibiotics (Gaskins et al., 2002). It is hypothesized that reductions in gut microbial diversity lessen the burden on the immune system, allowing more allocation to growth and development (Gaskins et al., 2002). Future studies could explore impacts of the pond water treatments on tadpole immune function.

The increased body mass could also be related to increased digestive efficiency. Future investigations into the products of gut-dwelling bacteria can provide more information related to tadpole size. For example, gut-derived metabolites such short-chain fatty acids (SCFAs) can directly or indirectly interface with vagal and enteric neuron afferents, which can influence host satiety and feeding behavior (Cryan et al., 2019; Warne and Dallas, 2022). A future study could evaluate the metabolomic profile of the tadpole gut microbiota to determine metabolite production that could be tied to host satiety, as well as investigate/track feeding frequency and behavior.

Questions remain as to whether these increases in tadpole body size are beneficial or detrimental. In larval tadpoles, increased body size is correlated with fitness and survival. Specifically, larger tadpole body size at metamorphosis can improve their long term-viability, including enhanced locomotory performance, better success at catching prey, enhanced growth rate and survival under varying seasonal conditions, and maintenance of size advantage over conspecifics that were smaller at metamorphosis (Cabrera-Guzmán et al., 2013).

Plasma CORT

Interestingly, pond water treatment did not influence plasma CORT levels across any of our studies (**Table 5.2**). This is surprising, as comprehensive reviews of the MGB axis describe a modulatory effect of the gut microbiota on the development of the HPA/I axis, which controls circulating levels of GCs (Cowan et al., 2020; Cryan et al., 2019). It is noteworthy that the seminal work describing a link between the gut microbiota and HPA/I axis take place in GF mice, which are a highly artificial system (Cryan et al., 2019). Some potential explanations are that the required microbes for the coordination and development of this neuroendocrine axis could have been present ubiquitously, or on the egg masses that were not sterilized as part of our experiments. Additionally, much of the seminal work describing this relationship invoked significant stressors such as restraint stress (Cowan et al., 2020; Cryan et al., 2019), which may have been greater than any stress experienced by tadpoles as a result of the pond water treatment.

Future work can investigate differences in gene expression related to the stress axis. In GF mice, there is differential mRNA expression encoding for hippocampal NMDA and 5-HT_{1A} receptors (Neufeld et al., 2011), as well as reduced brain-derived neurotrophic factor expression levels in the cortex and hippocampus compared to conventionally raised mice (Sudo et al., 2004), which are integral to the function of HPA/I axis.

Effects of Temperature

In Aim 2, I collaborated with Drs. Samantha Fontaine and Kevin Kohl to demonstrate that aspects of the gut microbiota are predictors of tadpole brain size and shape (**Table 5.1 – 5.2**). Temperature-induced changes in brain size and architecture could be due to shifts in host metabolism and digestive efficiency (Feder and Burggren, 1992; Fontaine et al., 2022; Gillooly et al., 2001), and similar results have been seen in other ectotherms exposed to warmer water temperatures (Závorka et al., 2020). A recent literature review suggested that increasing temperatures can stimulate neuronal activity in ectotherms, but the applicability of this hypothesis to other ectotherms and thermal thresholds of this mechanism aren't clear (Beltrán et al., 2021). As cognitive testing nor behavior was conducted in Chapter 2, it is unclear if these changes are detrimental or beneficial to tadpoles.

While the effects of rising temperature on wildlife animal health and physiology are relatively well studied, increasing global temperatures can have an indirect impact on wildlife animal health through the manipulation of environmental microbial communities. A recent study found that aquatic bacterial communities respond quickly to increases in water temperature that fall within an optimal thermal threshold, while these communities were much slower to adapt to decreasing temperatures (Bååth and Kritzberg, 2024). Interestingly, this adaptation rate of aquatic microbial communities to increasing temperatures occurs exponentially more quickly than terrestrial and soil-dwelling microbial communities, which remained unchanged after two months of temperature variation exposure (Barcenas-Moreno et al., 2009; Donhauser et al., 2020). This suggests that larval amphibians that develop exclusively in freshwater environments are more susceptible to temperature-mediated shifts in aquatic microbial communities compared to terrestrial wildlife.

Perhaps the most prominent issue that tethers climate change and aquatic microbial communities is the topic of harmful algal blooms (HABs). Recent studies have suggested that increasing global temperatures and increased heat wave frequency have warmed marine water temperatures closer to ranges that support optimal growth of HABs (Gobler, 2020). These trends have expanded into local freshwater sources such as Lake Erie, which have experienced increased proliferation of aquatic cyanobacterial communities that drive HABs (Watson et al., 2016). These cyanobacterial algal blooms in freshwater systems are linked to oxygen loss and increased hypoxia, eutrophication and loss of nutrients, and increased abundance of cyanobacteria-produced neurotoxins, all of which greatly impact wildlife (Watson et al., 2016). Future studies should investigate these cyanobacteria driven HABs and evaluate direct and indirect effects on wildlife, such as aquatic microbial community restructuring due to cyanobacterial proliferation.

Exposure to Predation Stress

As discussed in Chapter 4, tadpoles were exposed to both predation-derived chemical cues and exogenous CORT. Surprisingly, effects of these stressor treatments were minimal when compared to previous experiments using these treatments (**Table 5.1 – 5.4**). In Chapter 4, I discussed at length potential explanations for this lack of treatment effect. Here, I would like to discuss big-picture takeaways and the ecological relevance of stressors on tadpole development and fitness, and some potential avenues for future research.

By using larval amphibians to study the MGB axis, I can evaluate host-microbe interactions in more ecologically relevant scenarios. Thus, there are amendments to my predation stress experimental design that I could incorporate in future studies to better take advantage of the amphibian model. Larval amphibians are often studied in mesocosms, which are outdoor,

semi-natural ponds that better reflect the pond ecosystem compared laboratory housing. In the future, I could incorporate pond water treatments into mesocosms to evaluate the replicability of these treatments in more complex and realistic scenarios. Further, I could add live predators (in cages) which would provide a more realistic form of predation stress, because it would allow for tadpole visual recognition of a feeding predator paired with chemical cues produced from a feeding event. This experimental design would (1) better test if predation stress influences the composition of the tadpole associated microbiota, and (2) evaluate if development in a depleted aquatic microbial environment interacts with predation stress in phenotypes of interest. Additionally, I would more frequently test for whole body CORT as opposed to my previous experimental designs. My previous experiments tested whole body CORT over the duration of six consecutive days at the same time each day, which could have impacted my findings based on the diel cycle of circulating GCs (Buckingham, 2006).

Next, to get a better idea of treatment effect on fitness, I would incorporate trials with live predators to evaluate tadpole escape performance and survival. Predator trials have been used in tadpole work to study phenotypic plasticity; tadpoles that exposed to constant predator exposure during development later exhibit increased tail size and display enhanced escape performance when encountering a live predator (Fraker et al., 2021). I would like to use live predators to test whether tadpoles that develop in autoclaved pond water and exhibit increased locomotory activity are actually more susceptible to capture compared to tadpoles raised in natural pond water. Additionally, as tadpoles exposed to exogenous CORT had larger tail muscles, I would like to evaluate if this phenotypic plasticity modulated by stress hormones can increase tadpole escape performance.

Future Directions

Questions remain as to whether changes seen in the gut microbiota, brain development, behavior, and other physiological endpoints as a result of development in autoclaved pond water are beneficial or detrimental to host health. The increase in tadpole body mass seen in all my studies is generally believed to increase fitness. However, the potential reduced ability to evade predators (Aim 3) suggests reduced fitness. Furthermore, the loss of gut microbial biodiversity is generally considered detrimental to host health. The loss of biodiversity and overabundance of closely related species is commonly associated with increased intrusion success of extrinsic bacterial species and can predict susceptibility to enteric pathogens (Stecher et al., 2010). My dissertation was not designed to evaluate immunity and disease resistance, but a previous study found that reduced tadpole gut diversity was associated with increased infection with parasitic worms (Knutie et al., 2017). Future studies are needed to better understand the fitness consequences of the changes I have demonstrated.

Another future direction is whether the phenotypic effects seen throughout my dissertation would persist throughout ontogeny and metamorphosis. A purpose of using larval amphibians for this work was due to life-history characteristics and an ability to manipulate the composition of the gut microbiota during critical developmental windows. Previous studies that manipulated the larval amphibian microbiota post-hatching discovered that these early life disruptions are associated with life-lasting deficits in host immunity, disease resistance, development, and physiological and metabolic function (Knutie et al., 2017; Warne et al., 2017; Warne et al., 2019), and this is consistently seen in mice as well (Cox et al., 2014). While no work has been done to see if microbially-induced changes in neurodevelopment persist throughout metamorphosis in amphibians, previous work in our lab has shown that larval amphibian exposure to pesticides lead to changes in brain architecture that carry over through

metamorphosis (McClelland, 2020). This is exciting, as it provides fruitful avenues for future research. Some potential experiments could explore the ability of the amphibian microbiota to be 'rescued' after development in autoclaved pond water or evaluating if treatment induced changes in neurodevelopment and behavior persist throughout metamorphosis.

An important future direction is to determine if the dramatic effects of raising tadpoles in autoclaved pond water in the laboratory would be reflected in tadpoles in the wild. Ponds differ in microbial communities due to natural and anthropogenic factors. It would be important to determine if more subtle differences in the aquatic microbial communities across ponds have fitness related consequences on the development of resident larval amphibians. Further, while autoclaving has been referenced as a very practical form of sterilization to eliminate all forms of microbial life (Yoo, 2018), it is possible that autoclaving can affect water chemistry in ways that were not controlled or tested for in this work. Future works could test for autoclave effects by filtering natural pond water to maintain water chemistry, or these microbes could be filtered out and added back into autoclaved pond water.

Conclusion

My findings of potential evidence of the amphibian MGB axis are important for many reasons. My work is the first to show that development in a depleted aquatic microbial environment influences the composition of the amphibian associated microbiota, and those changes are associated with drastic alterations in brain development and potentially fitness related behaviors. I believe that my results are ecologically relevant, as these aquatic microbial communities are significant drivers of the amphibian microbiota throughout development. This is important, as aquatic microbial communities are influenced heavily by environmental factors such as temperature (Bååth and Kritzberg, 2024) and are more susceptible to change than

terrestrial and soil microbial communities (Barcenas-Moreno et al., 2009). Additionally, the seeding of freshwater ecosystems with microbially modulating contaminants and other local factors create an ecological scenario where tadpoles are receiving the majority of colonizing microbes from variable sources (Stocker et al., 2024). Due to the importance of this colonization process and an evolutionarily developed reliance on harboring symbiotic microbes (Cox et al., 2014; McFall-Ngai et al., 2013; McFall-Ngai, 2015), it creates a scenario where aquatic wildlife may or may not harbor a full complement of microbes that are vital to their development and health based on which pond they hatch and develop within. As seen in this dissertation, that can create scenarios where tadpoles exhibit alterations in their size and developmental rate, neurodevelopment, and behavior, which can impact their ability to reach metamorphosis and survive. As such, I believe my work supports the need to incorporate host-microbe interactions into wildlife conservation efforts that include aquatic microbial communities into assessments of freshwater ecosystem health (Trevelline et al., 2019).

Table 5.1: Measures of brain and behavioral endpoints across studies. Arrows represent significant increases or decreases in the associated endpoints based on dependent variables compared to controls. Hyphens represent nonsignificant effects; NA denotes the endpoint was not evaluated. APW represents autoclaved pond water. Temperature represents increasing temperatures as a dependent variable. Pred. Cues represent predator-derived chemical cue exposure. CORT represents exogenous corticosterone exposure.

	Ch. 2		**Ch. 3**	**Ch. 4**		
	APW	Temperature	APW	APW	Pred. Cues	CORT
Relative Brain Mass	–	↑	↑	↓	–	
Relative Brain Shape						
TW	–	–	–	–	–	
TL	–	–	–	–	–	
OTW	↑	↑	–	↓	↓	
OTL	↑	↑	–	–	–	
DW	–	–	–	↓	↓	
DL	–	–	–	–	–	
MW	–	–	↓	↓	↓	
Behavior Assays						
Baseline	NA	NA	–	–	–	
Glass Vial - Empty	NA	NA	↓	–	–	
Glass Vial – Frog Brittle	NA	NA	↓	–	–	
Food Slurry	NA	NA	_	NA	NA	
Alarm Pheromones	NA	NA	NA	↑	–	

Table 5.2: Measures of physiological and gut microbial endpoints across studies. Arrows represent significant increases or decreases in the associated endpoints based on dependent variables compared to controls. Hyphens represent nonsignificant effects; NA denotes the endpoint was not evaluated. APW represents autoclaved pond water. Temperature represents increasing temperatures as a dependent variable. Pred. Cues represent predator-derived chemical cue exposure. CORT represents exogenous corticosterone exposure. For Chapter 2, all endpoints described below were measured in Fontaine et al. 2022.

	Ch. 2 – Fontaine et al. (2022)		**Ch. 3**	**Ch. 4**		
	APW	Temperature	APW	APW	Pred. Cues	CORT
Physiological Endpoints						
Body Mass	↑	↑	↑	↑	–	–
Gosner Stage	↑	↑	↑	↑	–	–
Plasma CORT	NA	NA	–	–	–	–
Body Morphology	NA	NA	NA	NA	–	Yes
Organ Development	NA	NA	NA	NA	–	–
Gut Microbiota						
No. Observed ASVs	↓	↓	↓	↓	–	–
Shannon Diversity Index	↓	–	↓	↓	–	–
Faith's Phylogenetic Diversity	↓	↓	↓	↓	–	–
Bray-Curtis	Yes	Yes	Yes	Yes	Yes	Yes
PERMDISP	Yes	Yes	No	No	No	No

Table 5.3: Associations between brain and behavioral endpoints and gut microbial alpha diversity metrics across studies. Pos. represents a positive association between endpoints and alpha diversity metrics. Neg. represents a negative association between endpoints and alpha diversity metrics. Hyphens represent nonsignificant effects. ASV represents the number of observed ASVs, Shannon represents Shannon Diversity Index, and PD represents Faith's Phylogenetic Diversity. GLMs were used with measurements of alpha diversity as predictor variables and endpoints as response variables.

	Chapter 2			**Chapter 3**			**Chapter 4**		
	ASV	Shannon	PD	ASV	Shannon	PD	ASV	Shannon	PD
Relative Brain Mass	-	-	-	-	**Pos.**	-	-	-	-
Relative Brain Shape									
TW	-	-	-	-	-	-	-	-	-
TL	-	-	-	-	-	-	-	-	-
OTW	**Neg.**	-	**Neg.**	-	-	-	-	-	-
OTL	**Neg.**	-	**Neg.**	-	-	-	-	-	-
DW	-	-	-	-	-	-	-	-	-
DL	-	-	-	-	-	-	-	-	-
MW	-	-	-	-	**Pos.**	-	-	-	-
Behavior Assays									
Baseline	NA	NA	NA	-	-	-	**Neg.**	-	**Neg.**
Glass Vial - Empty	NA	NA	NA	-	-	-	-	**Neg.**	-
Glass Vial – Frog Brittle	NA	NA	NA	**Pos.**	**Pos.**	**Pos.**	-	**Neg.**	-
Food Slurry	NA	NA	NA	**Pos.**	-	**Pos.**	NA	NA	NA
Alarm Pheromones	NA	NA	NA	NA	NA	NA	-	-	**Neg.**

Table 5.4: Relative abundances of gut bacterial phyla and genera that were significantly associated with brain and behavioral endpoints across studies. Pos. represents a positive association between endpoints and bacterial taxa. Neg. represents a negative association between endpoints and bacterial taxa. Hyphens represent nonsignificant effects. Statistical testing was conducted using MaAsLin2. P-values were corrected using the BH FDR method. I did not find any significant associations between body mass and relative abundances of bacterial taxa.

	Chapter 2			**Chapter 3**			**Chapter 4**		
	Brain Mass	Brain Shape	Behav-ior	Brain Mass	Brain Shape	Behavior	Brain Mass	Brain Shape	Behavior
Phyla									
Chlamydiae	-	**Neg.**	-	-	-	-	-	-	-
Acidobacteria	-	**Neg.**	-	-	-	-	-	-	-
Dependentiae	-	**Neg.**	-	-	-	-	-	-	**Neg.**
WPS.2	-	**Neg.**	-	-	-	-	-	-	-
Chloroflexi	-	**Neg.**	-	-	-	-	-	-	-
Planctomycetes	-	**Neg.**	-	-	-	-	-	-	-
Patescibacteria	-	**Neg.**	-	-	-	**Pos.**	-	-	-
Cyanobacteria	-	**Neg.**	-	-	-	-	**Pos.**	-	-
Firmicutes	-	**Neg.**	-	-	-	**Neg.**	-	-	-
Halanaerobiaeota	-	**Neg.**	-	-	-	-	-	-	-
Dadabacteria	-	**Neg.**	-	-	-	-	-	-	-
Gemmatimonadetes	-	**Neg.**	-	-	-	-	-	-	-
Spirochaetes	-	**Neg.**	-	-	-	-	-	-	-
Proteobacteria	-	-	-	-	-	**Pos.**	-	-	-
Bdellovibrionota	-	-	-	-	-	**Pos.**	-	-	-
Deinococcota	-	-	-	-	-	-	-	**Neg.**	-

Bacteroidota	-	-	-	-	-	-	-	-	**Neg.**
Actinobacteriota	-	-	-	-	-	-	-	-	**Pos.**
Genus									
Clostridium.sensu.stricto	-	**Pos.**	-	-	-	**Neg.**	-	-	-
Brevundimonas	-	-	-	-	-	**Pos.**	-	-	-
Thermoactinomyces	-	-	-	-	-	-	-	-	**Neg.**
Planococcus	-	-	-	-	-	-	-	-	**Neg.**
Roseiarcus	-	**Neg.**	-	-	-	-	-	-	**Neg.**
Paenibacillus	-	**Neg.**	-	-	-	-	-	-	**Neg.**
Staphylococcus	-	-	-	-	-	-	-	-	**Pos.**
Ancylobacter	-	**Neg.**	-	-	-	-	-	-	**Pos.**

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