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Release of captive-raised Eastern hellbenders (Cryptobranchus alleganiensis) to test the success of a chytrid vaccine and new cage design

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Abstract of Thesis

Researchers and managers commonly apply captive-raising and reintroductions of animals to offset losses due to worldwide amphibian declines. Recent declines in the Eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) have resulted in several reintroductions that have had little success. There is evidence that chytridiomycosis (chytrid), a disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), may negatively affect hellbenders post-release. Further, excessive post-release movement may result in movement away from suitable habitat and increased risk of predation which can have negative effects on the survival of released hellbenders. Caging captive-raised animals can be effective for limiting excessive post-release movement. This study tested a new chytrid vaccine and release method involving a new temporary cage design. Twenty captive-raised hellbenders were released into a stream in the Allegheny River drainage in June 2017. Half of these hellbenders were vaccinated. Five vaccinated and five unvaccinated hellbenders were released into cages that were removed in September 2017. The other half (five vaccinated, five unvaccinated) were released directly into the stream. Hellbenders were located daily using radio telemetry and tested for *Bd* weekly for the remainder of the study period. Overall, the 118-day study resulted in 30% survival. The vaccine was unsuccessful; all hellbenders tested positive for *Bd* at some point during the summer. After cage removal, caged hellbenders moved as much as uncaged, but this caging method may have contributed to greater survival for hellbenders in the caged treatment group. These findings suggest that chytridiomycosis is a major issue for survival of head-started hellbenders in NYS, and that caging during release may require further investigation.

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Release of captive-raised Eastern hellbenders (*Cryptobranchus alleganiensis*) to test the success
of a chytrid vaccine and new cage design

A Thesis in
Biology

by

Megan Coventry Kocher

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Dedication

I dedicate this work to the loving memory of my great grandparents who believed in me from the start. They inspired me to continue in my schooling and generously aided financially in this pursuit. If only they were here to witness the completion of my degree so I could thank them profusely.

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Introduction

Habitat loss, climate change, pollution, and disease threaten amphibian populations around the world (Blaustein and Bancroft 2007) resulting in biodiversity declines since the 1960s (Houlahan *et al.* 2000). Amphibian populations are currently more threatened than birds or mammals, with 43.2% of amphibian species experiencing diminishing population sizes (Stuart *et al.* 2004). These alarming and rapid declines have made amphibians an important focus for species conservation. Conservationists have attempted to create and maintain viable, genetically diverse, self-sustaining amphibian populations via the reintroduction of captive-bred (head-started) animals into natural or reconstructed habitats (Griffiths and Pavajeau 2008). However, some researchers question whether head-starting programs are successful in sustaining amphibian populations (Dodd and Seigel 1991). Much of this controversy arises due to the lack of post-release monitoring (Armstrong and Seddon 2008). A successful reintroduction requires low post-release dispersal rates, high survival and reproduction rates, and the appropriate habitat to support and sustain the species of interest (Armstrong and Seddon 2008).

Disease is a major factor contributing to the limited success of amphibian reintroductions. Chytridiomycosis, a disease caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*), is likely the cause of mass amphibian mortalities around the globe (Berger *et al.* 1998, Skerratt *et al.* 2007, Bodinof *et al.* 2011). Chytridiomycosis (chytrid) was described by Berger *et al.* (1998) and has since been studied in many frog and some salamander species. *Batrachochytrium dendrobatidis* has been detected on all continents where amphibians occur (Skerratt *et al.* 2007), but *Bd* was most likely introduced to North America in the second half of the twentieth century (Bodinof *et al.* 2011). Recently, chytrid has become a concern relating to populations of North America's largest aquatic salamander, the Eastern hellbender (Bales *et al.*

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2015, Seeley *et al.* 2016). The Eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) is one of two subspecies, ranging from southern New York to northern Georgia and westward to Missouri (U.S.). Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*), the second of the two subspecies, are limited to southeastern Missouri and northeastern Arkansas (U.S.) (Figure 1) (Nickerson and Mays 1973).

Hellbenders are bound to specific habitats: cool, highly oxygenated, fast flowing streams. These aquatic salamanders spend most of their time under large, flat cover rocks that provide shelter from predators. A slimy mucous secreted by the skin reduces friction and allows the salamander to navigate well in tight rock crevasses. This secretion may also prevent excessive water loss while aiding in the diffusion of gases between the skin and water (Nickerson and Mays 1973).

Hellbenders possess external gills for their first two years of life (Nickerson and Mays 1973). Adult hellbenders depend heavily on cutaneous respiration but are capable of switching to pulmonary respiration when in anoxic conditions (Guimond and Hutchison 1973). Hellbenders can 'drown' if denied access to air when conditions are anoxic (Ultsch 2012). However, the lungs of hellbenders are underdeveloped and may have more of a hydrostatic purpose because they are largely inefficient for gas exchange (Guimond and Hutchison 1973). Hellbenders have special adaptations that allow for cutaneous breathing to be the most efficient and primary mode of respiration. A dorsoventrally flattened body with exaggerated folds of skin along its sides helps increase the surface area through which gases are exchanged. Cutaneous capillaries penetrate the epidermis and extend into the surface cell layer, which reduces the gas diffusion distance between the water and the hellbenders blood. Behavioral adaptations, such as rocking or

swaying, also help to reduce the boundary layer and replenish the oxygen supply in the surrounding water (Guimond and Hutchison 1973).

Hellbenders may be particularly sensitive to the chytridiomycosis disease because of their unique life history and dependence on skin as a functional tool for survival. *Batrachochytrium dendrobatidis* zoospores infect the skin of amphibian hosts and potentially disrupts gas exchange and releases toxins, which may be fatal (Berger *et al.* 2005). *Bd* is known to disrupt cutaneous gas exchange in frogs. Oxygen consumption in *Litoria raniformis* decreased by almost half after one week of chytrid infection (Carver *et al.* 2010). Once the integument of the hellbender is infected, chytrid may cause occasional epidermal sloughing and mild hyperkeratosis (Berger *et al.* 1998, Davidson *et al.* 2003, Bodinof *et al.* 2011). Hyperkeratosis, resulting from irritation, is the thickening of the outermost layer of skin by the production of keratin. Hellbenders may also become a blue-gray color when infected (Bodinof *et al.* 2012b). These symptoms ensue shortly after exposure and continue to worsen throughout the infection (Davidson *et al.* 2003). Although some studies have shown mortality as a result of chytrid infection (Berger *et al.* 1998), others suggest there may be a difference between captive-raised salamanders and wild salamanders. Symptoms of the disease have subsided in some field collected salamanders approximately four months after infection (Davidson *et al.* 2003).

Batrachochytrium dendrobatidis tends to be more prevalent in undisturbed habitat (Becker *et al.* 2012), which is typically where hellbenders are found. Higher densities of canopy cover result in lower water temperatures and greater *Bd* prevalence and intensity (Becker *et al.* 2012). The optimal growth temperature range for *Bd* is 17-25°C (Kilpatrick *et al.* 2010), which overlaps with the typical temperatures of hellbender habitat (<20°C) (Nickerson and Mays 1973). The earliest detections of *Bd* on hellbenders was found on five of 22 sampled Ozark

hellbenders in Missouri in 1969 (Bodinof *et al.* 2011). A study conducted from 2009-2010 found *Bd* prevalence of 26% in 96 hellbenders sampled from the Little and Hiwassee River in Tennessee (Souza *et al.* 2012). Another study has detected *Bd* in some populations of the Eastern hellbender in New York, Pennsylvania, Ohio and Virginia (Bales *et al.* 2015). A recent 2016 study evaluated *Bd* in 42 Eastern hellbenders from four sites in West Virginia and found prevalence to be 52% (Seeley *et al.* 2016).

Ozark and Eastern hellbender populations are declining primarily due to habitat degradation (Mayasich *et al.* 2003). Ozark hellbenders are listed as a federally endangered species (USFWS 2011a, USFWS 2011b). The Eastern hellbender is not federally listed but has a protective status in 12 of the 16 states within its native range (Mayasich *et al.* 2003). In recent years, specific Eastern hellbender populations in the Allegheny drainage region of New York State have suffered declines (Foster *et al.* 2009). Hellbender head-starting and release programs have become a popular attempt by the New York State Department of Environmental Conservation (NYSDEC) to counteract these rapid declines.

In 2009, a nest of more than 1000 eggs was collected from the Allegheny drainage. More than 600 of the eggs were brought to the Buffalo Zoo (McMillan *pers. comm.*). These hellbenders were raised in captivity and have been released into the wild in groups since 2011 (McMillan *pers. comm.*). Early releases had minimal success but results and findings have been continually built upon to improve each proceeding release. Boerner (2014) released 18 of these captive-raised hellbenders in 2013, which resulted in low survival. A third of these hellbenders were released using temporary caging but all had escaped from the cages, and some hellbenders had beached themselves on the stream bank. One beached hellbender from the study was swabbed for infection and had tested positive for chytrid. The beaching behavior described may

be consistent with symptoms of the infection. Hellbenders may rely more heavily on pulmonary gas exchange when infected, and perhaps beaching is an attempt at obtaining more oxygen. However, the beached hellbenders and those that moved excessively became more susceptible to predation (Boerner 2014). In the summer of 2014 another group of hellbenders, from the same nest collected in 2009, were released into modified cages. These hellbenders did not escape and many survived through the winter (Rothrock *pers. comm.*).

Caging captive-raised animals can be effective for limiting excessive post-release movement (Semlitsch 2002), which can lead to increased risk of predation or movement away from suitable habitat (Stamps and Swaisgood 2007). However, it is important to use a cage design that works best for the organism being released. Boerner (2014) used cages that were entirely submerged below the water's surface and easy to escape. Completely denying hellbenders air access can result in drowning, especially if they become oxygen stressed (Ultsch 2012). I will implement a new cage design, for released hellbenders, to address these issues. A taller cage will allow hellbenders to surface for air. We predict that mortality will decrease if hellbenders stay contained within the cage and also have the ability to rely on pulmonary gas exchange.

Preventing chytridiomycosis in salamanders by vaccination is a relatively new opportunity. Currently there are no published studies that use a vaccine to prevent chytrid infection in hellbenders. However, a chytrid vaccine has been studied in three frog species, using a dead strain of *Bd* to build resistance to the fungus (McMahon *et al.* 2014). Researchers at Cornell University's Animal Health Diagnostic Center (AHDC) have recently applied these methods to hellbenders in a lab study (Bunting *pers. comm.*). The success of this lab study was inconclusive (Bunting and Ossiboff *pers. comm.*) therefore, this newly developed vaccine will be

applied to the hellbenders in the present field study. This vaccine should increase the survival of the captive-raised, released hellbenders by decreasing the likelihood of *Bd* infection.

With this research, I intend to find answers to the unknowns that have resulted from previous hellbender releases (Boerner 2014, Rothrock *pers. comm.*). The three main objectives are to:

1. Determine the success of a new cage design for hellbender releases.

2. Determine the success of a chytrid vaccine developed at Cornell AHDC.

3. Monitor post-release movement of the hellbenders.

The new cage design and chytrid vaccine should both increase the chance of survival for released hellbenders. Close monitoring of the released hellbenders will help to find information that would otherwise go unknown. Altogether, these objectives should lead to answers that will aid in future hellbender releases and head-starting programs.

Materials and Methods

Study Area

Captive-raised hellbenders were released in a small stream within the Allegheny River drainage of New York State (NYS; Figure 2). The precise location will remain undisclosed due to the protected status of the Eastern hellbender in NYS. This release site was chosen by officials in the New York State Department of Environmental Conservation (NYSDEC) because it presumably contains suitable hellbender habitat and known, but declining, native hellbender occupancy. The streambed is primarily dominated by cobble, gravel, and silt with some areas of exposed

limestone and shale bedrock. Large cover rocks are dispersed throughout the stream with the highest density within the release area. Some large cover rocks in the study site were previously placed by the New York State Department of Transportation in collaboration with NYSDEC to improve hellbender habitat. The stream remains cool year-round and contains a mixture of pools, runs, and riffles. The riparian zone is largely forested, dominated by maple and pine. Some stretches of the stream bank are within agricultural corn fields or pasture for grazing livestock. Most of the surrounding land is private so permission was obtained from local landowners to access the stream from their properties.

Study Animals

The twenty hellbenders used in this study were hatched from an egg mass collected in 2009 from a stream within the Allegheny River drainage. They were reared at the Buffalo Zoo in Buffalo, NY. The hellbenders were approximately eight years of age at the beginning of the study and sex was unknown. Institutional Animal Care and Use Committee (IACUC) approval was attained for this study (#39 approved 5 May 2017) as well as a NYSDEC permit (#1641).

Prior to release, ten of the hellbenders, chosen at random, were vaccinated with an inactivated strain of *Batrachochytrium dendrobatidis* (*Bd*) to protect against chytrid infection at the Cornell University Animal Health Diagnostic Center (AHDC), Ithaca, NY. The remaining hellbenders, left unvaccinated to serve as a control, were similarly treated, but without the vaccine, to undergo similar stressors of the vaccination procedure. The vaccine was administered orally during an 85-day protocol involving four separate treatments (see Appendix A for details).

Following the vaccination procedure and a two-week recovery period, all hellbenders underwent surgery for transmitter implantation. This process was also completed at the AHDC, using techniques similar to Boerner (2014) (also see Stouffer *et al.* 1983). The transmitters were Advanced Telemetry Systems (Isanti, MN, U.S.A.) model F1170 with a slow pulse rate (Pulser R: 30 ppm, Pulser W: 15 ms). The transmitters were fully encapsulated with waterproof electrical resin and weighed approximately 4.2 grams each. This weight falls below the maximum recommended transmitter:animal mass ratio of 3-5% outlined by Brown *et al.* (2011); the average weight of the hellbenders was 450g prior to release.

Release and Monitoring Methods

Five vaccinated and five unvaccinated hellbenders were released into cages, one hellbender per cage. The remaining hellbenders (five vaccinated and five unvaccinated) were released directly into the stream under suitable cover rock near each cage. The cages were built following a five-sided NYSDEC design (1.2 x 1.2 x 0.9m) that allowed for access to the natural stream bed substrate as well as surface air (Figures 3, 4). During large storm events, the cages were submerged; however, the water depth at the release site remained low for most of the study period. The cages were installed throughout May-June 2017. Cages were placed in flat areas of the streambed and placed approximately equidistant to each other (Figure 4). Each side of the cage was buried 5-8 cm into the substrate. The cages were staked down with rebar pounded approximately 0.5 meter into the streambed. GPS coordinates were recorded for each cage. The hellbenders were released into the stream on 29 June 2017 after 2-4 weeks of recovery in the lab after transmitter implantation surgery. Due to healing complications, two of the twenty

hellbenders were released on 20 July 2017. These two hellbenders were in separate treatment groups.

The weight of each hellbender was recorded prior to release and every 2-4 weeks (to minimize handling time) during the study period (June 2017 – October 2017). In the field, weight measurements were taken by placing each hellbender into a clean mesh weigh bag using vinyl gloves and weighed using a handheld Pesola spring scale. Gloves were changes and the bags were sterilized with bleach and rinsed thoroughly between hellbenders. Each hellbender was swabbed for *Bd* zoospores once every week. The swabbing procedure (see Appendix B for details) included maneuvering the hellbender into a small holding tub, holding it with gloves, and wiping over the ventral surfaces including the feet, tail, and stomach with a sterile rayon swab. Each swab was placed in a dry 2 mL screw top tube then stored at -80°C until they were shipped on ice to the analysis lab (Hyatt *et al.* 2007). Gloves, holding bins, and water were changed between hellbenders. Skin color changes (often a sign of chytrid infection) and general appearance were also monitored each time a hellbender was handled. Photographs were taken to document any abnormalities.

In addition to weight and health assessments, all twenty hellbenders were located with radio telemetry. Throughout the study period (June 2017 – October 2017), the hellbenders were located using a Communications Specialists (Orange, CA, U.S.A.) receiver (model R1000) and a Telonics rubber "H" type antenna (model RA23). From the day of release until the end of August 2017 the hellbenders were located (but not handled) daily. Cages were removed from the stream on 11 September 2017. Following cage removal, the remaining hellbenders (caged and uncaged) were tracked daily for one week (to assess post-release movements) after which they were tracked once weekly until 24 October 2017. Air temperature, water temperature, precipitation,

and percent moon illumination (for that night) were recorded every day. The moon illumination

data were obtained from the US Naval Observatory website.

If a deceased hellbender was found within a day or two of its death, the body was

collected and sent to AHDC. Cornell Animal Health and Diagnostic Center had an AHDC

pathologist conducted necropsies on the dead hellbenders. At the completion of the study, any

surviving hellbenders were left in place with the transmitters still intact. The reintroduction of

these hellbenders was considered successful.

Chytrid infection was measured at the AHDC using the swabs collected in the field. DNA

was isolated from the swabs to complete an enumeration of *Bd* zoospores by qPCR (see

Appendix C for details). Chytrid swab results were then analyzed for differences over time and

for hellbender treatment groups.

Statistical and Data Analysis

Measurements of chytrid, measured by *Bd* zoospore load in ITS-1 copies per swab, were

converted to a 0/1 scale of low/high chytrid due to a clear break in the dataset at 20000 ITS-1

copies per swab. Hellbenders with average *Bd* loads above 20,000 ITS-1 copies per swab were

considered to have high chytrid and hellbenders with loads below 20,000 ITS-1 copies per swab

were considered to have low chytrid.

We analyzed survival as a function of chytrid, caging and vaccination using a generalized

linear model (GLM) assuming a binomial error distributions (quasi-binomial if overdispersed) in

the RStudio integrated development environment (RStudio Team Version 1.1.414). The

coefficients for the fitted GLM models were estimated using analysis of deviance (ANODEV).

ANODEV is a maximum likelihood approach used with GLMs fit using an analysis of variance (ANOVA) model with a Chi-square test. The ANOVA function uses a Wald chi-square test to calculate Type II P-values. A caging by vaccination interaction term was included.

Hellbender movement was quantified by distance traveled in the stream. This was calculated using the "riverdist" package (Tyers 2017) in RStudio. Parameters such as single movement distance range, cumulative distance, average daily distance, and sedentariness were calculated to help describe post-release hellbender movements during the study period (29 June – 24 October). For hellbenders in the caged treatment group these parameters were calculated using data collected after the cages were removed from the stream, during which the hellbenders were free to move (11 September – 24 October). Hellbender sedentariness was calculated using the ratio of 0 m movements to number of observations (Bodinof *et al.* 2012a).

A principle component analysis (PCA) was performed in RStudio using the "FactoMineR" package (Husson *et al.* 2019) to determine if any variables covaried with average distance and daily number of hellbender movements. Moon illumination covaried with total number of daily hellbender movements. Further analysis of the relationship between hellbender movement and moon illumination resulted in a 'wedge-shaped' residual pattern, so a 90th quantile regression was used to determine if moon illumination was a limiting response. This was performed using the "quantreg" package (Koenker *et al.* 2019). Air and water temperature (°C), precipitation (cm), days since release (#), and *Bd* load (ITS-1 copies per swab) did not covary with hellbender movement in the PCA and therefore was not analyzed further.

Average distances traveled in three directions (upstream, downstream, and lateral movement) were analyzed with an Analysis of Variance (ANOVA) using the "car" package (Fox *et al.* 2019. Three Student's *t-tests* were performed, assuming unequal variances, to detect

differences in mean distance moved by hellbenders (m), mean weight change (g), and mean *Bd* load (ITS-1 copies per swab) between each respective treatment group.

Results

Survival

The duration of the study period (29 June 2017 – 24 October 2017) resulted in 30% survival of the released hellbenders. The first nine confirmed mortalities occurred during the last two weeks of August, 8-9 weeks after release. Two more hellbenders were confirmed dead during the first two weeks of September, 10-11 weeks after release. The last confirmed death occurred during the first week of October, 15 weeks after release. One hellbender was not found after the second day of release and was assumed dead because the last known location had minimal cover and was surrounded by animal tracks. Of the six survivors, three were in the caged/vaccinated treatment group, two were in the caged/not vaccinated group, and one was in the not caged/not vaccinated group. A caging x vaccination interaction term ($p = 0.083$) indicated that the effects of caging and vaccination on survival were nonadditive as survival was low with high chytrid whether the hellbenders were caged or not, but caged hellbenders with low chytrid had higher survival than uncaged hellbenders (Figure 5). For the five deceased hellbenders in the caged treatment group, the final cause of death was determined to be chytridiomycosis for four of the five hellbenders. The other individual was found to have small amounts of *Bd* on its skin, however the primary cause of death was skin saprolegniasis resulting from *Saprolegniasis sp.* (a water mold). Bodies of deceased hellbenders that were not caged were unable to be collected and

diagnosed for mortality. However, daily tracking allowed for direct observation of specific events of predation in two cases (Table 1).

Health

All released hellbenders, except the one early disappearance, tested positive for *Bd* at some point throughout the summer. The onset of *Bd* began 4-6 weeks after release (Figure 6). Highest average *Bd* loads occurred during week seven. Vaccination treatments resulted in no difference in mean *Bd* load (ITS-1 copies per swab) ($t = -0.97$, $n = 87$, $p = 0.335$, Figure 7). Caged hellbenders gained slightly more weight (percent change from starting weight) than hellbenders that were not caged ($t = 2.12$, $n = 11$, $p = 0.058$, Figure 8).

Movement

PCA results showed a correlation between percent moon illumination and total number of daily hellbender movements (Figure 9). Quantile regression indicated that hellbender movements increased with increased moon illumination (*coeff.* $= 0.031$, $SE = 0.018$, $t = 1.727$, $p = 0.088$, Figure 10). There was no correlation between air temperature, water temperature, precipitation, number of days since release, and hellbender movement (neither total number of daily movements nor mean daily distance traveled).

Individual hellbender cumulative distances traveled are shown in Figure 11. Caging treatments had no effect on mean distance moved in the first 20 days of freedom ($t = 1.84$, $n = 14$, $p = 0.087$, Figure 12). The average daily distance traveled by uncaged hellbenders during the

entire study period (29 June 2017 – 24 October 2017) was 38 ± 16m ($n = 10$). The average daily distance traveled by caged hellbenders after the cages were removed (11 September 2017 – 24 October 2017) was 185 ± 92m ($n = 5$) (Table 2). During the first 20 days that both treatment groups were able to move (29 June 2017 – 18 July 2017 for uncaged and 11 September 2017 – 30 September 2017 for caged), caged hellbenders were slightly more sedentary (0.76) than uncaged hellbenders (0.69). All hellbenders in the study moved greater distances downstream than upstream ($F(2,108) = 5.87$, $p = 0.009$) whereas lateral distance moved did not differ from upstream ($p = 0.814$) or downstream distances ($p = 0.059$) (Figure 13).

Discussion

Past hellbender releases in New York have been largely unsuccessful, presumably due to high rates of post-release movement which may lead to increased predation events (Boerner 2014, McMillan *pers. comm.*). Wild hellbenders characteristically move very little (typically <30-40m), spending most of their life under one rock (Nickerson and Mays 1973, Foster *et al.* 2009). The hellbenders in this study made frequent movements, some of great distance, following their release. The range of observed single movement distances was 1-1839m, similar to Boerner (2014) (14-1892m). Average ($\pm$SE) daily distance of 87 ± 36m and an average cumulative distance of 1102 ± 267m (Table 2) were higher than those recorded in Boerner (2014) (11 ± 2m, 653 ± 138m, respectively). Most movements made were downstream, similar to findings of other hellbender releases (Bodinof *et al.* 2012a, Boerner 2014, Gates *et al.* 1985).

Previous work suggests that temporary caging reduced hellbender movement after release (Stamps and Swaisgood 2007), but the cages used in this study had no effect on hellbender

movement once the animals were released. Hellbenders in the caged treatment group moved more than uncaged hellbenders, and greater distances, once the cages were removed. The timing of cage removal in this study coincided with hellbender breeding season, which may have contributed to increased movement by the caged group of hellbenders. The only variable that seemed to positively correlate with movement was moon illumination. Interestingly, Boerner (2014) also found that hellbenders moved further and more frequently when moon illumination was high. This behavior is not typical for amphibians, as greater moon illumination makes predation by visual predators more likely (Lima and Dill 1990). This result should be investigated further as to whether this is a result of the light cycles used in captive raising or if this is typical behavior for wild hellbenders.

Although temporary caging was unsuccessful in reducing movement, it seemed that the cages may have offered protection from predators. Uncaged hellbenders were highly susceptible to predation, due to their frequent movements within the stream. Of the nine uncaged hellbender deaths, two deaths were confirmed predation events resulting most likely from great blue heron (*Ardea herodias*) and raccoon (*Procyon lotor*) attacks. However, hellbenders in cages survived better (5 caged survivors vs. 1 uncaged survivor) when their chytrid infections were lower. This is probably due to the added protection cages afforded during the early part of the study.

Captive-raised hellbenders lack predator avoidance behaviors. It has been demonstrated in the lab that hellbenders are capable of learning avoidance cues with some fish (*Oncorhynchus mykiss* and *Hypostomus plecotomus*) (Crane and Mathis 2011). In this study, major predators to hellbenders appeared to be birds and mammals and there are no documented studies involving recognition cues for these types of predators. Pre-release training is not well studied in amphibians, although it is better understood and more successful with other species. Whether

lack of predator avoidance behaviors in hellbenders is strictly due to captive-raising or from peculiar behaviors resulting from chytrid infection is unclear. However, predator-recognition training is something that could be included in hellbender captive-raising procedures to teach behaviors that may increase chances of survival (Crane and Mathis 2011).

Recent studies have reported detections of *Bd* on hellbender populations throughout their range (Bales *et al.* 2015, Bodinof *et al.* 2011, Seeley *et al.* 2016, Souza *et al.* 2012) however, I found no other studies that have conducted long term post-release monitoring of weekly *Bd* zoospore load. The monitoring in this study provides insight into the weekly progression of the disease. Chytridiomycosis infection appeared to fluctuate throughout the study period possibly due to the life cycle of the fungus living within the host's epidermal cells. However, the life cycle of *Bd* is not well understood. Our results show that *Bd* zoospores were first detected around four weeks after release. At week six, average *Bd* loads drastically increase. This increase in zoospore amount coincided with the first instances of hellbender mortalities, at least some of which were confirmed to be a result of chytridiomycosis. Regardless, all study hellbenders had some amount of *Bd* zoospores during the study.

Chytrid related mortalities in this study are likely due to the disruption of skin function, such as limited oxygen exchange, or the release of toxins from *Bd* zoospores (Berger *et al.* 2005). In the Boerner (2014) study, some hellbenders exhibited a beaching behavior and one of those hellbenders tested positive for chytrid. These hellbenders appeared to be oxygen stressed due to the increased blood flow to the retained gill slits and skin. In the current study, two hellbenders (both positive for chytrid infection, one caged and one uncaged) were observed surfacing for air on separate occasions, also indicative of oxygen stress, which most likely

resulted from their infections. The taller cage design allowed for hellbenders surfacing for air, without risk of predation, but both hellbenders that were observed surfacing still died.

In New York State, chytrid seems to be a major factor that limits the success of post-release hellbender survival (Boerner 2014). Captive-raised hellbenders are kept under sterile conditions in the lab (Dean *pers. comm.*, Felski *pers. comm.*) which may put them at higher risk of infection and disease once they are released into a natural stream setting. *Batrachochytrium dendrobatidis* has been detected in wild hellbender populations of NYS (Bales *et al.* 2015) but it is not clear if this disease is contributing to the local declines. Wild hellbenders may build up an immunity or tolerance to infection as juveniles that are exposed to the fungus early on. Successful releases will require methods of captive raising to mimic more natural conditions so hellbenders can better acclimate to their new habitat.

Conclusions

The goal of this study was to successfully reintroduce captive-raised Eastern hellbenders into native wild habitat and monitor them, while implementing two new strategies that were predicted to increase the chances of a successful release (higher survival): a vaccine for chytridiomycosis and a new caging release method. The results suggest that the chytrid vaccine was not successful at preventing infection for the hellbenders in this field study. Every released individual tested positive for *Batrachochytrium dendrobatidis* during the study period and, for at least four of those individuals, the infection was fatal. The new cage design and method of release intended to reduce excessive post-release movement in order to lower the chance of predation events and movement away from appropriate habitat. The results presented here suggest that temporary

caging, although successful with some amphibian species (Semlitsch 2002), may not reduce total post-release hellbender movement. However, more caged hellbenders survived the duration of the study period than uncaged, possibly because of protection from predators during a significant portion of the study. Regardless of these seemingly ineffective treatments, this study still resulted in greater hellbender survival than most past hellbender releases in New York State.

Captive raising and releasing of Eastern hellbenders is still in its early stages compared to head-starting programs of other species. If hellbender conservation efforts are to be successful, predator recognition training, early exposure to *Bd*, or even new chytrid vaccines may be useful for increasing released hellbender survival. The observed hellbender movement and the response to natural moon illumination suggests that captive-raising may require conditions that mimic the natural environment. Moreover, the findings here suggest that more research is required to fine-tune the art of hellbender raising and releasing.

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Table 1: Fate of each released Eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) by the end of the study period (24 October 2017).

ID	Treatment		Fate	Details
8625	Caged	Vaccinated	Dead (8-17)	Necropsy results – Chytridiomycosis (multifocal dermatitis and hyperkeratosis)
4791	Caged	Vaccinated	Alive	
7994	Caged	Vaccinated	Dead (9-1)	Necropsy results – Chytridiomycosis (multifocal dermatitis and hyperkeratosis)
5138	Caged	Vaccinated	Alive	
4250	Caged	Vaccinated	Alive	
5858	Caged	Not Vaccinated	Dead (8-24)	Necropsy results – Chytridiomycosis (multifocal hyperkeratosis and hyperplasia)
5087	Caged	Not Vaccinated	Alive	
5585	Caged	Not Vaccinated	Dead (8-26)	Necropsy results – Chytridiomycosis (multifocal hyperkeratosis and hyperplasia), Mycotic ulcerative dermatitis (possibly *Saprolegnia sp*)
6928	Caged	Not Vaccinated	Dead (9-11)	Necropsy results – Skin saprolegniasis (also possibly emaciation/starvation, some *Batrachochytrium dendrobatidis* fungus found in small amounts)
7326	Caged	Not Vaccinated	Alive	
5401	Not Caged	Vaccinated	Dead (8-17)	Body not found. Transmitter located but not recovered. Assumed transmitter washed downstream after death occurred.
5150	Not Caged	Vaccinated	Dead (8-16)	Body not found, transmitter recovered.
7770	Not Caged	Vaccinated	Dead (8-20)	Body not found, transmitter not recovered. Transmitter sound faded out as blue heron flew away, confirmed predation.
4885	Not Caged	Vaccinated	Dead (10-6)	Body not found, transmitter recovered.
8405	Not Caged	Vaccinated	Disappeared (6-30)	Lost after second day tracking (6/30), last found at shallow spot with blue heron and raccoon tracks.
8754	Not Caged	Not Vaccinated	Dead (9-11)	Body collected, transmitter recovered, no necropsy performed due to decomposed state of carcass.

7150	Not Caged	Not Vaccinated	Dead (8-17)	Body collected, transmitter recovered. Found previous day with severe puncture wounds and broken jaw from attempted predation. No necropsy performed since cause of death was known.
7414	Not Caged	Not Vaccinated	Alive	
4938	Not Caged	Not Vaccinated	Dead (8-22)	Body not found, transmitter not recovered. Seen attempting to swim and coming up for air, having trouble righting itself. Assumed dead after unable to locate again.
5628	Not Caged	Not Vaccinated	Dead (8-24)	Partial body collected, transmitter recovered.

Table 2: Parameters describing post-release movements of captive-raised Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*). Values describing movement of uncaged animals were calculated using data collected from the entire study (29 June 2017 – 24 October 2017). Values describing movement of caged animals were calculated using data collected after the cages were removed (11 September 2017 – 24 October 2017). Sedentariness was calculated using data from the first 20 days that both treatment groups were able to move (uncaged: 29 June 2017 – 18 July 2017, caged: 11 September 2017 – 30 September 2017). Numbers in parentheses denote sample size.

Treatment	Total number of movements	Range of single distances (m)	Average cumulative distance (m)	Average daily distance (m)	Sedentariness[a]
Caged	16	1-1839	1329.00 ± 705.15 (*n* = 5)	184.92 ± 92.38 (*n* = 5)	0.76
Not Caged	95	1-1032	988.70 ± 225.69 (*n* = 10)	37.97 ± 16.36 (*n* = 10)	0.69
All	111	1-1839	1102.13 ± 266.50 (*n* = 15)	86.95 ± 35.64 (*n* = 15)	0.72

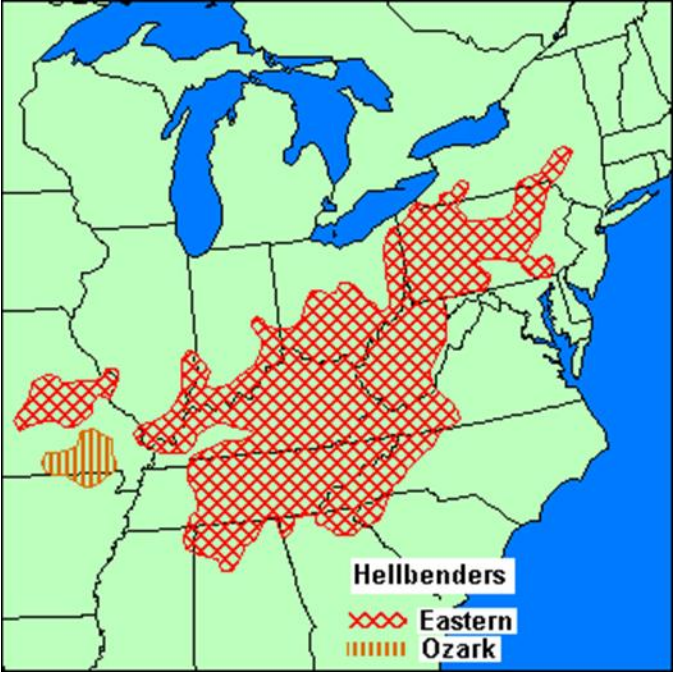[a]Sedentariness is the ratio of 0 m-movements to number of observations.

Figure 1: Map showing the native range of both Eastern and Ozark hellbenders. (New York State Department of Environmental Conservation)
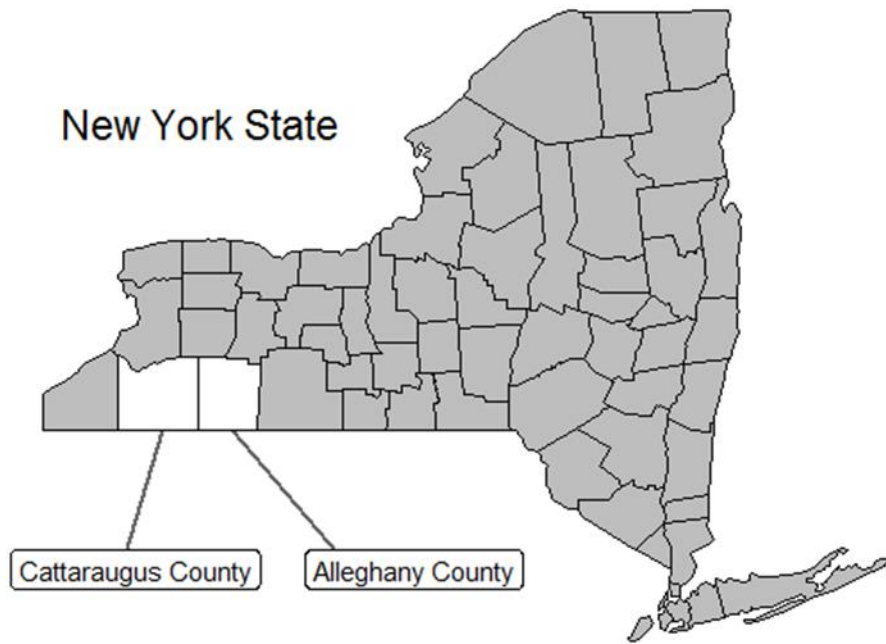
Figure 2: Map of New York State showing Allegany and Cattaraugus counties in which the Allegheny River drainage is contained.

Figure 3: Photograph of cage design used for the caging release method of captive-raised Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*). Dimensions of this 5-sided cage are 1.2m x 1.2m x 0.9m. The hinged door was secured with zip-ties when closed between sampling dates.
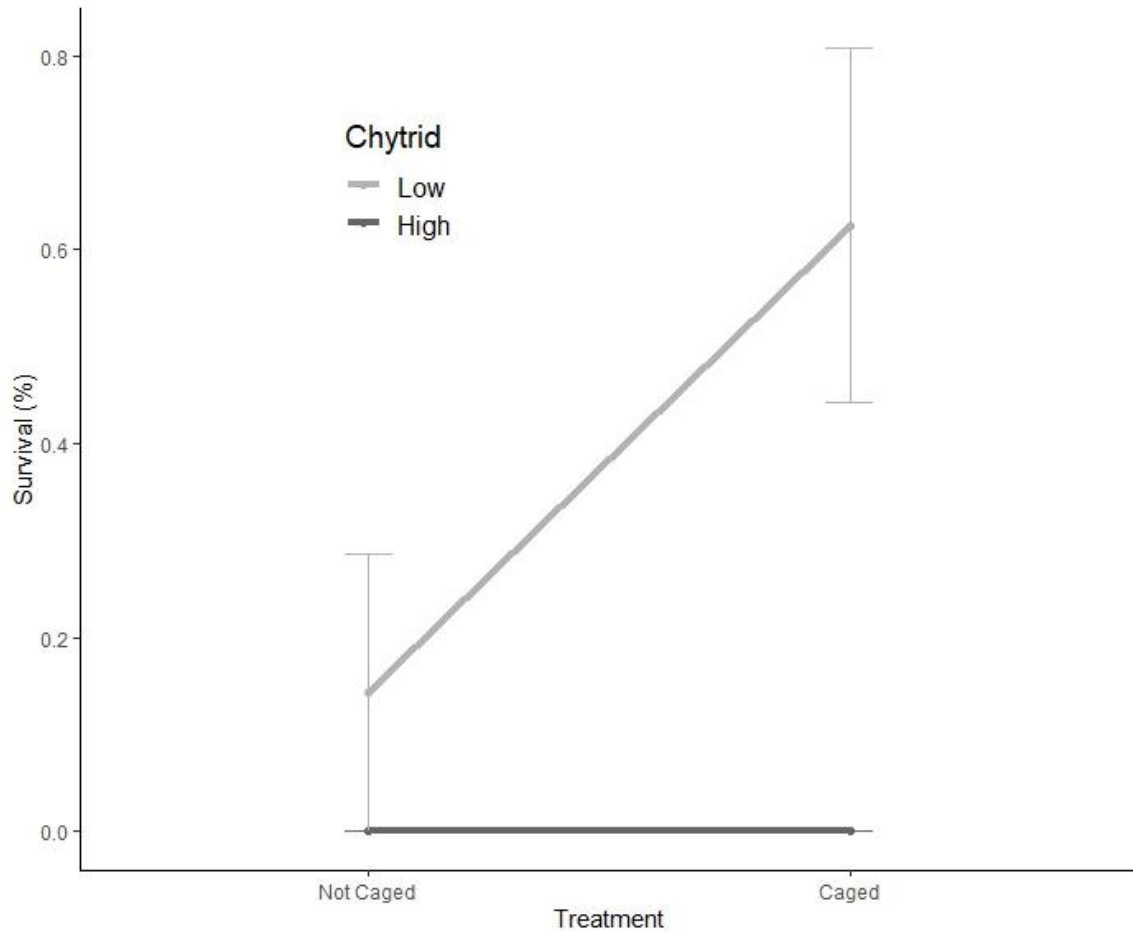
Figure 4: Photograph of cages used for the caging release method of captive-raised Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) installed in the study area of the stream.

Figure 5: Interaction plot of released Eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) survival. Figure displays a caging x vaccination interaction term (*p* = 0.083) indicating that the effects of caging and vaccination on survival were nonadditive.
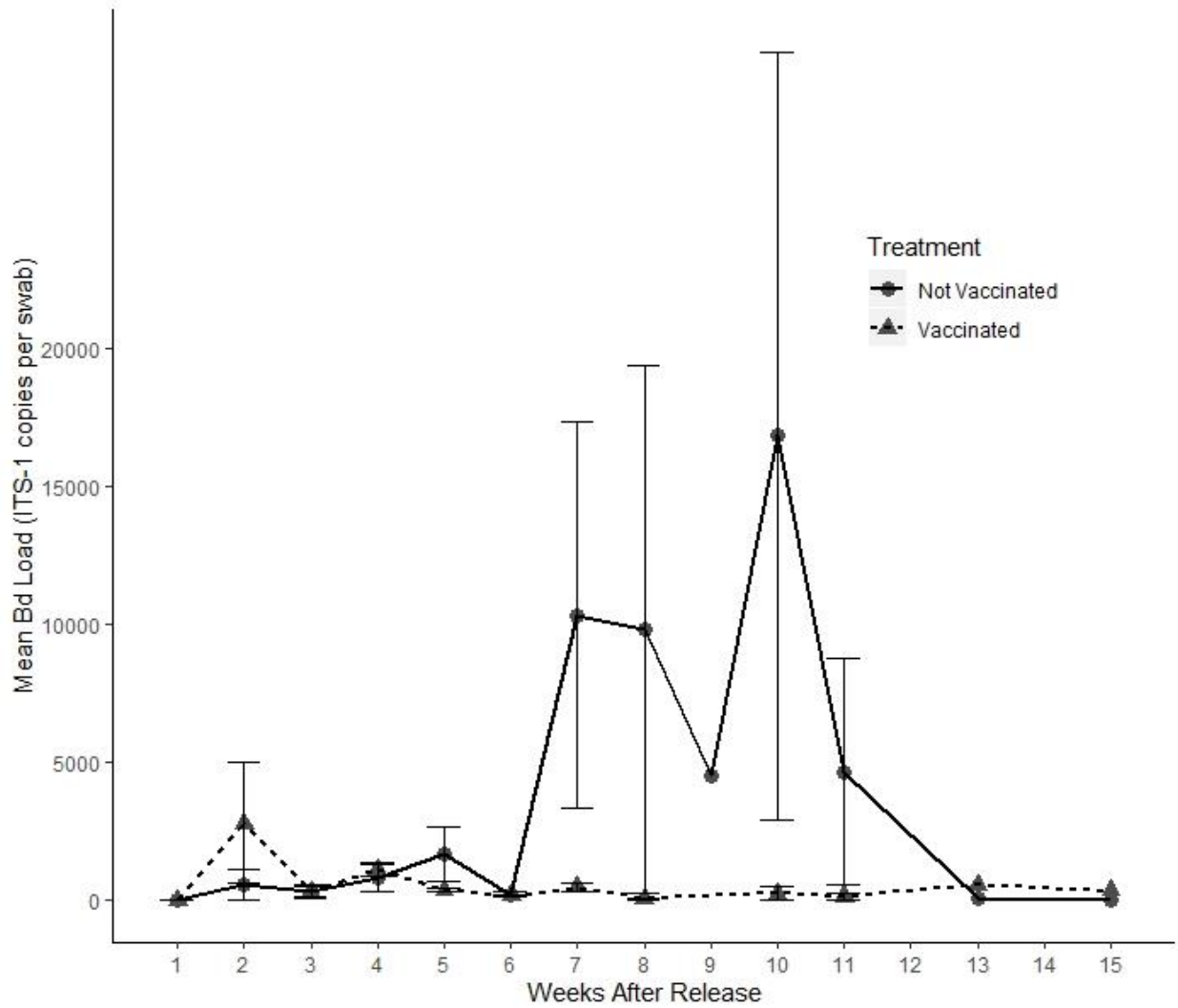
Figure 6: Mean *Batrachochytrium dendrobatidis* (*Bd*) load for vaccinated and unvaccinated released Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*). Figure displays onset of *Bd* infection approximately 4-5 weeks after initial release into the stream. Error bars represent standard error.
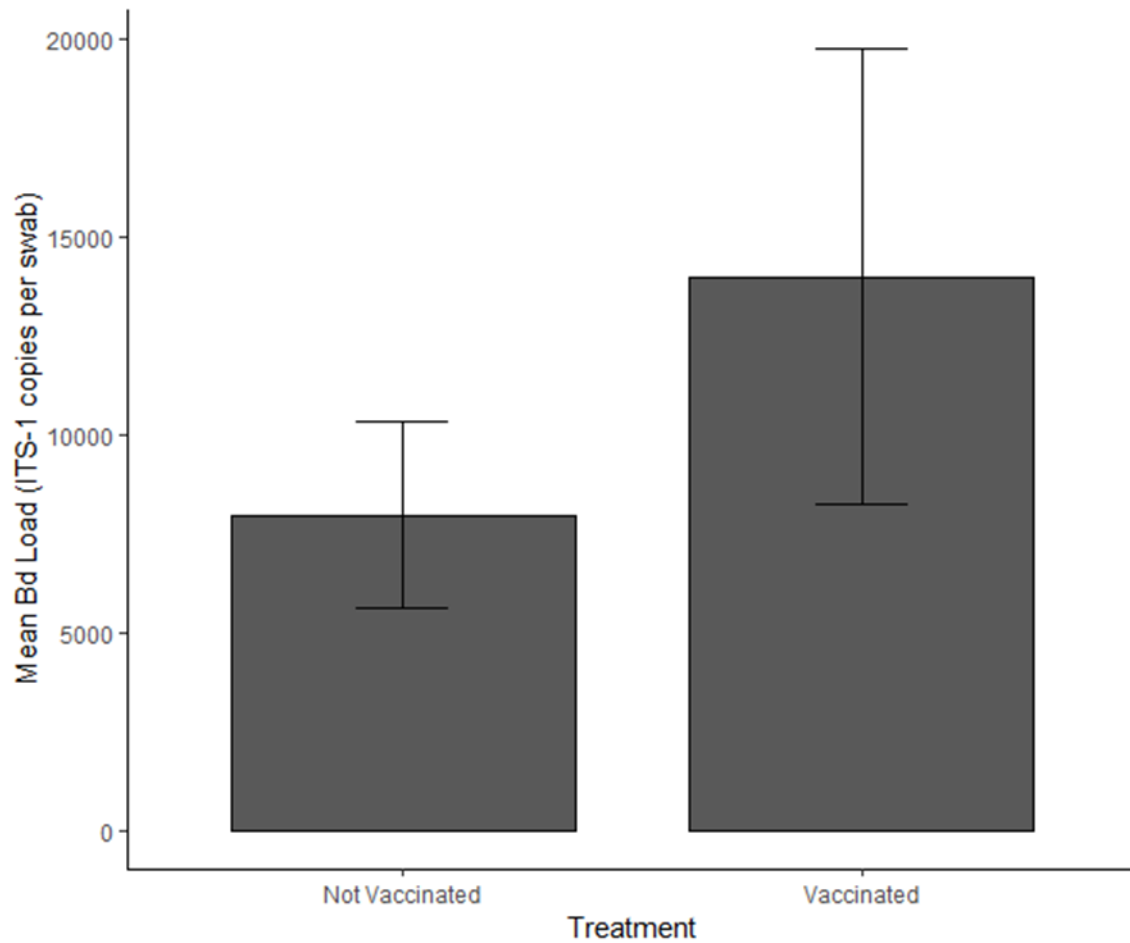
Figure 7: Mean *Batrachochytrium dendrobatidis* (*Bd*) loads recorded for released Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) for two treatment groups, vaccinated and not vaccinated. Data includes all *Bd* swabs taken within the study period (29 June 2017 – 24 October 2017). There was no difference in *Bd* loads between vaccinated and not vaccinated hellbenders ($p = 0.3353$). Error bars represent standard error.
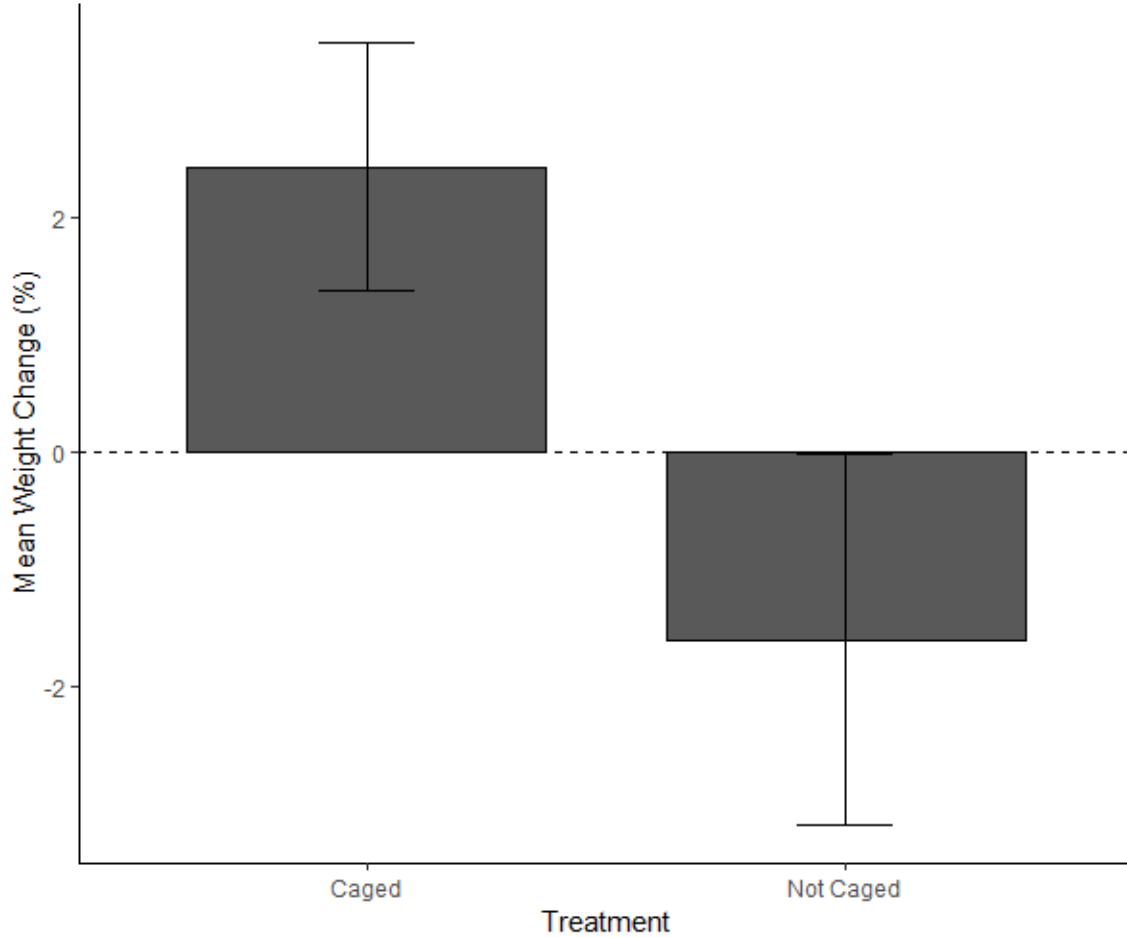
Figure 8: Mean change in body mass as a percentage of starting mass of released Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) for two treatment groups, caged and not caged. Data includes all weights recorded within the study period (29 June 2017 – 24 October 2017). Caged hellbenders gained slightly more weight than uncaged hellbenders ($p = 0.0575$). Error bars represent standard error.
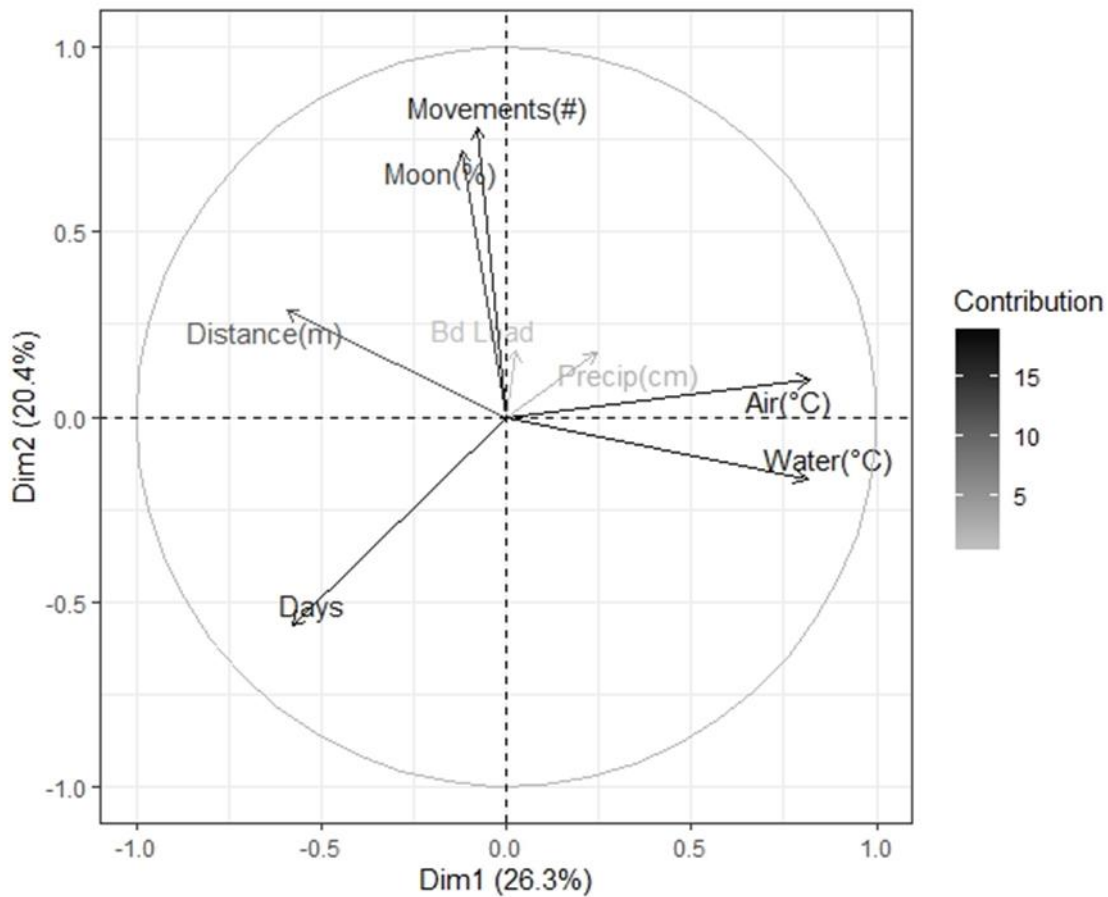
Figure 9: Principle component analysis of variables that may affect post-release movement of captive-raised Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*). The mean distance (m) calculation includes movements made by hellbenders that could move (ie. Excludes the "0" values from caged hellbenders while they were caged). (Days = Number of days since release, Moon(%) = Percent moon illumination each day, Distance(m) = Mean distance moved by uncaged hellbenders each day in meters, Movements(#) = Total number of movements made by all hellbenders each day, *Bd* Load = Mean *Batrachochytrium dendrobatidis* zoospore loads for all hellbenders each week in ITS-1 copies per swab, Precip(cm) = Total precipitation each day in centimeters, Air(°C) = Air temperature each day, Water(°C) = Water temperature each day)
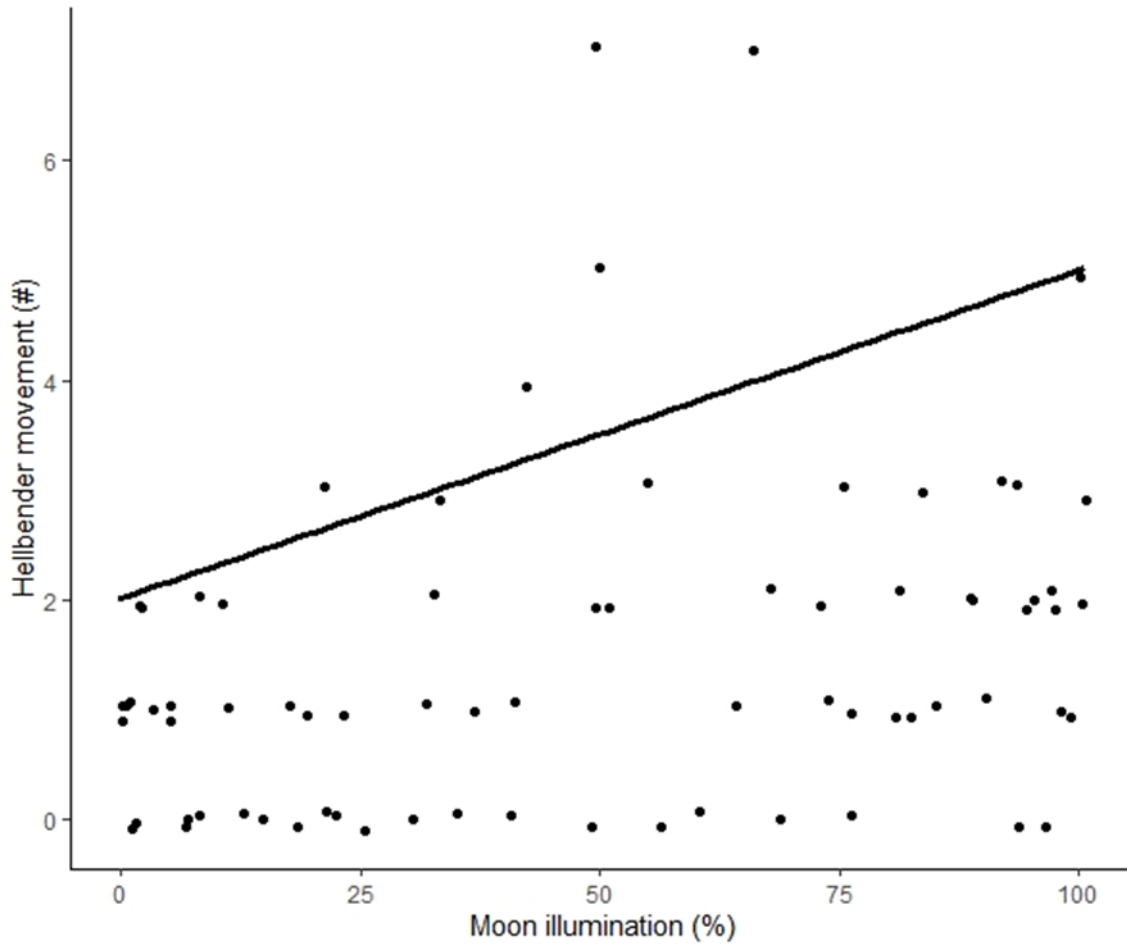
Figure 10: Ninetieth quantile regression of total number of Eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) movements per day and percent moon illumination. Moon illumination had a slight effect on greater number of hellbender movements ($p = 0.088$). The percent moon illumination was 36% on the first night after they were released.
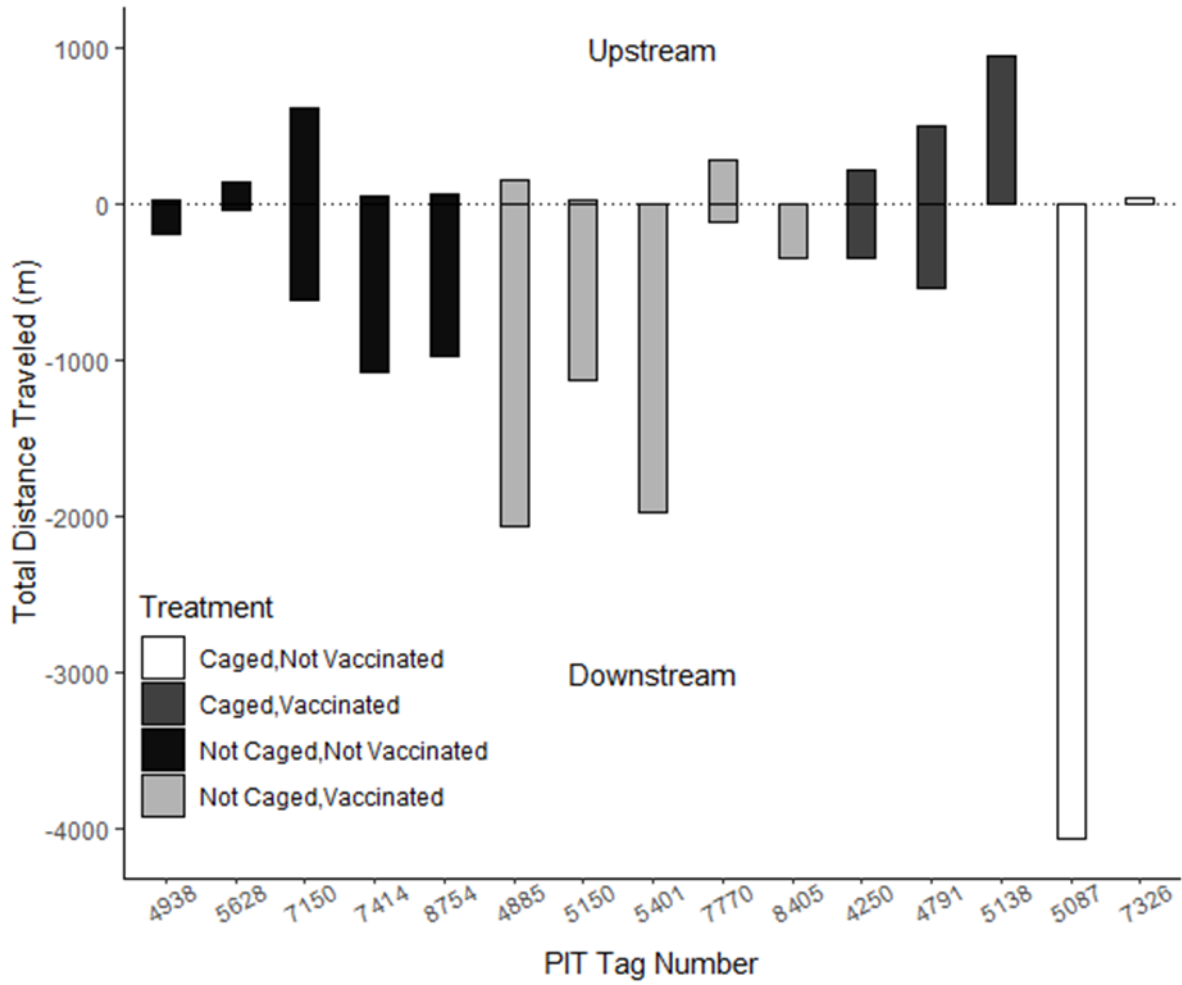
Figure 11: Total distance traveled in meters by each Eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) for the entire study period (29 June 2017 – 24 October 2017). Positive values represent total distance traveled upstream and negative values represent total distance traveled downstream.
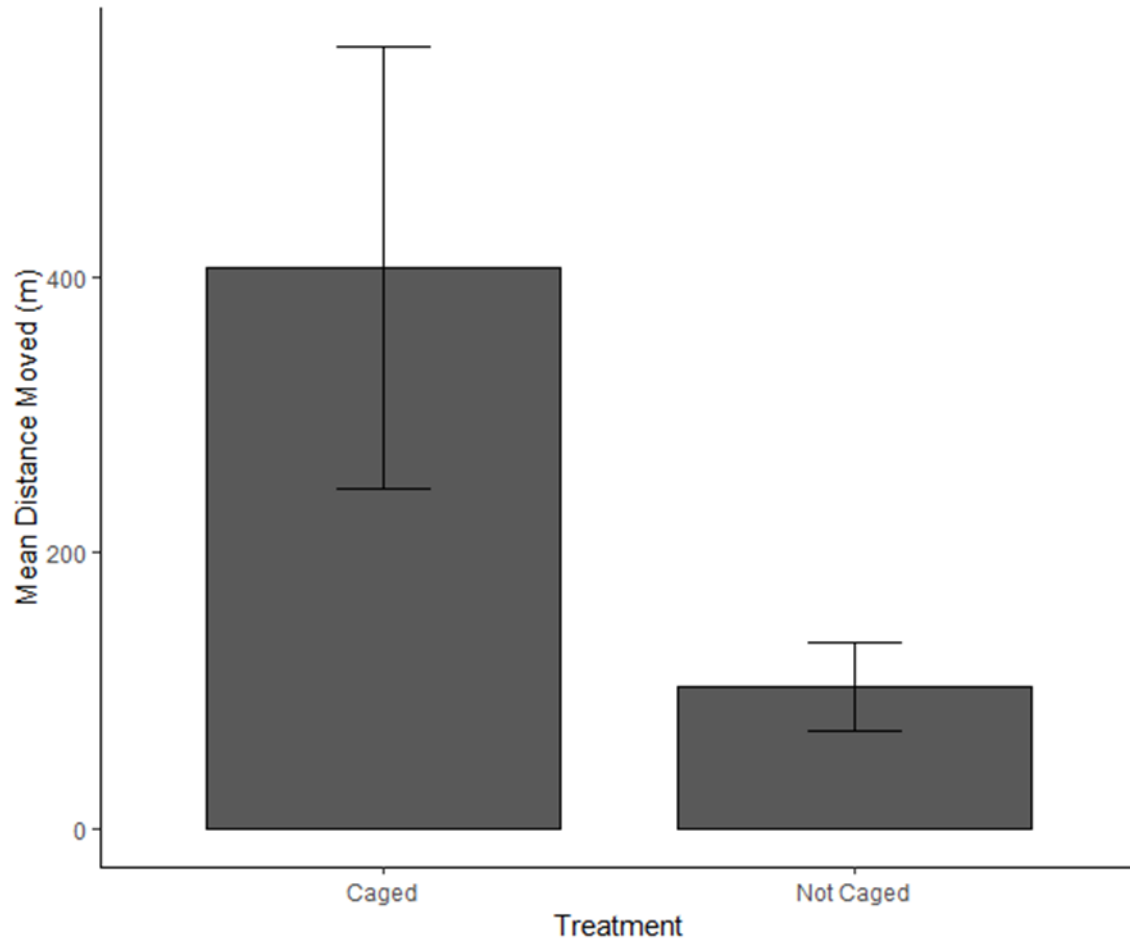
Figure 12: Mean distance traveled (meters) by Eastern hellbenders *(Cryptobranchus alleganiensis alleganiensis)* from two treatment groups, caged and not caged. Data for the hellbenders not caged were collected from the first 20 days after their release into the stream (29 June 2017 – 18 July 2017). Data for the caged hellbenders were collected from the first 20 days after cages were removed from the stream (11 September 2017 – 30 September 2017). There was no difference between mean distance traveled by the two caging treatments ($p = 0.0847$). Error bars represent standard error.
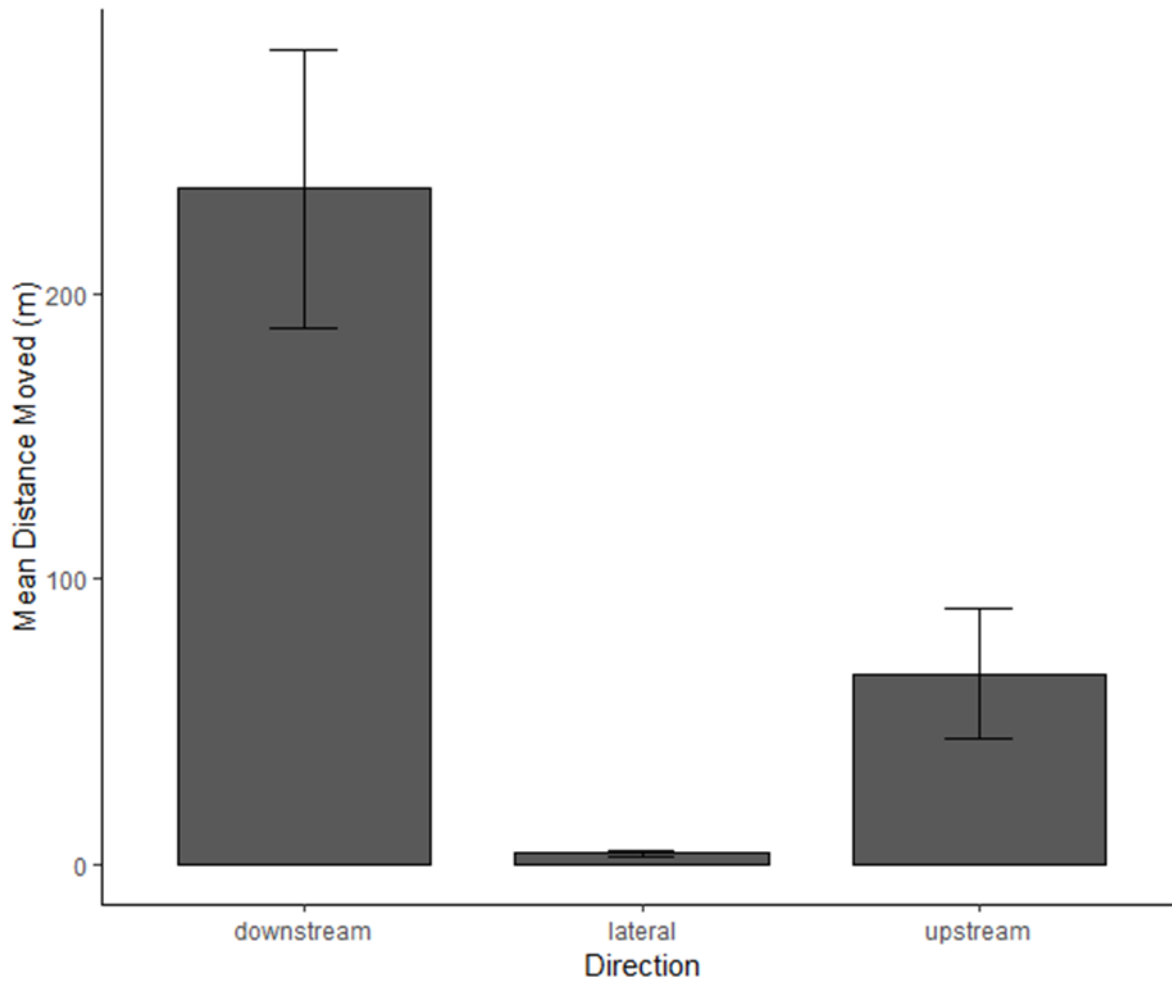
Figure 13: Mean distance traveled by released Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) in three directions: downstream, upstream, and lateral movement. Data includes all hellbender movements within the study period (29 June 2017 – 24 October 2017). Mean downstream distance traveled was significantly higher than mean upstream distance traveled ($p = 0.0086$). Error bars represent standard error.

Appendix A

Vaccination Treatment Protocol:

• Day 1: Animals will be briefly manually restrained using a paper towel and the following samples will be collected:

  - A skin swab for pretreatment *Bd* quantitative PCR

  - Physical measurements - Each individual hellbenders body weight will be taken using a gram scale with a tared plastic bin lined by a disposable plastic liner that can be changed between animals.

  - Hellbenders will then be experimentally or mock treated with an oral (0.5ml) deionized water plus or minus liquid nitrogen killed *Batrachochytrium dendrobatidis* zoospores. Each 0.5ml vaccination will have $1x10^6$ zoospores. This will be done using a pipettor and a plastic pipette tip, so as to not damage their teeth. After administration, the animals will be maintained in individual, plastic sterilite bins with a small amount of water and a paper towel for 2 hours to permit adsorption. After the adsorption period, hellbenders will be rinsed with tank water and returned to their respective tanks.

• Day 22: Animals will be manually restrained and all procedures as performed on Day 1 will be repeated.

• Day 43: Animals will be manually restrained and all procedures performed on Day 1 will be repeated.

• Day 64: Animals will be manually restrained and all procedures as performed on Day 1 will be repeated.

• Day 85: Animals will be ready for transfer back to the New York State Department of Environmental Conservation (NYSDEC) and arrangements will be made to give them back. After this animals will be maintained at Cornell as usual until the NYSDEC can take them for their summer field project.

• Pre-release to NYSDEC: Animals will be manually restrained and all procedures as performed on Day 1 will be repeated.

Appendix B

<u>Amphibian Chytrid and Ranavirus Swabbing Protocol:</u>

1. Preferably, capture amphibians by hand. Wear vinyl gloves when swabbing animals and change gloves between animals. If you are using a dip net, be aware that *Batrachochytrium dendrobatidis* zoospores could be caught on the net and transferred between individuals, therefore, use different nets whenever possible, or disinfect the net as often as you can (there is no perfect solution to this problem).

2. Swab the underside or ventrum of adult/metamorphs 30 times. Remember you are in effect scraping small amounts of tissue from the skin. Some pressure must be applied, but do not hurt the animal.

   - For frogs: Areas to target are the inguinal areas, thighs, and webbing between the toes. Standardized swabbing is best: 5 swabs each on R/L inguinal region, 5 swabs on each of 4 feet.
   - For salamanders: Areas to target include the underside of the tail and the back side of each of the limbs. Swab the back of each leg 5 times (20 total), the underside of the tail 5 times and the underside of the pelvic region 5 times for a total of 30.

3. Break swab ~3cm from tip and drop into screw cap tube. The swab stick should not touch or bump against the top of the vial. Screw the cap on the vial and store in a cool or preferably cold place. Label with some kind of identifying code that links the sample to the data sheet.

4. It is best to keep the samples cool and placed as soon as possible in a 4 °C freezer. Avoid extreme high temperature and direct sunlight.

5. Repeat process a second time and store second swab in a separate vial.

Appendix C

<u>Swab Preparation:</u>

1. If the swab sample is in Amies, Port-a-cul, or other bacterial transport media, inform the lab manager or lab director, as it generally is an unacceptable sample.
2. If the swab is entirely covered in feces, it should be processed as a fecal sample.
3. **If the swab is dry, the swab must be processed within 24 hours as described as follows:**

    3.1.1   If the swab is older than one week, proceed, otherwise skip to step 3.2.

    3.1.2   Add 1000 µl of media of media to swab in a 1.5ml labeled tube. Break the end of the swab to be able to seal the tube, and vortex well.

    3.1.3   Use boiling caps and incubate for 10 min at 96-97°C, then vortex well.

    3.1.4   Incubate for 45 min at 37°C, then proceed to step 3.3, transferring as much media as possible.

    3.2 Add 1.5 to 2 ml of DMEM using a transfer pipette, and close the lid of the tube.

    3.3 Vortex the tube vigorously. Then, leave at room temperature for one hour (or as long as possible if a STAT). Vortex the tube vigorously again, then use a pipette to transfer approximately 1 ml of media to a 1.5 ml tube labeled with the accession and item number.

    3.4 If there is more than one dry swab from the same animal, pool them by following step 3.1 and 3.2 but instead of transferring 1 ml of liquid, transfer (1 ml divided by the number of swabs) from each tube to a single 1.5 ml labeled tube.

    3.5 Make sure put a red dot on the 1.5ml tube cover for dry swab samples. The red dot is a sign of dry swab sample type for Virology reference if viral isolation is needed in the future.

4. **If the sample is not dry and already contains liquid:**

    4.1 Add the appropriate amount of DMEM so that there is at least 1 to 1.5 ml of liquid. Vortex the tube vigorously. Use a pipette to transfer approximately 1 ml of media to a 1.5 ml tube labeled with the accession and item number.

    4.2 If there is more than one swab with liquid from the same animal, pool them by following step 4.1 and then instead of transferring 1 ml of liquid, transfer (1 ml divided by the number of swabs) from each tube to a single 1.5 ml labeled tube.

5. Place the prepped sample in the designated rack in the sample refrigerator for the 1840 Extraction.

Extraction Process:

Total nucleic acid was extracted from 175 ul of swab suspension or negative control media using a magnetic bead based automated procedure (AM1840, Thermo Fisher) per the manufacturer's instructions with an additional mechanical lysis step using zirconia beads. An exogenous control (MS2 phage) was added to the lysis buffer to monitor inhibition (Dreier *et al.* 2005, Yan *et al.* 2019). Samples were eluted in 90 ul.

Real-time PCR was performed on the ABI 7500-FAST platform using The ITS-1 oligonucleotide sequences published by Boyle *et al.* 2004 without modification.

F primer (0.9 µM final)	**R primer** (0.9 µM final each)	**Probe** (0.15 µM final)
CCT TGA TAT AAT ACA GTG TGC CAT ATG TC	AGC CAA GAG ATC CGT TGT CAA A	6FAM CGA GTC GAA CAA AAT MGBNFQ

The reaction was performed with Path-ID Multiplex One-Step RT-PCR Kit (Thermo Fisher 4442137) with the following conditions: 48°C for 10min, 95°C for 3min, followed by 40 cycles of 95°C for 15sec then 62°C 1min. Five microliters of the nucleic acid template were used in a total reaction volume of 20 ul. Commercial copy number standard DNA was used for interpolation (Pisces Molecular).

Boyle, D., Boyle, D., Olsen, V., Morgan, J., and A. Hyatt. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms 60:141-148.

Dreier, J., Stormer M., and K. Kleesiek. 2005. Use of bacteriophage MS2 as an internal control in viral reverse transcription-PCR assays. Journal of Clinical Microbiology. 43(9):4551-7.

Yan, L., Toohey-Kurth K. L., Crossley B. M., Bai J., Glaser A. L., Tallmadge R. L., and L. B. Goodman. Inhibition monitoring in veterinary molecular testing. Journal of Veterinary Diagnostic Investigation.