

**ADVANCING EASTERN HELLBENDER CONSERVATION THROUGH
NOVEL HEAD-STARTING TECHNIQUES**

by

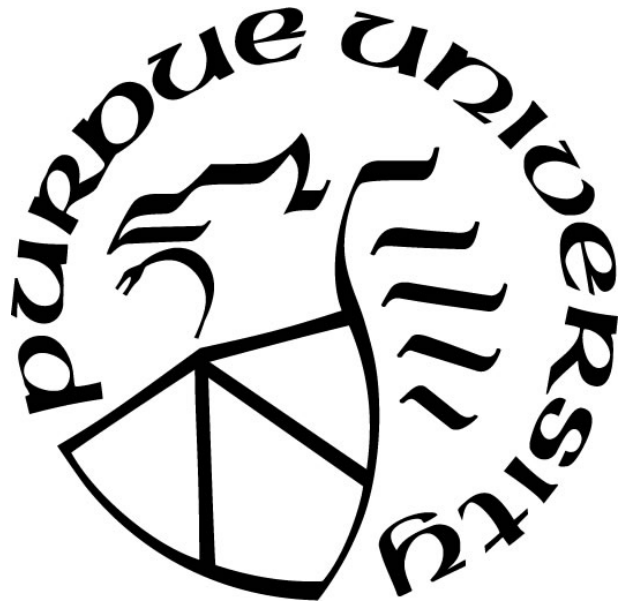
Erin K. Kenison

A Dissertation

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy



Department of Forestry and Natural Resources

West Lafayette, Indiana

May, 2018

ProQuest Number: 10809150

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 10809150

Published by ProQuest LLC (2018). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

**THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL**

Dr. Rod Williams, Chair

Department of Forestry and Natural Resources

Dr. Elizabeth Flaherty

Department of Forestry and Natural Resources

Dr. William Hopkins III

Virginia Polytechnic Institute, Department of Fish and Wildlife Conservation

Dr. Jason Hoverman

Department of Forestry and Natural Resources

Approved by:

Dr. Robert Wagner

Head of the Graduate Program

*I dedicate this in loving memory of my Nanny, who was always so proud of my accomplishments,
even if I was “working too hard”.*

ACKNOWLEDGMENTS

I want to first acknowledge my parents, Frank and Pamela Kenison. Thank you for your unwavering love, support, and encouragement throughout my graduate career. Having you as role models has taught me the merits of hard work, patience, and positive thinking. Thank you for always having confidence in me, for motivating me to continue through frustration, and for praising all my accomplishments. You have made me into the successful woman I am today.

A special thank you to my advisor Dr. Rod Williams. Rod, thank you for your understanding and encouragement, for pushing me to continually set higher goals for myself, and for shaping me into a confident and capable professional. Thank you to all my committee members. Drs. Elizabeth Flaherty, Jason Hoverman, Williams Hopkins, and Catherine Searle, it has been an honor to work with you. Your willingness to share your expertise, provide advice, and encourage my growth as a student has helped me become a better ecologist.

I want to acknowledge all the people that helped me collect data, construct experimental systems, and rear hellbenders: Nick Burgmeier, Becca Busse, Zach Compton, Lexi Eiler, Obed Hernández-Gómez, Todd Houser, Jake Linder, Emily McCallen, Kelsey Rearick, Maraiiah Russell, Sarah Sloan, Brian Tornabene, Paige Weldy, and Veronica Yager. Your efforts are truly invaluable. I also want to highlight Robert Rode for his aquaculture expertise. Bob, thank you for your continuous ability to solve problems and for providing guidance throughout my research.

I am forever grateful to members of the Williams lab. Emily McCallen, Obed Hernández-Gómez, Nick Burgmeier, and Becca Busse, you always kept my spirits up and encouraged me as a scientist - Emily and Obed, you two will continue to be the best lab mates of all time! And a sincere thank you to my graduate school comrades and fellow PAWS goers, you were always a source of support, hilarity, and friendship.

I owe the most thanks to Brian Tornabene. Thank you for being my biggest advocate and the most supportive partner throughout my Ph.D. Thank you for your continuous patience, for always believing in me, and for teaching me how to put the important things in life before my work. Brian, you are the reason why I will be able to finish this mighty accomplishment with a smile on my face and love in my heart. Thank you, thank you, thank you!

TABLE OF CONTENTS

LIST OF TABLES	vii
LIST OF FIGURES	viii
ABSTRACT	xi
CHAPTER 1. REARING CAPTIVE EASTERN HELLBENDERS (<i>CRYPTOBRANCHUS A. ALLEGANIENSIS</i>) WITH MOVING WATER IMPROVES SWIM PERFORMANCE	1
1.1 Abstract	1
1.2 Introduction	2
1.3 Methods	5
1.3.1 Study species and environmental enrichment	5
1.3.2 Morphologic comparisons	6
1.4 Results	8
1.4.1 Morphologic comparisons	8
1.4.2 Swim performance trials	9
1.5 Discussion	10
1.5.1 Conclusions	13
1.6 References	18
CHAPTER 2. TRAINING FOR TRANSLOCATION: PREDATOR CONDITIONING INDUCES BEHAVIORAL PLASTICITY AND PHYSIOLOGICAL CHANGES IN CAPTIVE EASTERN HELLBENDERS (<i>CRYPTOBRANCHUS ALLEGANIENSIS ALLEGANIENSIS</i>)	24
2.1 Abstract	24
2.2 Introduction	25
2.3 Materials and Methods	27
2.3.1 Study animals	27
2.3.2 Phase 1. Predator conditioning	27
2.3.3 Phase 2. Exposure trials	28
2.3.4 Statistical analyses	30
2.4 Results	32
2.4.1 Phase 1. Predator conditioning	32

2.4.2 Phase 2. Exposure trials.....	33
2.5 Discussion	33
2.6 References:	44
CHAPTER 3. A NOVEL BIOAUGMENTATION TECHNIQUE EFFECTIVELY INCREASES THE SKIN-ASSOCIATED MICROBIAL DIVERSITY OF CAPTIVE EASTERN HELLBENDERS.....	51
3.1 Abstract	51
3.2 Introduction	52
3.3 Methods	54
3.3.1 Experimental design.....	55
3.3.2 Skin microbiota	56
3.3.3 Hematology	58
3.3.4 Statistical Analyses.....	58
3.4 Results	60
3.4.1 Skin microbiota	60
3.4.2 Environmental microbiota.....	62
3.4.3 Hematology	62
3.5 Discussion	63
3.6 References	79

LIST OF TABLES

Table 1.1 Model estimates from six swim performance responses measured for control and treatment hellbenders. Test statistics are t values for swim time, initial distance, and time to move. Test statistics are z values for number of attempts, probability of needing motivation, and probability of completing trial. All trials were completed in November 2016 with 119 eastern hellbenders (*Cryptobranchus a. alleganiensis*); bolded values indicate significant differences. . 14

Table 2.1. MANOVA table reporting the results of a multivariate comparison evaluating differences in the number of eastern hellbenders (*Cryptobranchus a. alleganiensis*) observed active, stationary, or floating while outside of tile hides in unconditioned and conditioned treatment tanks. Repeated measures ANOVA table for univariate comparisons of each behavior, while accounting for repeated observations through time. These data provide evidence for significant differences, by treatment, date, and a treatment by date interaction, such that conditioned hellbenders reduced their time active and floating during the conditioning period compared to unconditioned individuals..... 39

Table 3.1 Adonis and Anosim test results for differences between beta diversity measures of treatment and control individuals. Beta diversity tests were conducted with unweighted UniFrac and Bray-Curtis distance matrices. The R values indicate the amount of variation explained by treatment..... 70

Table 3.2 The most common Blue River water OTUs, their phyla, and their relative abundance on the skin of treatment hellbenders (*Cryptobranchus a. alleganiensis*). Phyla are abbreviated as: Actinobacteria (Act), Armatimonadetes (Arm), Bacteroidetes (Bac), Cyanobacteria (Cya), Proteobacteria (Pro), Verrucomicrobia (Ver), and Unassigned OTU (un). Darker colors indicate higher relative abundance. Common Blue River water OTUs are found sparingly on hellbender skin and they do not tend to become relatively abundant until week six after hellbenders had been placed directly into the river for seven days. 71

LIST OF FIGURES

- Figure 1.1 Schematic of the artificial stream used for swim performance trials. Water entered into the stream channel through the pipe on the far left side, which was placed above the upstream tile hide. Water passed by a flow meter, dissipated downstream, emptied into two adjoining sumps, and then recirculated by the attached water pump. The hellbenders (*Cryptobranchus alleganiensis*) were placed at the bottom of the stream channel for each trial and observed for a maximum of ten minutes, using the black notches (1 notch = ~5 cm) to measure initial upstream movement during that time. 15
- Figure 1.2 Fine-scale tail morphology growth rates of hellbenders (*Cryptobranchus a. alleganiensis*) from control and environmental enrichment treatment raceways. Growth rate estimates for tail length (A.) and tail depth (B.) are units per year, presented with 95% confidence intervals. Individuals from the treatment raceways have 33% slower change in tail depth through time compared to those from the control raceways. An asterisk denotes significant differences at an alpha level of 0.05. 16
- Figure 1.3 Differences in swim time (A.), number of attempts (B.), and probability of needing motivation (C.) between eastern hellbenders (*Cryptobranchus a. alleganiensis*) reared without (control) and reared with (treatment) elevated water velocities, across three swim performance trials. By the third trial, treatment hellbenders were quicker to complete the trial, required fewer attempts to reach an upstream tile hide, and were less likely to need manual motivation to move. Model estimates with 95% confidence intervals are presented; an asterisk denotes significant differences at an alpha level of 0.05. 17
- Figure 2.1 Linear relationship between routine metabolic rate and proportion of time moving in respirometer chambers. The added regression line shows the strong relationship between general activity (i.e., turning, rocking, or moving) and oxygen consumption during exposure trials 40
- Figure 2.2 Behavioral observations of unconditioned and conditioned hellbenders (*Cryptobranchus a. alleganiensis*) in rearing tanks during the 30-day conditioning period. Hellbenders were counted whenever observed outside of tile hides and categorized as active, stationary, or floating. The mean number of hellbenders observed active or floating through time depended on conditioning treatment, such that fewer conditioned individuals were observed active or floating through the duration of the conditioning period compared to unconditioned individuals ($p < 0.05$). There were no significant treatment or time effects on stationary behavior 41
- Figure 2.3 The probability of moving within respirometer chambers during exposure to varying levels of risk/kairomone concentrations. Activity was measured as proportion of time turning, rocking the body, or moving the tail during the 30-minute exposure trial. Conditioned hellbenders (*Cryptobranchus a. alleganiensis*) increased their probability of moving when exposed to water without kairomones, but had a lower probability of moving when they were presented with low and high concentrations of predator kairomones compared to unconditioned individuals ($p < 0.05$). Estimates are back-transformed and presented with standard errors. There

were significant differences between conditioned and unconditioned individuals at each of the three exposure levels. Letters indicate significant differences across exposure levels ($p < 0.05$) 42

Figure 2.4 Oxygen consumption ($\text{mL O}_2\text{h}^{-1}$) of conditioned and unconditioned hellbenders (*Cryptobranchus a. alleganiensis*) exposed to varying levels of kairomone concentrations, after accounting for mass and proportion of time active during the exposure trials. Estimates are presented as marginal means with standard errors. Conditioned individuals had consistently lower oxygen consumption at each of the exposure levels compared to unconditioned individuals, but the rate of oxygen consumption did not differ across exposure levels. Asterisks denote significant differences between conditioning treatments ($p < 0.05$)43

Figure 3.1. Alpha diversity comparisons including species richness (A.) and Shannon diversity (B.) of hellbender (*Cryptobranchus a. alleganiensis*) skin microbiota from control ($n = 18$) and treatment individuals ($n = 18$) over the five-week experimental period in captivity. Treatment individuals were exposed to increasing amounts of river water from weeks two through five, and were exposed to undiluted river water at week five. There were no significant differences in alpha diversity at week one, between control and treatment individuals. However, there was a significant ($\alpha < 0.05$) interaction between week and treatment group, such that treatment hellbenders had quicker increases in species richness and Shannon diversity compared to control individuals. All skin swab samples were taken between 28 June and 29 July, 2016.72

Figure 3.2. Alpha diversity comparisons, species richness (A.) and Shannon diversity (B.), of hellbender (*Cryptobranchus a. alleganiensis*) skin microbiota from control ($n = 18$) and treatment individuals ($n = 18$) during weeks four and five in captivity, and week six following river release. Treatment individuals were exposed to 10% river water in week four, undiluted river water at week five, and all individuals were placed in the river for seven days before week six sampling. The dotted line indicates when all control and treatment hellbenders were released into the river. Treatment hellbenders had a large increase in richness and diversity between weeks four and five, but no change between week five and week six. Control individuals did not differ between weeks four and five, but had a rapid increase in species richness and Shannon diversity following river release. All skin swab samples were taken between 22 July and 5 August, 2016. Estimates are back-transformed and presented with standard error bars. Asterisks denote significant ($\alpha < 0.05$) differences between control and treatment individuals 73

Figure 3.3. PCoA plots depicting beta diversity comparisons between treatment and control hellbenders (*Cryptobranchus a. alleganiensis*) at weeks one, five, and six. Gray points depict control individuals (squares = unweighted UniFrac, circles = Bray-Curtis) and black points indicate treatment individuals (squares = unweighted UniFrac, circles = Bray-Curtis). Ellipses illustrate overlap in community composition between the two hellbender groups. There is strong overlap between control and treatment individuals at the beginning of the experiment and after all hellbenders are released into the river (weeks one and six). However, there is a strong differentiation between groups at week five, when treatment hellbenders are bathed in undiluted river water.74

Figure 3.4. PCoA plots depicting beta diversity comparisons of microbiota on the skin of treatment and control hellbenders (*Cryptobranchus a. alleganiensis*) over the six-week experimental period. The color gradient is indicative of experimental week, with week one being depicted as light gray and week six being depicted as black. Square points indicate control

individuals and circular points are used for treatment individuals. I have presented unweighed UniFrac and Bray-Curtis methods, both of which show a distinct separation in beta diversity at week six compared to weeks 1-5, regardless of group..... 75

Figure 3.5. Relative abundance of dominant phyla found on the skin of control (C) and treatment (T) hellbenders (*Cryptobranchus a. alleganiensis*) at each of the six experimental weeks. Skin microbiota was dominated by Proteobacteria, followed by Acidobacteria, Actinobacteria, Bacteroidetes, and Firmicutes, regardless of treatment. Species in the Firmicutes phylum were only detected on the skin after control and treatment hellbenders were released into the river.... 76

Figure 3.6. Linear trend lines from raw white blood cell counts for control (n = 18) and treatment hellbenders (*Cryptobranchus a. alleganiensis*) (n = 18) across the six experimental weeks. Counts are presented as proportion lymphocytes and proportion neutrophils. There was a significant ($\alpha < 0.05$) difference between groups at week one as well as an interaction between week and treatment group. Treatment hellbenders had a faster increase in the proportion of lymphocytes than control individuals, and oppositely, reduced the proportion of neutrophils across the five weeks in captivity compared to control hellbenders. Point estimates in week six are plotted with standard errors. Asterisks indicate significant differences between groups. The dotted line indicates when all control and treatment hellbenders were released into the river in cages, and then removed for sampling at week six. Blood was collected between June 28th and August 5th, 2016..... 77

Figure 3.7. Linear trends for changes in neutrophil to lymphocyte (N:L) ratios, estimated from white blood cell counts, for control (n = 18) and treatment hellbenders (*Cryptobranchus a. alleganiensis*) (n = 18) across the five-week experimental period in captivity. Treatment individuals had diminishing N:L ratios as they were exposed to increasing amounts of river water from weeks two through five, which was different than control individuals. However, they had an increase in N:L ratios in week six after they were released in the river. Point estimates are presented with standard errors and asterisks indicate significant ($\alpha < 0.05$) differences between groups. The dotted line indicates when all control and treatment hellbenders were released into the river in cages, and then removed for sampling at week six. Blood was collected between June 28th and August 5th, 2016. The Y-axis is back-transformed for ease of interpretation. 78

ABSTRACT

Author: Kenison, Erin, K. PhD

Institution: Purdue University

Degree Received: May, 2018

Title: Advancing Eastern Hellbender Conservation through Novel Head-Starting Techniques.

Major Professor: Dr. Rod Williams

Translocations often use captive-reared animals to help bolster or re-establish wild populations. Captive-rearing provides safety and ample amounts of food so animals can be released as larger, older individuals. However, captive environments are highly dissimilar from wild conditions and may deprive animals of experiences that promote normal development. Naïveté to natural stimuli can result in inappropriate or dangerous behaviors following release, elevated predation in the wild, increased disease susceptibility, and ultimately, failed translocation efforts. Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) have experienced precipitous declines over the past few decades in Indiana and have been reduced to a single, low-density population in the Blue River. Captive-rearing and translocations are underway; however, previous efforts have had variable success, leaving this population extremely vulnerable to extirpation.

I examined the efficacy of using semi-natural conditions in a captive setting to advance eastern hellbender head-starting techniques and improve standard rearing practices. I developed novel forms of environmental enrichment, predator conditioning, and bioaugmentation wherein I reared hellbenders with riverine-like water current (Chapter 1), predator kairomones (Chapter 2), and free-flowing river microbiota (Chapter 3). My goal was to combat captive-reared hellbenders naïveté to wild conditions and better prepare hellbenders to specific threats they will encounter in a natural river.

Hellbenders are commonly reared in aquaria that lack environmental stimuli and because of their inexperience with moving water may be more susceptible to downstream movement following release. I investigated the effects of rearing hellbenders with and without moving water on their body morphology and swim performance (Chapter 1). After 18 months, I found hellbenders reared in moving water had more shallow tails and better swim performance during simulated flood events. My data suggest that the addition of water current to hellbender rearing

environments produces a streamlined tail form, acclimates hellbenders to moving water, and improves their ability to reach upstream refugia.

Although some anti-predator behaviors are innate, many animals are brought into captivity at young ages before they have ever encountering a predator. Captive hellbenders identify predatory fish as threats, but may be more vulnerable to predation and stress without previous exposure to them. I investigated the use of predator conditioning to prepare hellbenders, behaviorally and physiologically, to the presence of a common riverine predator, largemouth bass (*Micropterus salmoides*, Chapter 2). I reared hellbenders for 30 days with and without continuous exposure to largemouth bass kairomones and heterospecific alarm cues. I found conditioned hellbenders became less active and had lower metabolic rates compared to unconditioned individuals. These data demonstrate that predator conditioning induces behavioral avoidance tactics that likely reduce predator detection and physiological changes that lower energetic demands.

Captive environments often lack natural microbial reservoirs, have filtration systems that remove excess microbes, and are maintained in hygienic ways to prevent the spread of disease. Subsequently, captive hellbenders may have depauperate microbial populations, lack symbiotic bacterial species, and subsequently may have increased disease susceptibility and reduced immune potential compared to wild conspecifics. In order to prepare hellbenders for natural free-flowing bacteria found in wild environments, I devised a novel bioaugmentation method. I exposed three-year-old eastern hellbenders to river water in captivity, before releasing them into their natal river in Indiana (Chapter 3). I found treatment hellbenders increased alpha diversity and had distinct differentiation in the community composition on their skin after they were exposed to undiluted river water in captivity. My data showed strong evidence that hellbenders' skin is being colonized by rare environmental OTUs and exposing hellbenders to river water is as an effective reservoir for bacterial colonization as release into the river. I also found suggestive evidence of an immune response among treatment hellbenders following river release, which may provide protection against the colonization of opportunistic species. If pre-exposed hellbenders have an advantage over control individuals because of microbial diversity and immune responses, then this novel technique could have beneficial effects on future translocation efforts.

This dissertation provides critical information to advance head-starting techniques for hellbender captive-rearing programs. The presence of water velocities, predator kairomones, and free-floating river microbiota are all representative of natural conditions and effectively induced morphologic plasticity, altered behavior, and physiological changes that could positively influence hellbender survival upon release into the wild. Future work is needed to explore the effects of these novel methods, separately and in combination, on hellbender movement, habitat use, health, and survival following translocation into the wild. If rearing hellbenders with semi-natural conditions and environmental stimuli in captivity can improve the success of future translocations, incorporating these novel methods into head-start programs could have profoundly positive effects on hellbender conservation initiatives in Indiana and throughout the nation.

CHAPTER 1. REARING CAPTIVE EASTERN HELLBENDERS (*CRYPTOBRANCHUS A. ALLEGANIENSIS*) WITH MOVING WATER IMPROVES SWIM PERFORMANCE

The work in this chapter has been previously published. Kenison, E.K. and R.N. Williams. 2018. Rearing captive eastern hellbenders (*Cryptobranchus a. alleganiensis*) with moving water improves swim performance. *Applied Animal Behaviour Science* 202:112-118. <https://doi.org/10.1016/j.applanim.2018.01.013>

1.1 Abstract

Translocations often use captive-reared animals to help bolster or re-establish wild populations. However, captive environments are highly dissimilar from wild conditions and may deprive animals of experiences that promote normal development. Captive-rearing and translocation efforts are underway for eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*). Yet, hellbenders reared in aquaria that lack stimuli often make long-distance downstream movements following release, perhaps because of their naïveté to riverine environments. I altered standard captive techniques and reared juvenile hellbenders with (treatment) and without (control) water current for 18 months. I quantified morphological plasticity and swim performance as a function of rearing environment to assess the value of environmental enrichment in hellbender head-start programs. I compared broad-scale growth rates for mass, snout-vent length, and total length, but found no difference between treatment and control hellbenders (mass difference = 0.1 g/month, $P = 0.596$; snout-vent length difference = 0.01 cm/month, $P = 0.360$; total length difference = 0.01 cm/month, $P = 0.533$). I also examined fine-scale tail morphology measurements and found treatment individuals developed more shallow tails that grew 49% slower than control individuals during the rearing period (mean difference = 0.86 mm/month, $P = 0.017$). I interpret this as evidence of either energy expenditure or phenotypic plasticity as more streamlined tail forms are found in lotic systems. Moreover, I found water current to be positively associated with hellbenders' swimming ability. After three swim trials, treatment hellbenders were 46% quicker in their swim time ($P = 0.033$), required 29% fewer upstream attempts ($P = 0.012$), and were 60% less likely to need manual motivation to make it to an upstream tile hide ($P = 0.010$). Moreover, treatment hellbenders tended to improve

these responses linearly through time ($P = 0.016$) compared to control individuals that showed no improvement across the three trials ($P = 0.075$). Together, my data suggest that the addition of water current to hellbender rearing environments does not have any detrimental impact on hellbender body morphology, but rather, acclimates hellbenders to moving water and improved their ability to reach upstream refugia. I advocate incorporating water velocities, representative of natural conditions, into hellbender captive-rearing programs. Rearing animals with semi-natural conditions in captivity may better prepare animals for and potentially improve the success of future translocations. This advancement to standard rearing techniques may positively influence the preservation of wild hellbender populations throughout the nation.

1.2 Introduction

Translocations and reintroductions are popular conservation strategies designed to bolster or re-establish wild populations. Although popular, fewer than half of translocations are classified as successful (Griffith et al., 1989; Fischer and Lindenmayer, 2000; Germano and Bishop, 2009). Many translocations release young age classes as they are easier to transport, can be collected in greater numbers, and will be less likely to demonstrate homing tendencies following release (Germano and Bishop, 2009). However, animals in their early life stages are at the greatest risk of mortality (Haskell et al., 1996). Therefore, focus has been directed toward head-start programs where animals are reared in captivity during their most vulnerable stage to reduce rates of mortality.

Head-starting provides safety and ample amounts of food to ensure rapid growth, but can still be inadequate in preparing individuals for natural living conditions (Alberts, 2007). Artificial rearing environments are highly dissimilar from wild conditions and can deprive animals of experiences and stimuli that promote the development of necessary behavioral skills and morphological responses to environmental stressors (McPhee and Carlstead, 1990; Hard et al., 2000; Kelley et al., 2005). Captive-reared animals may lack developed muscle tone, endurance, and sprint speeds making them morphologically distinct from wild populations (Young and Cech, 1993; Ward and Hilwig, 2004; Connolly and Cree, 2008). Moreover, individuals may adopt behaviors that are advantageous in captivity, but maladaptive in the wild, further hindering translocation success (e.g., Stoinski et al., 2003).

To combat morphological and behavioral deficiencies, some head-starting programs have incorporated environmental enrichment techniques (see Bashaw et al., 2016; Biggins et al., 1998; Salvanes et al., 2013). Environmental enrichment aims to mimic nature by introducing captive animals to live prey, incorporating more natural refugia, structural complexity, and microhabitat characteristics, and training them to adopt appropriate survival skills prior to release (Biggins et al., 1998; Ward and Hilwig, 2004; Alberts, 2007). For example, black-footed ferrets (*Mustela nigripes*) raised with live prey are more successful at locating and killing prey compared to untrained conspecifics (Vargas and Anderson, 1999). Captive Siberian polecats (*Mustela eversmani*) have heightened antipredator responses when trained with predator models and aversive stimuli (Miller et al., 1990). Also, hatchery-reared fishes reared with moving water have increased growth, weight, and muscle mass, and after only 50 days of exercise conditioning show similar trait values and performance to wild individuals (Young and Cech, 1993, 1994; Ward and Hilwig, 2004). Therefore, animals exposed to simulated natural conditions in captivity may be better equipped (i.e., behaviorally, morphologically, and physiologically) for introductions into the wild (Berejikian et al., 2000).

Head-start programs for eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) have been established to combat precipitous population declines. Captive-rearing efforts for hellbenders are effective at facilitating growth, but hellbenders are reared from egg to juvenile in aquarium tanks that lack stimuli. Subsequently, static aquarium environments may leave juvenile hellbenders naïve to natural conditions (e.g., riverine water velocities, predator cues, or complex habitat). Stamps and Swaisgood (2007) argue that captive conditions could be important determinants of post-release movement as translocated animals are more likely to leave release sites if they don't encounter cues, physical features, or microhabitat similar to their captive environment. Some hellbender rearing facilities incorporate substrate and structural complexity more similar to natural environments, but environmental enrichment has never been used in preparation for hellbender translocations.

It is unknown whether translocation failure is correlated with the head-starting environment, but previous hellbender augmentations have had variable success (17–72% survival over six months; Bodinof et al., 2012; Boerner, 2014; Kraus et al., 2017). Hellbenders are fairly sedentary throughout the year, usually moving infrequently and only relatively short distances between shelter rocks (27.5 m), if at all (Burgmeier et al., 2011). Yet, 70–100% of

captive-reared individuals released to the wild show downstream dispersal, 40–60% making abrupt long-distance movements permanently away from core habitat (50–1800 m), and many becoming completely lost following flood events (Bodinof, 2010; Kraus et al., 2017). Long-distance, post-release movements are particularly adverse to translocations, and are positively correlated with mortality as a large proportion of deaths can be attributed to dispersal away from high quality habitat (Moehrenschlager and Macdonald, 2003; Stamps and Swaisgood, 2007; Bodinof, 2010). Moreover, unintentional or intentional long-distance movements can negatively influence survival rates as individuals expend excessive amounts of energy and become more susceptible to predation while outside of refugia (Ward and Hilwig, 2004; Bodinof, 2010).

Amphibians exhibit considerable phenotypic plasticity, expressing changes in behavior, morphology, life history, or physiology in response to predators, competitors, or their rearing environment (Wilbur, 1987; Relyea and Werner, 2000; Relyea and Hoverman, 2003). Exposing juvenile hellbenders to natural water current in a captive setting may induce behavioral plasticity such as swimming skills, navigation techniques, and endurance. Moreover, hellbenders naturally have an oar-like tail, which they rely on to orient their body and propel them during bouts of sprint swimming. Although their primary mode of locomotion is through lateral undulation, exposure to elevated water velocity may lead to more swimming, which may induce plastic changes such as a wider and more utilitarian tail against water current. A variety of enrichment techniques (e.g., structural complexity, variability in prey, and natural vegetation) have been found to be effective in reversing maladaptive phenotypes and may be beneficial for hellbenders' development (Biggins et al., 1999; Ahlbeck and Holliland, 2012; Hyvärinen and Rodewald, 2013). Therefore, a combination of captive-rearing efforts that introduce semi-natural conditions, as well as provide safety and food to juveniles may be the most viable solution to acclimate and prepare hellbenders for release into the wild.

I investigated whether juvenile hellbenders exhibit morphological plasticity in their body and tail and examined the swim performance of hellbenders in simulated, flood-like conditions as a function of rearing environments with and without elevated water velocity. I predicted that hellbenders reared with moving water would grow faster, would have longer and deeper tails, and would have better upstream swimming ability against flood-like water velocity. This project has the potential to advance current rearing techniques for eastern hellbenders, provide valuable information for captive facilities, and positively influence future translocation efforts.

1.3 Methods

1.3.1 Study species and environmental enrichment

Hellbenders are North America's largest salamander, growing up to 74 cm in length (Petranka, 1998). They reside in cool, fast-flowing rivers and are distributed throughout the Midwest and southeastern areas of the United States (Mayasich and Phillips, 2003; Petranka, 1998). Although some healthy populations occur in parts of North Carolina, Virginia, West Virginia, and Tennessee (usually associated with the Appalachian mountain range and preserved forests), hellbender populations have suffered drastic population declines over the past few decades (Mayasich and Phillips, 2003). Threats such as sedimentation, water pollution, and human misconceptions have reduced available habitat, lead to disease or illness, and extirpated local populations (Mayasich and Phillips, 2003; Wheeler et al., 2003). In Indiana, hellbenders are only found in a single river system and a recent population viability analysis reported complete extirpation by 2035 if no management action was taken (Burgmeier et al., 2011; Unger et al., 2013). Reproduction was last documented in Indiana in 2015, but there has been no evidence of hellbender recruitment for the past 20 years (Kern, 1984). This suggests geriatric hellbenders (living up to 30-years of age) are the remaining stronghold to the wild population. The population viability analysis found that positive changes in early life-stage survival and the number of reproductively viable females (above the age of six) could increase population growth (Unger et al., 2013). Therefore, efforts to improve juvenile survival are critical to the persistence of Indiana's remaining wild population.

I created captive environments with and without water current at Purdue University's Aquaculture Research Laboratory in West Lafayette, Indiana, USA. I constructed a system with six polyethylene raceways (Pentair Filtration sump – S207095, Pentair Aquatic Eco-systems, Apopka, FL, USA: 1.4 x 0.6 x 0.6 m, L x W x H): three treatment raceways with elevated water velocity (0.2–0.3 m/sec) and three controls with slow flow (0–0.05 m/sec). I designed my raceway system as a part flow-through, part recirculating system to remove solid waste, sterilize water, and reduce iron levels. I maintained velocities between 0.2–0.3 m/sec in the treatment raceways because hellbenders are naturally found in riffles and runs varying in flow rate from 0.16–0.34 m/sec (Bodinof et al., 2012). Control raceways were maintained between 0– and 0.05 m/sec to mimic standard rearing conditions that lack moving water. I collected a single clutch of hellbender eggs from southern Indiana, USA in 2013. I reared these individuals in 132 liter

aquarium tanks with simple PVC and tile hides and no added substrate for two years. I randomly selected 120, two-year olds for this experiment, all of which weighed on average 30.6 g (SE \pm 0.46 g). I recorded spot patterns from the dorsum and tail of each of these hellbenders for unique identification and randomly assigned 20 to each treatment or control raceway ($n = 120$).

I fed hellbenders twice weekly with equal amounts of black worms (*Lumbriculus variegatus*) or crayfish (*Orconectes rusticus*). I standardized the amount of food between treatments at each feeding event. Diets varied monthly depending on availability of worms and crayfish, but all raceways received the same type of food and at comparable amounts of biomass: 35–40 g of worms and 20–25 crayfish per raceway. I always made sure to place worms behind the inflowing water pipe to prevent flushing them out of the raceways with water current. I also provided multiple shelter rocks (30 x 50 cm flagstone rocks) and assorted gravel substrate in raceways.

1.3.2 Morphologic comparisons

I reared all hellbenders in raceways for 18 months, from May 2015 to December 2016, and collected morphological measurements and lateral photographs at the beginning and end of the enrichment period. Hellbenders are slow-growing species, yet I predicted 18 months would be enough time to observe changes in size. Moreover, it would allow me to rear animals until they were nearly four years old, which is a standard age to release hellbenders back into the wild (Kraus et al., 2017). I measured broad-scale snout-vent length (SVL; cm), total length (TL; cm), and mass (g), being sure to identify each individual during morphologic measurements. I also placed hellbenders into a V-shaped, glass holding device to take a standardized lateral photograph to measure fine-scale tail length and depth (Olympus camera - SP-57OUZ, Olympus, Center Valley, PA, USA).

I calculated growth rates (end-beginning/18 months) for all broad-scale body morphology measurements (SVL, TL, and mass) and fine-scale tail morphology measurements (tail length and depth). I used ImageJ software to measure tail length, from vent to tail tip, and tail depth, at the deepest point between the dorsal and ventral sides of the tail, with lateral photographs of all the hellbenders (Schneider et al., 2012). I compared broad-scale growth rates between treatment and control individuals using univariate linear regressions and included ‘raceway’ in my model to account for multiple hellbenders within an individual raceway. I conducted univariate

ANOVAs for fine-scale growth and included raceway and mass. I included the growth rate for mass as a covariate in fine-scale comparisons to control for size variation across individuals (Hoverman and Relyea, 2012). I report mean differences and estimated growth rates. I ran all analyses in Program R version 3.3.2 and assigned an alpha level of 0.05 (R Core Team, 2016).

1.3.3 Swim performance trials

To evaluate hellbenders' ability to navigate and endure high flow events, we tested the swim performance of all hellbenders ($n = 119$ because of a previous mortality) in an artificial stream following 18 months of environmental enrichment. We used a 2.4 x 0.3 x 0.3 m (L x W x H) polycarbonate trough with water depth of 25 cm, a flow meter and tile hide placed at the upstream end, and a screen to catch hellbenders at the downstream end (Fig. 1; similar to Bestgen et al., 2010). We did not include gravel substrate in the artificial stream because we wanted to mimic bedrock sections of a natural river and motivate individuals to swim upstream rather than remain stationary. We constructed our stream system so that a centrifugal pump (Sequence[®] 1000 Series – Model 6100SEQ23, MDM Incorporated, Colorado Springs, CO, USA) pulled water from two large polyethylene sumps, pushed water past the flow meter and down the length of the stream channel, and then recirculated water through the stream back into the holding sumps. This allowed for maximized flow to be continually pumped into the channel.

Prior to our experimental trials, we introduced each of the 119 hellbenders to the stream channel individually and allowed them eight minutes to freely explore and acclimate to the system at low water velocities (~ 0.5 m/sec, Ward et al., 2003). These acclimations occurred once, 7-9 days prior to trials. An acclimation period is common in performance trials as this allows individuals to become oriented to the testing environment and, in this case, the presence of the upstream tile hide (Bestgen et al., 2010). Following the acclimation period, we evaluated hellbenders' swim performance across three trials, with two rest days between each event (Adolph and Pickering, 2008). We designed our study with three trial events in order to observe possible changes and improvement through time (Adolph and Hardin, 2007; Irschick and Meyers, 2007; Kupferberg et al., 2011).

We concentrated on the ability of hellbenders to endure flood-like velocity conditions and move upstream against current; therefore, we opened the gate valve to achieve and maintain a maximum velocity around 1.46 m/sec (nearly five times faster than the velocity in treatment

raceways). At the beginning of each trial period, we identified individuals by spot patterns and then placed a single hellbender at the bottom of the artificial stream. We recorded time to start moving, initial distance moved upstream before the hellbender fell back or turned around, the number of upstream attempts, and swim time (i.e., time when hellbenders started moving until the end of the trial). Time ended if hellbenders successfully completed the trial by making it under the upstream tile hide or if ten minutes passed. If a hellbender did not move or complete the trial after five minutes we motivated them with soft taps on their tail and repeatedly turned them to face upstream after failed attempts (Shaffer et al., 1991). Once the trial was completed or ten minutes had passed, we returned them to their designated raceways.

We compared time to start moving, initial distance moved upstream, swim time, number of upstream attempts, likelihood of requiring motivation, and probability of successfully completing the trial and making it to the tile hide, as a function of rearing environment. Due to non-normal data, we conducted generalized linear mixed regressions with penalized quasi-likelihood, used a Gaussian distribution, and log link (R package ‘MASS’) for continuous (i.e., measures of time and distance) response variables. We used generalized linear regressions with appropriate link functions for Poisson (i.e., number of attempts) and binomial (i.e., used motivation and successfully made it to tile hide) responses, as these did not meet the assumptions of penalized quasi-likelihood. We tested full models that included treatment, trial, and an interaction between treatment and trial as our fixed effects. We included individual nested within raceway as our random effects to account for repeated measures. We compared the treatment effect at each trial and changes across trials through time. We back-transformed estimates to report means and mean differences. We ran all analyses in Program R version 3.3.2 and assigned an alpha level of 0.05 (R Core Team, 2016).

1.4 Results

1.4.1 Morphologic comparisons

All hellbenders increased in size throughout the experiment, without differences in broad-scale growth rates for mass, snout-vent length, or total length between treatments (mass difference = 0.1 g/month, t value = -0.58, $df = 1$, $p = 0.596$; snout-vent length difference = 0.01 cm/month, t value = 1.03, $df = 1$, $p = 0.360$; total length difference = 0.01 cm/month, t value =

0.68, $df = 1$, $p = 0.533$). There were no differences among these measurements, likely because they were highly correlated with one another (correlation values >0.75 , p value <0.001). I found no differences in fine-scale tail length or depth at the beginning of the experiment (t value = 0.453, $df = 1$, $p = 0.651$ and t value = -1.495, $df = 1$, $p = 0.136$, respectively). Moreover, changes in tail length were similar among treatment and control hellbenders (mean difference = 1.6 mm/month, t value = -2.29, $df = 1$, $p = 0.084$; Figure 1.2a). However, fine-scale growth rates in tail depth were significantly different in treatment compared to control hellbenders (mean difference = 0.86 mm/month, t value = -3.93, $df = 1$, $p = 0.017$). More specifically, treatment hellbenders had 49% slower change in their tail depth through time (95% CIs = 24–60%), with significantly more shallow tails at the end of the enrichment period compared to control individuals (t value = -3.19, $df = 1$, $p = 0.033$; Figure 1.2b).

1.4.2 Swim performance trials

Treatment and control hellbenders were similar in their initial distance, time to move, and probability of completing each trial (Table 1.1). However, by the third trial conditioned hellbenders were 46% faster in their swim time (mean difference = 69 s, $p = 0.033$), required 29% fewer upstream attempts to reach the upstream hide (mean difference = 1.1 attempts, $p = 0.012$), and were 60% less likely to need motivation to move (mean difference = 0.22, Table 1.1, Figure 1.3). Moreover, there were linear changes through time, such that treatment hellbenders became 20% faster at each trial (95% CI = 7–30%, mean estimate = 37.5 s per trial, t value = -2.94, $df = 1$, $p = 0.004$). Treatment hellbenders also reduced their number of attempts by 18% (95% CI = 9–25%, mean estimate = 0.57 attempts per trial, t value = -3.82, $df = 1$, $p < 0.001$) and their probability of needing motivation by 41% through time (95% CI = 35–48%, mean estimate = 0.094 per trial, t value = -2.39, $df = 1$, $p = 0.017$). Alternatively, control hellbenders showed no improvement in swim time (mean estimate = 24.5 s per trial, t value = -1.79, $df = 1$, $p = 0.075$), reduction in the probability of needing motivation (mean estimate = 0.05 per trial, t value = -1.14, $df = 1$, $p = 0.255$), or any significant change in number of upstream attempts across trials (mean estimate = -0.13 attempts per trial, t value = 0.81, $df = 1$, $p = 0.417$).

1.5 Discussion

I found no detrimental effects of environmental enrichment on hellbender growth. Rearing fish with moving water can increase growth among some salmonids; but unlike fish, hellbenders do not continually swim and generally only come out of refugia to forage (see Davison, 1997). Instead, hellbenders' growth rates increased at comparable rates between treatments, which is similar to exercise conditioned goldfish (*Carassius auratus*), chub (*Leuciscus cephalus*), nace (*Chondrostoma nasus*), and branded wrasse (*Notolabrus fucicola*) that show no change in growth compared to conspecifics reared without moving water (Davison and Goldspink, 1978; Lackner et al., 1988; Hinterleitner et al., 1992; Sanger, 1992; Davison, 1994). Although body morphology was similar between treatments, I found hellbenders reared with elevated water velocity exhibited more shallow tails compared to control hellbenders. Most vertebrates expend more energy while swimming and perhaps more shallow tails was a result of energy use rather than energy storage. Fish reared at higher velocities have reduced whole body fat content and salamanders, including hellbenders, commonly store energy and deposit fat in their tails (Ogata and Oku, 2000; Wells, 2007; Personal Communication). Elevated energy expenditure might have reduced the available stores and subsequently affected average tail depth.

Alternatively, shallow tails are evidence of phenotypic plasticity as animals reared in heterogeneous environments have a greater chance for plasticity to occur. The presence of water current may have induced a more streamlined tail, which is common among salamanders naturally residing in lotic systems (Wells, 2007; Haad et al., 2011). Larvae that live in ponds typically have large tail fins that extend up the back, whereas larvae that live in brooks and rivers tend to have small, shallow tail fins that end at the body (Wells, 2007). A shallow tail is thought to reduce drag, similar to streamlined body forms. For example, shallow-bodied crucian carp (*Carassius carassius*) reared without predatory pike (*Esox lucius*) experience 32% less drag while swimming compared to deep-bodied conspecifics reared with pike (Bronmark and Miner, 1992). Regardless, of the mechanism inducing more shallow tails, treatment individuals had improved swimming ability, which suggests deeper tails are not necessarily indicative of increased maneuverability. Often, body condition or body mass are used to assess locomotor traits, but size may be an unreliable metric of locomotion potential (Perez-Tris et al., 2004; Vervust et al., 2008). Instead, swim performance is more likely a function of experience. Exercised and unexercised spikedace (*Meda fulgida*) have comparable length measurements, but

have up to 40% improvement in swim performance following swim training (Ward and Hilwig, 2004). Rearing efforts are usually tailored to magnify size and expedite growth, but rather, should be focused on experience and performance skills.

As seen in my results, hellbenders rearing with water current demonstrated improved swim performance. By the third swim trial, treatment hellbenders had a faster swim time, were less likely to need motivation, and they required fewer attempts to reach the upstream tile hide compared to control individuals. Previous exposure to water current may have improved endurance and allowed individuals to become better acclimated to riverine conditions. Similarly, fish reared in tanks with elevated water velocity have faster swim speeds, swim further, and are less susceptible to fatigue than fish reared with lower current (Pearson et al., 1990; Young and Cech, 1993; Ward and Hilwig, 2004). Fishes that have been exercise conditioned with moving water are thought to have greater stamina than unconditioned individuals (Hammond and Hickman, 1966; Lackner et al., 1988; Ward and Hilwig, 2004). Treatment hellbenders in my experiment may have perceived water current as a novel experience, therefore needing motivation to move, been less physically prepared, becoming fatigued after two trials, and subsequently, were less likely to reach the upstream tile hide by the third trial.

Not only were treatment hellbenders quicker to complete the swim challenge by the third swim trial, but they showed significant improvement in their swimming skills, navigation, and motivation through time. Conditioning, training, or imprinting techniques to improve performance have been successful among many animals, including amphibians (Burghardt, 2013). For example, salamanders (*Salamandra salamandra*) can recognize and preferentially choose prey objects after foraging experience, and tadpoles (*Rana lessonae* and *Rana esculenta*) show advantageous predator avoidance behaviors after previous exposure to predator stimuli (Luthardt-Laimer, 1983; Semlitsch and Reyer, 1992). There is also support across taxa that variable environments aid in developing flexible and plastic behaviors (Braithwaite and Salvanes, 2005). Hellbenders have an innate ability to swim, as all individuals were able to make it to the upstream tile hide at least once; however, I observed behavioral plasticity among some of the treatment individuals that were more successful and quicker to make it upstream. These individuals swam along the bottom of the artificial stream rather than at the water surface and required fewer attempts to reach the tile hide. This behavior might be advantageous following

release into the wild to reduce the likelihood of hellbenders being swept downstream and the potential for predator attacks at the water surface.

My study provides evidence that environmental enrichment can better acclimate individuals to riverine-like environments and corroborates evidence that pre-release training can improve skills such as swimming, navigating current, and finding refuge. Previous hellbender translocations have documented abrupt long-distance-downstream dispersal and lowered survival when individuals disperse more than 50 m from their core release habitat (Bodinof et al., 2012). Animals that move more become prone to exhaustion, being swept downstream during flood events, or occupying low-quality habitat – ultimately leading to translocation failure. Stocked razorback suckers (*Xyrauchen texanus*) have high downstream movement when they are initially released into river systems, likely because rearing stream-adapted fishes in standing water adversely impacts their ability to survive in lotic environments (Brooks, 1986; Ward and Hilwig, 2004). Hellbender salamanders are found in fast-flowing, well-oxygenated rivers; therefore, it makes intuitive sense that these animals would be at a disadvantage when reared in aquarium tanks. My findings suggest that advancing captive-rearing techniques to include environmental enrichment may have positive effects on translocation success.

I found that rearing two-year old hellbenders with moving water for 18 months was successful in producing advantageous changes in swim performance. Hellbenders are usually released into the wild between the ages of three and six; therefore, future work should aim to better understand the importance of starting age, duration, and intensity of water velocity when using environmental enrichment techniques prior to release efforts (Bodinof et al., 2012; Kraus et al., 2017; Personal Communication). Furthermore, additional work should investigate how rearing hellbenders with elevated water velocities influences survival, habitat use, and movement following their translocation into the wild. If hellbenders maintain upstream swimming skills once released, are able to withstand elevated and variable flow regimes, and are capable of returning to refuge objects, they may have a higher probability of survival even if they are unintentionally moved downstream. Incorporating other semi-natural conditions beyond water velocity (e.g., predator cues and riverine microbiota) into captivity to mimic animals' release environments may have profound effects on translocation success and the future of imperiled populations in the wild.

1.5.1 Conclusions

Environmental enrichment has been used effectively with a variety of vertebrate animals, but has never been used as a method to improve the success of hellbender translocation projects (Ward and Hilwig, 2004). This study provides substantial evidence that incorporating riverine-like water velocities into the captive-rearing environment improves hellbender swim performance. Moreover, this acclimation to moving water came without morphological trade-offs in weight or length growth rates. This information has strong implications for current hellbender rearing facilities, but also for endangered, imperiled, or at risk species that have struggled to transition from captive to wild environments. If animals reared in more natural environments prior to release prove to do better in the wild than captive individuals that are untrained and inexperienced, then altering standard rearing techniques to include semi-natural conditions can positively influence translocation efforts and the preservation of wild populations in the future.

Table 1.1 Model estimates from six swim performance responses measured for control and treatment hellbenders. Test statistics are t values for swim time, initial distance, and time to move. Test statistics are z values for number of attempts, probability of needing motivation, and probability of completing trial. All trials were completed in November 2016 with 119 eastern hellbenders (*Cryptobranchus a. alleganiensis*); bolded values indicate significant differences.

Response		Control	Treatment	Test statistic	P
Swim time (sec)	Trial 1	198.53	155.58	-1.47	0.145
	2	145.28	121.75	-0.81	0.419
	3	149.6	80.49	-2.16	0.033
Number of attempts	Trial 1	3.47	3.77	0.08	0.535
	2	3.52	2.89	-0.19	0.161
	3	3.73	2.63	-2.51	0.012
Probability of needing motivation	Trial 1	0.465	0.333	-0.55	0.158
	2	0.327	0.264	-0.74	0.462
	3	0.362	0.146	-2.59	0.010
Initial distance (cm)	Trial 1	145.52	146.25	0.11	0.913
	2	160.77	157.58	-0.48	0.635
	3	161.93	154.68	-1.08	0.282
Time to move (sec)	Trial 1	60.35	70.07	0.64	0.521
	2	53.02	39.86	-0.86	0.394
	3	54.33	47.97	-0.42	0.673
Probability of completing the trial	Trial 1	0.95	0.92	-0.63	0.528
	2	0.95	1.00	0.01	0.999
	3	0.88	0.98	1.51	0.130

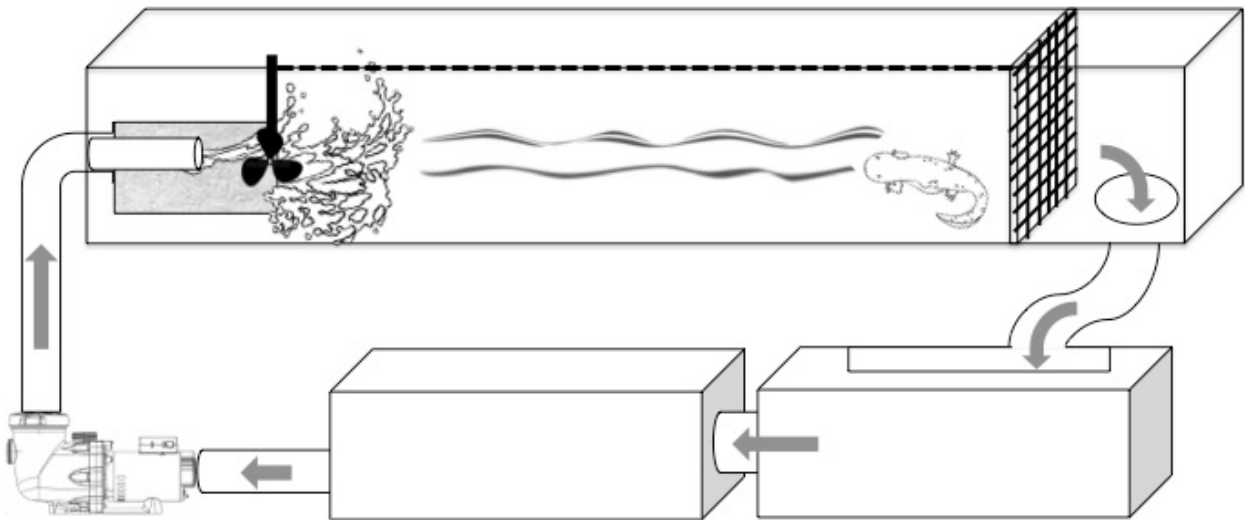


Figure 1.1. Schematic of the artificial stream used for swim performance trials. Water entered into the stream channel through the pipe on the far left side, which was placed above the upstream tile hide. Water passed by a flow meter, dissipated downstream, emptied into two adjoining sumps, and then recirculated by the attached water pump. The hellbenders (*Cryptobranchus a. alleganiensis*) were placed at the bottom of the stream channel for each trial and observed for a maximum of ten minutes, using the black notches (1 notch = ~5 cm) to measure initial upstream movement during that time.

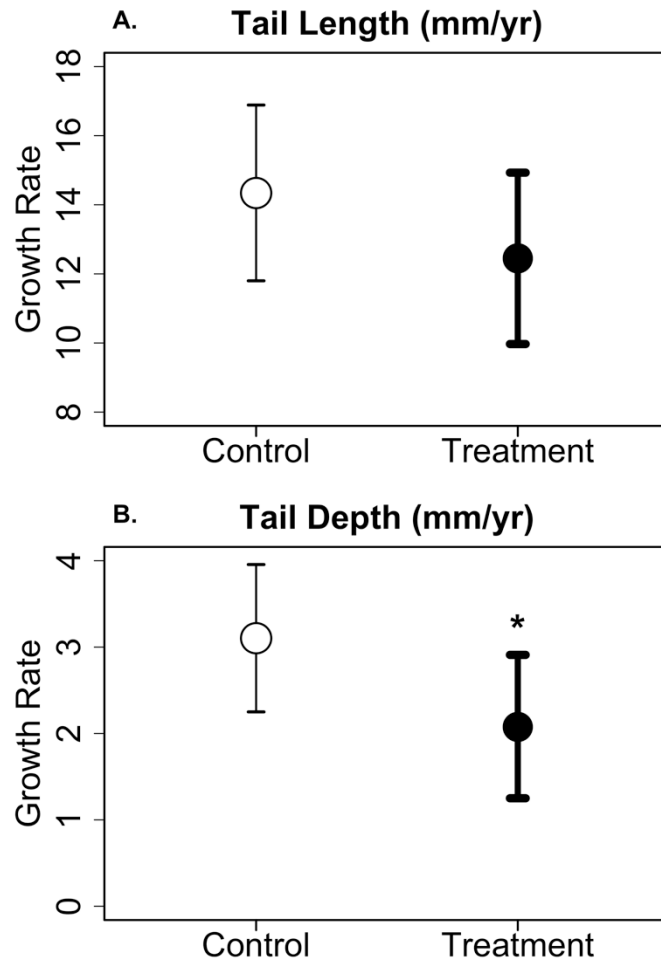


Figure 1.2. Fine-scale tail morphology growth rates of hellbenders (*Cryptobranchus a. alleganiensis*) from control and environmental enrichment treatment raceways. Growth rate estimates for tail length (A.) and tail depth (B.) are units per year, presented with 95% confidence intervals. Individuals from the treatment raceways have 33% slower change in tail depth through time compared to those from the control raceways. An asterisk denotes significant differences at an alpha level of 0.05.

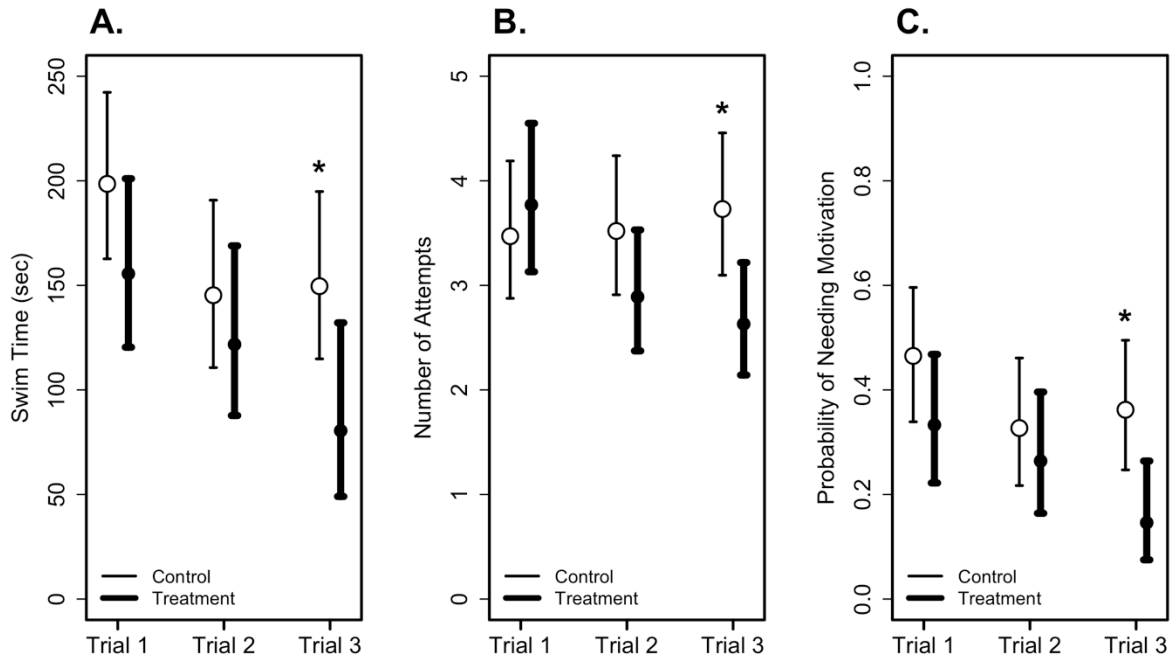


Figure 1.3. Differences in swim time (A.), number of attempts (B.), and probability of needing motivation (C.) between eastern hellbenders (*Cryptobranchus a. alleganiensis*) reared without (control) and reared with (treatment) elevated water velocities, across three swim performance trials. By the third trial, treatment hellbenders were quicker to complete the trial, required fewer attempts to reach an upstream tile hide, and were less likely to need manual motivation to move. Model estimates with 95% confidence intervals are presented; an asterisk denotes significant differences at an alpha level of 0.05.

1.6 References

- Adolph, S.C., Hardin, J.S., 2007. Estimating phenotypic correlations: correcting for bias due to intraindividual variability. *Funct. Ecol.* 21, 178–184. doi:10.1111/j.1365-2435.2006.01209.x
- Adolph, S.C., Pickering, T., 2008. Estimating maximum performance: effects of intraindividual variation. *J. Exp. Biol.* 211, 1336–43. doi:10.1242/jeb.011296
- Ahlbeck, I., Holliland, P.B., 2012. Rearing environment affects important life skills in pikeperch (*Sander lucioperca*). *Boreal Environ. Res.* 17, 291–304.
- Alberts, A.C., 2007. Behavioral considerations of headstarting as a conservation strategy for endangered Caribbean rock iguanas. *Appl. Anim. Behav. Sci.* 102, 380–391. doi:10.1016/j.applanim.2006.05.037
- Bashaw, M.J., Gibson, M.D., Schowe, D.M., Kucher, A.S., 2016. Does enrichment improve reptile welfare? Leopard geckos (*Eublepharis macularius*) respond to five types of environmental enrichment. *Appl. Anim. Behav. Sci.* 184, 150–160. doi:10.1016/j.applanim.2016.08.003
- Berejikian, B.A., Tezak, E.P., Flagg, T.A., LaRae, A.L., Kummerow, E., Mahnken, C.V.W., 2000. Social dominance, growth, and habitat use of age-0 steelhead (*Oncorhynchus mykiss*) grown in enriched and conventional hatchery rearing environments. *Can. J. Fish. Aquat. Sci.* 57, 628–636. doi:10.1139/f99-288
- Bestgen, K.R., Mefford, B., Bundy, J.M., Walford, C.D., Compton, R.I., 2010. Swimming performance and fishway model passage success of Rio Grande silvery minnow. *Trans. Am. Fish. Soc.* 139, 433–448. doi:10.1577/T09-085.1
- Biggins, E., Godbey, J.L., Hanebury, L.R., Luce, B., Marinari, P.E., Matchett, M.R., Vargas, A., 1998. The effect of rearing methods on survival of reintroduced black-footed ferrets. *J. Wildl. Manage.* 62, 643–653.
- Biggins, D.E., Vargas, A., Godbey, J.L., Anderson, S.H., 1999. Influence of prerelease experience on reintroduced black-footed ferrets (*Mustela nigripes*). *Biol. Conserv.* 89, 121–129. doi:10.1016/S0006-3207(98)00158-X
- Bodinof, C.M., 2010. Translocation and Conservation of Hellbenders (*Cryptobranchus alleganiensis*) in Missouri. University of Missouri.

- Bodinof, C.M., Briggler, J.T., Junge, R.E., Mong, T., Beringer, J., Wanner, M.D., Schuette, C.D., Ettlign, J., Millspaugh, J.J., 2012. Survival and body condition of captive-reared juvenile Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) following translocation to the wild. *Copeia* 2012, 150–159. doi:10.1643/CH-11-024
- Boerner, J.A., 2014. Comparison of Movement Patterns in Captive-Released Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) Using Three Different Release Methods. PhD Thesis. State University of New York Buffalo State.
- Braithwaite, V.A., Salvanes, A.G. V., 2005. Environmental variability in the early rearing environment generates behaviourally flexible cod: implications for rehabilitating wild populations. *Proc. R. Soc. B* 272, 1107–1113. doi:10.1098/rspb.2005.3062
- Brönmark, C., Miner, J.G., 1992. Predator-induced phenotypical change in body morphology in crucian carp. *Science* (80-.). 258, 1348–1350. doi:10.1126/science.258.5086.1348
- Brooks, J.E., 1986. Annual reintroduction and monitoring report for razorback sucker *Xyrauchen texanus*, in the Gila River basin, Arizona, 1985. Albuquerque.
- Burghardt, G.M., 2013. Environmental enrichment and cognitive complexity in reptiles and amphibians: concepts, review, and implications for captive populations. *Appl. Anim. Behav. Sci.* 147, 286–298. doi:10.1016/j.applanim.2013.04.013
- Burgmeier, N.G., Unger, S.D., Sutton, T.M., Williams, R.N., 2011. Population status of the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) in Indiana. *J. Herpetol.* 45, 195–201. doi:10.1670/10-094.1
- Connolly, J.D., Cree, A., 2008. Risks of a late start to captive management for conservation: phenotypic differences between wild and captive individuals of a viviparous endangered skink (*Oligosoma otagense*). *Biol. Conserv.* 141, 1283–1292. doi:10.1016/j.biocon.2008.02.026
- Davison, W., 1994. Exercise training in the banded wrasse *Notolabrus fucicola* affects muscle fibre diameter, but not muscle mitochondrial morphology. *N. Z. Nat. Sci.* 21, 11–16.
- Davison, W., 1997. The effects of exercise training on teleost fish, a review of recent literature. *Comp. Biochem. Physiol. Part A Physiol.* 117, 67–75. doi:10.1016/S0300-9629(96)00284-8
- Davison, W., Goldspink, G., 1978. The effect of training on the swimming muscles of the goldfish (*Carassius auratus*). *J. Exp. Biol.* 74.

- Fischer, J., Lindenmayer, D.B., 2000. An assessment of the published results of animal relocations. *Biol. Conserv.* 96, 1–11. doi:10.1016/S0006-3207(00)00048-3
- Germano, J.M., Bishop, P.J., 2009. Suitability of amphibians and reptiles for translocation. *Conserv. Biol.* 23, 7–15. doi:10.1111/j.1523-1739.2008.01123.x
- Griffith, B., Scott, J.M., Carpenter, J.W., Reed, C., 1989. Translocation as a species conservation tool: status and strategy. *Science* (80-.). 245, 477–480.
- Haad, B., Candiotti, F.V., Baldo, D., 2011. Shape variation in lentic and lotic tadpoles of *Melanophryniscus* (Anura: Bufonidae). *Stud. Neotrop. Fauna Environ.* 46, 91–99. doi:10.1080/01650521.2011.593124
- Hammond, B.R., Hickman, C.P.J., 1966. The effect of physical conditioning on the metabolism of lactate, phosphate, and glucose in rainbow trout, *Salmo gairdneri*. *J. Fish. Res. Board Can* 23, 65–83.
- Hard, J.J., Berejikian, B.A., Tezak, E.P., Schroder, S.L., Knudsen, C.M., Parker, L.T., 2000. Evidence for morphometric differentiation of wild and captively reared adult coho salmon: a geometric analysis. *Environ. Biol. Fishes* 58, 61–73. doi:10.1023/A:1007646332666
- Haskell, A., Graham, T.E., Griffin, C.R., Hestbeck, J.B., 1996. Size related survival of headstarted redbelly turtles (*Pseudemys rubriventris*) in Massachusetts. *J. Herpetol.* 30, 524–527. doi:10.2307/1565695
- Hinterleitner, S., Huber, M., Lackner, R., Wieser, W., 1992. Systemic and enzymatic responses to endurance training in two Cyprinid species with different life styles (Teleostei: Cyprinidae). *Can. J. Fish. Aquat. Sci.* 49, 110–115.
- Hoverman, J.T., Relyea, R.A., 2012. The long-term impacts of predators on prey: inducible defenses, population dynamics, and indirect effects. *Oikos* 121, 1219–1230. doi:10.1111/j.1600-0706.2011.19792.x
- Hyvärinen, P., Rodewald, P., 2013. Enriched rearing improves survival of hatchery-reared Atlantic salmon smolts during migration in the River Tornionjoki. *Can. J. Fish. Aquat. Sci.* 70, 1386–1395. doi:10.1139/cjfas-2013-0147
- Irschick, D.J., Meyers, J.J., 2007. An analysis of the relative roles of plasticity and natural selection in the morphology and performance of a lizard (*Urosaurus ornatus*). *Oecologia* 153, 489–499. doi:10.1007/s00442-007-0726-y

- Kelley, J.L., Magurran, A.E., Macías-García, C., 2005. The influence of rearing experience on the behaviour of an endangered Mexican fish, *Skiffia multipunctata*. *Biol. Conserv.* 122, 223–230. doi:10.1016/j.biocon.2004.07.011
- Kern, W.H.J., 1984. The Hellbender, *Cryptobranchus alleganiensis*, in Indiana. Indiana State University.
- Kraus, B.T., McCallen, E.B., Williams, R.N., 2017. Evaluating the survival of translocated adult and captive-reared, juvenile eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*). *Herpetologica* 73, 271–276. doi:10.1655/HERPETOLOGICA-D-16-00009
- Kupferberg, S.J., Lind, A.J., Thill, V., Yarnell, S.M., 2011. Water velocity tolerance in tadpoles of the foothill yellow-legged frog (*Rana boylei*): swimming performance, growth, and survival. *Source: Copeia* 2011, 141–152.
- Lackner, R., Weisler, W., Huber, M., Dalla Via, J., 1988. Responses of the intermediary metabolism to acute handling stress and recovery in untrained and trained *Leuciscus Cephalus* (Cyprinidae, Teleostei). *J. Exp. Biol.* 140, 393–404.
- Luthardt-Laimer, G., 1983. Ontogeny of preferences to visual prey stimulus parameters in salamanders. *J. Herpetol.* 17, 221–227.
- Mayasich, J., Phillips, C., 2003. Conservation Assessment for Eastern Hellbender, USDA Forest Service, Eastern Reg.
- McPhee, M.E., Carlstead, K., 1990. The importance of maintaining natural behaviors in captive mammals, in: *Effects of Captivity on the Behavior of Wild Mammals*. pp. 303–313.
- Miller, B., Biggins, D., Wemmer, C., Powell, R., Calvo, L., Hanebury, L., Wharton, T., 1990. Development of survival skills in captive-raised Siberian polecats (*Mustela eversmanni*) II: predator avoidance. *J. Ethol.* 8, 95–104.
- Moehrensclager, A., Macdonald, D.W., 2003. Movement and survival parameters of translocated and resident swift foxes *Vulpes velox*. *Anim. Conserv.* 6, 199–206. doi:10.1017/S1367943003251
- Ogata, H.Y., Oku, H., 2000. Effects of water velocity on growth performance of juvenile Japanese flounder *Paralichthys olivaceus*. *J. World Aquac. Soc.* 31, 225–231. doi:10.1111/j.1749-7345.2000.tb00357.x

- Pearson, M.P., Spriet, L.L., Stevens, E.D., 1990. Effects of spring training on swim performance and white muscle metabolism during exercise and recovery in rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* 149.
- Pérez-Tris, J., Díaz, J.A., Tellería, J.L., 2004. Loss of body mass under predation risk: cost of antipredatory behaviour or adaptive fit-for-escape? *Anim. Behav.* 67, 511–521. doi:10.1016/j.anbehav.2003.06.008
- Petranka, J.W., 1998. Salamanders of the United States and Canada. Smithsonian Books.
- R Core Team, 2016. R: A Language and Environment for Statistical Computing. R Found. Stat. Comput. Vienna, Au, <http://www.R-project.org/>.
- Relyea, R.A., Hoverman, J.T., 2003. The impact of larval predators and competitors on the morphology and fitness of juvenile treefrogs. *Oecologia* 134, 596–604. doi:10.1007/s00442-002-1161-8
- Relyea, R.A., Werner, E.E., 2000. Morphological plasticity in four larval anurans distributed along an environmental gradient. *Copeia* 2000, 178–190.
- Salvanes, A.G.V., Moberg, O., Ebbesson, L.O.E., Nilsen, T.O., Jensen, K.H., Braithwaite, V. a, 2013. Environmental enrichment promotes neural plasticity and cognitive ability in fish. *Proc. R. Soc.* 280, 20131331. doi:10.1098/rspb.2013.1331
- Sanger, A.M., 1992. Effects of training on axial muscle of two cyprinid species: *Chondrostoma nasus* (L.) and *Leuciscus cephalus* (L.). *J. Fish Biol.* 40, 637–646. doi:10.1111/j.1095-8649.1992.tb02611.x
- Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675.
- Semlitsch, R.D., Reyer, H.-U., 1992. Modification of anti-predator behaviour in tadpoles by environmental conditioning. *J. Anim. Ecol.* 61, 353–360.
- Shaffer, H.B., Austin, C.C., Huey, R.B., 1991. The consequences of metamorphosis on salamander (*Ambystoma*) locomotor performance. *Physiol. Zool.* 64, 212–231.
- Stamps, J.A., Swaisgood, R.R., 2007. Someplace like home: experience, habitat selection and conservation biology. *Appl. Anim. Behav. Sci.* 102, 392–409. doi:10.1016/j.applanim.2006.05.038

- Stoinski, T.S., Beck, B.B., Bloomsmith, M.A., Maple, T.L., 2003. A behavioral comparison of captive-born, reintroduced golden lion tamarins and their wild-born offspring. *Behaviour* 140, 137–160.
- Unger, S.D., Sutton, T.M., Williams, R.N., 2013. Projected population persistence of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) using a stage-structured life-history model and population viability analysis. *J. Nat. Conserv.* 21, 423–432. doi:10.1016/j.jnc.2013.06.002
- Vargas, A., Anderson, S.H., 1999. Effects of experience and cage enrichment on predatory skills of black-footed ferrets (*Mustela nigripes*). *J. Mammal.* 80, 263–269. doi:10.2307/1383226
- Vervust, B., Lailvaux, S.P., Grbac, I., Van Damme, R., 2008. Do morphological condition indices predict locomotor performance in the lizard *Podarcis sicula*? *Acta Oecol* 34, 244–251. doi:10.1016/j.actao.2008.05.012
- Ward, D.L., Hilwig, K.D., 2004. Effects of holding environment and exercise conditioning on swimming performance of Southwestern native fishes. *North Am. J. Fish. Manag.* 24, 1083–1087. doi:10.1577/M03-175.1
- Ward, D.L., Schultz, A.A., Matson, P.G., 2003. Differences in swimming ability and behavior in response to high water velocities among native and nonnative fishes. *Environ. Biol. Fishes* 68, 87–92. doi:10.1023/A:1026031128486
- Wells, K.D., 2007. *The Ecology and Behavior of Amphibians*. University of Chicago Press.
- Wheeler, B.A., Prosen, E., Mathis, A., Wilkinson, R.F., 2003. Population declines of a long-lived salamander: a 20+-year study of hellbenders, *Cryptobranchus alleganiensis*. *Biol. Conserv.* 109, 151–156.
- Wilbur, H.M., 1987. Regulation of structure in complex systems: experimental temporary pond communities. *Ecology* 68, 1437–1452. doi:10.2307/1939227
- Young, P.S., Cech, J.J.J., 1993. Effects of exercise conditioning on stress response and recovery in cultured and wild young-of-the-year striped bass, *Morone saxatilis*. *Can. J. Fish. Aquat. Sci.* 50, 2094–2099.
- Young, P.S., Cech, J.J.J., 1994. Optimum exercise conditioning velocity for growth, muscular development, and swimming performance in young-of-year striped bass (*Morone saxatilis*). *Can. J. Fish. Aquat. Sci.* 51, 1519–1527.

CHAPTER 2. TRAINING FOR TRANSLOCATION: PREDATOR CONDITIONING INDUCES BEHAVIORAL PLASTICITY AND PHYSIOLOGICAL CHANGES IN CAPTIVE EASTERN HELLBENDERS (*CRYPTOBRANCHUS ALLEGANIENSIS ALLEGANIENSIS*)

The work in this chapter has been previously published. Kenison, E.K. and R.N. Williams. 2018. Training for translocation: predator conditioning induces behavioral plasticity and physiological changes in captive eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*). Diversity 10(13):1-15. doi:10.3390/d10010013

2.1 Abstract

Translocations are stressful, especially when captive animals are naïve to natural stimuli. Captive eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) identify predatory fish as threats, but may be more vulnerable to predation and stress because of inexperience with them. I investigated the use of predator conditioning to prepare hellbenders, behaviorally and physiologically, for the presence of a common predator, largemouth bass (*Micropterus salmoides*). I reared hellbenders for 30 days with and without continuous exposure to largemouth bass kairomones and heterospecific alarm cues and found conditioned hellbenders became less active compared to unconditioned individuals ($p = 0.017$). After conditioning, I exposed hellbenders to water, a low concentration of kairomones, or a high concentration of kairomones in a closed respirometer system. I measured activity within respirometer chambers and routine metabolic rate. I found unconditioned hellbenders exposed to low and high concentrations of kairomones were 41% and 119% more active than conditioned animals ($p = 0.002$ and $p < 0.001$). Moreover, conditioned individuals had on average 6.5% lower metabolic rates across all three kairomone concentrations compared to unconditioned individuals ($p = 0.017$). My data suggest that predator conditioning induces behavioral avoidance tactics and physiological changes that could improve future translocation efforts for hellbenders and other imperiled species.

2.2 Introduction

Translocations are inherently stressful for animals (Teixeira et al. 2007; Dickens et al. 2009, 2010). Not only is transportation and release into a novel environment challenging, but stress is further exacerbated by exposure to additional threats that are typically absent from the captive environment. Stimuli such as stochastic weather conditions, contaminants, pathogens, and predators are novel to captive-reared animals and can magnify the stress of translocations (Engelsma et al. 2003; Breves and Specker 2005; Teixeira et al. 2007; Polednik et al. 2008). Increased stress is correlated with reduced reproductive potential, increased disease susceptibility, altered energy expenditure, irregular dispersal movements, and increased predation risk (Killen and Brown 2006; Van Dievel et al. 2016). Subsequently, stress (in a variety of forms) is a leading cause of translocation mortality (Aarts and Van Schagen 2006; Teixeira et al. 2007; Chipman et al. 2008; Dickens et al. 2009, 2010).

Animals reared in captivity are often naïve to predators and can lack experiences in predator detection and appropriate avoidance responses (Crane and Mathis 2011). Subsequently, captive-reared animals have higher mortality rates than wild or predator-conditioned conspecifics (Kellison et al. 2000). For example, captive-reared partridges (*Perdix perdix*) have a 69% chance of predator-mediated mortality following reintroductions because of inappropriate, or absent, antipredator behaviors (Parish and Sotherton 2007). Predators can lethally remove individuals from the population, but can also cause considerable physiological and psychological stress in their prey (Pfeiffer 1962). Tadpoles (*Rana clamitans* and *R. catesbeiana*) reared with caged dragonfly (*Anax junius*) predators have increased mortality rates because the presence of a predator can cause physiological stress that leads to death even without a physical encounter (Werner and Anholt 1996). Predator detection is associated with an increase in heart rate, elevated respiration, and the release of stress hormones (i.e., glucocorticoids), which are all components of the fight or flight response (Fraser and Gilliam 1992; Sapolsky 2002; Slos and Stoks 2008; Steiner and Buskirk 2009). Although acute fright responses are adaptive in responding to predators, chronic activation of the fright response becomes maladaptive during extended exposure or in combination with other stressful stimuli (e.g., translocations; Lankford et al. 2005).

Repeated predator-exposure events can familiarize an individual to reoccurring threats and prepare them physiologically to not respond with continuous activation of their acute stress

response (Figueiredo et al. 2003). Furthermore, repeated exposures can train animals to appropriately assess their level of risk and better balance predator avoidance with energy allocation (Chivers and Mirza 2001; Petersson et al. 2015). Fright responses are energetically costly; therefore, some fish exposed to predators conserve energy during low stress events to reserve resources for more threatening scenarios (Brown et al. 2005). Moreover, animals conditioned to chronic stress or living in areas with high densities of predators have more transient responses to threatening stimuli, exhibit lower levels of circulating stress hormones, and recover from acute stress events quicker (Woodward and Smith 1985; Young and Cech 1993; Brown et al. 2005). Although presenting predators to naïve prey can be initially stressful, prey species experience repeated predator exposure events in the wild and must alter their physiological demands and avoidance strategies to successfully coexist with predators. If predator conditioning prior to release can reduce predator-mediated stress and prepare captive animals to identify and avoid novel predators, perhaps animals will be better able to manage energetic costs associated with the stress of transportation and wild release (Dickens et al. 2010).

Eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) translocations, from captivity to the wild, have resulted in variable levels of success (17-72% survival over 6 months; Bodinof et al. 2012; Boerner 2014; Kraus et al. 2017). Translocation failures have been attributed to disease, long distance dispersal, and predation, which are all inherently linked with stress. Hellbenders are fully aquatic and reside in rivers with a diverse array of predatory fish species (see Carnahan 2001) and thus, an abundance of predator kairomones – chemical cues emitted by predatory species. Largemouth bass (*Micropterus salmoides*) live sympatrically with hellbenders in some rivers and are capable of consuming large prey items, such as 1-3 year old hellbenders (Gall 2008). Young, captive hellbenders respond to predatory fish kairomones with altered behavior, suggesting they accurately identify kairomones as stressful stimuli and have innate recognition to predators, such as largemouth bass (Gall and Mathis 2010; Crane and Mathis 2011). Translocations into predator-rich environments could exacerbate stress from transport and release and become detrimental to hellbenders; however, there has been no research to identify whether hellbenders have physiological responses to predators. In order to increase translocation success, it is important that hellbenders are able to identify, assess, and respond to predatory risk with advantageous avoidance behaviors, but without activation of physiologically costly stress responses and metabolic demands. I investigated the ability of

juvenile hellbenders to detect predators, their foraging and behavioral responses over acute periods of exposure, and their physiological response to low and high levels of risk after being conditioned to largemouth bass kairomones. I predicted that conditioning captive, juvenile hellbenders with predator kairomones would improve their ability to detect predators, increase their use of refugia and behavioral avoidance strategies, and reduce physiological stress.

2.3 Materials and Methods

2.3.1 Study animals

Eastern hellbender salamanders are threatened or endangered throughout much of their range in the central and southeastern United States. They are state-endangered in Indiana, USA and restricted to a single river system (Burgmeier et al. 2011). In efforts to preserve and bolster this wild population, researchers have been collecting hellbender clutches from the wild, head-starting them in captivity, and releasing them back into their natal river at older and larger age classes. I collected one wild clutch of eggs, all at least half siblings, in 2015. These individuals (n = 122) were reared in multiple aquarium tanks with sterilized water, tile hides, limited stimuli, and standardized food regimes for the first two years of their life. I haphazardly selected 48 of these individuals for this project, as all of them were completely naïve to predator kairomones. All animal handling procedures were reviewed and approved by Purdue University's Animal Care and Use Committee (protocol number 1406001094, approved 05/2017).

2.3.2 Phase 1. Predator conditioning

I reared two-year-old eastern hellbenders for 30 days after randomly assigning them to one of two conditioning treatments, with (conditioned, n = 24) or without (unconditioned, n = 24) continuous exposure to largemouth bass (*Micropterus salmoides*) kairomones (similar to Steiner and Buskirk 2009). I housed three largemouth bass in separate tanks directly above conditioned treatment tanks. I created a gravitational flow-through design, such that water from tanks with predators entered into the hellbender treatment tanks directly below them (similar to Kats et al. 1988). All tanks continually received fresh filtered and ultraviolet-sterilized well water ($20^{\circ} \pm 2^{\circ}\text{C}$); however, because conditioning tanks also received predator tank water, they were continually exposed to low concentrations of predator kairomones. I fed bass live larval tiger salamanders (*Ambystoma tigrinum*) each day in order to provide salamander alarm cues –

chemical cues emitted by prey during stress, disturbance, or attack – in conjunction with predator kairomones. Predator conditioning that combines predator kairomones with damage-released alarm cues, or some kind of aversive stimuli, can facilitate predator recognition and reduces the likelihood of naïve prey becoming habituated to kairomones (Barbosa and Castellanos 2005). It was important to provide an amphibian warning signal because amphibians elicit stronger responses when predators are fed amphibian prey (Chivers and Mirza 2001; Ferland-Raymond and Murray 2008; Schoeppner and Relyea 2009). However, I was unable to sacrifice hellbenders because of their endangered conservation status and I required more than 180 prey individuals. Subsequently, I used larval tiger salamanders because I could easily acquire multiple egg clutches prior to the experiment and this provided me a source of amphibian alarm cues.

I weighed all hellbenders at the beginning and end of the conditioning period; all hellbenders were comparable in size between the two treatments (mean = 42.2g, Standard Deviation \pm 8.8g, $t = -0.69$, $p = 0.491$). I housed eight hellbenders per tank with a total of six tanks. I conducted behavioral observations 21 times over the 30 conditioning days, or 5–6 times a week during daytime hours (0800-1700 hr). I conducted scan sampling to count the number of individuals outside of tile hides (Altmann 1974). Of those individuals outside of refugia, I classified and counted the number of individuals actively moving, stationary, or floating in the tank. I selected these three groupings, as they categorized behaviors commonly observed outside of the tile hides in captivity. I provided each hellbender tank 20 g of black worms (*Lumbriculus variegatus*), twice weekly. At each feeding event I recorded the time for at least one hellbender to start eating and noted the total number of individuals emerging from hides to feed within 10 minutes of providing food. At the beginning of each week, prior to feedings, I removed and weighed any worms that were remaining in the tanks to estimate overall consumption.

2.3.3 Phase 2. Exposure trials

Following 30 days of conditioning, I randomly assigned hellbenders to three exposure treatments for a full 2x3 factorial design: conditioned or unconditioned treatments crossed with control (no kairomones), low risk (low concentration of largemouth bass kairomones), or high risk (high concentration of largemouth bass kairomones) exposures. This design allowed me to compare physiological responses of hellbenders chronically exposed to low risk and then 1) released from predator threat, 2) maintained in a chronic low risk environment, or 3) exposed to

a novel high risk environment. I was also able to compare the response of unconditioned, naïve individuals exposed to these three levels of risk.

Predators are physiologically demanding to prey species. Their physical or chemical presence increases circulating glucocorticoid levels, induces altered behaviors, and changes respiration rates (Kats et al. 1988; Maher et al. 2013; Hall and Clark 2016). Metabolic rate is directly tied to the stress response; therefore, it provides a reliable metric for a physiological response (Sapolsky 2002). I used largemouth bass as my focal threat and then exposed all hellbenders to predator kairomones within respirometer chambers to measured changes in routine metabolic rate. I measured aquatic oxygen consumption using a Loligo Systems closed respirometer (Viborg, Denmark). The system consisted of four cylindrical glass chambers, each with a Witrox 4 for oxygen and temperature readings. The four chambers were connected to two pumps each via impermeable plastic tubing. The first pump moved fresh water into the chambers while the second pump recirculated water past the oxygen sensor that recorded readings every 30 seconds. All oxygen sensors were calibrated to 0% O₂ using sodium sulfite-treated water and 100% O₂ using fully aerated water (Burraco et al. 2013). I submerged all chambers, tubing, and pumps in a large 180-gallon sump full of UV sterilized water (Alvarez et al. 2006). In order to add predator kairomones for the low and high risk exposure treatments, I added predatory fish directly to the respirometer holding sump: one fish in 75 gallons of water for low risk and three fish in 75 gallons of water for high risk. Adding additional predators increases the concentration of kairomones in the water and therefore, could increase perceived levels of risk (Relyea 2004). I allowed the fish to swim around the holding tank for one hour, removed the fish, and then started the hellbender respirometer trials. I did not provide any alarm cues during the exposure trials, only predator kairomones.

Prior to exposure trials, I fasted all hellbenders for 48 hours to reach a post-absorptive state (Alvarez et al. 2006; Orlofske and Hopkins 2009; Kearney et al. 2016). I also acclimated hellbenders in open circuit respirometers for five minutes then created a closed, recirculating circuit for each individual chamber (Burraco et al. 2013). Hellbenders are primarily nocturnal; therefore, I conducted all experiments during daylight hours and kept overhead lights on in the experimental room to reduce activity. I conducted experiments over two days, between 1000 and 1600 hr, wherein I tested all individuals exposed to well water and then all individuals exposed to largemouth bass kairomones to avoid contamination between groups. I started with low risk

exposures and then tested high risk exposures within the same day. Again, I elected not to randomize my testing order to avoid contamination across the three risk levels. I ran trials with a single hellbender in each of the four chambers for 30 minutes. I restricted the sampling time to 30 minutes to avoid creating hypoxic conditions ($< 3 \text{ mgO}_2 \text{ L}^{-1}$) within the closed system while still allowing enough time to detect reductions in oxygen concentrations (Srean et al. 2017). I maintained equal temperatures across trials, which were comparable to hellbenders' rearing environment and to previous studies (Guimond and Hutchison 1973). I used the software package AutoResp to detect oxygen consumption of each chamber in real time ($\text{mL O}_2 \text{ h}^{-1}$, Loligo Systems, Viborg, Denmark).

Subtle changes in behavior can cause increases in metabolic rate; therefore, I wanted to account for any activity within the chamber during my exposure trials. There were no hides added to respirometer chambers because of limited space, which allowed me to fully observe each hellbender throughout the duration of the exposure trials. I scan sampled all four respirometer chambers every minute and recorded whether hellbenders were moving as a binary response. Moving could include turning around, rocking body, or swaying tail. I then combined the total number of times a hellbender was observed active during the 30-minute trial to calculate proportion of time active.

2.3.4 Statistical analyses

2.3.4.1 Phase 1. Predator conditioning

I made statistical comparisons of refuge use and tank behavior between conditioned and unconditioned individuals over the 30-day conditioning period. I tested for differences in the number of individuals outside of tile refugia during each behavioral observation using a generalized linear mixed-effects model. I used the Poisson distribution, because I found no evidence of overdispersion after using the 'AER' package in R. I included 'treatment' and 'date' in my model, tested for an interaction between the two 'treatment*date', and accounted for repeated observations by also including 'rearing tank' nested within 'date' as random effects. I used a multivariate analysis of variance (MANOVA) to compare tank behavior of hellbenders observed outside of refuge between conditioned and unconditioned treatments. I centered and scaled the number of hellbenders active, stationary, or floating in the tank to meet the assumption of normality and then combined them as multivariate response variables (Conover 1999). I tested

for behavioral differences across time of day, but found time to be unimportant and removed it from all models (f value = 0.55, p = 0.647). I then conducted repeated measures ANOVAs to compare each tank behavior (i.e., active, stationary, and floating) between conditioning treatments. I included ‘rearing tank’ nested within ‘date’ to account for repeated measures through time and tested for ‘treatment’, ‘date’, and ‘treatment*date’ interaction effects.

I compared feeding behavior with two separate analyses, using generalized linear mixed-effects models that accounted for repeated observations through time (i.e., ‘rearing tank’ nested in ‘date’). I compared the time to start feeding between conditioned and unconditioned individuals using a Gaussian distribution, and the total number of individuals observed feeding using a Poisson distribution. In addition, I used linear mixed-effects models to test for differences in the amount of food eaten each week; again, I treated ‘rearing tank’ nested in ‘date’ as a random effect. I also conducted t-tests to compare the mass of conditioned and unconditioned individuals at the end of the conditioning period.

2.3.4.2 Phase 2. Exposure trials

I tested for differences in activity between conditioned and unconditioned hellbenders during predator exposure trials in respirometer chambers. I included proportion of time active as my response variable and conducted binomial logistic regressions using the total number of times hellbenders were observed active or stationary during the exposure trials. I compared the effects of conditioning, exposure level, and their interactions on the probability of moving.

I was interested in how my measure of activity related to metabolic rate; therefore, I conducted a linear regression with metabolic rate as a function of proportion of time active. I found proportion of time active to be highly significant (t value = 5.80, p < 0.001, Figure 2.1) and chose to include it in all metabolic rate comparisons as a way to account for activity in my metabolic rate models. I compared metabolic rate between conditioned and unconditioned individuals at each exposure treatment using analysis of covariance (ANCOVA) with ‘mass’ and ‘proportion of time active’ as covariates. I considered one individual hellbender to be an outlier, because it was double the mean weight of all other hellbenders, and removed it from metabolic rate comparisons. All variables met assumptions of normality except mass, which I log-transformed. I tested for a mass by treatment effect in the model, but did not find evidence for an interaction between treatment and mass (t value = -1.12, p = 0.271). I also tested for interactions

between conditioning treatment and exposure level, after correcting for mass and accounting for proportion of time active. I tested for any effects of time on the probability of moving within chambers or on metabolic rate, but excluded this variable from my final models, as it was not a significant predictor for either response (probability of moving: t value = -1.08, p = 0.286; metabolic rate: t value = -0.22, p = 0.824). I used program R, version 3.2.3, for all analyses with an alpha level of 0.05 (R Core Team 2016). I used package ‘emmeans’ to report marginal means and standard errors around metabolic rate estimates. All data files are stored on Purdue University’s Research Repository (<http://purr.purdue.edu>).

2.4 Results

2.4.1 Phase 1. Predator conditioning

I found no differences in the number of hellbenders outside of refugia between conditioning treatments (estimated difference = -0.08, z value = -0.60, p = 0.55); however, I did find significant differences across sampling days (estimate change per day = -0.07, z value = -5.12, p < 0.001). All hellbenders, regardless of their treatment, increased their refuge use during the length of the experimental period. Of the individuals that I observed outside of refugia, I detected significant multivariate differences in active, stationary, or floating behaviors between conditioning treatments (p < 0.001) as well as a treatment*date interaction (p = 0.012; Table 2.1). Furthermore, I detected behavioral differences between treatments depended on sampling date, such that conditioned individuals were less likely to be active or float in tanks, compared to unconditioned individuals, as the conditioning period progressed (Table 2.1, Figure 2.2).

I found no differences in the time to start feeding (difference = 0.69 minutes, t value = 1.00, p = 0.335) or the number of individuals observed feeding within a 10-minute observation period (estimated difference = 0.83 individuals, z value = -0.54, p = 0.587). Furthermore, there was no difference in the amount of worms eaten between treatments (estimated difference = 2.18 g, t value = 0.57, p = 0.581). However, hellbenders conditioned to largemouth bass kairomones were 11.9% larger (95% Confidence Interval = 0.03–24%) at the end of the conditioning period compared to unconditioned hellbenders (estimated difference = 5.53 g, t value = -2.02, p = 0.049).

2.4.2 Phase 2. Exposure trials

There were significant differences in the probability of moving during the predator exposure respirometer trials between conditioned and unconditioned individuals. Conditioned individuals were 15.4% more likely to move in respirometer chambers when exposed to water without predator kairomones (estimated difference in probability of moving = 0.06, z value = 2.90, $p = 0.004$; Figure 2.3). However, they were 28.9% and 54.3% less likely to move compared to unconditioned individuals in low and high exposure trials, respectively (estimated difference in probability of moving = 0.05, z value = -3.03, $p = 0.002$ and estimated difference in probability of moving = 0.10, z value = -4.29, $p < 0.001$; Figure 2.3).

Conditioned individuals had significantly lower metabolic rates compared to unconditioned individuals across all three exposure levels, even after accounting for proportion of time active in respirometer chambers (t value = -2.49, $p = 0.017$; Fig. 4). Conditioned hellbenders exposed to water without kairomones had 6.7% lower metabolic rates compared to unconditioned, control individuals (estimated difference in $\text{mL O}_2 \text{ h}^{-1} = 0.10$, t value = -2.49, $p = 0.017$; Figure 2.4). Furthermore, conditioned individuals exposed to low and high risk had metabolic rates 6.4% lower than unconditioned individuals (estimated difference in $\text{mL O}_2 \text{ h}^{-1} = 0.10$, t value = -2.49, $p = 0.017$; Figure 2.4).

2.5 Discussion

Predator conditioning induced predator avoidance behaviors and provided strong evidence that hellbenders perceive largemouth bass kairomones as a threat. Hellbenders conditioned to largemouth bass kairomones and heterospecific alarm cues for 30 days were less active outside of tile hides and never observed floating compared to control hellbenders that only received well water. All hellbenders increased their refuge use through the duration of the conditioning period, which suggests hellbenders became acclimated to the rearing tanks and reduced their exploratory behavior. However, conditioned individuals demonstrated behavioral plasticity with chronic exposure to predator kairomones by reducing their time moving outside of refuge through time. Some of the most common predator avoidance strategies, observed across a multitude of taxonomic groups, are decreased movement, freezing in place, or seeking out refuge. For example, less mobile voles (*Microtus agrestis*) have reduced rates of predator capture, small-

mouth salamanders (*Ambystoma texanum*) spend more time in refuge away from green sunfish (*Lepomis cyanellus*), and crayfish (*Orconectes rusticus*) freeze in place to be less conspicuous (Kats et al. 1988; Norrdahl and Korpimäki 1998; Kenison et al. 2017). Behavioral responses reduce the probability of being detected, encountering predators, or being captured, and are adaptive when coexisting with predators. Remaining stationary is especially beneficial for hellbenders in the presence of fish predators, because fish use a lateral line system to detect and locate prey through movement (Bleckmann and Zelick 2009). Alternatively, floating in the water column or at the water surface likely increases the risk of capture by an aquatic or terrestrial predator and potentially being swept downstream in riverine water currents. This maladaptive behavior is commonly observed among hellbenders in captivity and could be particularly threatening to hellbenders' survival in the wild. However, conditioned individuals were never observed floating in their tanks, suggesting predator conditioning induced plastic behaviors that will aid in predator avoidance rather than predator capture.

Animals can suffer reduced growth in the presence of predation risk if they face a trade-off between foraging to fulfill their energy needs and remaining inactive to avoid predation (i.e., the growth/predation tradeoff; McPeck 2004). Although conditioned hellbenders were less active in their tanks, I did not observe differences in foraging behavior or overall food intake. Moreover, conditioned individuals were able to gain more weight during the conditioning period. Inactivity is inherently linked to reduced energy expenditure, and can also be associated with higher somatic growth (Holopainen et al. 1997). For example, fish reared with predators have 80% greater mean weight than controls, because they expend less energy during periods of inactivity and are able to allocate resources beyond general maintenance costs (Holopainen et al. 1997). Conditioned hellbenders may have been more stealthy feeders, reducing extraneous exploratory behavior when foraging and instead, directing resources to growth. Additionally, some animals allocate resources to develop morphological defenses in the presence of predators, such as spines, crush-resistant shells, or body sizes that are beyond the gape limitation of predators (Brönmark and Miner 1992; Repka et al. 1995; Hoverman et al. 2005). Hellbender weight gain provides evidence that predator conditioning was not detrimental to growth and refutes arguments for a tradeoff between growth and behavioral avoidance of predation in this experiment. Furthermore, larger hellbenders may be more likely to survive in the wild and to survive longer following release, as size is often a positive predictor of post-release success (see Haskell et al. 1996).

Hellbenders are a slow-growing species, yet within 30 days conditioned hellbenders gained weight. This suggests that conditioned hellbenders were better able to direct resources toward growth and that this technique can effectively increase size prior to release.

Following the conditioning period, I found that both conditioned and unconditioned individuals reduced their level of activity when they were exposed to largemouth bass kairomones in respirometer chambers. These results substantiate others' work that amphibians, including hellbenders, have innate behavioral responses to predator kairomones (Kats and Dill 1998; Epp and Gabor 2008; Gall and Mathis 2010). Crane and Mathis (2011) found that larval hellbenders, 21-25 weeks old, increase their swimming when exposed to trout kairomones, which they interpreted as evidence of escape behavior. My findings differ from Crane and Mathis (2011), likely because I presented live amphibian prey to largemouth bass as food. Although their study elicited responses using hellbender slime as an alarm cue, they fed their fish a diet of floating trout feed. Disturbance cues released from injured or stressed prey induce predator defenses and phenotypic plasticity among conspecifics (Dewitt et al., 1998; Van Buskirk and Relyea, 1998; Relyea, 2004; Schoeppner and Relyea, 2005). However, cues from chewed and digested prey have an even stronger influence on predator avoidance strategies; tadpoles exposed to predators fed conspecifics reduce their activity by 30% compared to tadpoles only exposed to a starved predator (Schoeppner and Relyea, 2009b). I observed different predator avoidance behaviors, likely because of the diet of largemouth bass. Furthermore, in the wild, larvae are reared in a nest that is paternally guarded and remain for months after hatching (5-6 months, W. Hopkins personal comm.). Therefore, larvae between 21-25 weeks old may have underdeveloped predator avoidance strategies because of innate protection from potential predators in the nest. Alternatively, my design more closely mimicked a natural environment where two-year-old hellbenders would be free swimming in the river, continually exposed to predator kairomones, and needing to actively avoid predation. Conditioned hellbenders in my study responded with stronger reductions in activity than unconditioned individuals, suggesting that predator conditioning for captive hellbenders might reduce susceptibility to predation and possible sublethal effects following translocation to the wild. Conditioned and unconditioned hellbenders reduced their activity by 70.1% and 40.8%, respectively, when presented with kairomones from one largemouth bass, but I didn't observe further changes in chamber activity when exposed to high concentrations of kairomones. A threshold response following the addition

of one predator is similar among other amphibians (Van Buskirk et al. 2002; Schoeppner and Relyea 2008). Wood frog tadpoles (*Rana sylvatica*) reduce their activity by 38% when presented with a single predator, but then show no additional differences in activity when two, four, or six predators are presented (Schoeppner and Relyea 2008). Oppositely, pool frog tadpoles increase the proportion of inactive individuals by 22% when they are presented with a single caged-predator, but have no additional changes when three more caged-predators are added to the same holding tank (Van Buskirk et al. 2002). Regardless of the magnitude of risk, conditioned hellbenders had consistently lower chamber activity in the low and high concentration trials, which translates to higher energy savings compared to unconditioned individuals. This could quickly become useful if hellbenders need to flee or escape lethal predators leading to a survival advantage over predator-naïve hellbenders released into the wild (Brown et al. 2005; Steiner and Buskirk 2009).

Being able to assess and opportunistically respond to the presence or absence of risk supports the risk allocation hypothesis (Lima and Bednekoff 1999). This hypothesis suggests that animals decrease their levels of activity when they detect high risk, but increase their foraging or activity during bouts of perceived safety (Lima and Bednekoff 1999; Sih and McCarthy 2002). For example, snails (*Physa gyrina*) that are maintained at high levels of risk and then exposed to a pulse of safety increase their activity until the pulse of low risk passed (Sih and McCarthy 2002). Conditioned hellbenders exposed to water during the exposure trials moved more than unconditioned hellbenders in the respirometer chambers. This release from predator pressure may have been perceived as a bout of safety and induced more activity. I did not detect differences in activity between conditioned individuals in low and high exposure treatments. However, this might be attributed to the fact that I did not present salamander alarm cues in combination with the fish kairomones during the exposure trials. Hellbenders can recognize conspecific alarm cues and perceive it as an indicator of elevated risk (Crane and Mathis 2011). Therefore, adding hellbender slime or alarm cues from other salamanders could have exaggerated behavioral responses. Despite this, conditioned individuals had lower activity in the low and high exposure treatments compared to unconditioned individuals and demonstrate a conditioning benefit even in the absence of conspecific or heterospecific alarm cues.

I am the first to observe physiological responses to largemouth bass kairomones in eastern hellbenders and confirm that predator conditioning successfully minimizes hellbenders'

energetic demands. Conditioned hellbenders had on average 6.5% lower metabolic rates compared to unconditioned individuals, even after accounting for the effects of activity within the respirometer chambers. Similarly, common frog (*Rana temporaria*) tadpoles have 10% lower oxygen consumption rates after being exposed to predator kairomones for 30 days (Steiner and Buskirk 2009). In addition, Arabian toad (*Bufo arabicus*) tadpoles reared with continuous exposure to dragonfly (*Anax* sp.) larvae show a linear decrease in their respiration and had metabolic rates ~45% lower than controls after 21 days of conditioning (Barry and Syal 2013). Lower metabolic baselines correlate with lower energetic demand and better budgeting of available resources under chronic risk (Figueiredo et al. 2003; Steiner and Buskirk 2009). Maintaining a lower metabolic rate is highly advantageous in a risky environment, because animals are able to avoid excessive energy expenditure, minimize anti-predator costs over the long-term, and allocate resources towards growth, reproduction, and immune function rather than fright responses alone (Reed et al. 2003; Brown et al. 2005; Handelsman et al. 2013; Van Dievel et al. 2016). I did not see differences in routine metabolic rate across exposure treatments in either the conditioned or unconditioned individuals. This may be because 30-minute exposure periods were too short to elicit a physiological response among hellbenders or because hellbenders invest in physiological changes over chronic time periods rather than rapidly shifting metabolic responses over acute exposure events. Regardless, unconditioned individuals showed consistently higher oxygen consumption rates, leaving them at a physiological disadvantage, compared to conditioned individuals.

My results suggest that predator conditioning can beneficially prepare hellbenders for release into the wild by strengthening their avoidance behaviors and promoting energy savings through physiological changes. Growth, behavioral avoidance, risk assessment, and metabolic shifts that I observed among conditioned hellbenders are all evolutionary advantageous responses to predation risk (Barry and Syal 2013). Conditioned hellbenders elicited behavioral and physiological responses that reduce naïveté to predators, susceptibility to lethal attacks, sublethal effects, and additional stress during translocations and may ultimately, improve post-release survival and long-term persistence of wild hellbender populations. Future work will investigate the influence of predator conditioning on hellbender translocation success as others have shown predator conditioning to improve survival. For example, white seabream (*Diplodus sargus*) are nearly twice as likely to survive following wild release if they are conditioned to

conger eel (*Conger conger*; D'Anna et al. 2012). Furthermore, brook trout (*Salvelinus fontinalis*) have a 20% increase in survival during staged encounters with predatory pickerel following conditioning (Mirza and Chivers 2000). Animals often rely on previous encounters with predators to learn necessary avoidance strategies; however, these experiences can be stressful for and potentially lethal to prey (Griffin et al. 2000). Predator conditioning may effectively remove this dangerous learning period in the safety of a captive environment. Ultimately, predator conditioning enables animals to enter into risky environments with experiences and honed skills that will help them avoid predation. Future work can also explore conditioning techniques with other predators such as raccoons (*Procyon lotor*) or river otters (*Lontra canadensis*), which have been observed capturing and eating hellbenders in the wild (Boerner 2014). Although I was able to account for genetic differentiation in my project by only using hellbenders from a single clutch of eggs, exploring variation in predator responses within and among hellbender populations could also be beneficial. I found predator conditioning to be a low cost technique that required minimum amounts of time and effort to effectively induce behavioral and physiological changes among captive-reared hellbenders. Therefore, this method could be valuable to other imperiled vertebrates planned for translocation and at risk of wild predation. Captive-rearing programs should explore the potential for predator conditioning to prepare animals for wild release as this technique may have profound impacts on future translocation success.

Table 2.1. MANOVA table reporting the results of a multivariate comparison evaluating differences in the number of eastern hellbenders (*Cryptobranchus a. alleganiensis*) observed active, stationary, or floating while outside of tile hides in unconditioned and conditioned treatment tanks. Repeated measures ANOVA table for univariate comparisons of each behavior, while accounting for repeated observations through time. These data provide evidence for significant differences, by treatment, date, and a treatment by date interaction, such that conditioned hellbenders reduced their time active and floating during the conditioning period compared to unconditioned individuals.

MANOVA	Df	Pillai	<i>f</i> value	<i>p</i> value
Treatment	1, 122	0.128	5.88	< 0.001
Date	1, 122	0.318	18.61	< 0.001
Treatment*Date	1, 122	0.087	3.81	0.012

REPEATED MEASURES ANOVA	Active			Stationary			Floating		
	Df	<i>f</i> value	<i>p</i> value	Df	<i>f</i> value	<i>p</i> value	Df	<i>f</i> value	<i>p</i> value
Treatment	1	9.43	0.003	1	3.70	0.057	1	11.21	0.001
Treatment*Date	1	5.83	0.017	1	0.80	0.372	1	5.41	0.022

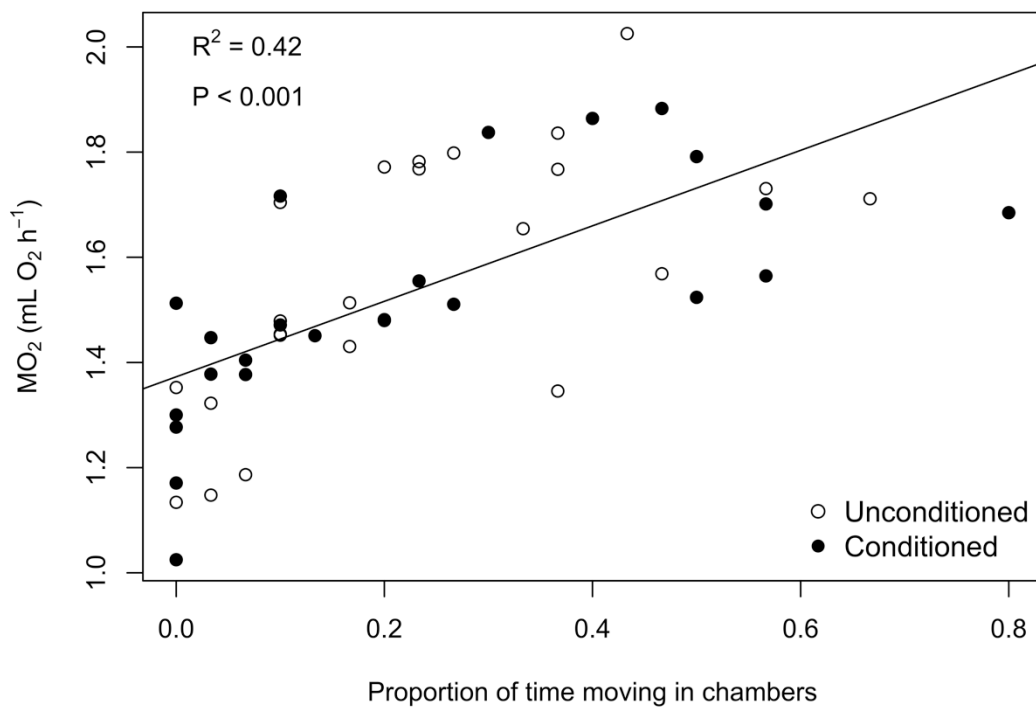


Figure 2.1. Linear relationship between routine metabolic rate and proportion of time moving in respirometer chambers. The added regression line shows the strong relationship between general activity (i.e., turning, rocking, or moving) and oxygen consumption during exposure trials.

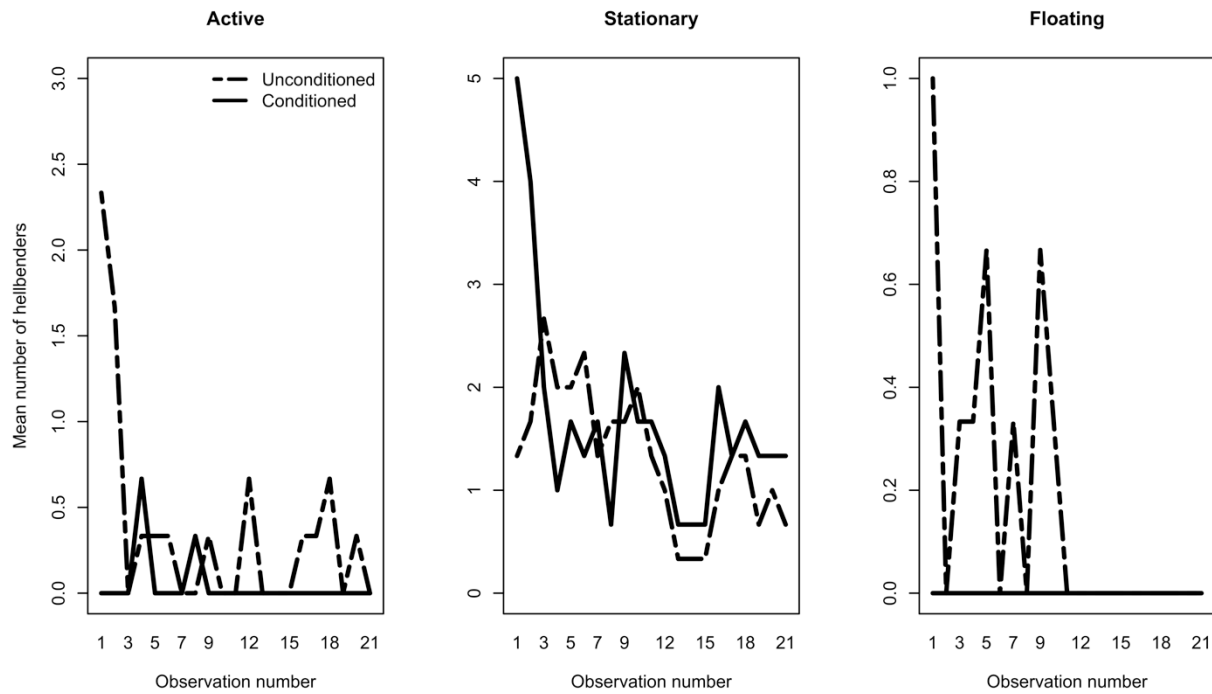


Figure 2.2. Behavioral observations of unconditioned and conditioned hellbenders (*Cryptobranchus a. alleganiensis*) in rearing tanks during the 30-day conditioning period. Hellbenders were counted whenever observed outside of tile hides and categorized as active, stationary, or floating. The mean number of hellbenders observed active or floating through time depended on conditioning treatment, such that fewer conditioned individuals were observed active or floating through the duration of the conditioning period compared to unconditioned individuals ($p < 0.05$). There were no significant treatment or time effects on stationary behavior.

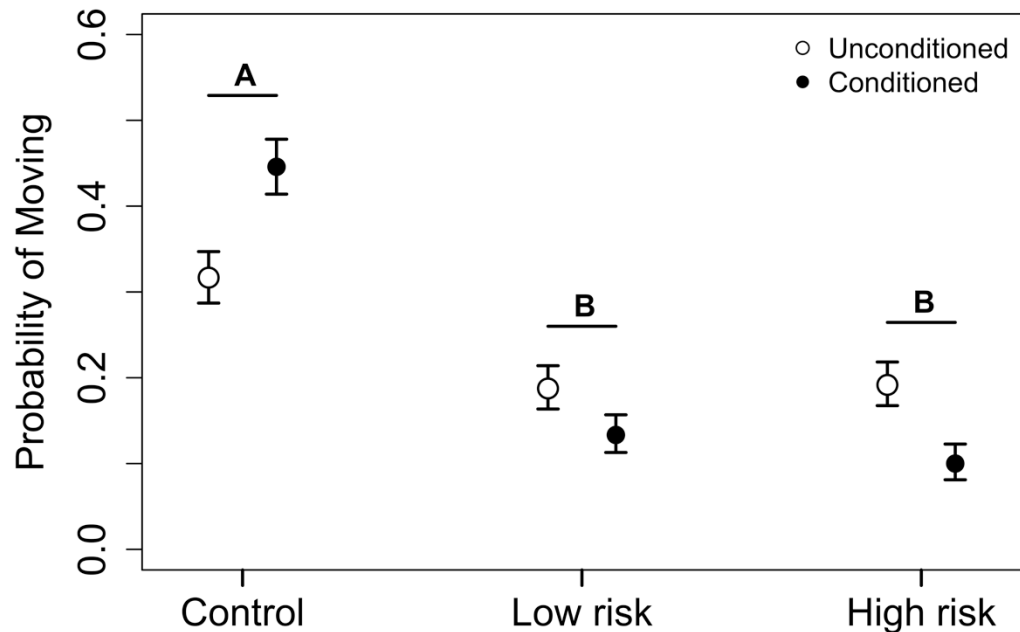


Figure 2.3. The probability of moving within respirometer chambers during exposure to varying levels of risk/kairomone concentrations. Activity was measured as proportion of time turning, rocking the body, or moving the tail during the 30-minute exposure trial. Conditioned hellbenders (*Cryptobranchus a. alleganiensis*) increased their probability of moving when exposed to water without kairomones, but had a lower probability of moving when they were presented with low and high concentrations of predator kairomones compared to unconditioned individuals ($p < 0.05$). Estimates are back-transformed and presented with standard errors. There were significant differences between conditioned and unconditioned individuals at each of the three exposure levels. Letters indicate significant differences across exposure levels ($p < 0.05$).

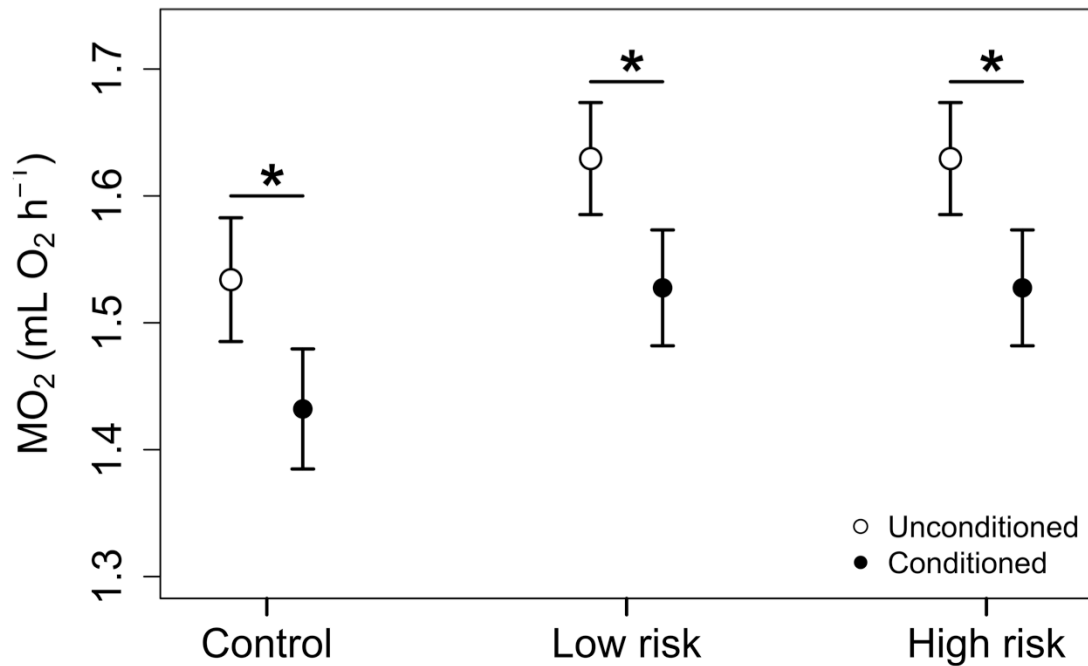


Figure 2.4. Oxygen consumption ($\text{mL O}_2\text{h}^{-1}$) of conditioned and unconditioned hellbenders (*Cryptobranchus a. alleganiensis*) exposed to varying levels of kairomone concentrations, after accounting for mass and proportion of time active during the exposure trials. Estimates are presented as marginal means with standard errors. Conditioned individuals had consistently lower oxygen consumption at each of the exposure levels compared to unconditioned individuals, but the rate of oxygen consumption did not differ across exposure levels. Asterisks denote significant differences between conditioning treatments ($p < 0.05$).

2.6 References:

- Aarts, L., Van Schagen, I., 2006. Driving speed and the risk of road crashes: a review. *Accid. Anal. Prev.* 38, 215–224. doi:10.1016/j.aap.2005.07.004
- Altmann, J., 1974. Observational study of behavior: sampling methods. *Behaviour* 49, 227–267.
- Alvarez, D., Cano, J.M., Nicieza, A.G., 2006. Microgeographic variation in metabolic rate and energy storage of brown trout: countergradient selection or thermal sensitivity? *Evol. Ecol.* 20, 345–363. doi:10.1007/s10682-006-0004-1
- Barbosa, P., Castellanos, I., 2005. *Ecology of predator-prey interactions*. Oxford University Press.
- Barry, M.J., Syal, S., 2013. Metabolic responses of tadpoles to chemical predation cues. *Hydrobiologia* 700, 267–276. doi:10.1007/s10750-012-1236-4
- Bleckmann, H., Zelick, R., 2009. Lateral line system of fish. *Integr. Zool.* 4, 13–25. doi:10.1111/j.1749-4877.2008.00131.x
- Bodinof, C.M., Briggler, J.T., Junge, R.E., Mong, T., Beringer, J., Wanner, M.D., Schuette, C.D., Ettlign, J., Millspaugh, J.J., 2012. Survival and body condition of captive-reared juvenile Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) following translocation to the wild. *Copeia* 2012, 150–159. doi:10.1643/CH-11-024
- Boerner, J.A., 2014. *Comparison of Movement Patterns in Captive-Released Eastern Hellbenders (Cryptobranchus alleganiensis alleganiensis) Using Three Different Release Methods*. PhD Thesis. State University of New York Buffalo State.
- Boonstra, R., Hik, D., Singleton, G.R., Tinnikov, A., 1998. The impact of predator-induced stress on the snowshoe hare cycle. *Ecol. Monogr.* 79, 371–394.
- Breves, J.P., Specker, J.L., 2005. Cortisol stress response of juvenile winter flounder (*Pseudopleuronectes americanus*, Walbaum) to predators. *J. Exp. Mar. Bio. Ecol.* 325, 1–7. doi:10.1016/j.jembe.2005.04.019
- Brönmark, C., Miner, J.G., 1992. Predator-induced phenotypical change in body morphology in crucian carp. *Science* (80-.). 258, 1348–1350. doi:10.1126/science.258.5086.1348
- Brown, C., Gardner, C., Braithwaite, V.A., 2005. Differential stress responses in fish from areas of high- and low-predation pressure. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 175, 305–312. doi:10.1007/s00360-005-0486-0

- Burgmeier, N.G., Unger, S.D., Sutton, T.M., Williams, R.N., 2011. Population status of the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) in Indiana. *J. Herpetol.* 45, 195–201. doi:10.1670/10-094.1
- Burraco, P., Duarte, L.J., Gomez-Mestre, I., 2013. Predator-induced physiological responses in tadpoles challenged with herbicide pollution. *Curr. Zool.* 59, 475–484.
- Carnahan, D.P., 2001. Blue river fisheries survey and game fish population estimates in Crawford, Harrison, and Washington counties, Fish management report.
- Chipman, R., Slate, D., Rupprecht, C., Mendoza, M., 2008. Downside risk of wildlife translocation. *Dev. Biol. (Basel)*. 131, 223–232. doi:10.1093/rfs/hhj035
- Chivers, D.P., Mirza, R.S., 2001. Importance of predator diet cues in responses of larval wood frogs to fish and invertebrate predators. *J. Chem. Ecol.* 27, 45–51. doi:10.1023/A
- Conover, W.J., 1999. Practical nonparametric statistics. Wiley.
- Crane, A.L., Mathis, A., 2011. Predator-recognition training: a conservation strategy to increase postrelease survival of hellbenders in head-starting programs. *Zoo Biol.* 30, 611–622. doi:10.1002/zoo.20358
- D'Anna, G., Giacalone, V.M., Vega Fernández, T., Vaccaro, A.M., Pipitone, C., Mirto, S., Mazzola, S., Badalamenti, F., 2012. Effects of predator and shelter conditioning on hatchery-reared white seabream *Diplodus sargus* (L., 1758) released at sea. *Aquaculture* 356–357, 91–97. doi:10.1016/j.aquaculture.2012.05.032
- DeWitt, T.J., Sih, A., Wilson, D.S., 1998. Cost and limits of phenotypic plasticity. *Trends Ecol. Evol.* 13, 77–81. doi:10.1111/j.1558-5646.2009.00647.x
- Dickens, M.J., Delehanty, D.J., Romero, L.M., 2009. Stress and translocation: alterations in the stress physiology of translocated birds. *Proc. R. Soc. B Biol. Sci.* 276, 2051–2056. doi:10.1098/rspb.2008.1778
- Dickens, M.J., Delehanty, D.J., Romero, L.M., 2010. Stress: an inevitable component of animal translocation. *Biol. Conserv.* 143, 1329–1341. doi:10.1016/j.biocon.2010.02.032
- Engelsma, M.Y., Hougee, S., Nap, D., Hofenk, M., Rombout, J.H.W.M., Van Muiswinkel, W.B., Lidy Verburg-van Kemenade, B.M., 2003. Multiple acute temperature stress affects leucocyte populations and antibody responses in common carp, *Cyprinus carpio* L. *Fish Shellfish Immunol.* 15, 397–410. doi:10.1016/S1050-4648(03)00006-8

- Epp, K.J., Gabor, C.R., 2008. Innate and learned predator recognition mediated by chemical signals in *Eurycea nana*. *Ethology* 114, 607–615. doi:10.1111/j.1439-0310.2008.01494.x
- Ferland-Raymond, B., Murray, D.L., 2008. Predator diet and prey adaptive responses: can tadpoles distinguish between predators feeding on congeneric vs. conspecific prey? *Can. J. Zool.* 86, 1329–1336. doi:10.1139/Z08-117
- Figueiredo, H.F., Bodie, B.L., Tauchi, M., Dolgas, C.M., Herman, J.P., 2003. Stress integration after acute and chronic predator stress: differential activation of central stress circuitry and sensitization of the hypothalamo-pituitary-adrenocortical axis. *Endocrinology* 144, 5249–5258. doi:10.1210/en.2003-0713
- Fraser, D.F., Gilliam, J.F., 1992. Nonlethal impacts of predator invasion: facultative suppression of growth and reproduction. *Ecology* 73, 959–970.
- Gall, B.G., 2008. Predator-prey interactions between hellbenders (*Cryptobranchus alleganiensis alleganiensis*) and *C. a. bishopi*) and native and nonnative fishes. Missouri State University.
- Gall, B.G., Mathis, A., 2010. Innate predator recognition and the problem of introduced trout. *Ethology* 116, 47–58. doi:10.1111/j.1439-0310.2009.01718.x
- Griffin, A.S., Blumstein, D.T., Evans, C.S., 2000. Review: training captive-bred or translocated animals to avoid predators. *Conserv. Biol.* 14, 1317–1326.
- Guimond, R.W., Hutchison, V.H., 1973. Aquatic respiration: an unusual strategy in the hellbender *Cryptobranchus alleganiensis alleganiensis* (Daudin). *Science* 182, 1263–1265. doi:10.1126/science.182.4118.1263
- Hall, A.E., Clark, T.D., 2016. Seeing is believing: Metabolism provides insight into threat perception for a prey species of coral reef fish. *Anim. Behav.* 115, 117–126. doi:10.1016/j.anbehav.2016.03.008
- Handelsman, C.A., Broder, E.D., Dalton, C.M., Ruell, E.W., Myrick, C.A., Reznick, D.N., Ghalambor, C.K., 2013. Predator-induced phenotypic plasticity in metabolism and rate of growth: Rapid adaptation to a novel environment. *Integr. Comp. Biol.* 53, 975–988. doi:10.1093/icb/ict057
- Haskell, A., Graham, T.E., Griffin, C.R., Hestbeck, J.B., 1996. Size related survival of headstarted redbelly turtles (*Pseudemys rubriventris*) in Massachusetts. *J. Herpetol.* 30, 524–527. doi:10.2307/1565695

- Holopainen, I.J., Aho, J., Vornanen, M., Huuskonen, H., 1997. Phenotypic plasticity and predator effects on morphology and physiology of crucian carp in nature and in the laboratory. *J. Fish Biol.* 50, 781–798. doi:10.1111/j.1095-8649.1997.tb01972.x
- Hoverman, J.T., Auld, J.R., Relyea, R.A., 2005. Putting prey back together again: integrating predator-induced behavior, morphology, and life history. *Oecologia* 144, 481–491. doi:10.1007/s00442-005-0082-8
- Kats, L.B., Dill, L.M., 1998. The scent of death: chemosensory assessment of predation risk by prey animals. *Écoscience* 5, 361–394. doi:10.1080/11956860.1998.11682468
- Kats, L.B., Petranka, J.W., Sih, A., 1988. Antipredator defenses and the persistence of amphibian larvae with fishes. *Ecology* 69, 1865–1870. doi:10.2307/1941163
- Kearney, B.D., Byrne, P.G., Reina, R.D., 2016. Short-and long-term consequences of developmental saline stress: impacts on anuran respiration and behaviour. *R. Soc. Open Sci.* 3, 150640. doi:10.1098/rsos.150640
- Kellison, G.T., Eggleston, D.B., Burke, J.S., 2000. Comparative behaviour and survival of hatchery-reared versus wild summer flounder (*Paralichthys dentatus*). *Can. J. Fish. Aquat. Sci.* 57, 1870–1877. doi:10.1139/cjfas-57-9-1870
- Kenison, E.K., Weldy, P.Y., Williams, R.N., 2017. There must be something in the water: assessing the behavioral responses of rusty crayfish (*Orconectes rusticus*) to fish and amphibian predator kairomones. *J. Ethol.* 1–8. doi:10.1007/s10164-017-0529-5
- Killen, S.S., Brown, J.A., 2006. Energetic cost of reduced foraging under predation threat in newly hatched ocean pout. *Mar. Ecol. Prog. Ser.* 321, 255–266.
- Kraus, B.T., McCallen, E.B., Williams, R.N., 2017. Evaluating the survival of translocated adult and captive-reared, juvenile eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*). *Herpetologica* 73, 271–276. doi:10.1655/HERPETOLOGICA-D-16-00009
- Lankford, S.E., Adams, T.E., Miller, R.A., Cech Jr., J.J., 2005. The cost of chronic stress: impacts of a nonhabituating stress response on metabolic variables and swimming performance in sturgeon. *Physiol. Biochem. Zool.* 78, 599–609. doi:10.1086/430687
- Lima, S.L., Bednekoff, P. a., 1999. Temporal variation in danger drives antipredator behavior: the predation risk allocation hypothesis. *Am. Nat.* 153, 649–659. doi:10.1086/303202

- Maher, J.M., Werner, E.E., Denver, R.J., B, P.R.S., 2013. Stress hormones mediate predator-induced phenotypic plasticity in amphibian tadpoles Stress hormones mediate predator-induced phenotypic plasticity in amphibian tadpoles. *Proc. R. Soc. B Biol. Sci.* 280, 1–9. doi:10.5061/dryad.1kf76
- McPeck, M.A., 2004. The growth/predation risk trade-off: so what is the mechanism? *Am. Nat.* 163, E88–111. doi:10.1086/382755
- McPeck, M.A., Grace, M., Richardson, J.M.L., 2001. Physiological and behavioral responses to predators shape the growth/predation risk trade-off in damselflies. *Ecology* 82, 1535–1545. doi:10.1890/0012-9658(2001)082[1535:PABRTP]2.0.CO;2
- Mirza, R.S., Chivers, D.P., 2000. Predator-recognition training enhances survival of brook trout : evidence from laboratory and field- enclosure studies. *Can. J. Zool.* 2208, 2198–2208.
- Norrdahl, K., Korpimäki, E., 1998. Fear in farmlands: how much does predator avoidance affect bird community structure? *J. Avian Biol.* 29, 79–85.
- Orlofske, S.A., Hopkins, W.A., 2009. Energetics of metamorphic climax in the pickerel frog (*Lithobates palustris*). *Comp. Biochem. Physiol. Part A* 154, 191–196. doi:10.1016/j.cbpa.2009.06.001
- Parish, D.M.B., Sotherton, N.W., 2007. The fate of released captive-reared grey partridges *Perdix perdix*: implications for reintroduction programmes. *Wildlife Biol.* 13, 140–149.
- Petersson, E., Valencia, A.C., Järvi, T., 2015. Failure of predator conditioning: an experimental study of predator avoidance in brown trout (*Salmo trutta*). *Ecol. Freshw. Fish* 24, 329–337. doi:10.1111/eff.12146
- Pfeiffer, W., 1962. The fright reaction of fish. *Biol. Rev.* 37, 495–511. doi:10.1111/j.1469-185X.1962.tb01333.x
- Poledník, L., Řehulka, J., Kranz, A., Poledníková, K., Hlaváč, V., Kazihnitkovi, H., 2008. Physiological responses of over-wintering common carp (*Cyprinus carpio*) to disturbance by Eurasian otter (*Lutra lutra*). *Fish Physiol. Biochem.* 34, 223–234. doi:10.1007/s10695-007-9180-3
- R Core Team, 2016. R: A Language and Environment for Statistical Computing. R Found. Stat. Comput. Vienna, Au, <http://www.R-project.org/>.

- Reed, D.H., Lowe, E.H., Briscoe, D.A., Frankham, R., 2003. Fitness and adaptation in a novel environment: effect of inbreeding, prior environment, and lineage. *Evolution* (N. Y). 57, 1822–1828. doi:10.1554/02-601
- Relyea, R.A., 2004. Fine-tuned phenotypes: tadpole plasticity under 16 combinations of predators and competitors. *Ecology* 85, 172–179.
- Repka, S., Walls, M., Kettle, M., 1995. Neck spine protects *Daphnia Pulex* from predation by *Chaoborus*, but individuals with longer tail spine are at a greater risk. *J. Plankton Res.* 17, 393–403. doi:10.1093/plankt/17.2.393
- Sapolsky, R.M., 2002. Endocrinology of the stress-response, in: Becker, J.B., Breedlove, S.M., Crews, D., McCarthy, M.M. (Eds.), *Behavioral Endocrinology*. MIT Press, Cambridge, pp. 409–450.
- Schoeppner, N.M., Relyea, R.A., 2005. Damage, digestion, and defence: The roles of alarm cues and kairomones for inducing prey defences. *Ecol. Lett.* 8, 505–512. doi:10.1111/j.1461-0248.2005.00744.x
- Schoeppner, N.M., Relyea, R.A., 2008. Detecting small environmental differences: risk-response curves for predator-induced behavior and morphology. *Oecologia* 154, 743–754. doi:10.1007/s00442-007-0862-4
- Schoeppner, N.M., Relyea, R.A., 2009a. When should prey respond to consumed heterospecifics? Testing hypotheses of perceived risk. *Copeia* 2009, 190–194. doi:10.1643/CE-08-041
- Schoeppner, N.M., Relyea, R.A., 2009b. Phenotypic plasticity in response to fine-grained environmental variation in predation. *Funct. Ecol.* 23, 587–594. doi:10.1111/j.1365-2435.2008.01525.x
- Sih, A., McCarthy, T.M., 2002. Prey responses to pulses of risk and safety: testing the risk allocation hypothesis. *Anim. Behav.* 63, 437–443.
- Slos, S., Stoks, R., 2008. Predation risk induces stress proteins and reduces antioxidant defense. *Funct. Ecol.* 22, 637–642. doi:10.1111/j.1365-2435.2008.01424.x
- Srean, P., Almeida, D., Rubio-Gracia, F., Luo, Y., Garcia-Berthou, E., 2017. Effects of size and sex on swimming performance and metabolism of invasive mosquitofish *Gambusia holbrooki*. *Ecol. Freshw. Fish* 26, 424–433. doi:10.1111/eff.12286
- Steiner, U.K., Buskirk, J. Van, 2009. Predator-induced changes in metabolism cannot explain the growth / predation risk tradeoff. *PLoS One* 4, 2–5. doi:10.1371/journal.pone.0006160

- Teixeira, C.P., Schetini De Azevedo, C., Mendl, M., Cipreste, C.F., Young, R.J., 2007. Revisiting translocation and reintroduction programmes: the importance of considering stress. *Anim. Behav.* 73, 1–13. doi:10.1016/j.anbehav.2006.06.002
- Van Buskirk, J., Muller, C., Portmann, A., Surbeck, M., 2002. A test of the risk allocation hypothesis: tadpole responses to temporal change in predation risk. *Behav. Ecol.* 13, 526–530. doi:10.1093/beheco/13.4.526
- Van Buskirk, J., Relyea, R.A., 1998. Selection for phenotypic plasticity in *Rana sylvatica* tadpoles. *Biol. J. Linn. Soc.* 65, 301–328.
- Van Dievel, M., Janssens, L., Stoks, R., 2016. Short- and long-term behavioural, physiological and stoichiometric responses to predation risk indicate chronic stress and compensatory mechanisms. *Oecologia* 181, 347–357. doi:10.1007/s00442-015-3440-1
- Werner, E.E., Anholt, B.R., 1996. Predator-induced behavioral indirect effects: consequences to competitive interactions in anuran larvae. *Ecology* 77, 157–169. doi:10.2307/2265664
- Woodward, J.J., Smith, L.S., 1985. Exercise training and the stress response in rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Biol.* 26, 435–447. doi:10.1111/j.1095-8649.1985.tb04283.x
- Young, P.S., Cech, J.J.J., 1993. Effects of exercise conditioning on stress response and recovery in cultured and wild young-of-the-year striped bass, *Morone saxatilis*. *Can. J. Fish. Aquat. Sci.* 50, 2094–2099.

CHAPTER 3. A NOVEL BIOAUGMENTATION TECHNIQUE EFFECTIVELY INCREASES THE SKIN-ASSOCIATED MICROBIAL DIVERSITY OF CAPTIVE EASTERN HELLBENDERS

3.1 Abstract

Captive environments are maintained in hygienic ways to prevent the spread of disease, but can also lack natural microbial reservoirs. As a result, captive animals often have depauperate host-associated microbial communities compared to conspecifics in the wild and may have increased disease susceptibility and reduced immune function. Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) are reared in captivity before being translocated to the wild. To prepare hellbenders for natural bacteria found in riverine environments, I devised a novel bioaugmentation method to increase the diversity of skin microbial communities and bolster host immune capacity within a captive setting. I exposed three-year-old hellbenders to increasing amounts of river water over five weeks before translocating them to the river. I swabbed skin for microbiota assays, drew blood for immune assessments, and collected samples of river water every week, before and after translocation. I genetically identified and phylogenetically compared bacteria collected from skin swabs and river water for alpha (species richness) and beta (community composition) diversity estimates. I found that treatment hellbenders exposed to undiluted river water in captivity had higher alpha diversity and distinct differentiation in the community composition on their skin, compared to control hellbenders only exposed to well water. I also found strong evidence that hellbender skin microbiota is host specific rather than environmentally driven and is colonized by rare environmental operational taxonomic units (OTUs) in river water. Exposing hellbenders to river water in captivity was an effective method to increase bacterial colonization and produced similar changes on the skin as release into the river. Treatment hellbenders had elevated neutrophil to lymphocyte (N:L) ratios without any change in alpha diversity upon release, which might suggest an immune response preventing the colonization of potential pathogenic species. This novel technique could have beneficial effects on future translocation efforts as increasing microbial diversity prior to release could be correlated with elevated disease resistance in the wild. Future work will be necessary to refine my methods, investigate the relationship between microbial diversity and hellbender

health, and understand how this novel bioaugmentation technique influences hellbenders' survival following translocation from captivity into the wild.

3.2 Introduction

Host microbial communities are important for metabolism, vitamin production, resilience to stress and environmental change, defense against pathogens, and immune responses (Woodhams et al. 2007; Turnbaugh et al. 2007; Loudon et al. 2014). Subsequently, a stable and diverse cutaneous microbiome is positively correlated with host health and immune function (Woodhams et al. 2011; Antwis et al. 2014; Loudon et al. 2014). Amphibians acquire diverse bacteria from their surrounding environment; being in direct contact with bacterial reservoirs can lead to colonization of rare or transient species (Loudon et al. 2014). Disturbances, such as removing eggs or animals from their natural environment and placing them into captivity, can be a major perturbation to amphibian skin microbiota (Redford et al. 2012; Loudon et al. 2014). Captive environments often lack natural microbial reservoirs, have filtration systems that remove microbes, and are maintained in hygienic ways to prevent the spread of disease. Thus, individuals brought to, or reared in, captivity quickly lose cutaneous microbe diversity because of aseptic rearing conditions and have little to no opportunity for environmental bacteria to re-colonize their skin (Antwis et al. 2014; Becker et al. 2014; Loudon et al. 2014). This results in significantly different skin microbial diversity among captive amphibians compared to wild conspecifics (e.g., golden frog [*Atelopus zeteki*] and fire-bellied toads [*Bombina orientalis*]; Becker et al. 2014; Bataille et al. 2016).

Without diverse skin microbiota and previous exposure to naturally occurring microorganisms, an individual's immune system may be underdeveloped and lack specialized responses to natural threats (Magnadóttir 2006; Richmond et al. 2009). Subsequently, animals in captivity likely have depauperate and atypical skin microbiota, naïve immune systems, and may be more susceptible to pathogens and disease following introduction to a natural system (Alberts 2007; Boyce et al. 2011; Loudon et al. 2014; Sabino-Pinto et al. 2016). To increase skin microbial diversity on captive animals, rearing programs are incorporating probiotics, pre-exposure, or bioaugmentation strategies (Moriarty 1999; Irianto and Austin 2002; Harris et al. 2009; Becker and Harris 2010; Merrifield et al. 2010; Woodhams et al. 2011). However, these techniques do not always increase disease resistance (e.g., *Atelopus* species; Becker et al. 2012),

often focus on exposing only one microorganism at a time, and are commonly directed toward resistance against the fungus *Batrachochytrium dendrobatidis*. However, amphibians are naturally exposed to a variety of bacterial, viral, and fungal organisms in soil and water; therefore, an environmental reservoir with a suite of microbes (6×10^{12} bacterial cells/m² of soil, 10×10^5 bacterial cells/mL of river water; Whitman et al. 1998) would better mimic natural conditions, compared to a single-species probiotic. Although a more complex bioaugmentation method has the potential to unintentionally introduce pathogens, presenting free-flowing microbes in captivity provides amphibians the opportunity to prime their immune systems to naturally occurring microorganisms prior to release. Furthermore, the safety of captivity allows animals to be monitored and treated if disease symptoms develop.

Captive-rearing efforts are underway to conserve wild populations of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*). These large, fully-aquatic salamanders historically occurred throughout much of the eastern and central United States (Mayasich and Phillips 2003). However, over the past few decades, hellbender populations have exhibited precipitous declines and are now listed as threatened or endangered throughout much of their range (Mayasich and Phillips 2003; Burgmeier et al. 2011). In Indiana, hellbenders are state-endangered and exist only in the Blue River in southern Indiana, making them extremely vulnerable to extirpation (Kern 1984; Wheeler et al. 2003; Unger et al. 2013). In captivity, hellbenders are reared in homogeneous environments that are nearly devoid of free-floating microbes present in a riverine environment; thus, limiting environmental transmission to the host-associated skin microbiome (Ettling et al. 2013). Furthermore, hellbenders experience parental care in the wild (Nickerson and Mays 1973), which can facilitate vertical transmission of skin microbiota from fathers to their offspring (Walke et al. 2011; Hughey et al. 2017). However, hellbenders are usually removed from the river as eggs before they have been exposed to river or parental microbiota as larvae (Personal comm., O. Hernández-Gómez; Kenison and Williams, In Press). Subsequently, captive hellbenders have distinctly different microbial communities than wild conspecifics (Hernández-Gómez, unpublished data). Furthermore, young hellbenders have weaker immune systems than adult hellbenders, making it especially important for them to diversify their skin microbiome and be exposed to potential pathogenic microbes before translocation (Hopkins et al. 2016). Without a diverse microbiome, novel microorganisms have the potential to negatively influence the immune system of naïve, captive-reared individuals following release into the wild

(Smith et al. 2009; Becker and Harris 2010; Boyce et al. 2011; Sabino-Pinto et al. 2016). This becomes a major concern for hellbender augmentation efforts in Indiana, where rapid population losses necessitate maximizing translocation success.

Translocation programs have been developed to stabilize declines and bolster remaining hellbender populations, but have had varying levels of success (17–72% survival, Bodinof 2010; Boerner 2014; Kraus et al. 2017). Hygienic captive-rearing environments, depauperate skin microbial communities, or increased susceptibility to pathogens in the wild may all influence the success of these programs (Redford et al. 2012; Becker et al. 2014). I developed a novel form of bioaugmentation to address the dissimilarities in skin microbial communities between captive and wild hellbenders. My primary goals were to investigate: a) whether exposure to river water in a captive setting could increase microbial diversity on the skin of captive-reared hellbenders, b) if communities found on the skin following inoculation resemble those found in river water, and c) how microbial diversity of pre-exposed and naïve hellbenders change following release into the wild. Secondarily, I examined the influence of river water exposure and translocation into the wild on hellbender immune parameters (i.e., white blood cell counts). Exposing hellbenders to river water, and its diverse microbiome, prior to release may be the most effective way to increase their cutaneous defenses against potentially pathogenic bacteria, prepare them immunologically for their first exposure to free-flowing bacteria, and facilitate a successful transition from captivity to the river.

3.3 Methods

I incrementally exposed captive hellbenders to river water to facilitate the colonization of naturally-occurring cutaneous microbiota and diversify symbiotic skin microbes in a captive setting, before I released them into their natal Indiana river. I altered the biotic environment in their tanks each week for five weeks to more closely mimic natural, riverine conditions in captivity. At the end of each experimental week, I collected skin microbiota samples to assess changes in alpha (species richness) and beta (community composition) diversity, and blood samples to assess physiological changes in the health or stress of hellbenders (Duncan et al. 1994; Solís et al. 2007; DuRant et al. 2015; Hopkins et al. 2016; Jimenez and Sommer 2016). Vertebrates produce leukocytes to defend against infection, inflammation, stress, and disease (Weiss and Wardrop 2010). Neutrophil to lymphocyte (N:L) ratios can act as a proxy for stress

or immune response (Davis and Maerz 2008; Davis and Maerz 2011; Hopkins et al. 2016). I also collected river water samples to compare similarities between hellbender skin and the riverine microbiome. Following the five-week experimental period in captivity, I released all hellbenders for seven days into cages within the river and then collected skin microbiota and blood samples: this totaled six sampling weeks between captivity and the field.

3.3.1 Experimental design

The hellbenders used for this study were collected as eggs from the Blue River in southern Indiana in 2013 and were reared at Purdue University's Aquaculture Research Laboratory without any exposure to Blue River water until the start of this experiment – three years after hatching. On 21 June 2016, I haphazardly chose 36 three-year-old hellbenders for this study. I identified each hellbender by unique markings on their dorsum and tail and randomly assigned them to six separate 132 L tanks (six hellbenders per tank). I housed all tanks on a two-tiered rack in a temperature-controlled room ($14^{\circ}\text{C} \pm 1^{\circ}\text{C}$). I provided hellbenders with tile hides as refugia and black worms (*Lumbriculus variegatus*) every three days as food. Half of the individuals were control individuals and only received UV-sterilized and filtered well water ($n = 18$) while treatment individuals ($n = 18$) received supplementary river water. I placed all treatment tanks on the bottom rack, with control tanks stacked above them, to reduce cross contamination between the treatment and control groups. I waited one week before adding river water, to collect baseline skin microbiota and hematology data as well as allow hellbenders to acclimate to their rearing tanks. I added 100 mL of river water to treatment tanks during the second week and increased the amount by an order of magnitude every week thereafter (100 mL, 1 L, 10 L, and then 100 L, respectively). Following the addition of river (treatment tanks) or filtered tap (control tanks) water, I maintained static conditions within all tanks for seven-day exposure periods. Previous studies have successfully inoculated amphibians with a probiotic bath for 2-48 hours; therefore, week-long exposures should provide ample time for bacterial colonization (Harris et al. 2009; Vrendenburg et al. 2011; Bletz et al. 2013). I retrieved new Blue River water for treatment tanks at the beginning of weeks two through five. I collected water in five gallon buckets, drove the water three hours to Purdue University in coolers, and then added it directly to treatment tanks. I conducted complete water changes for all treatment and control tanks at the beginning of each week.

After the last lab-sampling event (week five), I transported hellbenders (see Kenison et al. 2016) to the Blue River in southern Indiana. Treatment hellbenders were placed together in one cooler of the transport system, but were separated from control individuals for the duration of travel to the river. I placed all 36 hellbenders (18 treatment and 18 controls) into hardware mesh cages (1'x3'x3'), being sure to separate hellbenders by treatment and control groups, with tile hides for refuge, closed them securely, and let them remain in the river for seven days. The cages allowed me to collect hellbenders at the end of the experiment for one more sampling event rather than permanently releasing them into the river.

3.3.2 Skin microbiota

I swabbed the dorsum of each hellbender at the beginning of the experiment (28 June, 2016) and every week thereafter (6 July through 5 August, 2016) to collect skin-associated microbiota (similar to Loudon et al. 2014). I chose not to identify each individual based on skin spot patterns prior to swabbing to minimize handling time between animal collection and microbiota sampling. I handled hellbenders and sampled their microbiome following the protocol of Hernández-Gómez et al. (2017b); being sure to change gloves between individuals, rinse each hellbender with 1 L of autoclaved water, and rub sterile cotton-tipped swabs (Medline Industries Inc., Mundelein, Illinois) along their dorsum for 30 seconds. I stored the sample swabs in 1.5 mL microcentrifuge tubes. When I collected swabs in captivity, I immediately placed tubes in a -20° C freezer and moved them on ice to a -80° C freezer within two hours of collection. In the field, I stored swab tubes in liquid nitrogen and placed them in a -80° C freezer upon return to the laboratory within 24 hours of collection.

During each sampling event in captivity, I collected 1 L of water from each of the control and treatment tanks (n = 6) after swabbing. I also collected 2 L of river water directly from the Blue River each week when I retrieved water for treatment tank inoculations and following translocation to the river to assess the microbial community of the river through time. I stored water samples in a cooler and filtered them in an aseptic environment within 12 hours of collection using a Whatman glass microfiber filter (GE Healthcare, Chicago, IL). I stored all filters in 15 mL centrifuge tubes and placed them in a -80° C freezer until I isolated the DNA.

I extracted DNA from skin swabs using a PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA), following the modified protocol of Hernández-Gómez et al.

(2017b). I also used the PowerWater DNA Isolation Kit to extract DNA from water filters using the manufacturer's instructions. I used the primer set 27F/338R to amplify the 16S rRNA gene V2 region. I ran all samples in triplicate, each with 25 μL of reaction volume: 5 μL template DNA, 1X MyTaq Master Mix (Bioline, Tauton, MA), 0.7 μM of forward and reverse primers, and 6.5 μL of water (MoBio Laboratories, Inc., Carlsbad, CA). I ran the PCRs with 2 min at 95° C, 30 cycles for 45 sec at 94°C, 60 sec at 50°C, 90 sec at 72°C, and 10 min at 72°C. I pooled the triplicates from each sample, cleaned the PCR products with an UltraClean PCR Clean-up kit (MoBio Laboratories, Inc., Carlsbad, CA), and then performed a second PCR. My second PCR was used to prepare the sequencing library; I added on dual-index barcodes connected to Illumina sequencing adaptors to the end of amplicons (Hernández-Gómez et al. 2017b). I ran each PCR sample with 15 μL of reaction volume: 5 μL clean amplicons, 1X MyTaq Master Mix (Bioline, Tauton, MA), 0.7 μM of forward and reverse barcode primers, and 1.5 μL of water (MoBio Laboratories, Inc., Carlsbad, CA). I ran the second PCR with 2 min at 95° C, 5 cycles for 45 sec at 94°C, 60 sec at 65°C, 90 sec at 72°C, and 10 min at 72°C. I measured the DNA concentrations of my second PCR products using a Qubit Fluorometer 2.0 (Invitrogen Corp, Carlsbad, CA) and pooled my samples in equimolar amounts. I used the Reagent Kit V2 on a MiSeq machine (Illumina, Inc., San Diego, CA) at the Purdue Genomics Core Facility to sequence the sample pool and produce 250 bp paired-end reads. I processed raw sequence reads, assigned operational taxonomic units, and generated species abundance tables for all skin swabs and water samples following Hernández-Gómez et al. (2017). In brief, I implemented custom Phyton programs developed by Hernández-Gómez et al. (2017b) and the Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1 pipeline (Caporaso et al. 2011) to filter erroneous reads, cluster reads into operational taxonomic units (OTUs), and generate abundance based OTU tables using the open-open reference protocol (Rideout et al. 2014). To limit the inclusion of any OTUs derived from sequencing errors, such as base miscalls or chimeras, I filtered out OTUs that represented less than 0.005% of the total read count (Bokulich et al. 2013). To standardize OTU read depth across all samples, I rarified the OTU table to 1699 reads per sample.

3.3.3 Hematology

I drew blood from each hellbender following weekly skin microbiota swabbing. I was not restricted by handling time and therefore identified each hellbender by their unique spot patterns. I collected 0.1-0.2 mL of blood from their caudal vein using a 1cc pre-heparinized syringe. I collected blood at the end of each experiment week for a total of six blood draws between June and August, 2016. I made two blood smears for each individual and let them air dry. Once slides were dry, I stained them using a Wright-Giemsa stain kit (Volu-Sol) and examined them under a microscope with 40x magnification. I examined slides in a zigzag pattern to inspect as much of the slide as possible. I identified five types of leukocytes: lymphocytes, neutrophils, basophils, eosinophils, and monocytes. I counted leukocytes in each field of vision until I reached a total of 100 white blood cells. I only calculated the proportion of lymphocytes and neutrophils (number of specified blood cell/total leukocytes counted), because basophils, eosinophils, and monocytes each accounted for < 10% of white blood cells. I also calculated N:L ratios by dividing the total number of neutrophils by the number of lymphocytes counted per slide.

3.3.4 Statistical Analyses

3.3.4.1 Skin microbiota

I ran all analyses in program R version 3.3.2 (R Core Team 2016). I investigated differences in skin microbiota between treatment and control hellbenders across the five weeks in captivity and after release into the river at week six. I assessed differences in skin microbiome community richness (observed OTUs) and community diversity (Shannon diversity indices), both of which were calculated in QIIME using the relative-abundance based OTU table. I used generalized linear mixed-effects models to compare treatment and control groups, while accounting for repeated measures. I did not identify each hellbender prior to swabbing; therefore, I assigned 'rearing tank' as my random effect. I included 'week' and 'treatment' as my fixed effects, and tested for two-way interactions between 'treatment' and 'week'. I treated 'week' as a continuous and categorical variable to make linear and by-week comparisons. I used the package 'nlme' for mixed effects analyses and report estimates with standard errors.

I also tested for differences in skin microbiome community composition (beta diversity) by comparing the phylogenetic differences of OTUs between groups. I used the Newick phylogenetic tree with the OTU table in program R to create unweighted UniFrac and Bray-

Curtis distance matrices using the packages ‘GUniFrac’ and ‘vegan’. Unweighted distance matrices take into account the presence/absence of observed OTUs and incorporate phylogenetic distances while Bray-Curtis methods account for OTU abundance in dissimilarity distance matrices (Lozupone and Knight 2005; Hernández-Gómez et al. 2017b). I performed Adonis and Anosim tests (package ‘vegan’) using unweighted UniFrac and Bray-Curtis distances to partition the variation between control and treatment groups. I visualized differences in community composition between control and treatment groups at each week and community composition across weeks by plotting principle coordinate analyses (PCoAs) using unweighted UniFrac and Bray-Curtis distances (package ‘phyloseq’). I also compared the proportion of shared OTUs between control and treatment hellbenders to identify similarities between groups. I conducted a linear regression with a Gaussian distribution to detect changes in the number of shared OTUs through time. Lastly, I compared the composition of phyla that were found in relative abundances greater than 2% on the skin of control and treatment hellbenders during each of the six experimental weeks. I report percent differences and model estimates with standard errors.

3.3.4.2 Environmental microbiota

I also compared the microbial communities found on hellbenders to river water. I compared the number of OTUs control and treatment individuals shared with their tank water and with river water. I conducted linear regressions to test for differences in the number of OTUs control and treatment individuals shared with their tank and river water samples through time. Furthermore, I used relative abundance estimates from the river water OTU table to compare what, if any, bacterial species were shared between treatment individuals and river water. I also identified whether abundant bacterial species in river water were found in similar abundance on the skin of treatment hellbenders.

3.3.4.3 Hematology

I used generalized linear mixed-effects models to compare the proportion of neutrophils and lymphocytes across the five-week experimental period in captivity, and to evaluate differences between treatment and control groups at week six after river release. I accounted for repeated measures by nesting ‘individual’ within ‘rearing tank’ and assigning them as random effects. I ran models with a binomial distribution and logit link to account for proportional data. I

also compared N:L ratios between treatment and control individuals through time. I log-transformed N:L ratios to meet normality and conducted linear mixed-effects models with a Gaussian distribution. I included ‘individual’ nested within ‘rearing tank’ as my random effect to account for repeated measures. I back-transformed all model estimates and report proportions and percent differences with standard errors.

3.4 Results

3.4.1 Skin microbiota

I found evidence for differences in species richness on hellbender skin across the five-week experimental period in captivity that depended on group (Figure 3.1). There was no difference in species richness between groups at week one (estimated difference = 46.9 OTUs, t value = 2.12, $P = 0.091$). However, species richness on the skin of treatment hellbenders increased more quickly than species richness on the skin of control individuals, between weeks one and five (estimated slope difference = 20.1 OTUs per week, t value = 3.71, $P = 0.003$). Furthermore, by week five, when treatment hellbenders were exposed to undiluted river water, they had 22% greater richness than control individuals (estimated difference = 66.6 OTUs, t value = 3.35, $P = 0.001$; Figure 3.2). Treatment individuals had a 25% increase in species richness from week four to week five, but no change in richness between weeks five and six (Figure 3.2). In contrast, control individuals had a slower change in species richness across the first five weeks of the experiment. However, control individuals had a 28% increase in species richness between week five in captivity and week six in the river, which was similar to treatment individuals between weeks four and five (Figure 3.2). In week six, following caged-release into the river, treatment individuals had 15% fewer OTUs compared to control individuals (estimated difference = 51.0 OTUs, t value = 2.54, $P = 0.012$).

I found trends in Shannon diversity were similar to those in species richness through time (Figure 3.1). There was no difference in Shannon diversity at week one between groups (estimated difference = 0.58 H, t value = 1.70, $P = 0.164$). However, Shannon diversity on the skin of treatment individuals increased more rapidly compared to diversity on the skin of control individuals between weeks one and five (estimated slope difference = 0.24 H, t value = 2.82, $P = 0.005$; Figure 3.1). At week five, treatment individuals had 10% greater diversity than control

individuals (estimated difference = 0.63 H, t value = 2.01, $P = 0.046$; Figure 3.2). After caged-release into the river, both treatment and control individuals had similar Shannon diversity on their skin ($P = 0.167$; Figure 3.2).

I found distinct differentiation in community composition measurements between control and treatment hellbenders at each of the experimental weeks (Table 3.1). PCoA plots demonstrated overlapping community composition at week one, but distinct separation between groups at week five (Figure 3.3). In week five, the variation accounted for by treatment was nearly twice as much as in week one (week one: Adonis $R = 0.073$ and Anosim $R = 0.314$; week five: Adonis $R = 0.17$, Anosim $R = 0.77$ for unweighted UniFrac, Table 3.1). Following caged-release into the river, control and treatment hellbenders had overlapping microbial communities and little variation explained by group (week six: Adonis $R = 0.068$, Anosim $R = 0.162$ for unweighted UniFrac; Figure 3.3). I also found significant differences in beta diversity across all six weeks combined, with week explaining the most variation (unweighted Adonis $R = 0.268$, $P = 0.001$, Bray-Curtis Adonis $R = 0.342$, $P = 0.001$). PCoA plots demonstrated that the largest separation in community composition across weeks occurred between week six and weeks one through five (Figure 3.4).

Hellbender skin was dominated by bacteria from five different phyla (Figure 3.5). Proteobacteria was the most abundant phylum of both treatment and control groups and had the highest relative abundance across all six weeks (relative abundance > 13%). Bacteroidetes, Acidobacteria, and Actinobacteria were the three other most dominate phyla and were found on control and treatment groups during the first five weeks of the experiment. The relative abundance of these three phyla varied slightly across weeks; however, they were all detected with relative abundances > 2% during at least four of the six weeks. Control and treatment individuals gained an additional phylum, Firmicutes, after release into the river in week six.

Treatment and control hellbenders shared an average of 842 OTUs across the six experimental weeks (Standard Deviation [SD] ± 43). There were no differences in the number of shared OTUs between control and treatment individuals through time (estimated change per week = -0.2 OTUs, t value = -0.017, $P = 0.987$).

3.4.2 Environmental microbiota

Treatment hellbenders shared on average 411 OTUs ($SD \pm 94$) with their tank water and control individuals shared on average 423 OTUs ($SD \pm 70$) with their tank water during the experiment. There were no significant differences in the number of OTUs treatment or control individuals shared with their tank across the experimental period (estimated change per week = -2.3 OTUs, t value = -0.07, $P = 0.951$; estimated change per week = 3 OTUs, t value = 0.13, $P = 0.908$, respectively). In general, I did not observe strong similarities between the OTUs found on the skin of treatment individuals and the OTUs found in river water, and this trend did not change significantly as I incrementally supplemented more river water into treatment tanks (estimated change per week = -22.9 OTUs, t value = -0.84, $P = 0.489$). Between weeks two and four, treatment individuals shared on average 222 OTUs with river water and control individuals shared 169 OTUs with river water. However, I did detect a change in shared OTUs following exposure to undiluted river water in week five. Treatment individuals shared 33.7% more OTUs with river water compared to control individuals (222 OTUs versus 147 OTUs, respectively). In captivity, treatment hellbenders shared 13% of their OTUs with river water while control hellbenders only shared 9%. Of the most abundant OTUs in river water, I found low relative abundances on the skin of treatment individuals, especially during the first five weeks in captivity (Table 3.2).

3.4.3 Hematology

Control and treatment individuals had differences in lymphocyte counts at the beginning of the experiment and through time that depended on group (intercept: z value = -0.72, $P < 0.001$; interaction: z value = 21.37, $P < 0.001$; Figure 3.6). Lymphocyte counts increased linearly for treatment individuals across the five-week experimental period in captivity (z value = 11.42, $P < 0.001$), but decreased through time for control individuals (z value = -18.95, $P < 0.001$). After the river release at week six, treatment individuals had lower lymphocytes compared to control individuals (proportional difference = -0.146 lymphocytes, z value = 6.03, $P < 0.001$). There were also differences in neutrophil counts at week one and through time that depended on group (intercept: z value = 3.94, $P < 0.001$; interaction: z value = -18.03, $P < 0.001$; Figure 3.6). Treatment individuals decreased their neutrophil counts across the five weeks in captivity (z value = -4.94, $P < 0.001$), while control individuals increased neutrophils counts through time (z

value = 21.15, $P < 0.001$). Treatment individuals had more neutrophils following river release compared to control individuals (proportional difference = 0.150 neutrophils, z value = 6.58, $P < 0.001$). I found experimental week to be an important predictor for differences in N:L ratios between the treatment and control groups (t -value = 3.13, $P = 0.002$, Figure 3.7). Contrary to my expectations, I found control individuals increased their N:L ratio through time (estimated slope = 1.39, t value = 7.41, $P < 0.001$), while treatment individuals slightly decreased their N:L ratios across the five weeks in captivity (estimated slope = 0.89, t value = -2.64, $P = 0.009$). There were no differences in N:L ratios between groups at week one (estimated difference = 0.16, t value = -2.64, $P = 0.058$). However, treatment individuals had N:L ratios 60% higher than controls in week six (estimated difference = -0.611, t -value = 7.7, $P = 0.002$; estimated difference = 0.319, t -value = 3.64, $P = 0.022$, respectively).

3.5 Discussion

I developed a novel environmental inoculation technique that effectively increased hellbender skin microbial community richness and diversity in captivity. I observed the largest increase in alpha diversity on the skin of treatment hellbenders following exposure to undiluted river water (week five) and this change remained after hellbenders were released into the river (week six). Increases in alpha diversity are correlated with increased disease resistance and stability of the skin microbiome (Dillon et al. 2005; Becker et al. 2017). Increasing species richness increases the likelihood of the skin harboring anti-pathogenic bacterial species that can protect against disturbances (i.e., infections, Antwis et al. 2014). Although community composition on the skin of control hellbenders was comparable to treatment hellbenders following translocation into the river, elevating hellbenders' disease resistance, cutaneous microbiome stability, and immune protection prior to release could greatly improve their success in the wild. For example, control hellbenders naïve to the river had higher species richness on their skin following release, which may be evidence of poor cutaneous defenses and the establishment of opportunistic and potentially pathogenic species on their skin. Few studies have investigated the use of environmental reservoirs to increase alpha diversity, yet I provide clear evidence that inoculating hellbenders with river water, for five weeks, leads to a more specious microbial skin community in a captive setting. Furthermore, supplementary river water was as effective a reservoir for bacterial colonization as the river itself, because treatment individuals

exposed to undiluted water at week five had similar alpha and beta diversities as control individuals released into the river at week six. Being in constant and complete contact with an environmental reservoir quickly facilitated the immigration and colonization of new species on hellbender skin prior to release. For example, control and treatment hellbenders had distinctly different community compositions (i.e., beta diversity) at week five, but complete separation was lost within just seven days of all control and treatment hellbenders being released into the river. Eastern red-backed salamanders (*Plethodon cinereus*) that are in constant presence of a soil bacterial reservoir receive environmental transmission, maintain stable alpha diversity, and show differences in beta diversity after just seven days of exposure (Muletz et al. 2012; Loudon et al. 2014). These, and my study, clearly demonstrate that environmental reservoirs support the immigration of new bacteria and the establishment of numerous, potentially beneficial, species on the skin of amphibians (Loudon et al. 2014).

Although inoculation was successful, differentiations between skin bacterial communities in captivity and following river release suggest my river water reservoirs need to more closely match the microbial composition of a natural, riverine environment. I detected strong differences in the community composition of skin microbiota before and after translocation, regardless of treatment. This suggests that although undiluted river water is proficient at inoculating hellbenders, there is something distinct about the wild environment that is not fully transferred to or maintained in captivity. Although a river's bacterial community is extremely diverse, the species on hellbender skin may be dependent on what hellbenders are interacting with rather than the water alone (e.g., rocks, substrate, other organisms such as fish or mussels; Whitman et al. 1998; Walke et al. 2014; Rebollar et al. 2016). For example, the phylum Firmicutes was detected in high relative abundance on control and treatment hellbenders only after they were released into the river. This phylum is frequently found on the skin of wild hellbenders, and must thereby be abundant in or specific to their wild environment (Hernández-Gómez et al. 2017a). Alternatively, the same phylum (Firmicutes) is less common among captive eastern red-backed salamanders (*P. cinereus*) without a microbial reservoir, and is suspected to need regular seeding in order to remain present on the skin (Loudon et al. 2014). Thus, there must be some sources of bacteria in the wild that cannot thrive or be cultivated in captivity, or more simply, were missed during river water collection (Becker et al. 2014). Bacterial species initially present in experimental treatment water, transported from the Blue River, may have also died during the

seven-day bathing period. Rearing tanks were maintained with static water rather than a flow-through continually moving design, which may have selected for bacterial species that can thrive in stagnant, nonmoving environments rather than those reliant on lotic systems. Once bacteria are lost, for any variety of reasons, they become less and less common without a fresh reservoir for replenishment, and are thus less likely to colonize skin (Muletz et al. 2012; Bletz et al. 2013). Furthermore, more rare species are often at a higher risk of loss during stochastic events, such as temperature change or amphibian skin sloughing (Meyer et al. 2012; Bletz et al. 2013; Loudon et al. 2014). Therefore, bacteria may have colonized hellbender skin during the first 48 hours of inoculation, but were lost or unable to recolonize by the time I collected skin swabs.

Even with some dissimilarity between my inoculation environment and the wild river, I found that captive hellbenders had similar core microbial skin communities to wild con- and hetero-specifics. Wild-captured eastern hellbenders have core OTUs composed primarily of Proteobacteria, Actinobacteria, Firmicutes, Cyanobacteria, and Verrucomimicrobia (Hernández-Gómez et al. 2017a). Wild bullfrogs' (*Rana catesbeiana*) and red-spotted newts' (*Notophthalmus viridescens*) skin are dominated primarily by Proteobacteria, but also colonized by Actinobacteria, Firmicutes, and Bacteroidetes (Walke et al. 2014). The most abundant phylum on the skin of control and treatment hellbenders was Proteobacteria, followed by Actinobacteria, Bacteroidetes, Acidobacteria, and Firmicutes. These same patterns are seen among captive Panamanian frog species (*Agalychnis callidryas*, *Craugastor fitzingeri*, and *Dendropsophus ebraccatus*), with nearly 60% of their relative abundance accounted for by Proteobacteria, and lesser contributions by Actinobacteria, Bacteroidetes, and Firmicutes (Belden et al. 2015).

The similarities in dominant phyla between wild and captive hellbenders demonstrate that hellbenders have a core microbiota on their skin that is host specific and that remains comparable under variable conditions. Host specificity is a driving factor in shaping amphibian skin microbiota (McKenzie et al. 2012; Walke et al. 2014). Such that, species are significant predictors of amphibian skin bacterial diversity, rather than location or environment (McKenzie et al. 2012). I found control and treatment individuals to have the most shared OTUs with each other, regardless of the experimental week. The similarities in skin bacterial species may have been driven by the fact that control and treatment hellbenders likely cohabited in the same tank prior to the beginning of this experiment. All hellbenders would have been reared together after they first hatched, which is a critical time for microbial establishment (Bletz et al. 2013).

Hellbenders commonly lie on or beside one another, share tile hides, and interact with each other during feeding, these interactions may have facilitated horizontal transmission between conspecifics (Moran and Dunbar 2006). Furthermore, all hellbenders were fed the same black worm (*Lumbriculus variegatus*) diet. I wanted to maintain similar rearing conditions between groups, but diet can influence community composition in the gut, and ultimately, affect bacterial abundance and species richness on the skin (Antwis et al. 2014).

Even with similarities between control and treatment hellbenders, I found clear evidence that amphibian microbial skin communities do not always match the environment they are in, which is similar to other experimental studies (Kueneman et al. 2014; Walke et al. 2014; Sabino-Pinto et al. 2016). Treatment hellbenders had very few OTUs shared with tank or river water. Of the most dominant OTUs among treatment individuals, some were not even found in river water. Additionally, of the most abundant OTUs found within river water, very few had a relative abundance greater than 0.1% on the skin of hellbenders exposed to undiluted river water. Treatment hellbenders only shared 13% of their OTUs with river water while in captivity. Similarly, wild hellbenders that are continually exposed to river water only share 16% of their OTUs with the river environment (Hernández-Gómez et al. 2017a). Similar trends in environmental-host microbial patterns are seen among bullfrogs (*Rana catesbeiana*) and newts (*Notophthalmus viridescens*); their skin communities harbor OTUs that are generally in low abundance in the environment and the more abundant environmental OTUs are in low abundance on their skin (Walke et al. 2014). This further supports the hypothesis that amphibian skin is not simply a result of their environment, and instead corroborates that hellbender skin is colonized by rare, rather than abundant, environmental microbes (Walke et al. 2014).

Treatment individuals maintained N:L ratios, during the first five weeks in captivity, close to those detected among wild hellbenders (0.42 ± 0.06 ; Hopkins et al. 2016). They also had a steady increase in lymphocytes with increasing amounts of river water. Increases in lymphocytes often occur in response to chronic viral infections and chronic inflammation, but are not a result of corticosteroid-induced stress responses (Weiss and Wardrop 2010). Increasing lymphocyte counts indicate that treatment hellbenders may have been mounting an immune response to river water inoculations, but consistently low N:L ratios suggest they did not initiate concomitant stress responses. Conversely, the elevation in N:L ratios I observed among control individuals across the five weeks in captivity may have been suggestive of a strong stress or

immunological response. Unfortunately, I cannot provide reason for the distinct changes in leukocytes among control individuals or the differences in white blood cell counts at the beginning of the experiment between treatment and control individuals. Control individuals were maintained in standard rearing conditions without any exposure to natural microbes. The differences I observed were unlikely a result of human error, because the same person made all blood slides and all slides were read by a single observer. These estimates could have been evidence of random variation across hematologic metrics. Hellbenders can have large variations in their white blood cell counts; for example, of ten male hellbenders all captured from the same river in Missouri they were estimated to have $28\% \pm 21\%$ lymphocytes and $54\% \pm 31\%$ neutrophils (Solís et al. 2007).

Following translocation to the river, I found pronounced changes in neutrophils and lymphocyte counts as well as N:L ratios among control and treatment hellbenders. Only when treatment hellbenders were released into the river did their N:L ratios increase and become comparable to wild hellbenders coinfecting with trypanosomes and leeches (i.e., N:L ratios of about 0.71 and blood cell counts made up of 46% lymphocytes and 32% neutrophils; Hopkins et al. 2016). Neutrophils are produced in excess to protect the body from bacterial infections, and thus could have been beneficial to treatment hellbenders upon release (Mitruka and Rawnsley 1981). Treatment hellbenders had higher N:L ratios than control individuals following release into the river, which could help increase disease resistance. There was no accompanying change in alpha diversity, perhaps because treatment hellbenders had hematological responses that helped prohibit the establishment of potentially pathogenic bacteria on their skin. One limitation to using 16S rRNA sequencing to identify microbiota on the skin of hellbenders and in the river water is that it only assesses bacterial species (Hernández-Gómez et al. 2017b). Treatment hellbenders may have been responding to bacterial, fungal, viral, or protozoan species during river water exposure; however, I cannot confirm from my methods alone. At week six, opposite to treatment hellbenders, control individuals had a rapid reduction in N:L ratios. Their low N:L ratios with high measures of species richness on their skin might suggest lowered cutaneous defenses and the colonization of opportunistic species following release. Furthermore, control hellbenders were naïve to free-flowing river microbiota, which suggests they may have been immunosuppressed and unable to respond following only seven days of exposure, again leading to lowered N:L ratios. Although the differences I observed suggest the effects of river water

exposure on hellbenders' health are dependent upon treatment group, they are still difficult to explain mechanistically.

My novel form of river water inoculation was an effective bioaugmentation method that exposed captive hellbenders to natural microbes in a more complex design than usual, single-species probiotic methods. This method allowed me to inoculate multiple hellbenders at once, it required no additional expense in terms of probiotic testing and selection, and it produced distinct differences in alpha and beta diversity on the skin of captive hellbenders. Captive hellbenders are at a disadvantage being reared in an aseptic environment. Not only do they lack access to rare environmental OTUs, but their opportunity to acquire skin microbiota through vertical transmission is also eliminated when eggs hatch in captivity. Early life disruptions, like a transfer to captivity, can have profound effects on adult health and disease susceptibility (Knutie et al. 2017). However, I found river water exposure to be effective at altering hellbenders' microbial skin community in a way that increased the diversity of skin microbial communities. My novel methodology provides a feasible way to correct for skin microbiome perturbations and hygienic rearing environments, and adequately prepare hellbenders for translocation back into the wild.

If prior exposure to riverine microbiota has beneficial effects on hellbender health and survival following release, this method could have profoundly positive effects on future translocation projects. Future work should explore the use of real-time river water inoculation through stream side nurseries (similar to Peckarsky and McIntosh 1998). Stream side nurseries may be challenged by stochastic flood events, mammalian predators, or human poachers, but providing a place for hellbenders to acclimate to natural river conditions, be inoculated to free-flowing microbes, and become conditioned to predator kairomones could greatly improve the success of translocation efforts (Kenison and Williams 2018). Future studies are also necessary to refine my methods and investigate ways to optimize this technique in the safety of captivity. My seven-day exposure time with undiluted water was effective, but longer exposure periods or more frequent water changes could further increase alpha and beta diversity and make the skin microbiota of captive individuals more similar to conspecifics in the wild. Although I did not measure disease susceptibility because of hellbenders' conservation status, investigating disease dynamics and survival following permanent release into the river could provide more information about the relationship between microbial diversity and disease resistance for

hellbenders, and quantify the benefits this technique may have on translocation efforts. Although riverine bacteria is able to quickly colonize the skin of naïve hellbenders following release, prior exposure to free-flowing microbiota in the safety of captivity may be the most feasible way to develop strong cutaneous defenses and prime juvenile hellbenders' immune systems before they are moved from captivity to the wild environment. I suggest river water inoculation methods be incorporated into hellbender rearing programs so that we can better understand the long-term benefits of this technique and ultimately, adapt it to aid in the conservation and translocation of other captive amphibians into the wild.

Table 3.1. Adonis and Anosim test results for differences between beta diversity measures of the community composition of bacterial species on the skin of treatment and control individuals. Beta diversity tests were conducted with unweighted UniFrac and Bray-Curtis distance matrices. The R values indicate the amount of variation explained by treatment.

Week	Beta Diversity		Adonis R	P value	Anosim R	P value
	Test					
1	Unweighted		0.073	0.001	0.314	0.001
	Bray-Curtis		0.092	0.001	0.187	0.001
2	Unweighted		0.112	0.001	0.561	0.001
	Bray-Curtis		0.162	0.001	0.392	0.001
3	Unweighted		0.109	0.001	0.52	0.001
	Bray-Curtis		0.186	0.001	0.664	0.001
4	Unweighted		0.124	0.001	0.619	0.001
	Bray-Curtis		0.145	0.001	0.472	0.001
5	Unweighted		0.134	0.001	0.772	0.001
	Bray-Curtis		0.170	0.001	0.666	0.001
6	Unweighted		0.068	0.002	0.162	0.005
	Bray-Curtis		0.069	0.016	0.126	0.024

Table 3.2. The most common Blue River water OTUs, their phyla, and their relative abundance on the skin of treatment hellbenders (*Cryptobranchus a. alleganiensis*). Phyla are abbreviated as: Actinobacteria (Act), Armatimonadetes (Arm), Bacteroidetes (Bac), Cyanobacteria (Cya), Proteobacteria (Pro), Verrucomicrobia (Ver), and Unassigned OTU (un). Darker colors indicate higher relative abundance. Common Blue River water OTUs are found sparingly on hellbender skin and they do not tend to become relatively abundant until week six after hellbenders had been placed directly into the river for seven days.

Week 2	Week 3	Week 4	Week 5	Week 6
Bac - 0.00094827	Bac - 0.000623204	Bac - 0.000457786	Bac - 0.000163495	Bac - 0.094677541
Bac - 0.004545157	Cya - 0	Bac - 0	Pro - 0.002060035	Bac - 0.0568822
Bac - 0.009776993	Bac - 0.00270055	Bac - 0.012458309	Bac - 0.000588582	Bac 0.051164551
Pro - 0.019357792	Pro - 0.003773846	Pro - 0.007455366	Bac - 0	Pro - 0.017531321
Cya - 0.0000327	Bac - 0.000830939	Bac - 0.0000981	Pro - 0.00094827	Pro - 0.002017994
Cya - 0.0000327	Ver - 0.0000346	Pro - 0.003466091	Act - 0.000523184	Pro - 0.011519381
Ver - 0.000555883	Bac - 0	Cya - 0	Act - 0.000555883	Pro - 0.008828723
Cya - 0.0000654	Act - 0	Bac - 0.0000327	Bac - 0	Act - 0.006474397
Cya - 0.000653979	Pro - 0.007686182	Act - 0.0000654	Pro - 0.001013668	Bac - 0.01202388
Pro - 0.003302596	Bac - 0.04168542	Bac - 0.0000654	Act - 0.000490485	Bac - 0.012486337
Ver - 0.014158655	Bac - 0.003012152	Act - 0	Arm - 0.000719377	Bac - 0.003909863
Bac - 0.0000327	Bac - 0	Act - 0.0000327	Act - 0.000588582	Act - 0.004708652
Pro - 0.005983912	Bac - 0	Bac - 0	Bac - 0	Pro - 0.007020937
Act - 0.000752076	Pro - 0	Bac - 0	Cya - 0.001569551	Pro - 0.004666611
Act - 0.000294291	Act - 0	Bac - 0	Act - 0.0000327	Act - 0.004750694
Bac - 0.000523184	Bac - 0.007928539	Bac - 0	Act - 0.000130796	Bac - 0.012234087
Act - 0.00062128	Bac - 0.001904234	Bac - 0.000425087	Bac - 0	Bac - 0.003531489
un - 0.011640834	Act - 0.0000346	Act - 0	Bac - 0.000490485	Act - 0.003951904
Act - 0.000130796	Act - 0.000103867	Bac - 0.001961938	Act - 0.000228893	Bac - 0.00655848
Act - 0.0000327	Bac - 0	Act - 0	Cya - 0.0000654	Act - 0.001723703
Act - 0.000915571	Bac - 0.000346224	Cya - 0	Bac - 0.0000654	Bac - 0.003111074
Act - 0.000719377	Bac - 0.000450092	Cya - 0	Pro - 0.0000654	
Bac - 0.000228893				
Cya - 0.000523184				

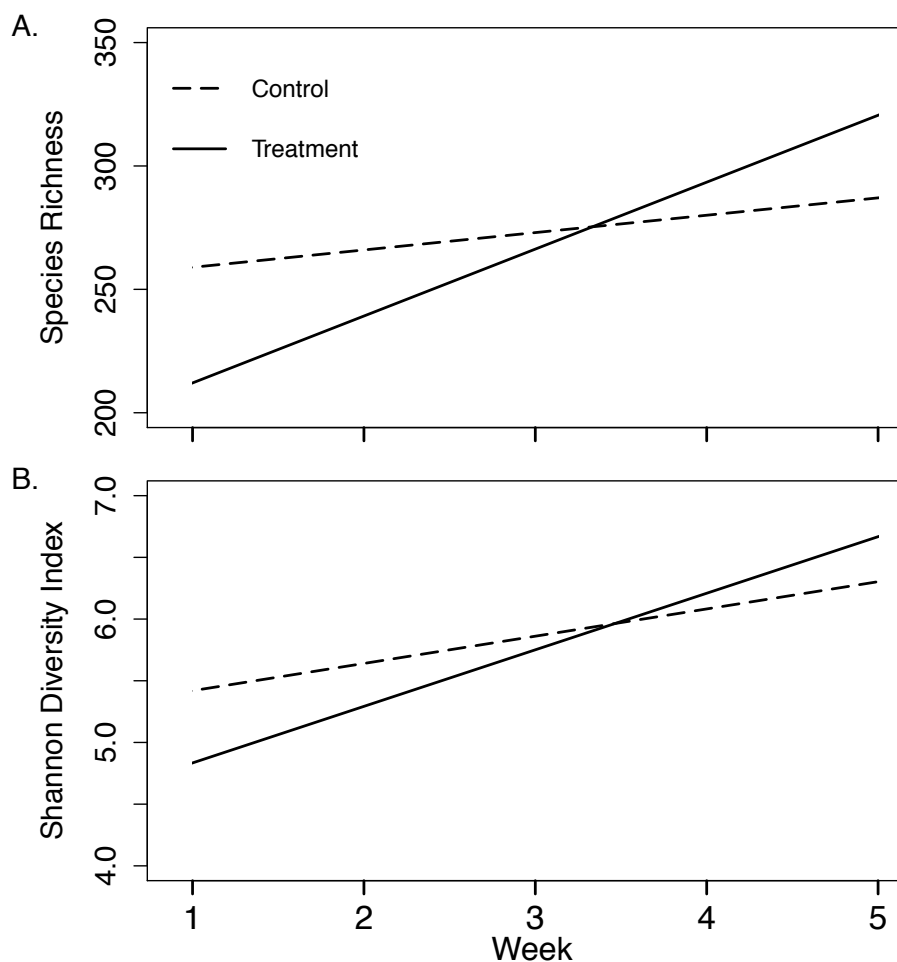


Figure 3.1. Alpha diversity comparisons including species richness (A.) and Shannon diversity (B.) of hellbender (*Cryptobranchus a. alleganiensis*) skin microbiota from control (n = 18) and treatment individuals (n = 18) over the five-week experimental period in captivity. Treatment individuals were exposed to increasing amounts of river water from weeks two through five, and were exposed to undiluted river water at week five. There were no significant differences in alpha diversity at week one, between control and treatment individuals. However, there was a significant ($\alpha < 0.05$) interaction between week and treatment group, such that treatment hellbenders had quicker increases in species richness and Shannon diversity compared to control individuals. All skin swab samples were taken between 28 June and 29 July, 2016.

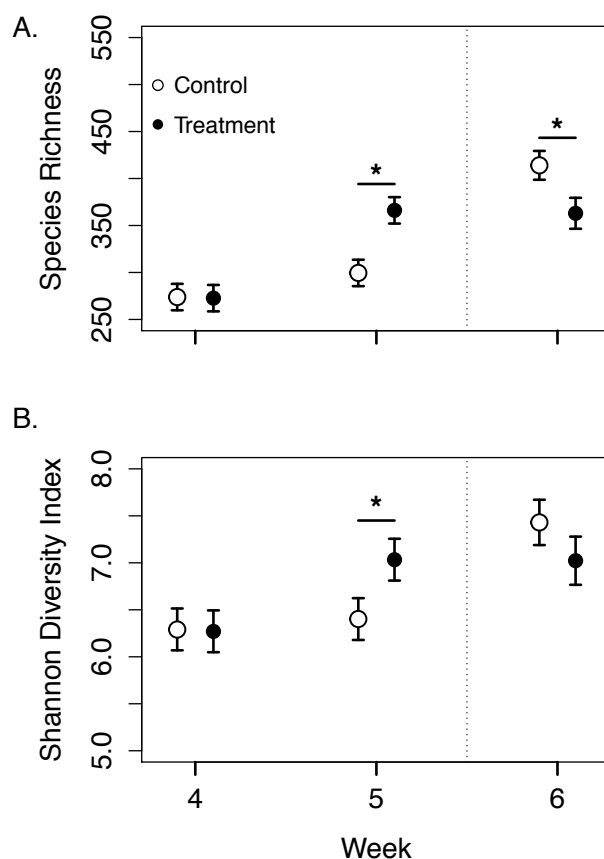


Figure 3.2. Alpha diversity comparisons, species richness (A.) and Shannon diversity (B.), of hellbender (*Cryptobranchus a. alleganiensis*) skin microbiota from control ($n = 18$) and treatment individuals ($n = 18$) during weeks four and five in captivity, and week six following river release. Treatment individuals were exposed to 10% river water in week four, undiluted river water at week five, and all individuals were placed in the river for seven days before week six sampling. The dotted line indicates when all control and treatment hellbenders were released into the river. Treatment hellbenders had a large increase in richness and diversity between weeks four and five, but no change between week five and week six. Control individuals did not differ between weeks four and five, but had a rapid increase in species richness and Shannon diversity following river release. All skin swab samples were taken between 22 July and 5 August, 2016. Estimates are back-transformed and presented with standard error bars. Asterisks denote significant ($\alpha < 0.05$) differences between control and treatment individuals

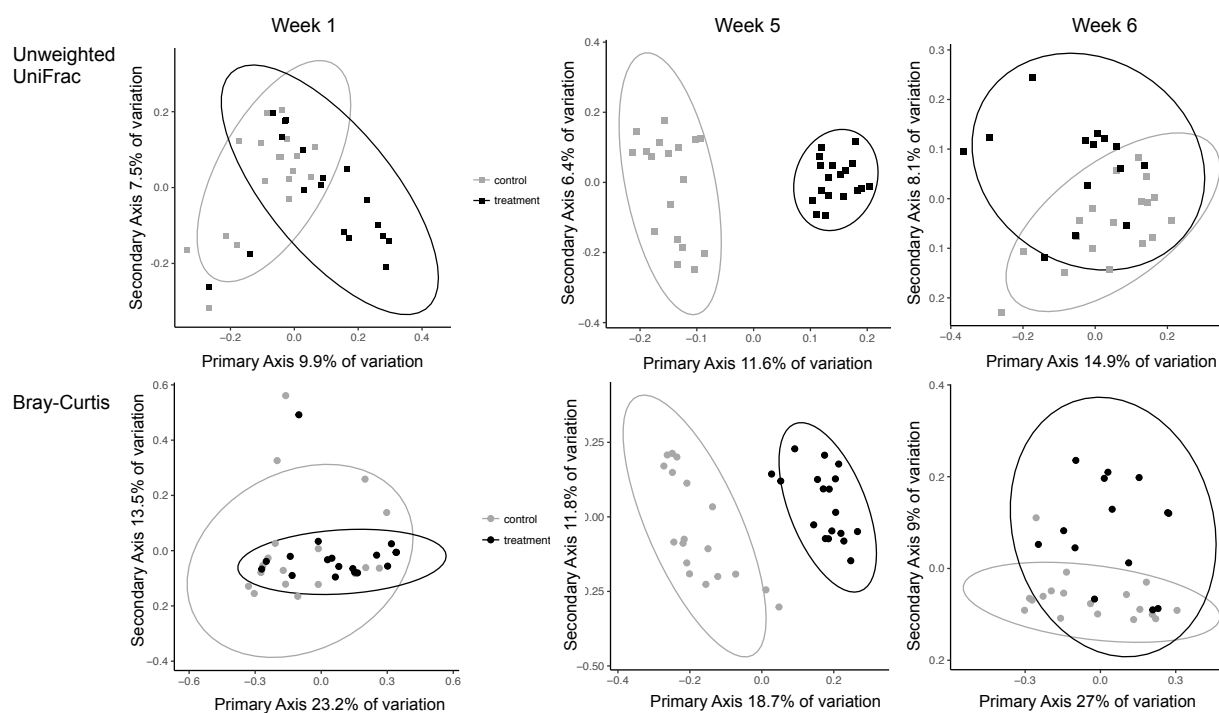


Figure 3.3. PCoA plots depicting beta diversity comparisons between treatment and control hellbenders (*Cryptobranchus a. alleganiensis*) at weeks one, five, and six. Gray points depict control individuals (squares = unweighted UniFrac, circles = Bray-Curtis) and black points indicate treatment individuals (squares = unweighted UniFrac, circles = Bray-Curtis). Ellipses illustrate overlap in community composition between the two hellbender groups. There is strong overlap between control and treatment individuals at the beginning of the experiment and after all hellbenders are released into the river (weeks one and six). However, there is a strong differentiation between groups at week five, when treatment hellbenders are bathed in undiluted river water.

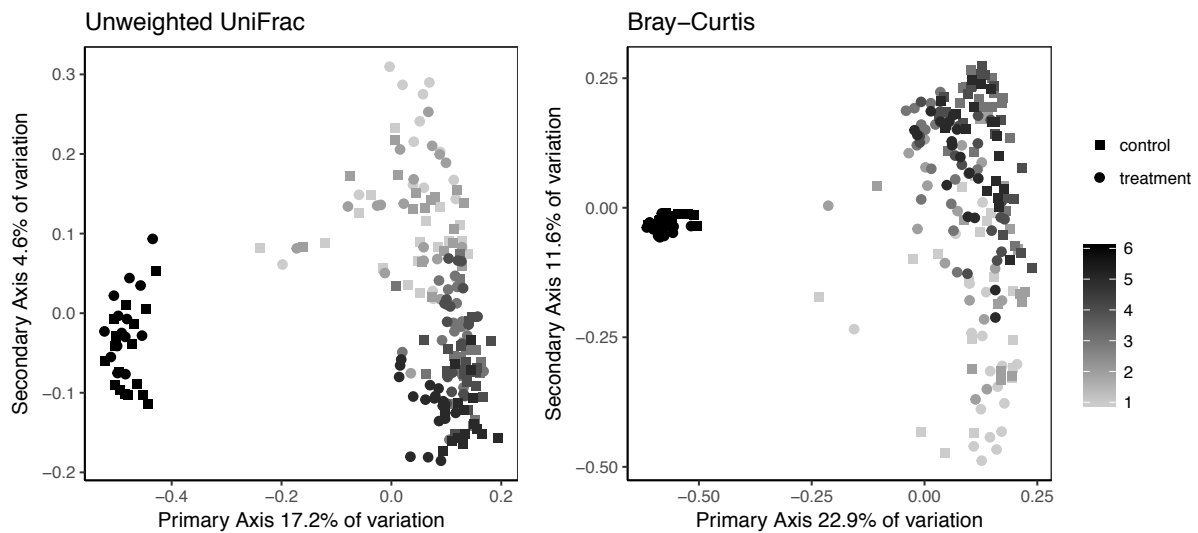


Figure 3.4. PCoA plots depicting beta diversity comparisons of microbiota on the skin of treatment and control hellbenders (*Cryptobranchus a. alleganiensis*) over the six-week experimental period. The color gradient is indicative of experimental week, with week one being depicted as light gray and week six being depicted as black. Square points indicate control individuals and circular points are used for treatment individuals. I have presented unweighed UniFrac and Bray-Curtis methods, both of which show a distinct separation in beta diversity at week 6 compared to weeks 1-5, regardless of group.

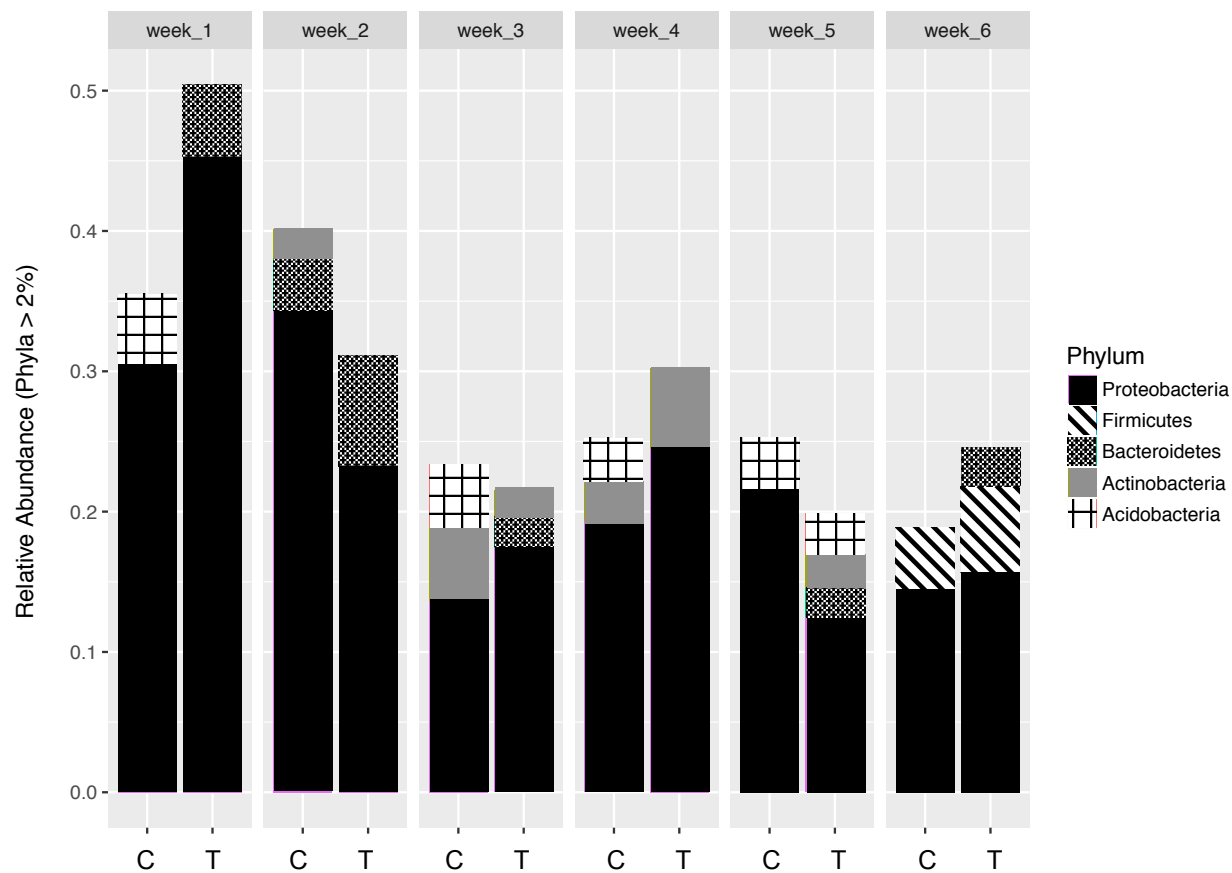


Figure 3.5. Relative abundance of dominant phyla found on the skin of control (C) and treatment (T) hellbenders (*Cryptobranchus a. alleganiensis*) at each of the six experimental weeks. Skin microbiota was dominated by Proteobacteria, followed by Acidobacteria, Actinobacteria, Bacteroidetes, and Firmicutes, regardless of treatment. Species in the Firmicutes phylum were only detected on the skin after control and treatment hellbenders were released into the river.

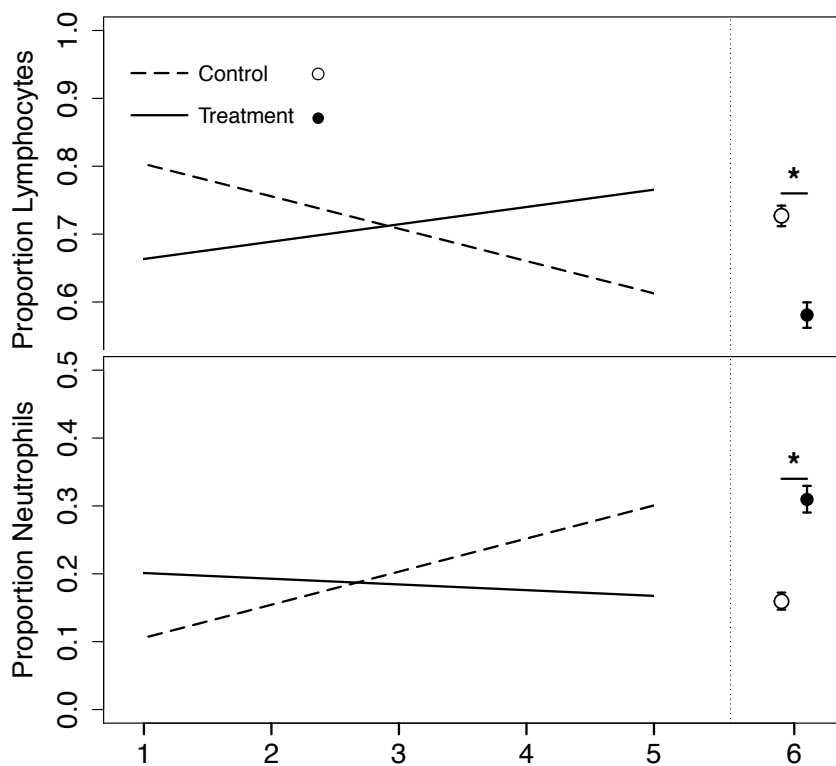


Figure 3.6. Linear trend lines from raw white blood cell counts for control ($n = 18$) and treatment hellbenders (*Cryptobranchus a. alleganiensis*) ($n = 18$) across the six experimental weeks. Counts are presented as proportion lymphocytes and proportion neutrophils. There was a significant ($\alpha < 0.05$) difference between groups at week one as well as an interaction between week and treatment group. Treatment hellbenders had a faster increase in the proportion of lymphocytes than control individuals, and oppositely, reduced the proportion of neutrophils across the five weeks in captivity compared to control hellbenders. Point estimates in week six are plotted with standard errors. Asterisks indicate significant differences between groups. The dotted line indicates when all control and treatment hellbenders were released into the river in cages, and then removed for sampling at week six. Blood was collected between 28 June and 5 August, 2016.

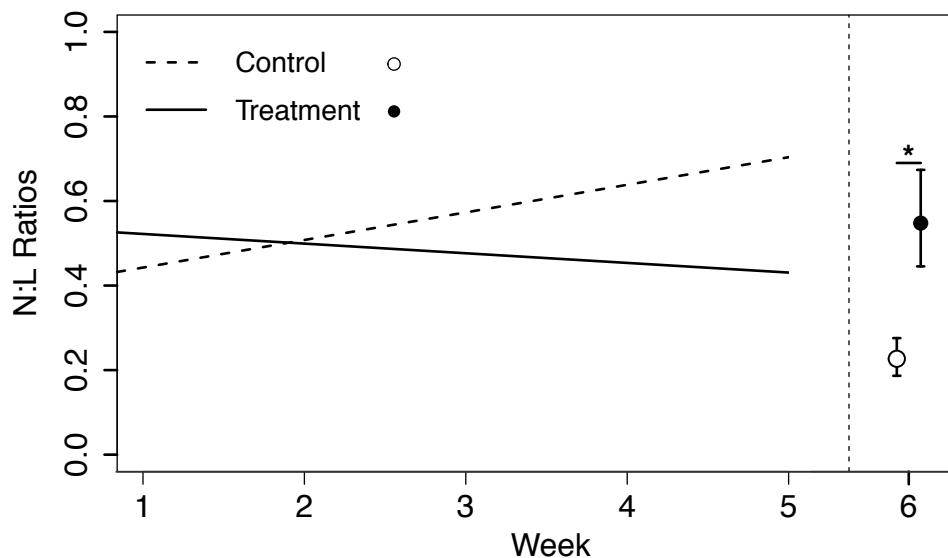


Figure 3.7. Linear trends for changes in neutrophil to lymphocyte (N:L) ratios, estimated from white blood cell counts, for control ($n = 18$) and treatment hellbenders (*Cryptobranchus a. alleganiensis*) ($n = 18$) across the five-week experimental period in captivity. Treatment individuals had diminishing N:L ratios as they were exposed to increasing amounts of river water from weeks two through five, which was different than control individuals. However, they had an increase in N:L ratios in week six after they were released in the river. Point estimates are presented with standard errors and asterisks indicate significant ($\alpha < 0.05$) differences between groups. The dotted line indicates when all control and treatment hellbenders were released into the river in cages, and then removed for sampling at week six. Blood was collected between 28 June and 5 August, 2016. The Y-axis is back-transformed for ease of interpretation.

3.6 References

- Alberts, A.C., 2007. Behavioral considerations of headstarting as a conservation strategy for endangered Caribbean rock iguanas. *Appl. Anim. Behav. Sci.* 102, 380–391. doi:10.1016/j.applanim.2006.05.037
- Antwis, R.E., Haworth, R.L., Engelmoer, D.J.P., Ogilvy, V., Fidgett, A.L., Preziosi, R.F., 2014. Ex situ diet influences the bacterial community associated with the skin of red-eyed tree frogs (*Agalychnis callidryas*). *PLoS One* 9, 1–8. doi:10.1371/journal.pone.0085563
- Bataille, A., Lee-Cruz, L., Tripathi, B., Kim, H., Waldman, B., 2015. Microbiome variation across amphibian skin regions: implications for chytridiomycosis mitigation efforts. *Microb. Ecol.* 71, 221–232. doi:10.1007/s00248-015-0653-0
- Becker, C.G., Longo, A. V, Haddad, C.F.B., Zamudio, K.R., 2017. Land cover and forest connectivity alter the interactions among host, pathogen and skin microbiome. *Proceedings. Biol. Sci.* 284, 20170582. doi:10.1098/rspb.2017.0582
- Becker, M.H., Harris, R.N., 2010. Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PLoS One* 5, 1–6. doi:10.1371/journal.pone.0010957
- Becker, M.H., Harris, R.N., Minbiole, K.P.C., Schwantes, C.R., Rollins-Smith, L.A., Reinert, L.K., Brucker, R.M., Domangue, R.J., Gratwicke, B., 2012. Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. *Ecohealth* 8, 501–506. doi:10.1007/s10393-012-0743-0
- Becker, M.H., Richards-Zawacki, C.L., Gratwicke, B., Belden, L.K., 2014. The effect of captivity on the cutaneous bacterial community of the critically endangered Panamanian golden frog (*Atelopus zeteki*). *Biol. Conserv.* 176, 199–206. doi:10.1016/j.biocon.2014.05.029
- Belden, L.K., Hughey, M.C., Rebolgar, E.A., Umile, T.P., Loftus, S.C., Burzynski, E.A., Minbiole, K.P.C., House, L.L., Jensen, R. V., Becker, M.H., Walke, J.B., Medina, D., Ibáñez, R., Harris, R.N., 2015. Panamanian frog species host unique skin bacterial communities. *Front. Microbiol.* 6, 1–21. doi:10.3389/fmicb.2015.01171

- Bletz, M.C., Loudon, A.H., Becker, M.H., Bell, S.C., Woodhams, D.C., Minbiole, K.P.C., Harris, R.N., 2013. Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.* 16, 807–820. doi:10.1111/ele.12099
- Bodinof, C.M., 2010. Translocation and Conservation of Hellbenders (*Cryptobranchus alleganiensis*) in Missouri. University of Missouri.
- Boerner, J.A., 2014. Comparison of Movement Patterns in Captive-Released Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) Using Three Different Release Methods. PhD Thesis. State University of New York Buffalo State.
- Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., Mills, D.A., Caporaso, J.G., 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods* 10, 57–9. doi:10.1038/nmeth.2276
- Boyce, W.M., Weisenberger, M.E., Penedo, M.C.T., Johnson, C.K., 2011. Wildlife translocation: the conservation implications of pathogen exposure and genetic heterozygosity. *BMC Ecol.* 11, 5. doi:10.1186/1472-6785-11-5
- Burgmeier, N.G., Unger, S.D., Sutton, T.M., Williams, R.N., 2011. Population status of the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) in Indiana. *J. Herpetol.* 45, 195–201. doi:10.1670/10-094.1
- Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U. S. A.* 108 Suppl 1, 4516–22. doi:10.1073/pnas.1000080107
- Davis, A.K., Maerz, J.C., 2011. Assessing stress levels of captive-reared amphibians with hematological data: implications for conservation initiatives. *J. Herpetol.* 45, 40–44.
- Davis, A.K., Maerz, J.C., 2008. Comparison of hematological stress indicators in recently captured and captive paedomorphic mole salamanders, *Ambystoma talpoideum*. *Copeia* 3, 613–617. doi:10.1643/CP-07-133
- Dillon, R.J., Vennard, C.T., Buckling, A., Charnley, A.K., 2005. Diversity of locust gut bacteria protects against pathogen invasion. *Ecol. Lett.* 8, 1291–1298. doi:10.1111/j.1461-0248.2005.00828.x

- Duncan, J., Praise, K., Mahaffey, E., 1994. *Veterinary Laboratory Medicine (Clinical Pathology)* Iowa State University Press.
- DuRant, S.E., Hopkins, W.A., Davis, A.K., Romero, L.M., 2015. Evidence of ectoparasite-induced endocrine disruption in an imperiled giant salamander, the eastern hellbender (*Cryptobranchus alleganiensis*). *J. Exp. Biol.* 218, 2297–2304. doi:10.1242/jeb.118703
- Ettling, J.A., Wanner, M.D., Schuette, C.D., Armstrong, S.L., Pedigo, A.S., Briggler, J.T., 2013. Captive reproduction and husbandry of adult Ozark hellbenders, *Cryptobranchus alleganiensis bishopi*. *Herpetol. Rev.* 44, 605–610.
- Harris, R.N., Brucker, R.M., Walke, J.B., Becker, M.H., Schwantes, C.R., Flaherty, D.C., Lam, B.A., Woodhams, D.C., Briggs, C.J., Vredenburg, V.T., Minbiole, K.P.C., 2009a. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi:10.1038/ismej.2009.27
- Harris, R.N., Lauer, A., Simon, M.A., Banning, J.L., Alford, R.A., 2009b. Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. *Dis. Aquat. Organ.* 83, 11–16. doi:10.3354/dao02004
- Hernández-Gómez, O., Hoverman, J.T., Williams, R.N., 2017a. Cutaneous microbial community variation across populations of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*). *Front. Microbiol.* 8, 1–16. doi:10.3389/fmicb.2017.01379
- Hernández-Gómez, O., Kimble, S.J.A., Briggler, J.T., Williams, R.N., 2017b. Characterization of the cutaneous bacterial communities of two giant salamander subspecies. *Microb. Ecol.* 73, 445–454. doi:10.1007/s00248-016-0859-9
- Hopkins, W.A., Fallon, J.A., Beck, M.L., Coe, B.H., Jachowski, C.M.B., 2016. Haematological and immunological characteristics of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) infected and co-infected with endo- and ectoparasites. *Conserv. Physiol.* 4, 1–16. doi:10.1093/conphys/cow002
- Hughey, M.C., Delia, J., Belden, L.K., 2017. Diversity and stability of egg-bacterial assemblages: The role of paternal care in the glassfrog *Hyalinobatrachium colymbiphyllum*. *Biotropica* 49, 792–802. doi:10.1111/btp.12461
- Irianto, A., Austin, B., 2002. Probiotics in aquaculture. *J. Fish Dis.* 25, 633–642. doi:10.1046/j.1365-2761.2002.00422.x

- Jiménez, R.R., Sommer, S., 2016. The amphibian microbiome: natural range of variation, pathogenic dysbiosis, and role in conservation. *Biodivers. Conserv.* 26, 763–786. doi:10.1007/s10531-016-1272-x
- Kenison, E.K., Olson, Z.H., Williams, R.N., 2016. A novel transport system for hellbender salamanders (*Cryptobranchus alleganiensis*). *Herpetol. Conserv. Biol.* 11.
- Kenison, E.K., Williams, R.N., 2018a. Rearing captive eastern hellbenders (*Cryptobranchus a. alleganiensis*) with moving water improves swim performance. *Appl. Anim. Behav. Sci.* 202, 112–118. doi:10.1016/j.applanim.2018.01.013
- Kenison, E.K., Williams, R.N., 2018b. Training for translocation: predator conditioning induces behavioral plasticity and physiological changes in captive eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) (*Cryptobranchidae*, *Amphibia*). *Diversity* 10, 13. doi:10.3390/d10010013
- Kern, W.H.J., 1984. The Hellbender, *Cryptobranchus alleganiensis*, in Indiana. Indiana State University.
- Knutie, S.A., Wilkinson, C.L., Kohl, K.D., Rohr, J.R., 2017. Early-life disruption of amphibian microbiota decreases later-life resistance to parasites. *Nat. Commun.* 8, 1–8. doi:10.1038/s41467-017-00119-0
- Kraus, B.T., McCallen, E.B., Williams, R.N., 2017. Evaluating the survival of translocated adult and captive-reared, juvenile eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*). *Herpetologica* 73, 271–276. doi:10.1655/HERPETOLOGICA-D-16-00009
- Kueneman, J.G., Parfrey, L.W., Woodhams, D.C., Archer, H.M., Knight, R., McKenzie, V.J., 2014. The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi:10.1111/mec.12510
- Loudon, A.H., Woodhams, D.C., Parfrey, L.W., Archer, H., Knight, R., McKenzie, V., Harris, R.N., 2014. Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *Int. Soc. Microb. Ecol. J.* 8, 830–840. doi:10.1038/ismej.2013.200
- Lozupone, C., Knight, R., 2005. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi:10.1128/AEM.71.12.8228–8235.2005

- Magnadóttir, B., 2006. Innate immunity of fish (overview). *Fish Shellfish Immunol.* 20, 137–151. doi:10.1016/j.fsi.2004.09.006
- Mayasich, J., Phillips, C., 2003. Conservation assessment for eastern hellbender, USDA Forest Service, Eastern Reg.
- McKenzie, V.J., Bowers, R.M., Fierer, N., Knight, R., Lauber, C.L., 2012. Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.* 6, 588–596. doi:10.1038/ismej.2011.129
- Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R.T.M., Bøggwald, J., Castex, M., Ringø, E., 2010. The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture* 302, 1–18. doi:10.1016/j.aquaculture.2010.02.007
- Meyer, E.A., Cramp, R.L., Bernal, M.H., Franklin, C.E., 2012. Changes in cutaneous microbial abundance with sloughing: Possible implications for infection and disease in amphibians. *Dis. Aquat. Organ.* 101, 235–242. doi:10.3354/dao02523
- Mitruka, B.M., Rawnsley, H.M., 1981. Clinical biochemical and hematological reference values in normal experimental animals and normal humans. Masson Pub. USA.
- Moran, N.A., Dunbar, H.E., 2006. Sexual acquisition of beneficial symbionts in aphids. *Proc. Natl. Acad. Sci. U. S. A.* 103, 12803–6. doi:10.1073/pnas.0605772103
- Moriarty, D.J.W., 1999. Disease control in shrimp aquaculture with probiotic bacteria. *Proc. 8th Int. Symp. Microb. Ecol. - Microb. Interact. Aquac. Dis.* 2013, 424123. doi:10.1155/2013/424123
- Muletz, C.R., Myers, J.M., Domangue, R.J., Herrick, J.B., Harris, R.N., 2012. Soil bioaugmentation with amphibian cutaneous bacteria protects amphibian hosts from infection by *Batrachochytrium dendrobatidis*. *Biol. Conserv.* 152, 119–126. doi:10.1016/j.biocon.2012.03.022
- Nickerson, M.A., Mays, C.E., 1973. The hellbenders: North American giant salamanders. *Biol. Geol.* 1, 1–106.
- Peckarsky, B.L., McIntosh, A.R., 1998. Fitness and community consequences of avoiding multiple predators. *Oecologia* 113, 565–576.
- R Core Team, 2016. R: A Language and Environment for Statistical Computing. R Found. Stat. Comput. Vienna, Au, <http://www.R-project.org/>.

- Rebollar, E.A., Simonetti, S.J., Shoemaker, W.R., Harris, R.N., 2016. Direct and indirect horizontal transmission of the antifungal probiotic bacterium *Janthinobacterium lividum* on green frog (*Lithobates clamitans*) tadpoles. *Appl. Environ. Microbiol.* 82, AEM.04147-15. doi:10.1128/AEM.04147-15
- Redford, K.H., Segre, J.A., Salafsky, N., Del Rio, C.M., Mcaloose, D., 2012. Conservation and the microbiome. *Conserv. Biol.* 26, 195–197. doi:10.1111/j.1523-1739.2012.01829.x
- Richmond, J.Q., Savage, A.E., Zamudio, K.R., Rosenblum, E.B., 2009. Toward immunogenetic studies of amphibian Chytridiomycosis: linking innate and acquired immunity. *Bioscience* 59, 311–320. doi:10.1525/bio.2009.59.4.9
- Rideout, J.R., He, Y., Navas-Molina, J.A., Walters, W.A., Ursell, L.K., Gibbons, S.M., Chase, J., McDonald, D., Gonzalez, A., Robbins-Pianka, A., Clemente, J.C., Gilbert, J.A., Huse, S.M., Zhou, H.-W., Knight, R., Caporaso, J.G., 2014. Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. *PeerJ* 2, e545. doi:10.7717/peerj.545
- Sabino-Pinto, J., Bletz, M.C., Islam, M.M., Shimizu, N., Bhujju, S., Geffers, R., Jarek, M., Kurabayashi, A., Vences, M., 2016. Composition of the cutaneous bacterial community in Japanese amphibians: effects of captivity, host species, and body region. *Microb. Ecol.* 72, 460–469. doi:10.1007/s00248-016-0797-6
- Smith, K.F., Acevedo-Whitehouse, K., Pedersen, A.B., 2009. The role of infectious diseases in biological conservation. *Anim. Conserv.* 12, 1–12. doi:10.1111/j.1469-1795.2008.00228.x
- Solís, M.E., Bandeff, J.M., Huang, Y.-W., 2007. Hematology and serum chemistry of Ozark and eastern hellbenders (*Cryptobranchus alleganiensis*). *Herpetologica* 63, 285–292. doi:10.1655/0018-0831(2007)63[285:HASCOO]2.0.CO;2
- Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R., Gordon, J.I., 2007. The human microbiome project. *Nature* 449, 804–10. doi:10.1038/nature06244
- Unger, S.D., Sutton, T.M., Williams, R.N., 2013. Projected population persistence of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) using a stage-structured life-history model and population viability analysis. *J. Nat. Conserv.* 21, 423–432. doi:10.1016/j.jnc.2013.06.002

- Vrendenburg, V.T., Briggs, C.J., Harris, R., 2011. Host-pathogen dynamics of amphibians chytridiomycosis: the role of the skin micro biome in health and disease, in: Olson, L., Choffnes, E., Relman, D., Pray, L. (Eds.), *Fungal Diseases: An Emerging Threat to Human, Animal, and Plant Health ...* - Institute of Medicine, Board on Global Health, Forum on Microbial Threats. National Academy Press, Washington, D.C., USA, pp. 342–355.
- Walke, J.B., Becker, M.H., Loftus, S.C., House, L.L., Cormier, G., Jensen, R. V., Belden, L.K., 2014. Amphibian skin may select for rare environmental microbes. *ISME J.* 8, 2207–2217. doi:10.1038/ismej.2014.77
- Walke, J.B., Harris, R.N., Reinert, L.K., Rollins-Smith, L.A., Woodhams, D.C., 2011. Social immunity in amphibians: evidence for vertical transmission of innate defenses. *Biotropica* 43, 396–400. doi:10.1111/j.1744-7429.2011.00787.x
- Weiss, D.J., Wardrop, K.J., 2010. *Schalm's Veterinary Hematology*, Sixth. ed. John Wiley & Sons.
- Wheeler, B.A., Prosen, E., Mathis, A., Wilkinson, R.F., 2003. Population declines of a long-lived salamander: a 20+-year study of hellbenders, *Cryptobranchus alleganiensis*. *Biol. Conserv.* 109, 151–156.
- Whitman, W.B., Coleman, D.C., Wiebe, W.J., Jun, N., Whitman, W.B., Coleman, D.C., William, J., 1998. Prokaryotes: the unseen majority. *Proc. Natl. Acad. Sci.* 95, 6578–6583.
- Woodhams, D.C., Bosch, J., Briggs, C.J., Cashins, S., Davis, L.R., Lauer, A., Muths, E., Puschendorf, R., Schmidt, B.R., Sheafor, B., Voyles, J., 2011. Mitigating amphibian disease: Strategies to maintain wild populations and control chytridiomycosis. *Front. Zool.* 8, 8. doi:10.1186/1742-9994-8-8
- Woodhams, D.C., Rollins-Smith, L.A., Alford, R.A., Simon, M.A., Harris, R.N., 2007. Innate immune defenses of amphibian skin: Antimicrobial peptides and more. *Anim. Conserv.* 10, 425–428. doi:10.1111/j.1469-1795.2007.00150.x