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The effects of electrofishing on different life stages of Ozark and eastern hellbenders

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THE EFFECTS OF ELECTROFISHING ON DIFFERENT LIFE STAGES OF OZARK (*CRYPTOBRANCHUS ALLEGANIENSIS BISHOPI*) AND EASTERN (*C. A. ALLEGANIENSIS*) HELLBENDERS

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Of the Requirements for the Degree

Master of Science, Biology

By

Stephanie K. Morrison

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ABSTRACT

Electrofishing, a common method of freshwater fish sampling, has been shown to negatively affect some fish species, but the effects on non-target species, such as hellbenders, have not been well studied. I tested effects of electrofishing on the behavior of several life stages of captive-reared Ozark (*Cryptobranchus alleganiensis bishopi*) and eastern (*C. a. alleganiensis*) hellbenders. Ozark hellbender eggs were exposed to different voltages in the laboratory, and embryos in higher voltages had higher incidences of twitching during exposures and higher numbers of morphological deformities after exposures. For hatchling Ozark hellbenders, which typically are sedentary, individuals moved more during exposure to higher voltages. Free-swimming larval eastern hellbenders were less active and spent more time twitching and immobilized during exposure to higher voltages. Immediately after exposures, larvae in the higher treatment groups were less active. Ozark hellbenders (~ 3-years old) exhibited a greater incidence of stress secretions, twitching, and immobilization during exposure to higher voltages. After exposures, they had lower righting reflex scores and longer latencies to right. Eastern hellbenders (~ 6-years old) were tested in both laboratory trials and in a natural river habitat. In laboratory trials, during exposure to higher voltages, individuals had greater incidences of stress secretions and spent more time twitching and immobilized. Following exposures to higher voltages, they had longer latencies to right and faster heartrates. During a double-shocking experiment, 6-year old eastern hellbenders spent more time twitching in the first shocking event and tended to spend more time immobilized in the second shocking event although this difference was not significant; latencies to secrete were longer in the second shocking event. In the river trials, shocked 6-year old had a higher incidence of stress secretions than controls. No behavioral differences appeared to persist after 3–5 months. Under the conditions of our experiments, exposure to voltages similar to those experienced during electrofishing caused at least short-term negative effects on hellbender behavior.

KEYWORDS: hellbender, *Cryptobranchus*, electrofishing, conservation, behavior, stress

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I dedicate this thesis to my father and mother, Kent and Amy Morrison.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: OVERVIEW

The following thesis examines the effects of electroshocking on the hellbender, a species of conservation concern, in two chapters that are designed to be independently publishable units. Chapter 2 is the study of how electrofishing affects early hellbender life stages (eggs, hatchlings, and larvae). Chapter 3 examines the effects of electrofishing on older hellbender life stages (3-years old and 6-years old). All individuals were tested under St. Louis Zoo IACUC Protocol #15-06 approved in September of 2016, and Ozark hellbender individuals were tested under Jeff Briggler, MDC under Federal Permit #TE73587A-2.

CHAPTER 2: THE EFFECTS OF ELECTROFISHING ON OZARK HELLBENDER (*CRYPTOBRANCHUS ALLEGANIENSIS BISHOPI*) EGGS AND HATCHLINGS AND EASTERN HELLBENDER (*C. A. ALLEGANIENSIS*) LARVAE

Introduction

Electrofishing is a method of aquatic sampling that uses electricity to stun and create taxis in fish so that they can be easily captured. Electrofishing is an excellent management tool that allows biologists to sample freshwater fish for assessment of species presence, abundance, and health (Graynoth et al., 2011). Older fishes are usually the target of electrofishing studies, but exposure of eggs, hatchlings, and younger life stages can occur during surveys.

Although there is variation among species and specific electrofishing methodologies (Oberlercher and Wazenbock, 2016), considerable research has shown that fish embryos experience negative effects from electrofishing. For example, in both rainbow trout (*Oncorhynchus mykiss*, Dwyer et al., 1993) and European whitefish (*Coregonus lavaretus*, Oberlercher and Wazenbock, 2016) the mortality rate of eggs was higher for shocked eggs, and the impact was greater at higher voltages. Developmental stage and egg size also can affect the level of electroshocking impacts. Mortality of electroshocked embryos of some salmonids declined after the emergence of eyes (*Salvelinus fontinalis,* Keefe et al., 2000) and/or pigmentation (*O. mykiss*, Simpson et al., 2016). Comparisons of several freshwater taxa (sunfish, catfish, trout, danios, shiners, perch, and suckers) indicated that species with increasing embryo diameter had higher levels of mortality (Bohl et al., 2010).

In addition to the effects of electrofishing on the embryos, survival of eggs produced by females who have been electroshocked can be affected. In Artic graylings (*Thymallus arcticus*),

eggs from electroshocked females had higher mortality rates than control eggs (Roach, 1999). Shocked razorback suckers (*Xyrauchen texanus*) expelled gametes during exposure to electrofishing, and unshocked fish had significantly more eggs hatch than the treatment groups exposed to electrofishing (Muth and Ruppert, 1997). Chinook salmon (*O. tshawytscha*) are commonly collected both via fish ladder and by electrofishing, but higher mortality rates of eggs were observed for females captured via by electrofishing (Huysman et al., 2018). For electroshocked females, there was also a significant correlation between amount of handling the females received and the survival of eggs (Huysman et al., 2018).

Electrofishing can also affect post-hatching stages. In steelhead trout (*O. mykiss*), a particularly sensitive stage to electrofishing was the swim-up larval stage, which is the stage where larvae are able to swim freely (Simpson et al., 2016). The most susceptible developmental period to electroshocking appears to be during the transition from larvae to juveniles for some species (*Leomis macrochirus, Micropterus salmoides, Ictalurus punctatus, Oreochromis niloticus;* Henry et al., 2003).

Most studies of effects of electrofishing on eggs and juveniles focus on fishes. However, nontarget species in other taxa could also be affected. For vertebrates, the most likely nonfish species in which eggs/hatchlings might be influenced are amphibians. Although many amphibians lay their eggs in fishless ponds, some species co-occur in lakes, streams or rivers with fishes where they might experience inadvertent exposure to electrofishing. However, few studies have tested for effects of exposure to electroshock on amphibian eggs and younger life stages (Gilbert et al., 2017).

One species that may be particularly likely to be exposed to electrofishing during fish censuses is the hellbender (*Cryptobranchus alleganiensis*), a large fully-aquatic salamander that

spends its life in fast-flowing streams (Nickerson and Mays, 1973). There are two currently-recognized subspecies, the Ozark (*C. a. bishopi*) and eastern (*C. a. alleganiensis*) hellbenders. All hellbenders are of conservation concern, with documented population declines throughout much of their hellbender's range (Trauth et al., 1992; Wheeler et al., 2003; Burgmeier et al., 2011). In Missouri, both the Ozark and eastern hellbender were listed as state endangered in 2003. The Ozark hellbender was listed as a federally endangered species in 2011 (USFWS) and the populations of eastern hellbenders in Missouri were recently proposed for a federally endangered status as a Distinct Population Segment (USFWS, 2019). When species are imperiled, additional stressors can exacerbate the problems that have led to the decline. In addition to the overall decline in numbers, low recruitment suggests problems with reproduction or survival of young (Wheeler et al., 2003; Briggler et al., 2007). Therefore, understanding the potential effects of electrofishing on eggs/hatchlings is important for conservation management decisions. Stable younger life stages are essential in hellbender population recovery (Unger et al., 2013).

The natural history of hellbenders is reviewed in Browne et al. (2013). Hellbenders are long-lived (> 30 years) and reach reproductive status at 4–6 years of age. Males defend spawning sites under cover under object (rocks and within bedrock crevices) where one or multiple females may spawn, producing about 200–550 eggs each. Males guard the eggs, which hatch after about 45–80 days, depending on water temperature. Larvae rely on their yolk sacs for food and retain their yolk sac for about two months after hatching and retain their gills for about 1.5–2 years.

The goal of this study was to determine the impacts electrofishing has on embryos and larvae of hellbenders. Ozark hellbender embryos and hatchlings (with yolk) and free-swimming

eastern hellbender larvae (post-yolk absorption) were exposed to a range of voltage and control treatments and measured their survival and behavior.

Exposure of Ozark Hellbender Eggs to Voltage Treatments

General Methods. Following are the general methods for testing the effects of electrofishing on Ozark hellbender eggs.

Individuals Tested. For embryos, each control (no voltage) and treatment group (low, medium-low, medium-high, high voltage; see below for specific voltages used) contained 100 individuals, with 500 total Ozark hellbenders tested once each. Eggs were shocked in clusters of 5 eggs, therefore there were 20 replicates of each shocking event. Individuals were selected from three clutches from the Current River collected during the fall of 2018; any eggs that appeared abnormal were excluded from selection. Eggs were maintained in vertical incubation trays as described by Civiello et al. (2018) with the Saint Louis Zoo's (SLZ) lab-created river water. This water is St. Louis city water that has been reconstituted with RO Right and Supperbuffer-dkh (Kent Marine) to achieve a target pH of 7.6–8.2 and total dissolved solids of 140–180 mS/cm for the study. The eggs were housed with no direct light and water temperature was 14.4 ± 1.1 ºC. Eggs were exposed to voltage or control treatments at stage 21 of development (Smith, 1912).

Eggs were exposed to the voltage or control treatments in groups of five, with exposures lasting for 20 s. Individuals were tested only once. Due to time constraints, only half of the eggs could be tested in one day. Therefore, treatment groups were divided in half so that half of the eggs in each treatment group was tested on a given day. The two days of testing took place approximately two weeks apart. On the first day of electrofishing eggs, 250 individuals from one clutch were exposed to treatments and on the second day on electrofishing eggs, 250 individuals

from the two other clutches were randomly mixed together and exposed to treatments. All eggs were at stage 21 as defined by Smith (1912), however the eggs that were tested on the second day of testing were a couple of days older than eggs tested on the first day. All eggs were placed in coolers and were randomly removed in clusters of five, tested, and then placed back into egg trays. Treatment order was randomized on each day of testing.

Laboratory Setup. A treatment aquarium, similar to the one used by Miranda and Dolan (2003), was constructed indoors at the SLZ with assistance from Missouri Department of Conservation (MDC) biologists with assistance by U. S. Fish and Wildlife Service (USFWS).

The rectangular test aquarium (L × W × D: 59 × 29 × 40 cm) without any substrate was fitted with plate electrodes set at either end of the tank such that a homogenous electric field was produced/maintained in the water. The electrodes ran parallel to each other, and the flow of the electric current ran perpendicular to the electrodes. That is, the voltage gradient (V/cm) was the same at all points between the plate electrodes. Thus, a hellbender placed inside the test aquarium was exposed to a known and uniform voltage gradient. The uniformity of the electrical field was verified with a voltage gradient probe and an oscilloscope. Voltage gradients (V/cm) were calculated as applied voltage divided by the distance in cm between the two plate electrodes. All settings not influencing target voltage were kept constant and consistent among treatments.

Experimental Treatments. Voltage treatments were selected with a range of voltage gradients to which various hellbenders may be exposed in the field. In preliminary studies by MDC biologist, the low (~0.2 V/cm), medium-low (~0.8 V/cm), and medium-high (~1.4 V/cm) voltage gradients were measured at various water depths. The high (~2.0 V/cm or greater) voltage gradients were only measured in shallow water (<15 cm). To determine the values to the

range of voltage gradients, a probe was constructed by MDC fisheries biologists to measure voltage exposures in the field. A typical electrofishing boat equipped with a Midwest Lake Electrofishing Systems Infinity Box (control box) was driven upstream over the voltage probe in a standard method to the extent practicable (e.g., defined path, speed, boat orientation, etc.). The probe consisted of 7 input pins for measuring voltage at different angles relative to electrical field lines. The voltage probe was tested on multiple rivers at various locations representing conditions typical of hellbender habitat (e.g., flow, gravel substrate, under rocks, water conductivity, etc.) to see what a hellbender would be exposed to. The voltage probe was attached to an oscilloscope located on the bank, allowing the absolute value of peak voltage to be recorded. The target power range for testing was based on the median range of American Fisheries Society (AFS) standards for freshwater fish sampling for a given water conductivity (Bonar et al., 2009). These data predicted that a hellbender could be exposed to a range of voltages approximately 20 s based on boat speed.

Field testing of voltage probes showed that hellbenders would experience two waves of voltage. The second peak of voltage being approximately 75% of the first peak. MDC biologists were able to simulate this experience in lab by turning the voltage dial up at a uniform pace until the peak was reached within 6–7 s, the peak was then held for 1–2 s, then voltage was decreased to almost 0 V/cm. The second peak would be initiated near 14–15 s, and voltage would return to 0 V/cm by approximately 20 s. Duration of exposure was approximately 20 s. See Appendix A for depiction of the voltage exposure experienced during the exposure to the high voltage treatment group. Treatment groups are defined as the maximum peak of voltage experienced (first peak).

Ozark hellbender eggs were exposed to the following treatments in the laboratory: control (0 V/cm), low (~ 0.2 V/cm), medium-low (~ 0.8 V/cm), medium-high (~ 1.4 V/cm), and high (~ 2.0 V/cm) voltage gradients. Water temperatures were 16.8–17.8 ºC (mean = 17.42, standard deviation = 0.377) on the first day of testing and 18.0–18.9 ºC (mean = 18.54, standard deviation = 0.261) on the second day of testing. Water used was SLZ lab created river water, which was the same as that used in maintenance containers except that a target of total dissolved solids of 250–260 mS/cm was used. Eggs were exposed to the voltage or control treatments in groups of five, with exposures lasting for 20 s.

Behavior During Shocking Events. Behavioral assessments were performed during exposure to voltage treatments.

Methods. Videos were made during the treatment exposure, with a GoPro camera providing a top view and an iPad providing a side view of the treatment aquarium. From the video of the 20 s voltage exposures, I recorded the number of embryos (out of the clutch of 5) that exhibited twitching. I defined twitching as a contraction of the head portion of the embryo towards the tail. There was an observable difference in the ability of the embryos to twitch between the two days of testing (embryos tested on the second day ~ 2 weeks later were a couple of days older). Twitching was only clearly observable for the eggs that were older, therefore twitching was not noted for individuals tested on the first day.

Minitab v. 17 was used for all statistical analyses and conclusions were based on a type-I error rate of 0.05. Data met the assumption of normality, and comparisons of means were made by One-Way ANOVAs.

Results. There was a significant difference among treatment groups for average number of embryos that twitched in each cluster of five eggs (One-Way ANOVA: $F = 213.40$, $p <$

0.0005; Fig. 1). No embryos in the control or low voltage treatments exhibited twitching, about half of the embryos in the medium-low voltage treatments exhibited twitching, and almost all individuals in the two highest voltage treatments (medium-high and high) exhibited twitching.

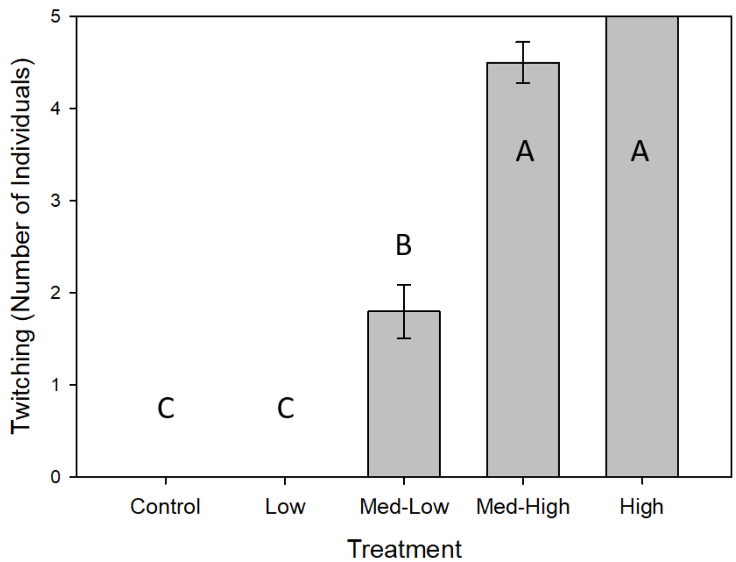

Figure 1. Number of individuals that twitched (mean ± 1 SE) out of the five eggs exposed together to control and electroshocking exposures. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 10 clusters of 5 eggs per treatment

Behavior Immediately After Shocking Events. Directly after exposure to electroshocking treatments, eggs were removed from the treatment tank and SLZ staff made notes on abnormalities.

Methods. After exposure to the electroshocking treatments, the clutches of 5 eggs were housed in individual compartments in treatment-specific incubation trays (described in Civiello et al., 2018) in the same groups of five eggs as in the exposures. These tanks contained SLZ lab created river water, which had the same characteristics as that used in the egg trays. There were no lights directly shining on the egg trays, and water temperature was 14.4 ± 1.1ºC.

Notes on number of eggs that hatched, ability to naturally hatch on their own, deformities, and mass at hatching were recorded by SLZ staff. If an individual was unable to hatch on its own, the egg was opened with tweezers by SLZ staff. This is standard practice to prevent embryos from being trapped in egg membranes. Once hatched, the individuals from each treatment group were housed together in specific "critter keeper" boxes (plastic boxes with lids containing slits for ventilation) and placed in a 189.3 L aquarium. Each aquarium contained two critter keepers.

To determine how quickly individuals began feeding after hatching, food (i.e., blackworms, *lumbriculus variegatus*) was placed in the center of the home box and the number of individuals that ate within 5 min was recorded. This process was repeated every 3–7 days until all individuals in the tank were observed feeding within 5 min.

Minitab v. 17 was used for all statistical analyses, and conclusions were based on a type-I error rate of 0.05. Comparisons between treatments for count data were made by a Chi-squared test. Comparisons of means were made by ANOVAs following logarithmic transformations (log(datum+1)) to correct for departures from normality.

Results. There was no significant difference in the percentage of total number of individuals that survived to hatching in each treatment group ($\chi^2 = 5.438$, $p = 0.245$; Fig. 2). There was also no difference in the number of eggs that were manually hatched versus naturally hatched ($\chi^2 = 7.278$, $p = 0.122$; Fig. 3A). However, there was a significant difference among treatments in the number of individuals with deformities ($\chi^2 = 12.328$, $p = 0.015$; Fig. 3B), with control individuals having fewer deformities than those in the voltage treatments. Deformities include embryos being stuck to the egg, yolk protrusions, pink spots on yolk, small head and tail buds, fuzzy tails, head deformities, curvy tails, short tails that were curved downward, "C"

shaped embryos, and “J” shaped embryos. There were no differences among treatments groups for masses at hatching (F = 0.55, $p = 0.697$; Fig. 4). There were not sufficient data to perform statistical comparisons of feeding behavior, however the two tanks that had the highest percentage of individuals that fed initially were both in the control group but as of February 19, 2019 all shocked groups were feeding and feeding (Fig. 5).

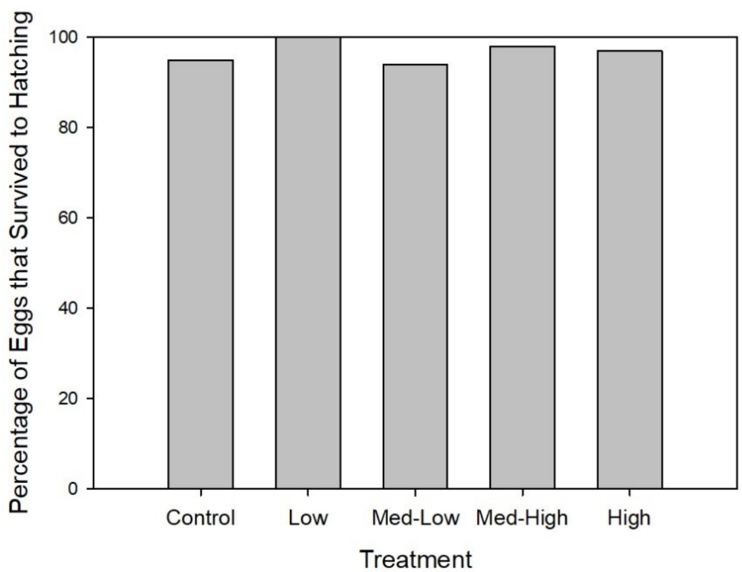

Figure 2. The percentage of eggs that survived to hatching in control and treatment groups; n = 100 per treatment

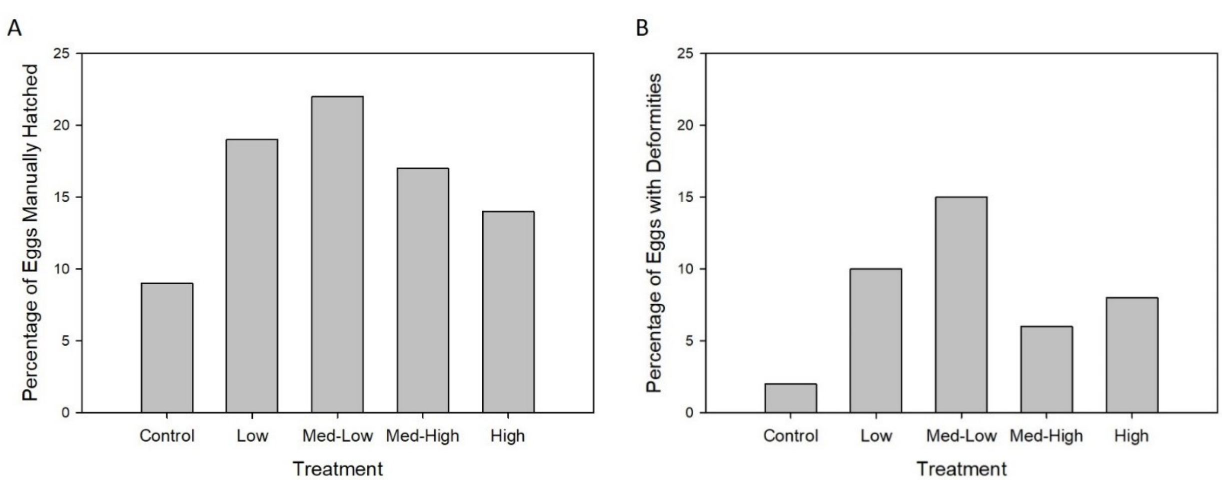

Figure 3. The percentage of eggs: **A.** that were manually hatched in control and treatment groups, and **B.** with deformities in control and treatment groups; n = 100 per treatment

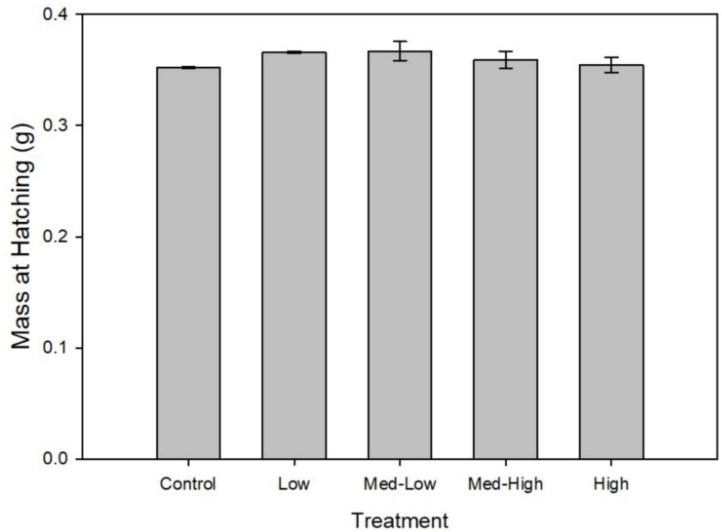

Figure 4. Mass at hatching (mean ± 1 SE) for individuals from control and electroshocked eggs; n = 100 per treatment

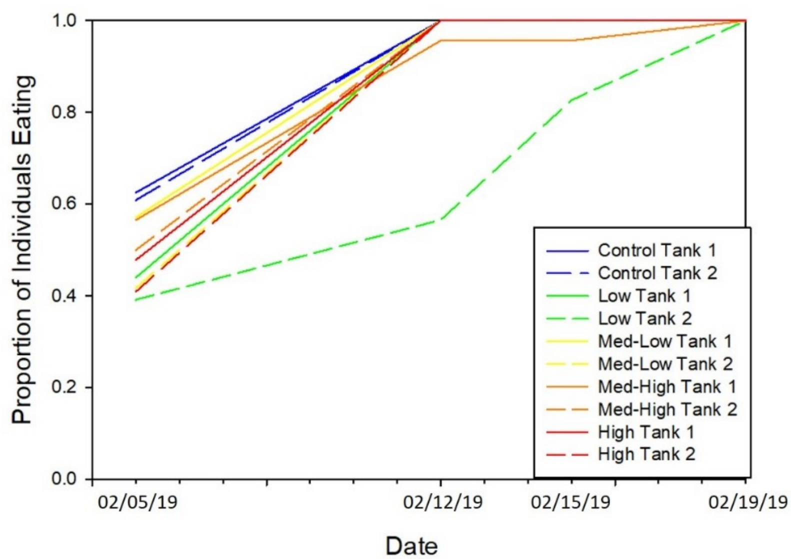

Figure 5. Proportion of individuals that ate within 5 min of food being put in their tank for individuals that hatched from control and electroshocked eggs; n = 100 per treatment

Behavior 5-month Post-Shocking Events. Five months after exposure to treatments, movement behaviors were assessed to test for long term effects of electrofishing.

Methods. Five months after eggs were exposed to electrofishing treatments, I tested the behavior of the larvae. Individuals that were tested were randomly selected from the critter keepers of larvae. A total of 20 individuals were randomly selected from each treatment group, with 10 being from the first day of testing and 10 being from the second day of testing that occurred ~ 2 weeks later. No additional voltage exposures were made. Behavioral trials took place in a testing chamber (L × W × D: 8 × 8 × 8 cm plastic dish) with 150 mL of lab-created river water that was the same as in their home tanks. The researchers making assessments were blind to treatments.

The testing chamber was placed on top of a 2 × 2 cm grid for movement measurements. Movement behaviors assessed were (1) latency to move, which was the time from the start of the trial until the individuals showed body movements (e.g., head turns, limb movement), and (2) number of lines crossed, with a cross counted when the individual's snout crossed a line. Movement trials terminated when 3 min had elapsed.

Minitab v. 17 was used for all statistical analyses and conclusions were based on a type-I error rate of 0.05. Comparisons of means were made by Two Way ANOVAs with treatment and shocking date as factors, following transformations to correct for departures from normality. Data were transformed using an aligned-rank transformation (Higgins and Tashtoush, 1994).

Results. There was no significant difference of latency to move among treatments (2-Way ANOVA: F = 1.02, $p = 0.399$; Fig. 6A) or shocking dates (2-Way ANOVA: F = 1.51, $p = 0.220$). Additionally, there was no significant interaction of treatment and shocking date on latency to move (2-Way ANOVA: F = 1.54, $p = 0.191$).

There was no significant difference in number of lines crossed among treatments (2-Way ANOVA: F = 0.81, $p = 0.519$; Fig. 6B). However, there was a significant effect of shocking date

(2-Way ANOVA: F = 5.43, $p = 0.021$; Fig. 7). There was no interaction effect between shocking date and treatment on number of lines crossed (2-Way ANOVA: F = 1.26, $p = 0.287$).

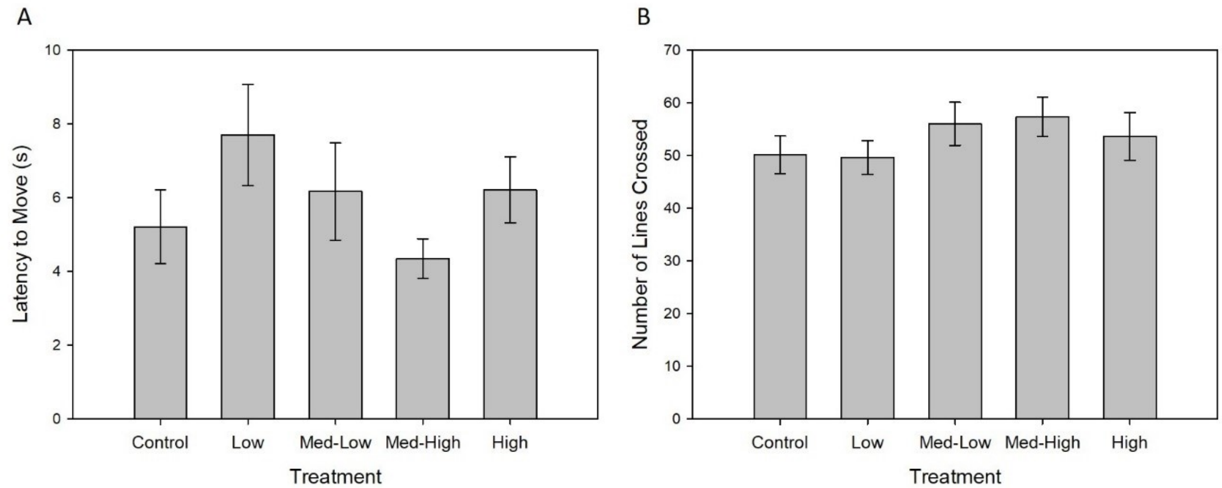

Figure 6. Movement behaviors (mean ± 1 SE) of larvae that were exposed to treatment as eggs during control and electroshocking exposures. **A.** Latency to move. **B.** Number of lines crossed; n = 20 per treatment

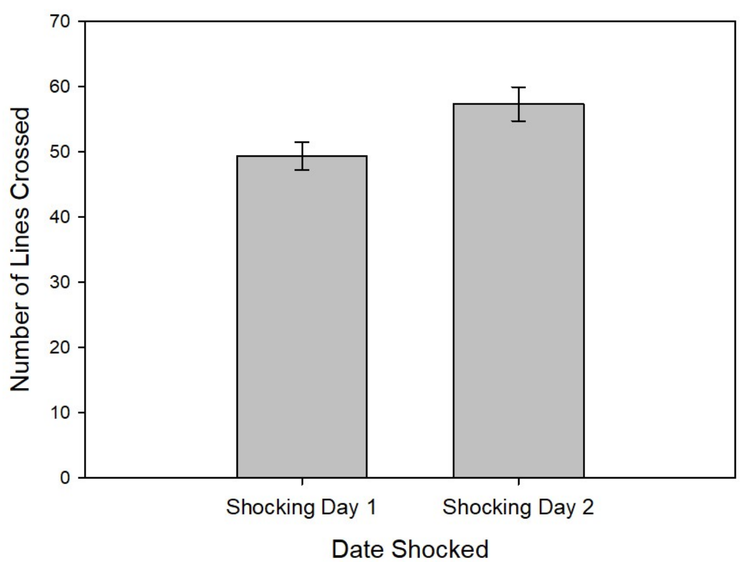

Figure 7. Number of lines crossed (mean ± 1 SE) of larvae that were exposed to treatment as eggs that were shocked on the first day testing eggs and the second day of testing eggs; n = 50 per date shocked

Exposure of Ozark Hellbender Hatchlings to Voltage Treatments

General Methods. Following are the general methods for testing the effects of electrofishing on Ozark hellbender hatchlings.

Individuals Used for Treatments. Each control and treatment group (see voltages below) contained 20 individuals, with 100 total Ozark hellbenders tested. Individuals were selected from a single clutch from the Current River which was collected during the fall of 2018 as eggs. Eggs were maintained in incubation trays until hatching as outlined in the previous experiment and were transferred to plastic critter-keeper boxes inside 189.3 L aquarium upon hatching. Water in these egg trays and hatchling tanks was the same SLZ lab-created river water as the previous experiment. Individuals used in the egg electroshocking study were not used in this study. Individuals were less than 1 month old and still had prominent yolk sacs (Table 1). Only hellbenders that appeared healthy were used in the experiment. Hatchlings with yolk were randomly selected from healthy individuals. Each treatment group was placed in a holding cooler until treatment exposure and behavioral tests larvae were returned to their home tanks. For testing, the water was reconstituted to have a target total dissolved solids of 250–260 mS/cm.

Table 1. Mass of hatchlings (g); n = 20 per treatment

Treatment	Mean	SD	Minimum	Maximum
Control	0.4535	0.1282	0.12	0.69
Low	0.4360	0.0742	0.32	0.64
Medium-Low	0.4550	0.1023	0.36	0.72
Medium-High	0.5090	0.1773	0.19	0.83
High	0.4885	0.1408	0.35	0.89

Laboratory Setup. The same treatment aquarium and methods were used as in the egg study for control and voltage exposures.

Experimental Treatments. Hatchling Ozark hellbenders were exposed to the following treatments in the laboratory: control (0 V/cm), low (~ 0.2 V/cm), medium-low (~ 0.8 V/cm), medium-high (~ 1.4 V/cm), and high (~ 2.0 V/cm) voltage gradients. Water temperatures were 19.8 – 20.4ºC (mean = 20.13, standard deviation = 0.17). Duration of exposure to voltage was 20 s, and individuals were exposed only once. To control for time of day, treatment groups were divided in half, with half of individuals in each treatment tested early in the day and half tested later in the day. Testing order was randomized within each testing block.

Behavior During Shocking Events. Behavioral assessments were made during exposure to treatment while hatchlings were in the treatment tank.

Methods. Videos were made during the treatment exposures using the same method as in the previous experiment. The small size of these individuals prevented accurate determination of whether movement was due to walking, swimming, or twitching. Therefore, only duration of movement was recorded.

Minitab v. 17 was used for all statistical analyses and conclusions were based on a type-I error rate of 0.05. Comparisons of means were made by ANOVAs following transformations to correct for departures from normality. Data were transformed using either the aligned-rank transformation (Higgins and Tashtoush, 1994), logarithmic transformation (log (datum+1)), or square root transformation.

Results. There was a significant difference among treatment groups for duration of movement during exposure to voltages (One-Way ANOVA: $F = 98.26$, $p < 0.005$; Fig. 8). The control and low voltage treatment groups moved the least, the medium-low voltage treatment group moved an intermediate amount, and the two highest voltage treatment groups moved the most.

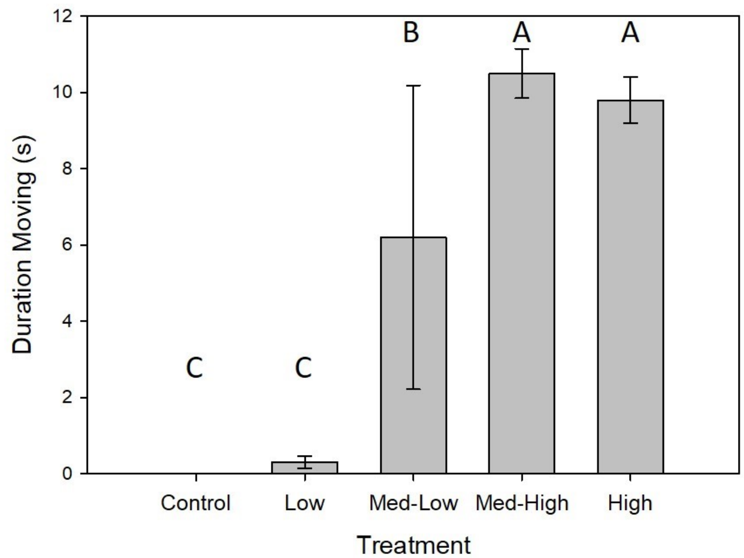

Figure 8. Duration of movement (mean ± 1 SE) of hatchlings with yolk during control and electroshocking exposures. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 20 per treatment

Behavior Immediately After Shocking Events. Immediately after exposure to treatment hatchlings were removed from the treatment tank and behavioral assessments occurred.

Methods. After exposures to treatments, the focal hellbender was transported to a testing chamber (circular plastic dish, 9 cm diameter, 35 mL of water) in a dip net. The water used was SLZ lab-created river water that was same as in the treatment aquarium. Each arena was rinsed between trials. The researchers making assessments were blind to the treatment.

Individuals were measured for mass and then the testing chamber was moved on top of a 2 × 2 cm grid for movement measurements. Behavioral measurements began 1–2 min after exposures and concluded 4–6 min after exposures. Movement behaviors assessed were (1) latency to move, which was the time from the start of the trial until the individuals showed any body movement (e.g., head movement, limb movement), (2) number of lines crossed, with a cross counted when the individual's snout crossed a line, and (3) number of movement bursts,

which was a bout of movement interrupted by > 1 s of immobility. Movement trials terminated when 3 min had elapsed.

Half of the individuals in each treatment group were randomly selected to assess heartrate, which was quantified by looking at the individual through a scanning microscope and counting the number of gill pulses for 1 min. At this stage in development the hatchlings' gills were so thin that when viewed under a dissecting microscope a single line of blood cells was visible in the gill arches that moved with each heart pulse and can serve as an approximation for heartrate. If possible, I counted the number of gill pulses in 1 min. If the individual moved out of the field of view and could not be relocated within approximately 3 s, the trial was ended and the beats per minute was extrapolated. Data were not collected if individuals moved too frequently to accurately count gill pulses or if pulse rates increased noticeably following movement.

Minitab v. 17 was used for all statistical analyses and conclusions were based on a type-I error rate of 0.05. Comparisons between treatments for presence/absence data were made by a Chi-squared test. Comparisons of means were made by ANOVAs following transformations to correct for departures from normality. Data were transformed using either the aligned-rank transformation (Higgins and Tashtoush, 1994) or logarithmic transformations (log(datum+1)).

Results. Post-shocking movement data were not significantly different among treatment groups: latency to move (One-Way ANOVA: F = 0.96, $p = 0.435$; Fig. 9), number of lines crossed (One-Way ANOVA: F = 0.73, $p = 0.572$; Fig. 10), and number of movement bursts (One-Way ANOVA: F = 0.60, $p = 0.662$; Fig. 11). There was also no significant difference among treatment groups for the number of individuals that moved ($\chi2 = 3.9994$, $p = 0.407$). Heart rate was not significantly different among treatment groups (One-Way ANOVA: F = 0.74, $p = 0.567$; Fig. 12). No mortality was observed in any treatment group.

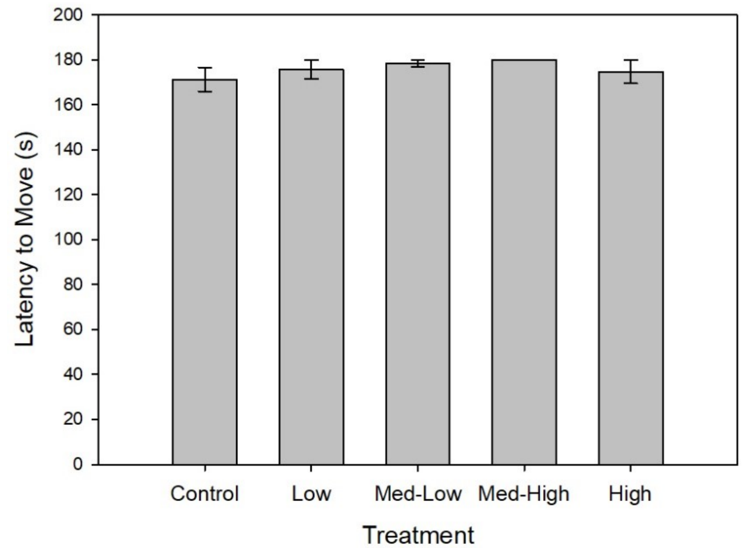

Figure 9. Latency to move (mean ± 1 SE) by hatchlings with yolk following control and electroshocking exposures; n = 20 per treatment

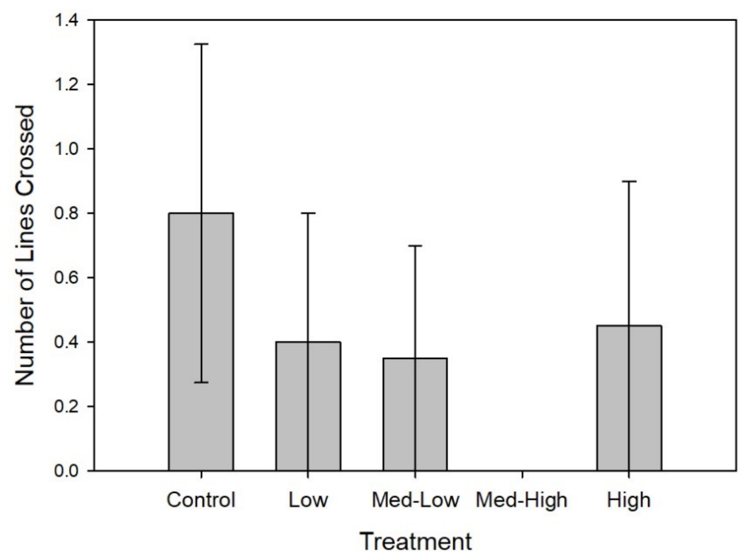

Figure 10. Number of lines crossed (mean ± 1 SE) by hatchlings with yolk following control and electroshocking exposures; n = 20 per treatment

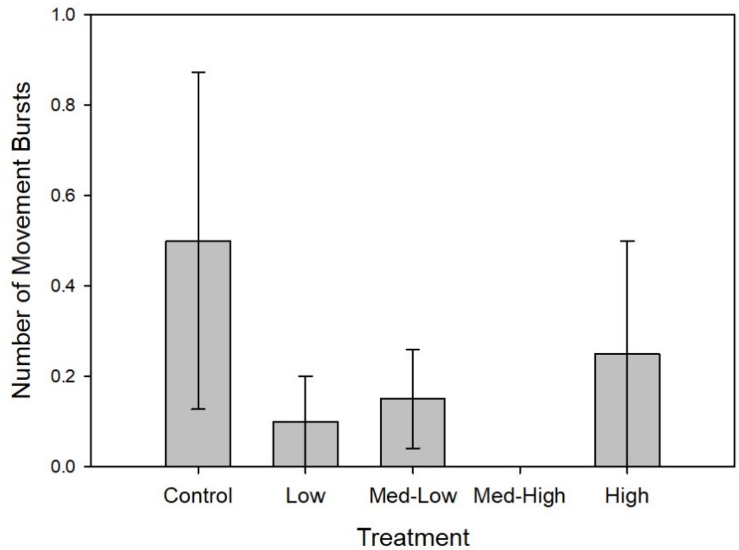

Figure 11. Number of movement bursts (mean ± 1 SE) by hatchlings with yolk following control and electroshocking exposures; n = 20 per treatment

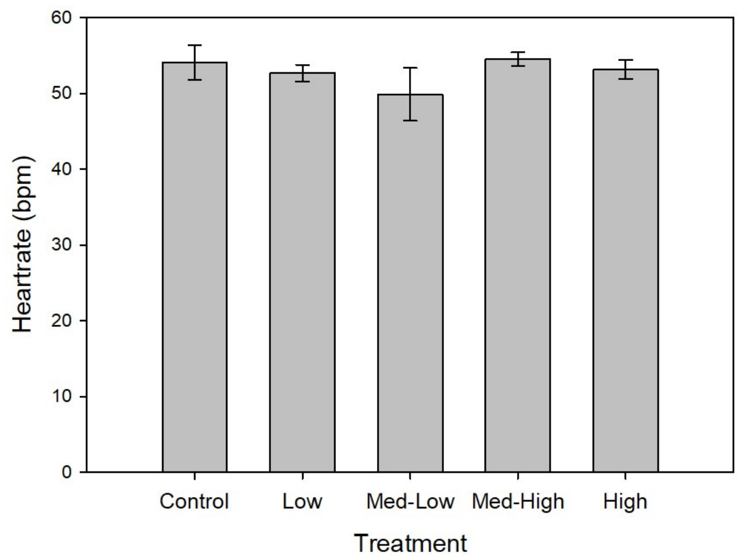

Figure 12. Heartrate (mean ± 1 SE), estimated via number of gill pulses, of hatchlings with yolk following control and electroshocking exposures; n = 20 per treatment

Exposure of Free-Swimming Eastern Hellbender Larvae to Voltage Treatments

General Methods. Following are the general methods for testing the effects of electrofishing on free-swimming eastern hellbender larvae.

Individuals Tested. For larval hellbenders, each control (no voltage) and treatment group (see below for specific voltages used) contained 20 individuals, with 100 total eastern hellbenders tested. Individuals were selected from two captive reared clutches from the Niangua River that were collected during the fall of 2018 as eggs. Eggs and larvae were maintained as in the previous experiment. Any hellbenders that exhibited apparent health issues were excluded from selection (Table 2). At the time of testing, free-swimming larvae were approximately 3 months old and had absorbed their yolk sacs.

Table 2. Mass (g) of free-swimming larvae; n = 20 per treatment

Treatment	Mean	SD	Minimum	Maximum
Control	0.0975	0.0476	0.04	0.22
Low	0.1180	0.0991	0.05	0.35
Medium-Low	0.0990	0.0568	0.05	0.22
Medium-High	0.0825	.0454	0.05	0.21
High	0.1100	0.0856	0.03	0.28

Laboratory Setup and Experimental Treatments. The same treatment aquarium and voltage treatment groups were the same as were used in the previous experiments. Water temperatures were 15.3 – 16.2º C (mean = 15.75, standard deviation = 0.303). Duration of exposure to voltage was 20 s, and individuals were tested only once. To control for time of day, treatment groups were divided in half, with half of individuals in each treatment tested early in the day and half tested later in the day. Testing order was randomized within each testing block.

Behavior During Exposures. During exposure to treatment behavioral assessments were conducted.

Methods. Videos were made during the treatment exposures as described for the previous experiment. Behaviors assessed were (1) duration of forward movement by either swimming (lateral undulation) or walking (movement of legs), (2) duration of twitching, rapid jerky movements and (3) duration of immobilization (same as tetany in fish: Lamarque, 1990), body in a stiff "C" shape (head bent toward tail) for greater than 1 s. If the movement was not easily differentiated between twitching or swimming, it was classified as swimming.

Minitab v. 17 was used for all statistical analyses and conclusions were based on a type-I error rate of 0.05. Comparisons of means were made by ANOVAs following transformations to correct for departures from normality. Data were transformed using the aligned-rank transformation (Higgins and Tashtoush, 1994).

Results. There was significantly shorter duration of forward movement in the highest two voltage treatments (One-way ANOVA: $F = 6.65$, $p < 0.0005$; Fig. 13).

Duration of twitching was also significantly different among treatment groups (One-Way ANOVA: $F = 92.74$, $p < 0.0005$; Fig. 14A). No individuals in the control or low voltage treatment groups experienced twitching, individuals in the medium-low voltage treatment showed an intermediate level of twitching and individuals in the two highest voltage treatment groups experienced the most twitching.

Duration of immobilization differed significantly among treatment groups as well (One-Way ANOVA: $F = 19.12$, $p < 0.0005$; Fig. 14B). Immobilization was absent/rare in all but the highest voltage treatment group. One individual in the medium-high treatment group became immobilized; however, the mean duration of immobilization was not different than the control,

low, and medium-low voltage treatment groups. The high voltage treatment group was immobilized significantly longer than all other treatment groups.

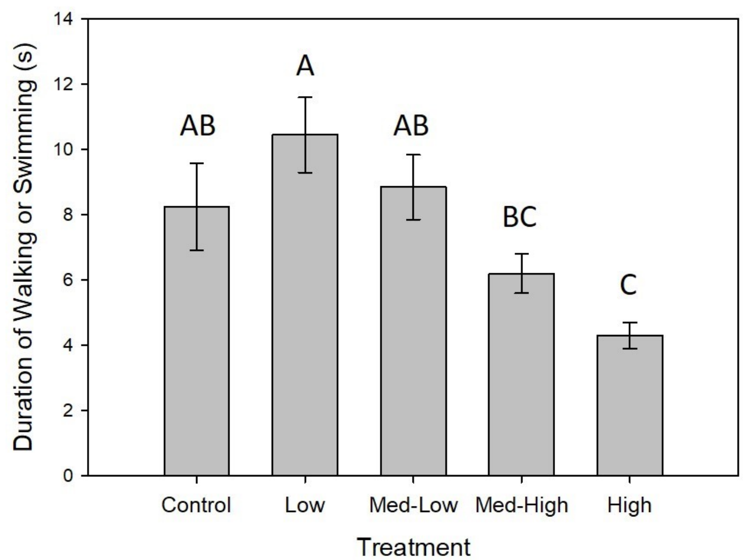

Figure 13. Duration of walking or swimming (mean ± 1 SE) of free-swimming larvae during control and electroshocking exposures. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 20 per treatment

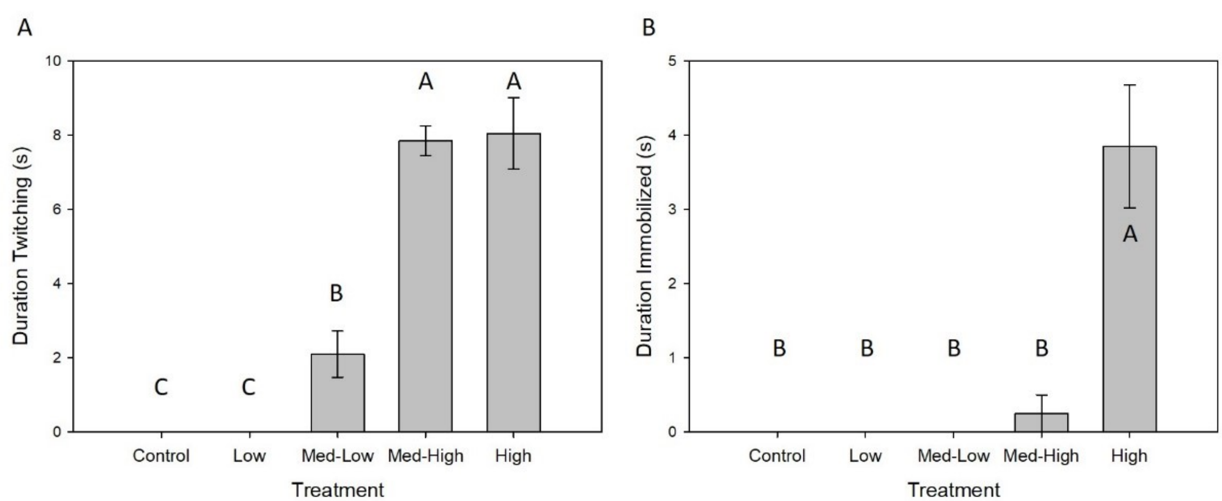

Figure 14. Duration mean ± 1 SE) of twitching (**A**) and immobilization (**B**) of free-swimming larvae during control and electroshocking exposures. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 20 per treatment

Behavior Immediately After Shocking. Immediately after exposure to treatment, free swimming larvae were removed from the treatment tank and behavioral assessments of movement were conducted.

Methods. After exposures to treatments, the focal hellbender was transported to a testing chamber (L × W × D: 8 × 8 × 8 cm plastic dish, 150 mL of water) in a dip net. The water used was SLZ lab-created river water. The water used was the same as in the treatment aquarium. The researchers making assessments were blind to the treatment. Trials began <1 min after exposure and concluded within 4 min after exposure.

Individuals were measured for mass, and then the chamber was placed on top of a 2 × 2 cm grid for measurements of movement. Movement behaviors assessed were latency to move and number of lines crossed, as described in the previous study. In addition, the number of times the individual opened its mouth was also recorded. Movement trials terminated when 3 min had elapsed.

Minitab v. 17 was used for all statistical analyses and conclusions were based on a type-I error rate of 0.05. Comparisons between treatments for presence/absence data were made by a Chi-squared test. Comparisons of means were made by ANOVAs following transformations to correct for departures from normality. Data were transformed using either the aligned-rank transformation (Higgins and Tashtoush, 1994) or a logarithmic transformation (log (datum+1)).

Results. There was no significant difference in latency to move among treatment groups (One-Way ANOVA: F = 0.40, $p = 0.805$; Fig. 15A). However, there was a significant difference among treatments for number of lines crossed (One-Way ANOVA: F = 4.68, $p = 0.002$; Fig. 15B). Activity was generally lowest in the highest voltage treatments. There was no significant difference among treatment groups in the number of times the free-swimming larvae opened

their mouth during the movement behavioral analyses (One-Way ANOVA: F = 1.64, $p = 0.170$; Fig. 16). No mortality was observed in any treatment group.

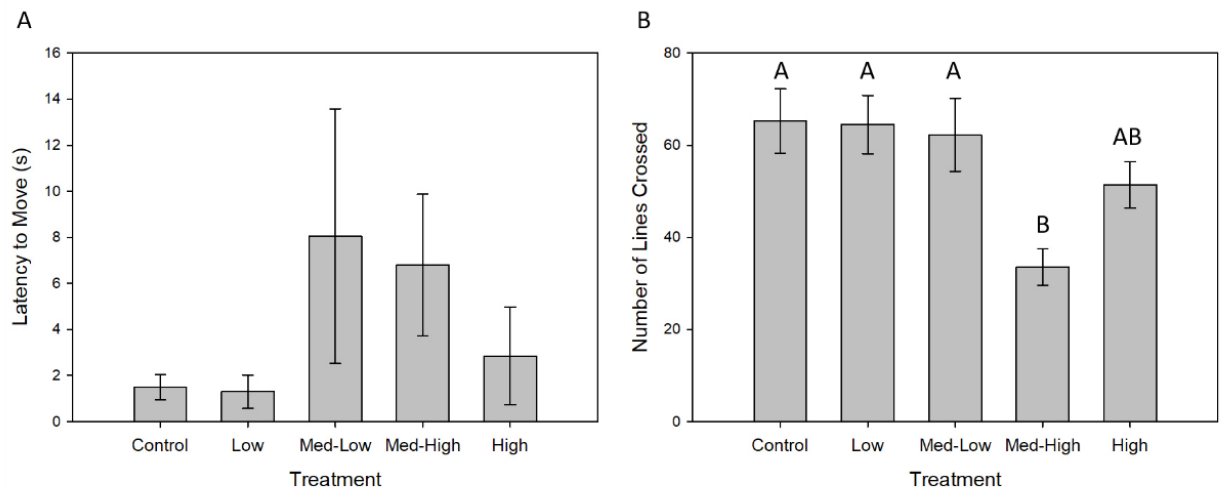

Figure 15. Movement responses (mean ± 1 SE) of free-swimming larvae following control and electroshocking exposures. **A.** Latency to Move. **B.** Number of Lines Crossed. Means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Test; n = 20 per treatment

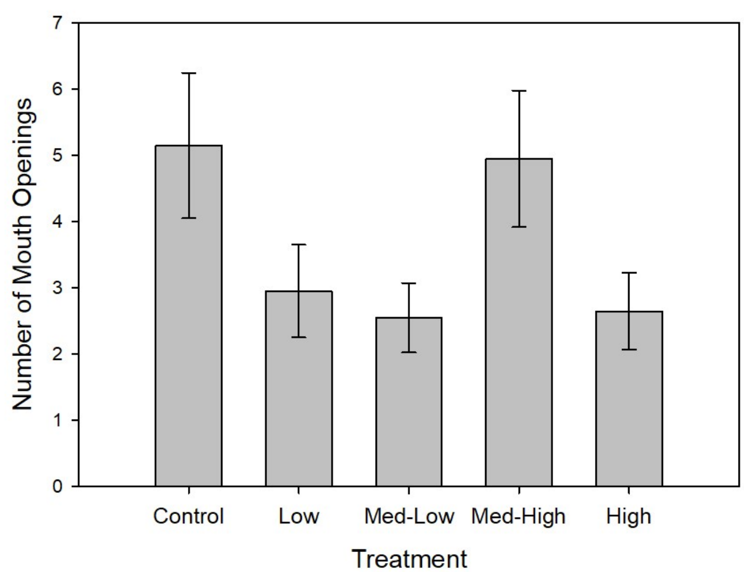

Figure 16. Number of mouth openings (mean ± 1 SE) of free-swimming larvae following control and electroshocking exposures; n = 20 per treatment

Discussion

Effects of exposure to electroshocking events for hellbender eggs and larvae occurred both during and immediately after shocking. Effects occurred in both subspecies and all age classes (embryos, hatchlings, free-swimming larvae).

Exposure of Ozark Hellbender Eggs to Voltage Treatments. During shocking of eggs, twitching of embryos (stage 21) was observed at all but the lowest voltage treatment and control, but only on the second event of testing. While the eggs tested on this day were still stage 21, they were several days older than the eggs previously tested. Although the time difference is short, it could account for the apparent delay in the onset on twitching because stage 21 is the stage at which spontaneous muscle movements first begin (Smith, 1912). The difference between the two testing days could also be due to clutch differences (one clutch was tested on the first day and two other clutches were tested on the second day) or, possibly, temperature differences (first day of testing = 17.4ºC; second day = 18.5ºC). It is common for adult fishes (Snyder, 2003) and juvenile hellbenders (Thesis, Chapter 3) to experience twitching during electroshocking exposures.

Overall, survival to hatching was relatively high (>90%) for all treatments. This result contrasts with that found in several species of fishes, where survival was generally lower in electroshock treatments (e.g., Muth and Ruppert, 1997; Keefe et al., 2000; and Cho et al., 2002). Egg survival in fishes sometimes is affected by the stage at which electroshocking occurs. For example, in cutthroat trout (Muth and Ruppert, 1997) and razorback suckers (Dwyer and Erdahl, 1995) survival is lowest when shocking occurs at early—mid development. The hellbender embryos in this study were fairly late in development when the electroshocking occurred (stage 21 at shocking, with hatching occurring at stage 23: Smith, 1912).

Deformities for hellbenders after exposure to electroshock treatments were significantly higher in the voltage treatment groups than the control (about 2% in the control and 6—15% in the voltage treatments). During their captive breeding program, embryos with deformities such as those seen in our study would be euthanized before hatching, although they were not euthanized in this study and were considered as "surviving" if they hatched. Shocked pre-eyed embryos of brook stickleback, *Culea inconstans*, also showed higher levels of morphological deformities (21.5%) compared to unshocked controls (7%) (Keefe et al., 2000). Some embryos had apparent difficulties in hatching and were assisted to leave the eggs by the SLZ staff (control = 9%; electroshocked treatment = 14—22%; $p = 0.122$). In nature, these embryos would not have survived the hatching stage.

There was no significant main effect of treatment on movement behavior of the larvae 5 months after the eggs were shocked. At this time, the eggs had hatched, and larvae had fully absorbed their yolk and were able to swim freely. The individuals that were exposed at the earlier date were less active. However, because there was no interaction between treatment and exposure dates, this difference cannot be attributed to shocking per se, but perhaps to general disturbance.

Exposure of Ozark Hellbender Hatchlings to Voltage Treatments. During exposures, hatchlings moved more when exposed to the higher voltage treatments but not to control and low voltage treatments, in which larvae showed little activity. At this stage in development, hatchlings are usually sedentary, relying on their yolk sacs for nutrition (Smith, 1912). Extra movement during exposure to higher voltages could be energetically costly for hatchlings and could make them more visible to predators. Not surprisingly, individuals in all treatments, including the control, showed very low activity levels immediately post-shocking, and there was

no difference among treatments. Low activity of newly hatched individuals is also common for larval fishes (Witzel and MacCrimmon, 1981; Olsson and Persson, 1986). I did not detect any differences in heartrate for hatchlings in the different treatments, which contrasts to the findings for 6-year old eastern hellbenders (Thesis, Chapter 3).

Exposure of Free-Swimming Eastern Hellbender Larvae to Voltage Treatments. By the free-swimming stage, the hellbender larvae had completely absorbed their yolk and were more active than the hatchlings with yolk. During electroshocking, general locomotory activity was significantly lower and twitching was higher in the highest voltage treatment groups. These free-swimming larvae also showed significantly more immobilization in the high voltage treatment group. The immobilization I observed was similar to "tetany" seen in electroshocked fishes, which is characterized as an extreme response that can lead to injury (Dolan and Miranda, 2004).

Immediately after shocking, general activity continued to be lower in the highest voltage treatment groups, indicating at least delayed recovery for these hellbenders. This result contrasts with that of zebrafish, *Danio rerio*, where electrofishing did not affect swimming performance (Teulier et al., 2018).

Conclusions. Because many hellbender populations in the wild, including the Missouri populations in this study, are experiencing a lack of recruitment (Briggler et al., 2007), investigation into potential anthropogenic effects on vulnerable early life stages is essential for conservation efforts. I found effects of electroshocking that are potentially alarming, particularly with respect to embryo deformities. I do not know whether effects might be more or less dramatic if the eggs were shocked at earlier stages of development. I also found some effects on activity that persisted for at least a short period after shocking, but these effects had disappeared

by 5 months post-shocking. It is not known whether changes in behavior have consequences for long term survival, for example, via effects on foraging success or predator avoidance.

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CHAPTER 3: THE EFFECTS OF ELECTROFISHING ON 3-YEAR OLD OZARK HELLBENDERS (*CRYPTOBRANCHUS ALLEGANIENSIS BISHOPI)* AND 6-YEAR OLD EASTERN HELLBENDERS (*C. A. ALLEGANIENSIS)*

Introduction

Electrofishing is a common and effective method for determining species presence and abundance in freshwater fish communities (e.g., reviews: Barbour et al., 1999; Portt et al., 2006; Copp, 2010). Although electrofishing can be a useful tool for fish management, negative behavioral, physiological and morphological effects have been reported for some fish species (Snyder, 2003; Bohl et al., 2010; Gharacheh, 2018). For example, 3–4 hours after electroshocking, cutthroat trout, *Oncorhynchus clarki,* sought cover, remained inactive, and did not feed (Mesa and Schreck, 1989). A blood analysis showed that there were also physiological stress responses (increased cortisol and lactic acid) and that some fish required up to 24 hours to recover. The propensity of electrofishing to produce injuries can vary substantially among species (Miranda and Kidwell, 2010).

Another potential negative consequences of electrofishing are hemorrhaging, and spinal injuries associated with the strong muscle contractions that result from exposure to the voltage levels that are common with this technique (Snyder, 2003). Because of their larger muscle mass, larger fish can be more susceptible to such injuries. For example, in some species post-incidence and severity of spinal injuries following electrofishing was positively correlated with length (Dalbey et al., 1996; Dolan and Miranda, 2004). However, size-dependent effects of electrofishing are not consistent across species; in five cyprinid fishes (*Rhodeus amarus*,

Leuciscus cephalus, *Alburnus alburnus*, *Barbus barbus*, and *Rutilus rutilus*) mortality decreased with fish length following electrofishing exposures (Janáč and Jurajda, 2011).

In addition to injuries, exposure to electrical stimuli via electrofishing could lead to negative effects related to stress, including behavioral, neuroendocrine, and immunological responses (Moberg, 2000), as well as hematological and biochemical changes (Barton and Grosh, 1996; Barton and Dwyer, 2005; Matsche et al., 2017). In a study by Awata et al. (2013) electroshocked fishes' cortisol levels returned to the same level as unshocked fish within 24 – 48 hours. Of these measures of stress, behavior is the least invasive for assessment; procedures such as blood collection for measurement of stress hormones can lead to additional physiological stress (Balcombe et al. 2004). Altered behavior is a common response to stress (Carr, 2002; Strand et al., 2007; Ricciardella et al., 2010; Trompeter and Langkilde, 2011; Bliley and Woodley, 2012).

Electrofishing has been shown to influence the behavior of several common fish species. After electrofishing a significant increase of brown trout (*Salmo trutta*) were found in upstream traps after electrofishing occurred downstream (Dunham et al., 2002). Additionally, feeding behavior can be altered after electrofishing. Bluegills, *Lepomis macrochirus*, had feeding responses after electrofishing that were dependent on age class. Juveniles reduced feeding for three hours and adults reduced feeding for up to 12 hours after electrofishing (Wahl et al., 2007). However, other studies on fish indicate a varied response among and within species after electrofishing (Fredricks et al. 2012).

Effects of electrofishing can also vary due to specific methodologies used in the process. For rainbow trout, *Oncorhynchus mykiss,* the frequency of spinal injuries increased with number of passes (Ainslie et al., 1998). The type of electrical waveform (AC vs different types of DC)

also influenced the effects, with AC being more lethal to larval stages (*O. mykiss*, Simpson et al., 2016). In a multi-year study with annual shocking events, spinal injuries accumulated over time, although there were differences among species in the magnitude of the effects (*Catostomus Catostomus*, *Salmo trutta*, *Salvelinus fontinalis*, *O. mykiss*, Kocovsky et al., 1997).

Because electrofishing can be more effective than other sampling methods for at least some parameters (e.g., Macnaughton et al., 2014; Smith et al., 2015; Bies et al., 2016; Dgebuadze and Bashinskiy, 2017), this method is a frequently recommended technique in spite of the risk of injury to some individuals (e.g., Le Pichon et al., 2017). However, the cost/benefit analysis may need to be reconsidered when species of conservation concern are affected (Snyder, 2003; Ellender et al., 2012; Bennett et al., 2016). In these cases, the benefits of electrofishing should be weighed against mortality costs to species of conservation concern, and mitigating measures (e.g., avoiding spawning sites, minimizing exposure levels) may be required (Nielsen, 1998; NOAA, 2000; Reynolds and Holliman, 2000; Bohl et al., 2009).

Relatively little data on effects of electrofishing on vertebrates other than fishes has been reported. Karssing et al. (2012) used electrofishing to estimate densities of tadpoles of cascade frogs, *Hadromophryne natalensis*, and reported that "no tadpoles were killed or injured" during the study, although they did not report how individuals were assessed for injuries. Lack of immediate mortality due to electrofishing in other amphibians (Maciolek and Timbol, 1980: *Bufo marinus, Rana rugosa, R. catesbeiana*; Williams et al., 1981: *Crypotobranchus alleganiensis*) has also been reported, but only cursory examinations for injury were made. Amphibians (adults and larvae) are likely to be stunned frequently as part of fish surveys (Allen and Riley, 2012). As part of their report, Allen and Riley (2012) corresponded with over 20 biologists who had shocked over 30,000 amphibians in total with no direct mortality observed, leading the authors to

conclude that amphibians are less vulnerable to electrofishing than fishes. However, potential injuries or other long-term effects of stress could be important, particularly for vulnerable species.

One potential nontarget species that may be exposed to electrical stimuli during censuses of fishes is the hellbender, a large permanently-aquatic salamander that cutaneously respires (Guimond and Hutchison, 1973) (Amphibia: Caudata: Cryptobranchidae). The two subspecies (eastern hellbenders: *Cryptobranchus alleganiensis alleganiensis* and Ozark hellbenders: *C. a. bishopi*) of hellbenders are of conservation concern throughout their range. Both subspecies occur and are listed as state endangered. Also, Ozark hellbenders are listed as a federally endangered species (USFWS, 2011). In Missouri, hellbender populations have declined an average of 77% between the 1980's and 2000, including both subspecies and multiple rivers (Wheeler et al., 2003). The cause of the decline is not clear, and a variety of factors have been suggested (Bodinof et al., 2011; Nickerson and Briggler, 2007; Mayasich et al., 2003). Regardless of the initial cause of the decline, additional stressors on already declining populations can increase risk of further decline or extinction (Salice, 2012). The effects of electrofishing on health, behavior and development of hellbenders are unknown and therefore cannot be ruled out as a factor that is contributing to the current declines.

The overall goal of this portion of the study was to determine whether there were negative consequences to electrofishing on two age classes of hellbenders: 3-year old Ozark hellbenders and 6-year old eastern hellbenders. Variables assessed for electroshocked and control hellbenders included (1) injuries (bone and bruising), (2) heart rate, a potential physiological sign of stress that can be significantly affected during and immediately after shocking in some species (Schreer et al., 2004), (3) presence of increased skin secretions (hellbenders frequently increase

their skin secretions following disturbance: Nickerson and Mays, 1973; Gall et al., 2010), and a variety of behavioral responses, which can indicate persistent deficits following acute stress (e.g., Lima, 1998). Ozark hellbenders were tested in the laboratory only and eastern hellbenders were tested under both laboratory and field conditions.

Laboratory Study of 3-Year Old Ozark Hellbenders and 6-Year Old Eastern Hellbenders: Single Shocking Event

General Methods. In these experiments, individual hellbenders were exposed to one of several levels of voltage or a control in the laboratory and then measured for several morphological and behavioral variables.

Test subjects. Ozark hellbenders (with absorbed gills; ~3 years old: Smith 1912) were collected as eggs in 2015 from the Current River and reared at the SLZ. Eastern hellbenders (~6 years old) were collected as eggs in 2011 from the Niangua River and hatched at Shepard of the Hills Hatchery and later transferred to the Saint Louis Zoo. Maintenance was by zoo staff as described in Bodinof et al. (2012). The water used in the hellbender systems is city water that was put through a reverse osmosis/deionization to remove all impurities. The water was then reconstituted with RO Right and Supperbuffer-dkh (Kent Marine) to achieve a target pH of 7.6–8.2 and target total dissolved solids of 140–180 mS/cm. For Ozark hellbenders, each control (no voltage) and treatment (low, medium-low, medium-high, high voltage; see below for specific voltages used) group contained 20 individuals, with 100 total Ozark Hellbenders tested, and individuals were assigned to treatment groups to minimize the body size differences among treatments (Table 3). For eastern hellbenders *C. a. alleganiensis*), only 25 individuals were available for testing, so each group contained five individuals that were randomly assigned to treatment groups (Table 4).

Table 3. Size of 3-year old Ozark hellbenders: mass (g), SVL (cm), and total length (cm); n = 20 per treatment

Variable	Treatment	Mean	SD	Minimum	Maximum
Mass (g)	High	31.60	9.01	16.00	48.50
	Medium-High	32.13	9.19	17.00	55.50
	Medium-Low	33.75	9.15	18.50	51.50
	Low	31.00	9.20	18.50	48.50
	Control	29.25	12.09	16.00	64.00
SVL (cm)	High	11.20	1.09	8.90	13.00
	Medium-High	11.19	1.18	9.30	13.20
	Medium-Low	11.45	1.23	9.40	13.50
	Low	11.24	0.98	9.50	13.00
	Control	10.85	1.29	9.00	13.90
Total Length (cm)	High	17.82	1.61	14.40	20.70
	Medium-High	17.93	1.56	15.90	20.70
	Medium-Low	18.14	1.70	14.90	21.40
	Low	17.95	1.51	15.60	20.70
	Control	17.43	1.99	14.40	22.10

Table 4. Size of 6-year old eastern hellbenders: mass (g), SVL (cm), and total length (cm); n = 5 per treatment

Variable	Treatment	Mean	SD	Minimum	Maximum
Mass (g)	High	307.6	45.9	250.0	377.0
	Medium-High	323.8	63.3	234.0	412.0
	Medium-Low	300.8	44.8	230.0	348.0
	Low	318.5	82.2	208.0	405.0
	Control	325.0	31.9	280.0	356.0
SVL (cm)	High	22.400	0.962	21.000	23.500
	Medium-High	22.100	0.742	21.000	23.000
	Medium-Low	22.700	1.681	20.000	24.500
	Low	22.700	2.110	20.000	25.000
	Control	23.600	1.084	22.500	25.000
Total Length (cm)	High	36.100	1.851	33.500	38.000
	Medium-High	36.100	1.342	35.000	38.000
	Medium-Low	36.70	2.91	32.00	39.00
	Low	36.60	3.36	33.00	40.00
	Control	37.900	1.710	36.500	40.000

Because Ozark Hellbenders are listed as state and federally endangered, authorization to expose these individuals to electrical treatment was granted by U.S. Fish and Wildlife Section 10 Permit for Research and Recovery and permits from the Missouri Department of Conservation. Water temperatures were 14.7 – 14.9º C (mean = 14.8, standard deviation = 0.10).

Laboratory Setup. A treatment aquarium, similar to the one used by Miranda and Dolan (2003), was constructed indoors at the SLZ with assistance from Missouri Department of Conservation (MDC) biologists with assistance by U. S. Fish and Wildlife Service (USFWS).

The rectangular test aquarium (L × W × D: 59 × 29 × 40 cm) was fitted with plate electrodes set at either end of the tank such that a homogenous electric field was produced/maintained in the water. The electrodes ran parallel to each other, and the flow of the electric current ran perpendicular to the electrodes. That is, the voltage gradient (V/cm) was the same at all points between the plate electrodes. Thus, a hellbender placed inside the test aquarium was exposed to a known and uniform voltage gradient. The uniformity of the electrical field was verified with a voltage gradient probe and an oscilloscope. Voltage gradients (V/cm) were calculated as applied voltage divided by the distance in cm between the two plate electrodes. All settings not influencing target voltage were kept constant and consistent among treatments.

Experimental Treatments. Voltage treatments were selected with a range of voltage gradients to which various hellbenders may be exposed in the field. In preliminary studies by MDC biologists, the low (~0.2 V/cm), medium-low (~0.8 V/cm), and medium-high (~1.2 V/cm) voltage gradients were measured at various water depths. The high (~2.0 V/cm or greater) voltage gradients were only measured in shallow water (<15 cm). To determine the values to the range of voltage gradients, a probe was constructed by MDC fisheries biologists to measure

voltage exposures in the field. A typical electrofishing boat equipped with a Midwest Lake Electrofishing Systems Infinity Box (control box) was driven upstream over the voltage probe in a standard method to the extent practicable (e.g., defined path, speed, boat orientation, etc.). The probe consisted of 7 input pins for measuring voltage at different angles relative to electrical field lines. The voltage probe was tested on multiple rivers at a various locations representing conditions typical of hellbender habitat (e.g., flow, gravel substrate, under rocks, water conductivity, etc.) to see what a hellbender would be exposed to. The voltage probe was attached to an oscilloscope located on the bank, allowing the absolute value of peak voltage to be recorded. The target power range for testing was based on the median range of American Fisheries Society (AFS) standards for freshwater fish sampling (Bonar et al., 2009). These data predicted that a hellbender could be exposed to range of peak voltages up to 2.0 V/cm for approximately 20 s.

Field testing of voltage probes showed that hellbenders would experience two waves of voltage. The second peak of voltage being 75% of the first peak. MDC biologists were able to simulate this experience in lab by turning the voltage dial up at a uniform pace until the peak was reached within 6–7 s, the peak was then held for 1–2 s, then voltage was decreased to almost 0 V/cm. The second peak would be initiated near 14–15 s, and voltage would return to 0 V/cm by 20 s. Duration of exposure was 20 s. See Appendix A for depiction of the voltage exposure experienced during the exposure to the high voltage treatment group. Treatment groups are defined as the maximum peak of voltage experienced (first peak).

Hellbenders were exposed to the following treatments in the laboratory: control (0 V/cm), low (~ 0.2 V/cm), medium-low (~ 0.8 V/cm), medium-high (~ 1.4 V/cm), and high (~ 2.0 V/cm) voltage gradients. Water temperatures were 16.8 – 17.8º C (mean = 17.42, standard

deviation = 0.377) on the first day of testing and 18.0–18.9 º C (mean = 18.54, standard deviation = 0.261). Water used was SLZ lab created river water, which was the same as that used in maintenance containers except that a target of total dissolved solids of 250–260 mS/cm was used.

Behavioral Assessment Methods. The methods for behavioral assessments during exposure to voltage treatments, immediately following exposure to voltage treatment and 3 months after exposure to voltage treatments are outlined subsequently.

Behavior during shocking events. Videos were made during the treatment exposure, with a GoPro camera providing a top view and an iPad providing a side view of the treatment aquarium. From the video of the 20-s voltage exposures, I recorded: (1) duration of walking: forward movement by limbs (2) duration of swimming: forward movement by lateral undulation, (3) twitching: rapid spasmodic movements (note: if the movement was not easily differentiated between twitching or swimming, it was classified as swimming), (4) duration and (5) latency immobilization: maintaining a "C" shape (head bent toward tail) for greater than one second (same as tetany in fish: Lamarque, 1990), (6) latency to secrete: a grayish-white cloud surrounds the hellbender, and (7) latency to gape: wide opening of the mouth.

Behavior immediately after shocking events. Because 3-year old juveniles and 6-year old subadult hellbenders differed in size, the testing arenas and water depth used for post-exposure behavioral trials were different for the two groups (L × W × D: 3-year old: 25.4 × 17.8 × 19.8 cm, water depth = 7 cm; 6-year old: 60.9 × 42.7 × 19.8 cm, water depth = 10 cm). The water used was the same as in the treatment aquarium. The researchers making assessments were blind to the treatment. Behavioral assessments began < 1 min after shocking and concluded within 6 min.

Ability to right was assessed using methods adopted from DiDonato and Bogdanik. (2011). Each hellbender was immediately placed upside down in the plastic container and was scored based on its ability to right itself, with 0 = the individual remained in the dorsal position the entire time, 1 = the individual attempted to right itself but was never successful, and 2 = the individual successfully righted itself. Successfully righting was defined as all four limbs being perpendicular to the bottom of the container. For individuals that successfully righted, latency to right was recorded. Righting trials ended after successful righting was achieved or if the maximum time of 3 min had passed.

Cover use was assessed immediately after righting reflex trials. Testing arenas were plastic boxes containing a square tile (30.5 × 30.5 cm) with a small PVC pipe glued to its bottom in one corner that acted as a refuge under which the hellbenders could retreat (3-year old Ozark hellbenders: 12.7 × 12.7 cm, 6-year old eastern hellbenders: 30.5 × 30.5 cm Fig. 17). These cover objects were the same as those provided to the hellbenders at the SLZ and so were familiar to the hellbenders. Orientation of the opening of the cover object (top left, bottom left, top right, or bottom right) was arbitrarily assigned. While holding the individual to one side of the arena, the cover object was added to one corner. Hellbenders typically occupy cover objects (e.g., flat rocks) in the river, particularly during the daytime (Nickerson and Mays, 1973); differences between controls and electroshocked treatments in cover use could be an indicator of stress.

Once the hellbender was released, I started a stopwatch and recorded latency to move, latency to touch the cover tile with any body part, and latency to move at least half of the body successfully under the cover tile, with a maximum possible score of 3 min. Because latency to move and latency to touch the cover data were frequently correlated (Appendix B), I combined into a movement index. I ranked these latency data and then calculated a movement index as the

mean of the ranks for each individual. Trials terminated when cover was reached or when 3 min had elapsed. Hellbenders also received a score based on their cover use success, with 0 = no movement toward cover, 1 = movement toward cover but not successfully moving underneath, and 2 = successfully using cover.

In addition, the number of surfaces and rocking behaviors were also recorded during this time. Surfacing was defined as the hellbender swimming to the surface and raising its open mouth above the surface apparently to inhale air (Nickerson and Mays, 1973). Rocking was defined as a still hellbender on the substrate performing rhythmic, lateral back-and-forth movements of the body, usually causing its lateral skin folds to move (Harlan and Wilkinson, 1981). Both rocking and surfacing to gulp are accessory means of increasing oxygen consumption and typically occur when animals are stressed or have performed vigorous activity (Harlan and Wilkinson, 1981; Settle et al., 2018).

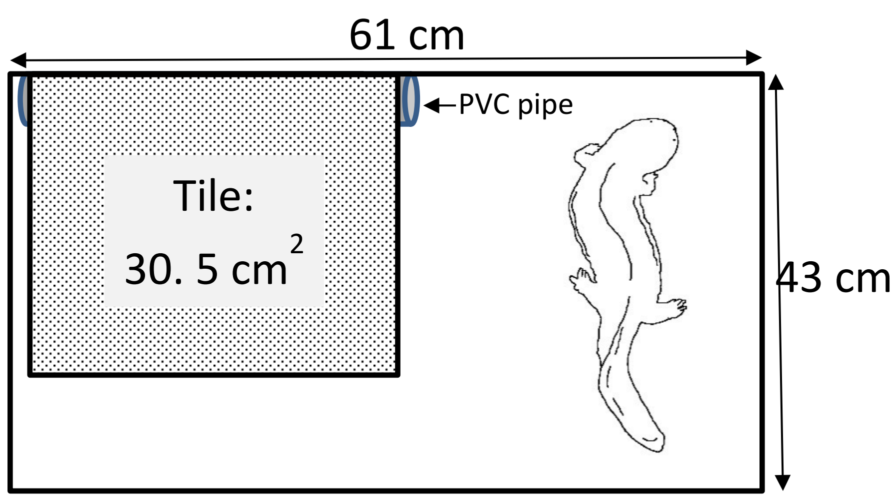

Figure 17. Diagram of the testing arena (20cm deep) used for the behavioral trials. The tile cover object was propped on top of a piece of PVC pipe to create a refuge space. The hellbender's head is 10 cm from the cover tile.

After the cover use trials, the hellbender was removed from the water and its heart rate was measured using a fetal Doppler heart rate monitor (Habeco) with an 8 MgHz probe. The hellbender was held so that its ventral side was facing up and its hind limbs were against a flat surface while the probe was held for approximately 2 min on the chest above the hellbender's heart. Ozark hellbenders (3-year old) were too small to accurately measure heartrate. X-rays were taken of 6-year old hellbenders pre- and post-treatment.

Behaviors 3-months post-shocking. Three months after electroshocking exposures, I re-tested hellbender behavioral responses to determine long-term persistence of effects on behavior. These follow-up behavioral analyses consisted of measuring the same behavior as in the immediate post-shocking behavioral analyses. Heartrate and secretion data were not collected during this experiment.

Statistical Analyses. Minitab v. 17 was used for all statistical analyses and conclusions were based on a type-I error rate of 0.05. Comparisons between treatments for presence/absence data were made by a Chi-squared test. Comparisons of means were made by ANOVAs following transformations to correct for departures from normality. Data were transformed using either the aligned-rank transformation (Higgins and Tashtoush, 1994) or a logarithmic transformation (log (datum+1)).

Results for 3-Year Old Ozark Hellbenders. Results for 3-year old Ozark hellbenders are listed subsequently in the order of testing.

Behavior during shocking events. Mean duration of walking differed among treatment groups ($F = 66.46$, $p < 0.0005$; Fig. 18A), with post-hoc Tukey's tests indicating that the highest two voltage treatments had significantly shorter durations of walking, the medium-low voltage treatment group having an intermediate duration, and the low and control group spending the

most time walking. Mean durations of swimming were also different among treatment groups (F = 3.93, $p = 0.005$; Fig. 18B), with individuals that were exposed to the three highest voltage gradients swimming less than the control group.

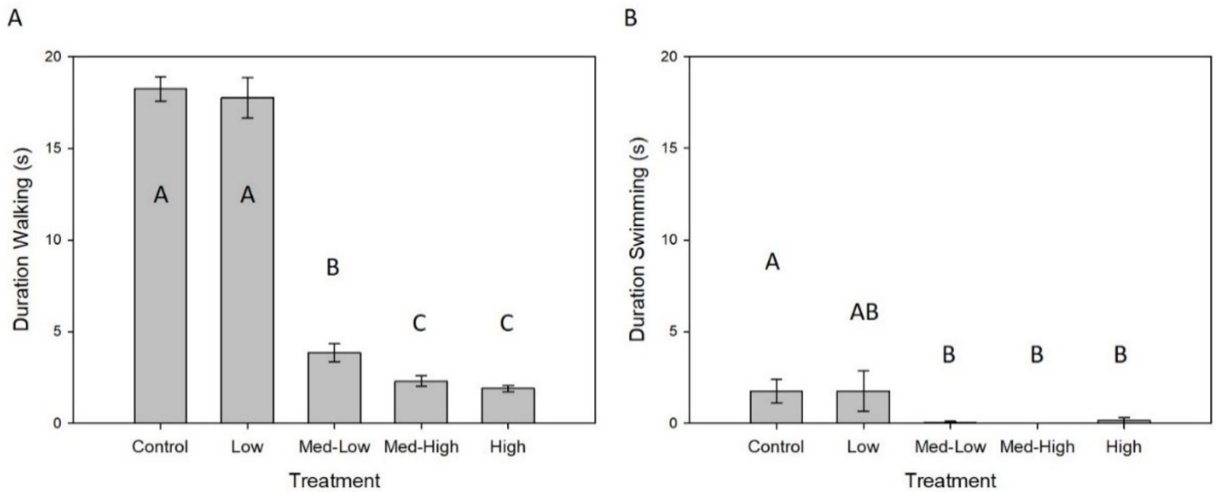

Figure 18. 3-year old Ozark hellbender activity during voltage exposures. **A.** Duration of walking (mean ± 1 SE) during control and electroshocking exposures. **B.** Duration of swimming (mean ± 1 SE) during control and electroshocking exposures. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 20 per treatment

Twitching also differed significantly among treatment groups, including both latency (F = 80.22, $p < 0.0005$, Fig. 19A) and duration (F = 117.73, $p < 0.0005$, Fig.19B). Hellbenders in the three highest voltage treatment groups began twitching significantly sooner (< 5 s) than the low voltage and control treatment groups, with only one individual exhibiting twitching in the low voltage treatment and none in the control treatment. Consequently, the control and low voltage treatment groups also spent the least time twitching, with the higher voltage treatment groups differing in twitching duration (medium-low > medium-high > high).

Latencies (F = 101.45, $p < 0.0005$, Fig.20A) and durations (F = 484.76, $p < 0.0005$, Fig. 20B) of immobilization were also significantly different among treatment groups. Hellbenders in control and low voltage treatments spent no time immobilized. In medium-low and medium-high

voltage treatments, hellbenders quickly became immobilized (< 5 s). More time was spent immobilized with increasing voltage treatment. Once immobilization occurred, anecdotally most individuals sank to the bottom of the treatment tank.

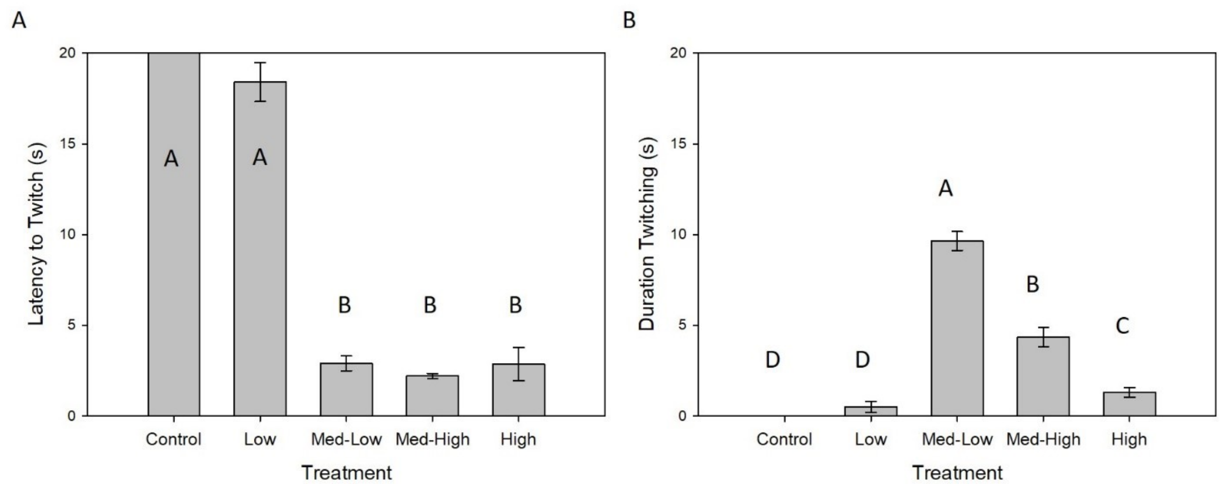

Figure 19. Twitching responses (mean ± 1 SE) for 3-year old Ozark hellbenders during control and electroshocking exposures. **A.** Latency to twitch **B.** Duration of twitching. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 20 per treatment

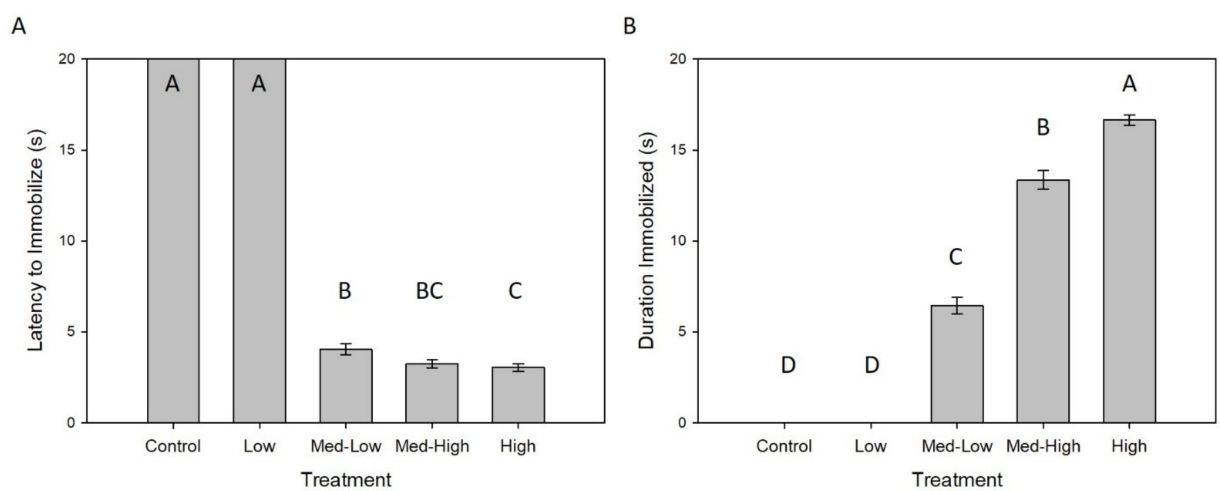

Figure 20. Immobilization responses (mean ± 1 SE) for 3-year old Ozark hellbenders during control and electroshocking exposures. **A.** Latency to immobilize **B.** Duration of immobilization. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 20 per treatment

Latency to secrete differed significantly among treatment groups (F = 67.56, p <0.0005, Fig. 21A). No secretions were observed for individuals in the control and low voltage treatment groups, and the fastest secretion latencies were observed for hellbenders in the higher voltage treatments. Latency to gape also differed significantly among treatment groups (F = 5.24, p = 0.001, Fig. 21B), with no gaping in the control and low voltage treatments and higher levels of gaping in the higher voltage treatments.

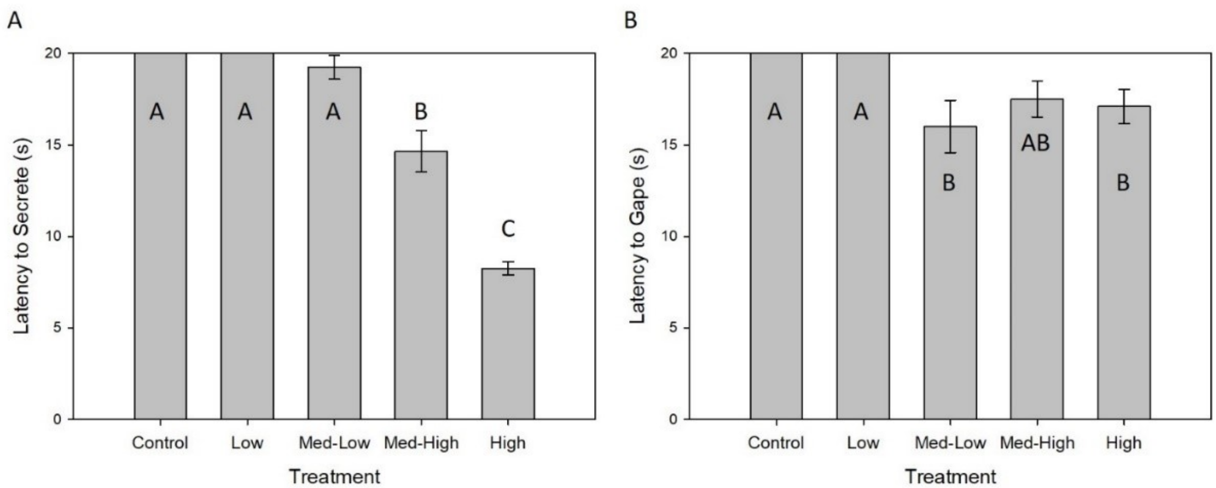

Figure 21. Secretion and gaping behavior by 3-year old Ozark hellbenders exposed to control and voltage treatments. **A.** Latency to secrete (mean ± 1 SE) of individuals during control and electroshocking exposures. **B.** Latency to gape (mean ± 1 SE) of individuals during control and electroshocking exposures. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 20 per treatment

<u>Behavior immediately after shocking events</u>. Mean righting scores differed among treatment groups (F = 3.36, p = 0.013; Fig. 22A), with all individuals in the control, low, and medium low voltage treatments righting themselves within 3 min, and five individuals failing to right within 3 min in the two highest voltage treatments (one individual in high voltage treatment group was not righted within 7 min). Mean righting latencies also differed among treatment groups (F = 16.53, p < 0.0005; Fig. 22B), with control and low voltage treatment hellbenders righting almost immediately and longer latencies with increasing voltage; for individuals in the

highest voltage treatment, it took over a minute on average for hellbenders to right themselves. There were no significant differences among treatment groups for any behaviors measured during the cover use behavioral assessment (Table 5).

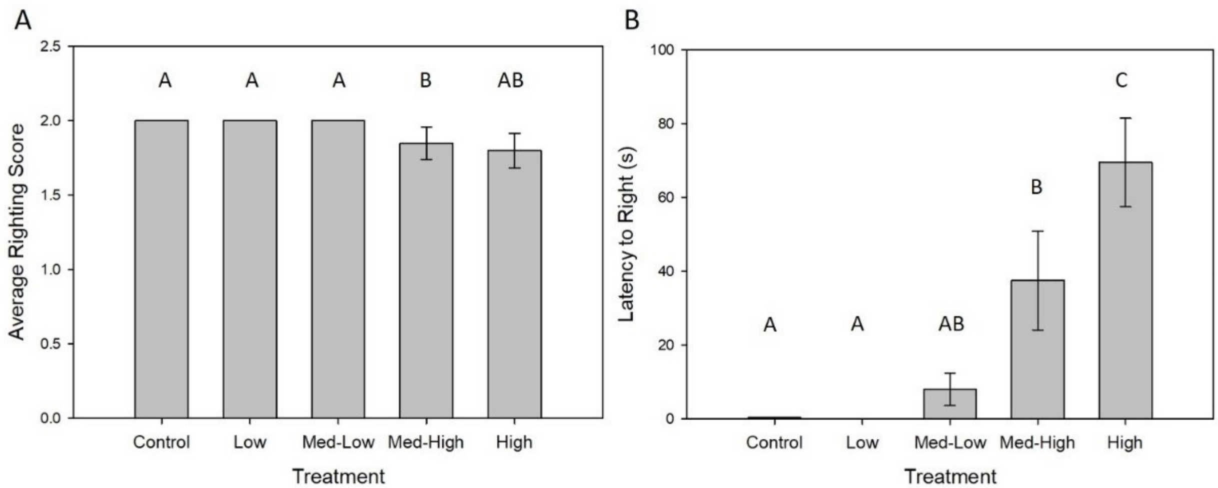

Figure 22. Righting responses (mean ± 1 SE) of 3-year old Ozark hellbenders following control and electroshocking exposures. **A.** Righting score **B.** Latency to right. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 20 per treatment

Table 5. One-Way ANOVA results for cover use behaviors of 3-year old Ozark hellbenders immediately after exposure to electrofishing; n = 20 per treatment

Behavior	Degrees of Freedom	F	*p*
Movement Rank	4, 99	1.77	0.141
Latency to cover	4, 99	1.41	0.237
Cover Score	4, 99	1.14	0.342

Rocking (n = 2) and surfacing (n = 1) observations were rare and not analyzed statistically. Rocking movements were made by one individual in the medium-high voltage treatment. Surfaces were made by one individual in the medium-high and high voltage treatments

Behavior 3-months Post-shocking. Mean latencies to right were not different among treatment groups (F = 2.22, $p = 0.072$; Fig.23). All individuals successfully righted themselves

almost immediately (within 3 s). Juveniles had significantly different latencies to successfully go under cover (F = 3.56, p = 0.009; Fig. 24) with the medium-low voltage gradient group achieving cover faster than the medium-high voltage gradient group. However, no other cover use behaviors were significantly different among treatment groups (Table 6). No mortality was observed in any treatment group.

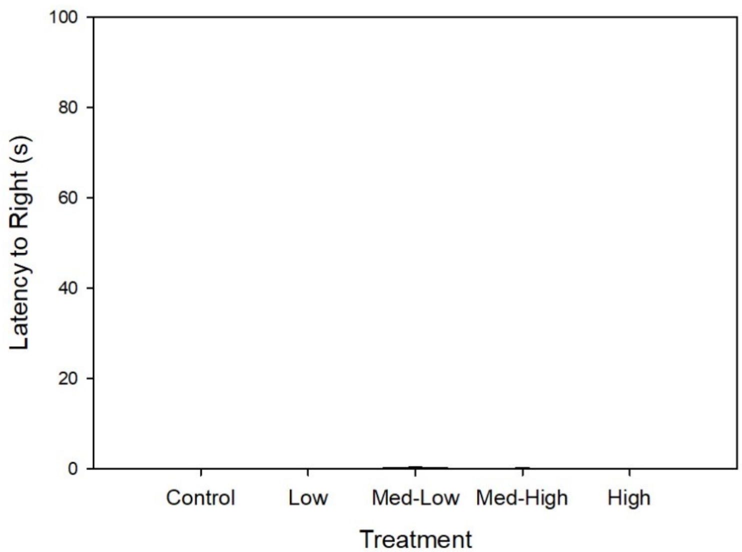

Figure 23. Latency to right (mean ± 1 SE) for 3-year old Ozark hellbenders 3 months after control and electroshocking exposures; n = 20 per treatment

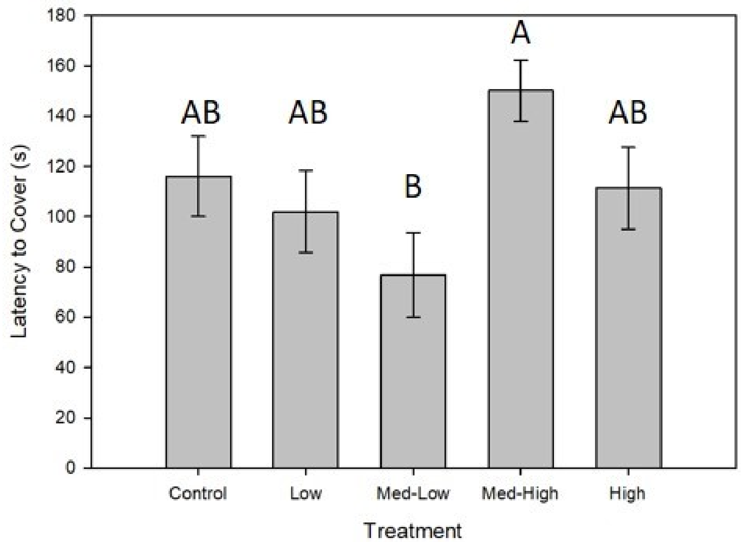

Figure 24. Latency to cover (mean ± 1 SE) for 3-year old Ozark hellbenders 3 months after control and electroshocking exposures. The sample means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Test; n = 20 per treatment

Table 6. One-Way ANOVA results for cover use behaviors of 3-year old Ozark hellbenders 3-months after treatment exposure; n = 20 per treatment

Behavior	Degrees of Freedom	F	*p*
Movement Rank	4, 99	1.80	0.136
Latency to cover	4, 99	3.56	0.009
Cover Score	4, 99	2.25	0.069

Results for 6-Year Old Eastern Hellbenders

Behavior during shocking events. Duration of walking (F = 10.42, $p < 0.0005$; Fig. 25A) and swimming (F = 5.05, $p = 0.006$; Fig. 25B) differed among treatment groups, with the most activity occurring in the control and low voltage treatments and very little activity at the higher voltage treatment levels.

Latency to twitch was different among treatment groups (F = 42.81, $p < 0.0005$, Fig. 26A). Hellbenders in the three highest voltage treatment groups experienced twitching sooner

than the low voltage and control treatment groups. Duration of twitching also significantly differed among treatment groups (F = 15.30, $p < 0.0005$, Fig. 26B). Individuals in the medium-low voltage treatment group spent more time twitching than all other groups

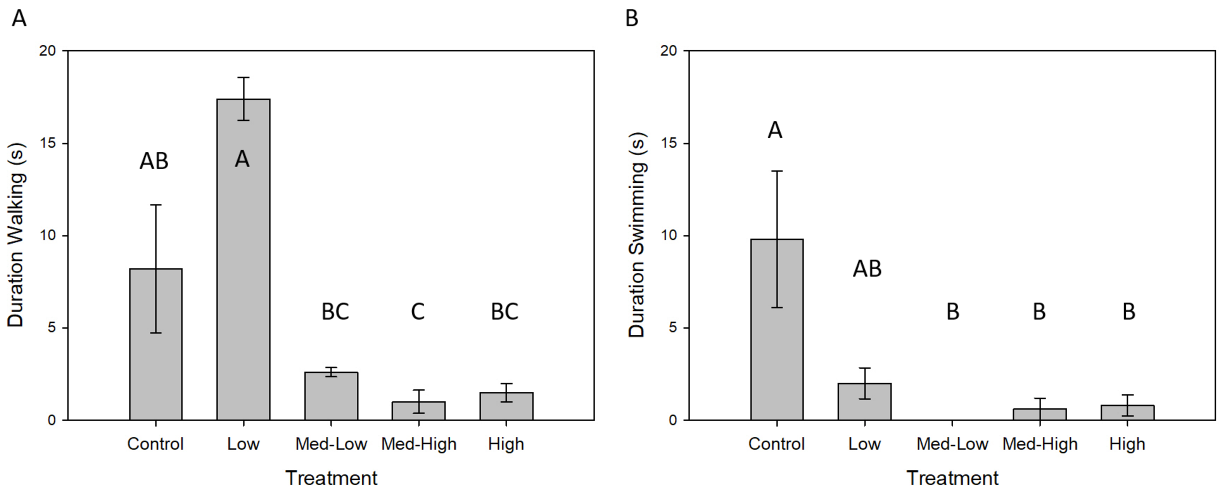

Figure 25. Walking and swimming activity by 6-year old eastern hellbenders in control and voltage treatments. **A.** Duration of walking (mean ± 1 SE) by eastern hellbenders during control and electroshocking exposures. **B.** Duration of swimming (mean ± 1 SE) by eastern hellbenders during control and electroshocking exposures. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 5 per treatment

Both latency (F = 21.71, $p < 0.0005$, Fig.27A) and duration (F = 194.91, $p < 0.0005$, Fig. 27B) of immobilization were significantly different among treatments. Hellbenders in the control and low voltage treatments did not experience immobilization. Individuals in the other treatments immobilized on average within the first 5 s, with the longest durations in the highest two voltage groups. Once immobilization occurred, anecdotally most individuals sank to the bottom of the treatment tank.

Latency for observance of secretions significantly differed among treatment groups (F = 22.25, $p < 0.0005$, Fig. 28A). Secretions were not observed for individuals in the control and low voltage treatment groups, but were visible for hellbenders in the three highest voltage treatment groups in less than 15 s. Gaping was only observed in the three highest voltage treatment groups,

but latency to gape did not significantly differ among treatment groups (F = 1.23, $p = 0.331$, Fig. 28B).

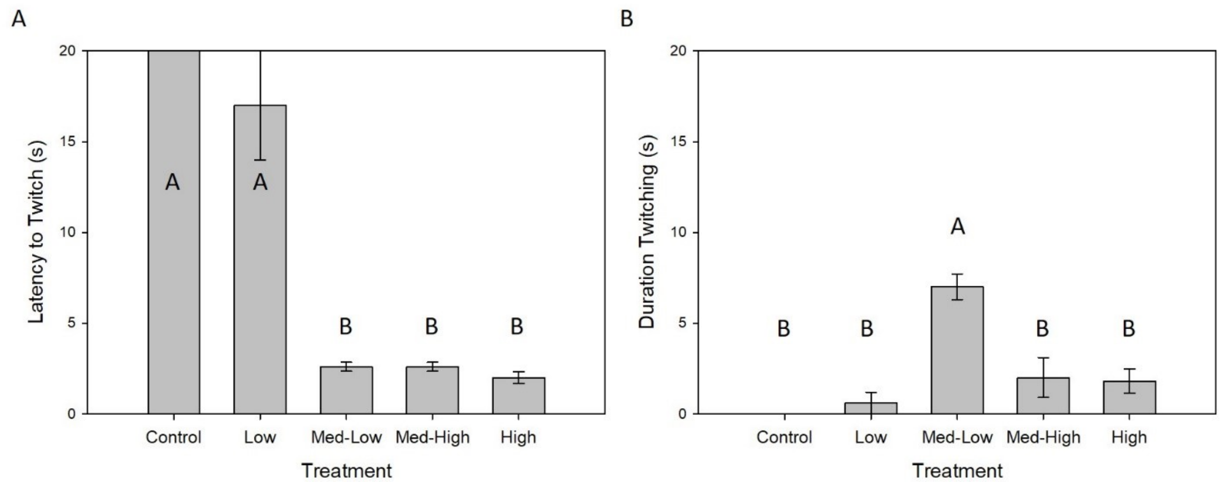

Figure 26. Twitching responses (mean ± 1 SE) of 6-year old eastern hellbender during control and electroshocking exposures. **A.** Latency to twitch **B.** Duration of twitching. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 5 per treatment

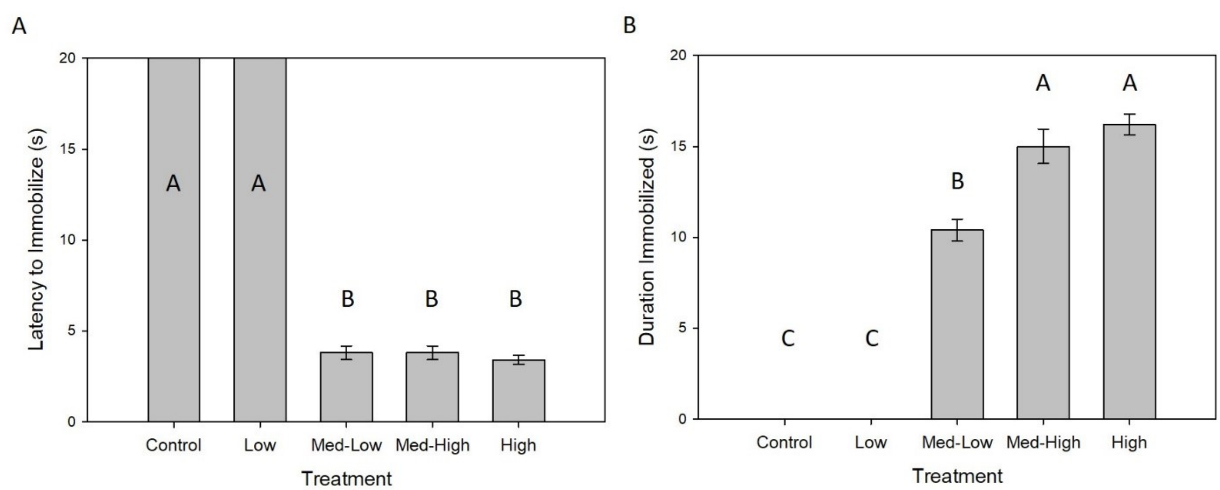

Figure 27. Immobilization for 6-year old eastern hellbenders responses (mean ± 1 SE) during control and electroshocking exposures. **A.** Latency to immobilize. **B.** Duration of immobilization. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 5 per treatment

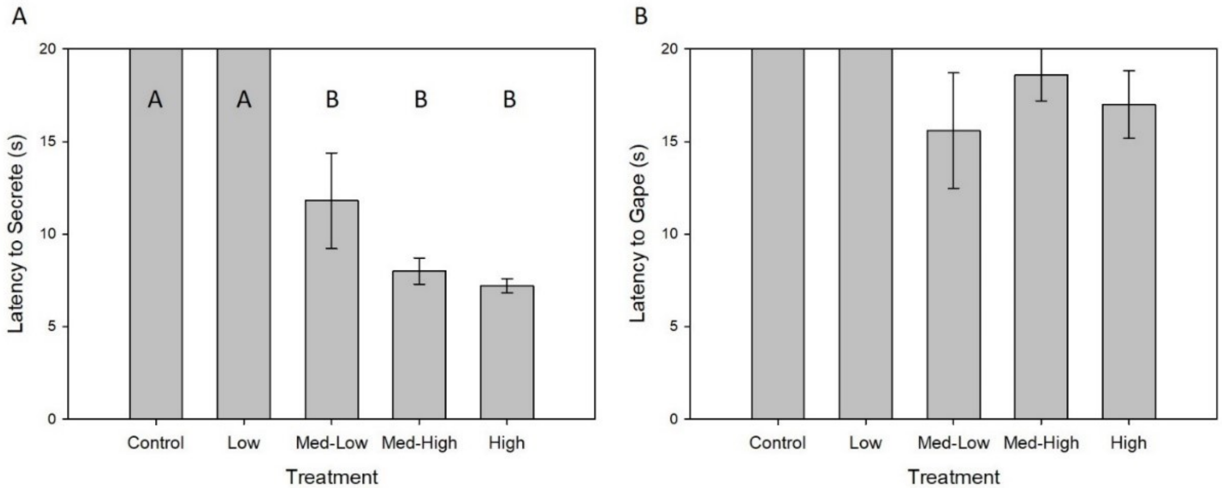

Figure 28. For 6-year old eastern hellbenders, latency to: **A.** secrete (mean ± 1 SE) and **B.** gape (mean ± 1 SE) during control and electroshocking exposures. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 5 per treatment

Behavior immediately after shocking events. The latency to right was significantly different among treatment groups (F = 11.92, $p < 0.0005$; Fig. 29), with no difference between the control and the two lowest voltage treatments, a moderate increase in latency in the next highest voltage treatment, and a large increase in the highest voltage treatment. All individuals successfully righted themselves within 3 min.

There was no significant difference among the treatment groups for the number of surfaces per individual (F = 2.15, $p = 0.121$), and only one individual rocked (medium-low voltage treatment group). However, because the statistical power was low due to small sample sizes, I combined the heartrate data for all electroshocked treatment groups (low, medium-low, medium-high, and high) and compared the combined data to the control group. Heartrates differed significantly between the control and electroshocked treatment groups (t = -2.57, p = 0.019; Fig. 30), with electroshocked individuals having an elevated heartrate. There were no significant differences among treatment groups for any behaviors recorded during the cover use

behavioral assessment (Table 7). No spinal abnormalities were present for any individuals after exposure to voltage treatment.

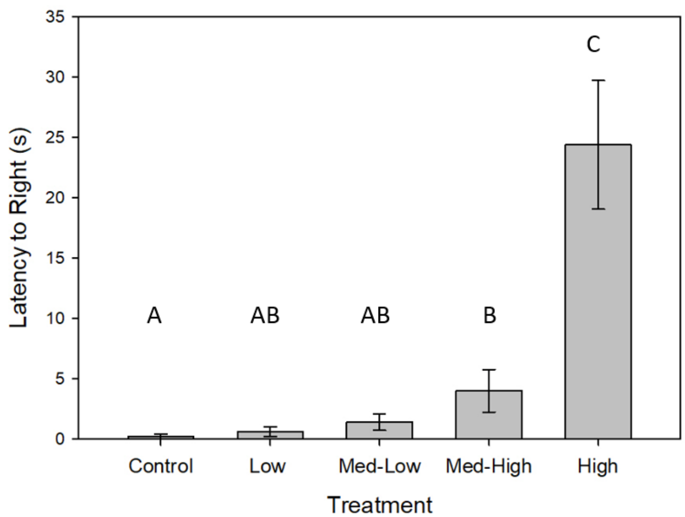

Figure 29. Latency to right (mean ± 1 SE) for 6-year eastern hellbenders following control and electroshocking exposures. The sample means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Test; n = 5 per treatment

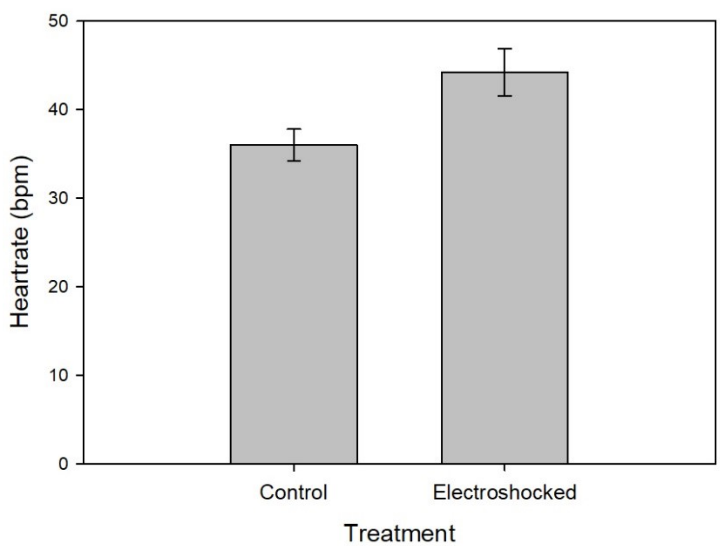

Figure 30. Heartrates (mean ± 1 SE) of 6-year old eastern hellbenders following control and electroshocking exposures; n = 5 control, 20 electroshocked

Table 7. One-Way ANOVA results for cover use behavior of 6-year old eastern hellbenders immediately after exposure to treatment; n = 5 per treatment

Behavior	Degrees of Freedom	F	*p*
Movement Rank	4, 24	0.37	0.825
Latency to cover	4, 24	2.64	0.113
Cover Score	4, 24	0.91	0.491

Behavior 3-months post-shocking. Mean latencies to right were not different among treatment groups (F = 0.87, p = 0.501; Fig. 31). All individuals successfully righted themselves almost immediately (within 3 s).

Mean movement rank was significantly different between treatment groups (F = 3.36, p = 0.029; Fig. 32), with the control and medium-low voltage treatment groups having a higher average movement rank (moved faster) then the low voltage treatment group. No other behaviors measured during the cover use trials were significantly different among treatment groups (Table 8). There was also no significant difference in the mean number of surfaces among treatment groups (F = 1.21, p = 0.340, Fig. 33). No mortality was observed in any treatment group.

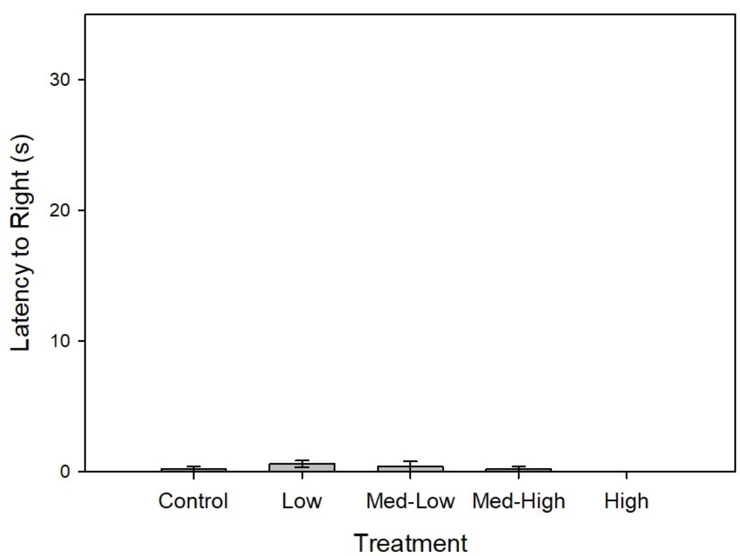

Figure 31. Latency to right (mean ± 1 SE) for 6-year old eastern hellbenders 3 months after control and electroshocking exposures; n = 5 per treatment

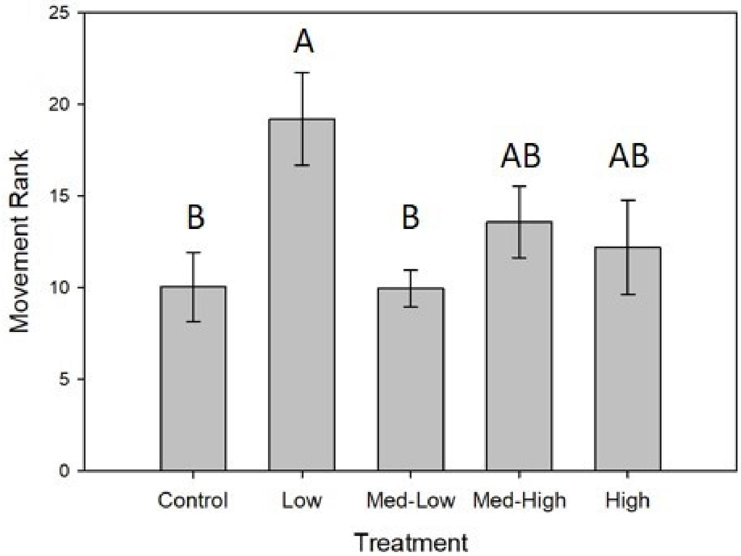

Figure 32. Movement rank (mean ± 1 SE) for 6-year old eastern hellbenders 3 months after control and electroshocking exposures. The sample means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Test; n = 5 per treatment

Table 8. One-Way ANOVA results for cover use behaviors of 6-year old eastern hellbenders 3-months after exposure to treatment; n = 5 per treatment

Behavior	Degrees of Freedom	F	*p*
Movement Rank	4, 24	3.36	0.029
Latency to cover	4, 24	0.23	0.919
Cover Score	4, 24	0.40	0.803

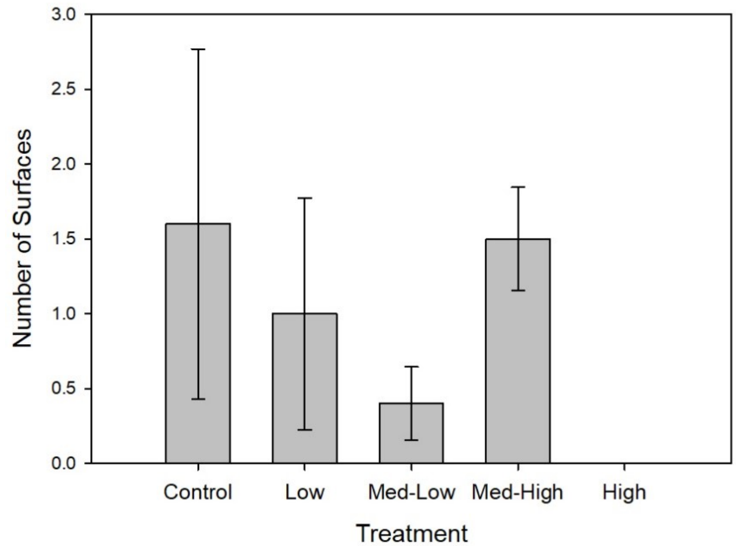

Figure 33. Number of surfaces responses (mean ± 1 SE) for 6- year old eastern hellbenders 3 months after control and electroshocking exposures; n = 5 per treatment

Laboratory Study of 6-Year Old Eastern Hellbenders: Double Shocking Event

General Methods. Eastern hellbenders that were 6-years old were exposed to a double electrofishing event. They were exposed to their assigned voltage treatment twice, 3 days apart, and behaviors assessed after each exposure.

Individuals Tested. Each control (no voltage) and treatment (low, medium-low, medium-high, high voltage; see below for specific voltages used) group contained 5 individuals, with 25 total Eastern Hellbenders tested. Individuals were selected from captive-reared eastern hellbenders at SLZ that were collected as eggs from the Big Piney River in 2013. Any hellbenders that exhibited apparent health issues were excluded from the study. Individuals were randomly assigned to treatment groups (Table 9).

Table 9. Size of 6-year old eastern hellbenders in double electrofishing study: mass, SVL, and total length; n = 5 per treatment

Variable	Treatment	Mean	SD	Minimum	Maximum
Mass (g)	High	503.6	91.4	358.0	590.0
	Medium-High	522.0	27.1	492.0	558.0
	Medium-Low	534.4	76.1	463.0	653.0
	Low	474.4	87.2	367.0	569.0
	Control	519.2	152.1	362.0	770.0
SVL (cm)	High	25.4	22.5	22.5	28.0
	Medium-High	25.8	25.5	22.5	26.0
	Medium-Low	25.8	23.0	23.0	26.0
	Low	24.8	24.0	24.0	26.0
	Control	25.1	21.5	21.5	29.0
Total Length (cm)	High	40.8	36.0	36.0	44.0
	Medium-High	41.5	40.5	40.5	43.0
	Medium-Low	40.4	38.5	38.5	42.0
	Low	39.9	37.5	37.5	42.5
	Control	40.1	44.0	34	44.0

Laboratory Setup, Experimental treatments. The treatment aquarium and voltage treatments were the same as used in the previous experiments. Individuals were exposed to voltage treatments once, and then re-exposed again with the same voltage three days later. Duration of exposure to voltage was 20 s. Water temperatures were 20.6–21.6º C (mean = 21.16, standard deviation = 0.434) on the first day, and 20.3–20.9 º C (mean = 20.56, standard deviation = 0.241) on the third day.

Behavior During Shocking Events. During exposure to voltage treatment behaviors were assessed.

Methods. Video recordings and analyses were as in the previous experiments. Minitab v. 17 was used for all statistical analyses and conclusions were based on a type-I error rate of 0.05. Comparisons of means were made using a 2-Way Repeated Measures ANOVAs following transformations to correct for departures from normality. The factors were (1) voltage/control treatment and (2) number of exposures (i.e, after the first exposure or the after second

exposure). Data were transformed using either the aligned-rank transformation (Higgins and Tashtoush 1994) or logarithmic transformation (log (datum+1)).

Results. Mean duration of walking differed among treatment groups (F = 17.16, $p <$ 0.0005; Fig. 34), with the most walking during the control and low voltage treatment groups. There was no significant effect of number of exposures (F < 0.005, $p = 0.969$) and no interaction between treatment and number of exposures (F = 1.27, $p = 0.313$). There was little swimming activity in any treatment, and so these data were not compared statistically.

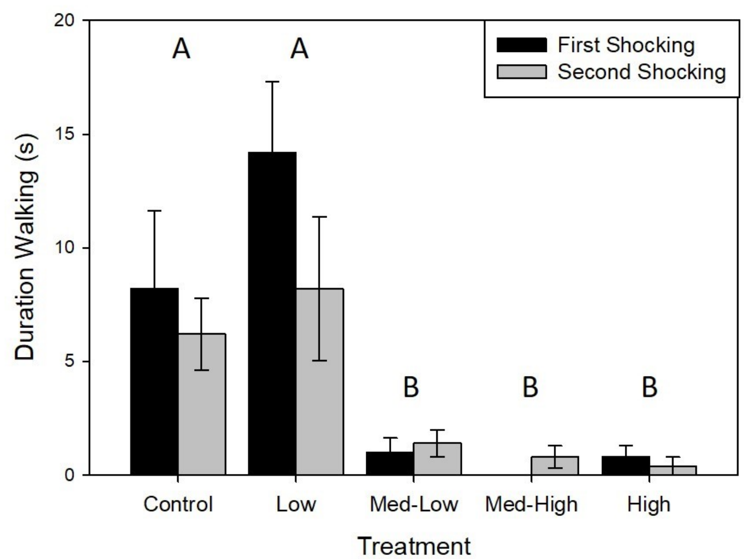

Figure 34. Duration of walking (mean ± 1 SE) by 6-year old eastern hellbenders during control and electroshocking exposures. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 5 per treatment

Latency to twitch was significantly different among treatment groups (F = 29.55, $p <$ 0.0005, Fig. 35A), with the control and low voltage treatment groups exhibiting the longer latencies than the higher voltage treatments. Latency to twitch differed between number of exposures (F = 9.28, $p = 0.006$), with individuals taking longer to twitch after the second exposure. There was no significant interaction between treatment and number of exposures (F =

1.08, $p = 0.391$). Duration of twitching also differed significantly among treatment groups (F = 22.83, $p < 0.0005$, Fig. 35B), with the control and low voltage treatments exhibiting the shortest duration of twitching and the medium-low voltage treatment group exhibiting the longest duration of twitching. Duration twitching was also significantly affected by number of exposures (F = 4.50, $p = 0.047$), with the first shocking event eliciting longer incidences of twitching. There was no significant interaction between treatment and number of exposures (F = 1.92, $p = 0.146$).

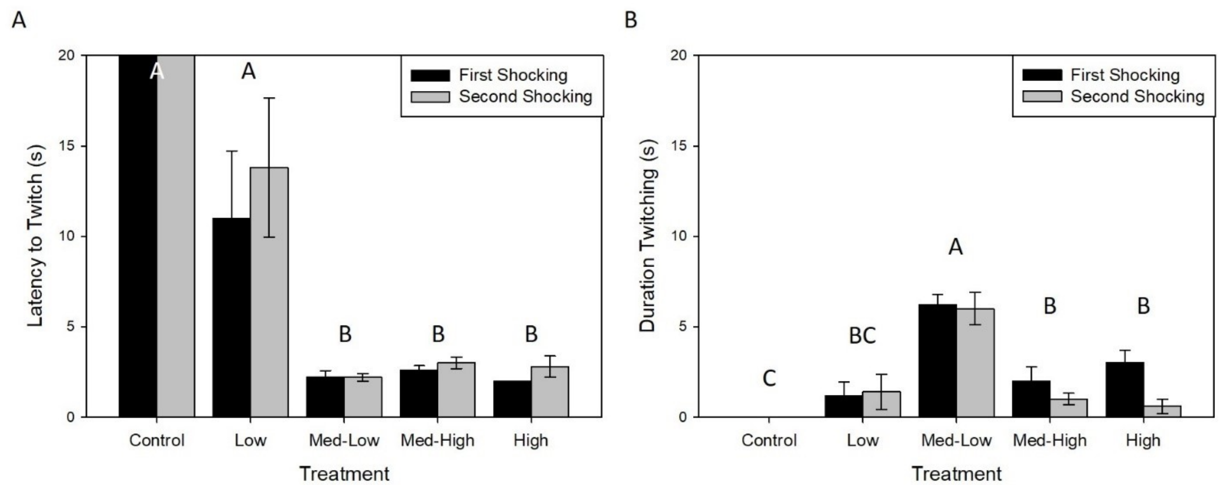

Figure 35. Twitching responses (mean ± 1 SE) of 6-year old eastern hellbenders during control and electroshocking exposures. **A.** Latency to twitch **B.** Duration of twitching. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 5 per treatment

Latency to immobilize differed (F = 56.92, $p < 0.0005$, Fig. 36A) among treatment groups, with low and control both showing no immobilization, and the three highest voltagae treatment groups quickly becoming immobilized (<5 s). There was no significant effect of number of exposures (F = 0.91, $p = 0.352$) and no interaction between treatment and number of exposures (F = 0.30, $p = 0.875$). Duration of immobilization also differed significantly among treatment groups (F = 62.20, $p < 0.0005$, Fig. 36B), with control and low voltage treatment groups spending no time immobilized, medium-low groups spending an intermediate time

immobilized, and medium-high and high voltage treatment groups spending the most time immobilized. There was no significant effect of number of exposures (F = 1.95, *p* = 0.178) and no interaction between treatment and number of exposures (F = 0.77, *p* = 0.557). Once immobilization occurred, anecdotally most individuals sank to the bottom of the treatment tank.

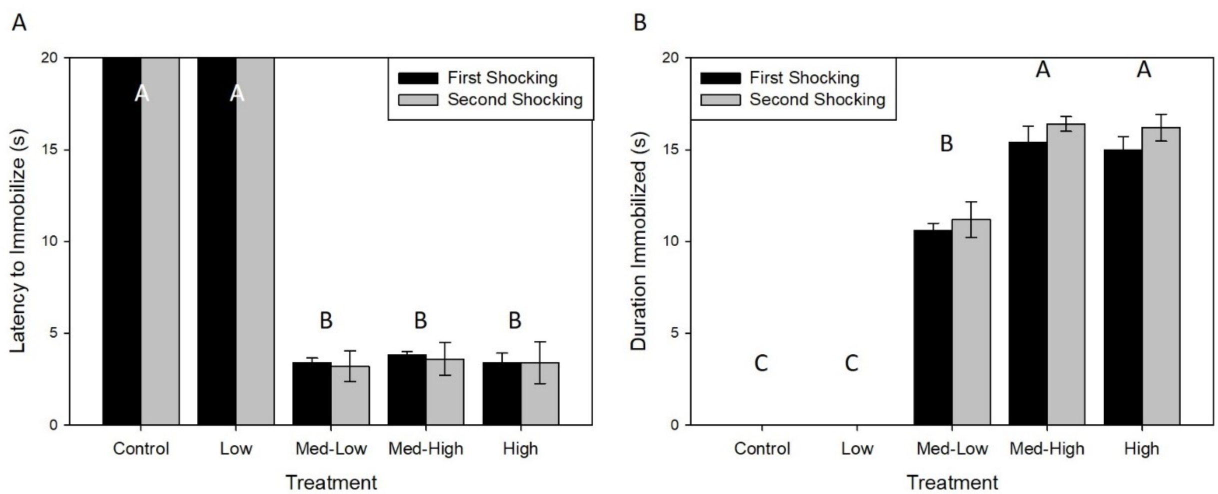

Figure 36. Immobilization responses (mean ± 1 SE) of 6-year old eastern hellbenders during control and electroshocking exposures. **A.** Latency to immobilize **B.** Duration immobilized. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 5 per treatment

For latency to secrete, treatment groups differed significantly (F = 45.83, *p* <0.0005; Fig. 37A). Individuals in the control and low voltage treatment groups did not secrete, whereas individuals in the higher voltage treatments secreted within 10 s. There was also a significant effect of number of exposures on latency to secrete (F = 9.52, *p* = 0.006), with the individuals taking longer to secrete during the second shocking event than the first. There was no significant interaction between treatment and number of exposures (F = 1.88, *p* = 0.153).

Latency to gape did not differ significantly among treatment groups (F = 1.75, *p* = 0.178; Fig. 37B). There was no effect of the number exposures (F < 0.005, *p* = 1.00) and no interaction between treatment and number of exposures (F = 0.14, *p* = 0.967).

One individual in the medium-low voltage treatment had a spinal abnormality after the second shocking exposure. A large bump was observed during exposure to shocking and during behavioral tests. An x-ray confirmed the presence of broken vertebra.

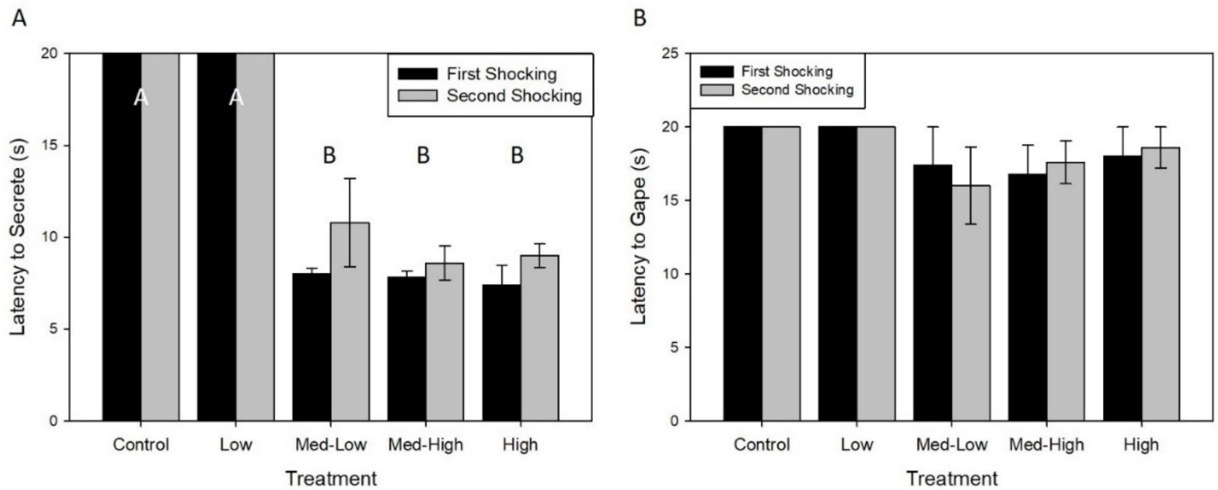

Figure 37. Latency (mean ± 1 SE to secrete) to **A.** secrete and **B.** gape (mean ± 1 SE) for 6-year old eastern hellbenders during control and electroshocking exposures. The means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Tests; n = 5 per treatment

Behavior Immediately After Shocking Events. Immediately after exposure to treatment, 6-year old eastern hellbenders were removed from the treatment tank and behaviors were measured as they were in the single electrofishing event study.

<u>Methods</u>. Behavior immediately after shocking events were measured the same as in the previous experiments. Statistical analysis was as in the video analyses for this experiment.

<u>Results</u>. All individuals successfully righted themselves within 3 min. However, the latency to right was significantly different among treatment groups (F = 1412.60, $p < 0.0005$; Fig. 38), with the two highest voltage treatments taking longer to right. There was no significant difference for latency to right for number of exposures (F = 0.98, $p = 0.333$) and no interaction between treatment and number of exposures (F = 0.73, $p = 0.580$).

In cover object trials, there was only one significant result, an interaction between treatment group and number of exposures for movement rank (F = 2.83, p = 0.037; Fig. 39), with the effect of number of exposures differing across treatments. All other results can be found in Table 10. For the number of surfaces per individual, there was no significant difference among the treatments (F = 2.05, p =0.128; Fig. 40), no difference based on number of exposures (F = 0.33, p = 0.570), and no interaction between treatment and number of exposures (F = 0.37, p = 0.826). No mortality was observed in any treatment group.

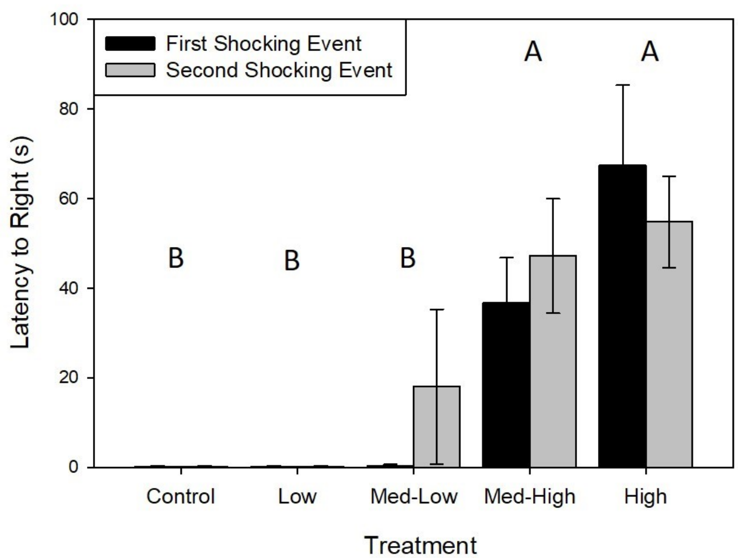

Figure 38. Latency to right (mean ± 1 SE) of 6-year old eastern hellbenders following control and electroshocking exposures for first and second shocking events. The sample means sharing the same letter are not significantly different based on Tukey's Multiple Comparison Test; n = 5 per treatment

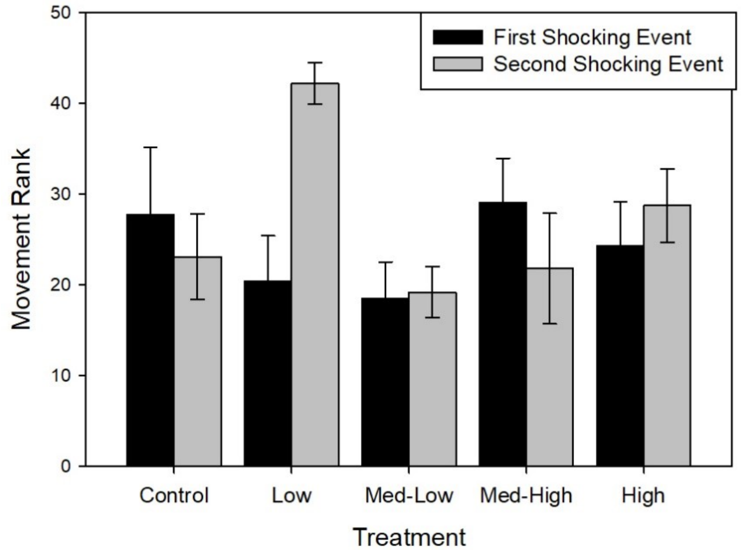

Figure 39. Movement rank (mean ± 1 SE) of 6-year old eastern hellbenders following control and electroshocking exposures for first and second shocking events; n = 5 per treatment

Table 10. Two-way Repeated Measures ANOVA results for cover use behaviors of 6-year old eastern hellbenders immediately after exposure in the double electrofishing study; n = 5 per treatment

Behavior	Factor	Degrees of Freedom	F	*p*
Movement Rank	Treatment	4, 49	1.69	0.171
	Shocking Event	1, 49	0.98	0.329
	Treatment*Shocking Event	4, 49	2.83	0.037
Latency to cover	Treatment	4, 49	1.47	0.248
	Shocking Event	1, 49	<0.0005	0.976
	Treatment*Shocking Event	4, 49	0.29	0.879
Cover Score	Treatment	4, 49	0.94	0.459
	Shocking Event	1, 49	0.36	0.558
	Treatment*Shocking Event	4, 49	0.19	0.941

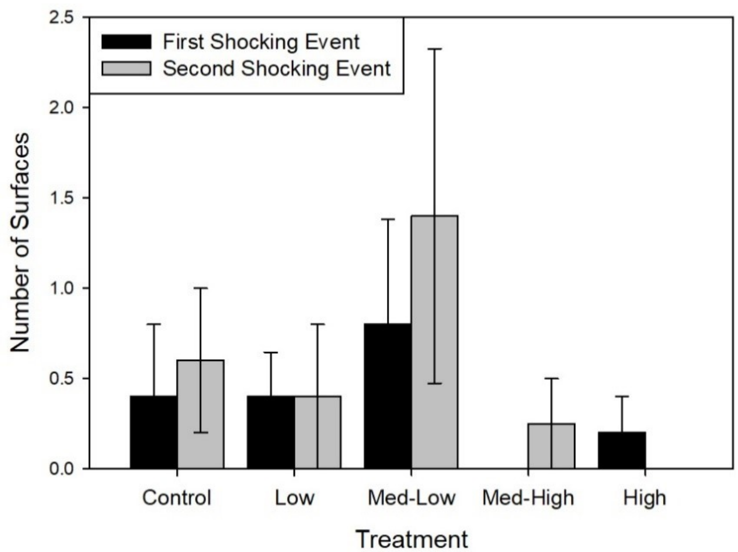

Figure 40. Surfacing behavior (mean ± 1 SE) of 6-year old eastern hellbenders following control and electroshocking exposures for first and second shocking events; n = 5 per treatment

Behavior 3-months After Exposures. The same behavioral assessments that were made immediately after exposure to treatment were conducted again, 3 months later, to assess for long term effects of electrofishing.

Methods. Approximately three months after electroshocking exposures, follow-up behavioral analyses were conducted. The follow-up behavioral analyses consisted of measuring the same responses as in the post-shocking behavioral analyses using the same methods and statistical analyses.

Results. All individuals successfully righted themselves almost immediately (within 3 s), and latency to right was not significantly different among treatment groups ($F = 0.18$, $p = 0.948$; Fig. 41). No cover use behaviors differed among treatment groups (Table 11). In addition, number of surfaces was not significantly different among treatment groups ($F = 0.48$, $p = 0.747$; Fig.42). No mortality was observed in any treatment group.

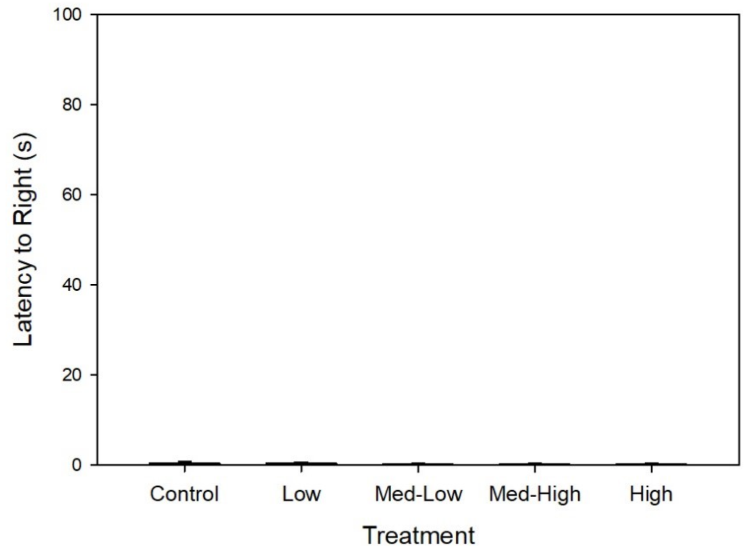

Figure 41. Latency to right (mean ± 1 SE) of 6-year old eastern hellbenders 3 months after control and electroshocking exposures in a double-shocking event; n = 5 per treatment

Table 11. One-Way ANOVA results for cover use behaviors of 6-year old eastern hellbenders 3-months after exposure to a double electrofishing event; n = 5 per treatment

Behavior	Degrees of Freedom	F	*p*
Movement Rank	4, 24	1.14	0.366
Latency to cover	4, 24	0.62	0.651
Cover Score	4, 24	0.48	0.747

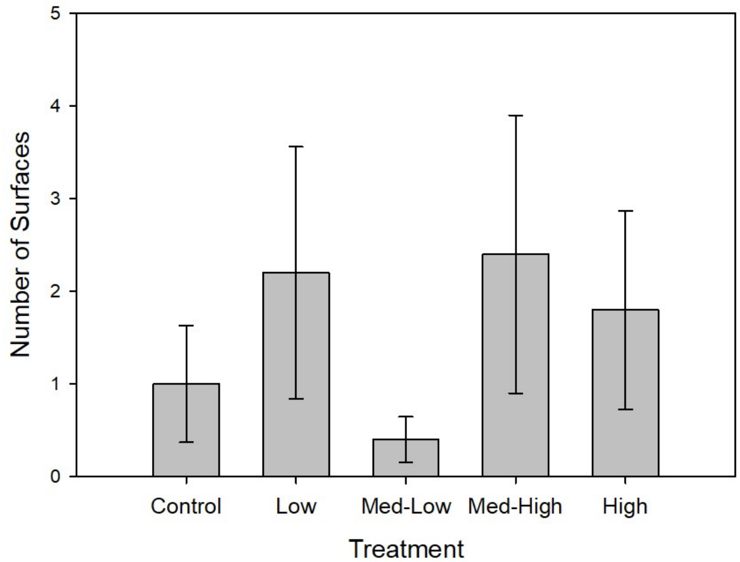

Figure 42. Number of surfaces (mean ± 1 SE) of 6-year old eastern hellbenders 3 months after control and electroshocking exposures in a double-shocking event; n = 5 per treatment

Field Study of 6-Year Old Eastern Hellbenders

Methods. In this experiment, hellbenders were placed under rocks in a river with suitable hellbender habitat, exposed to voltage treatment or to a control via boat-based electrofishing, and then measured for several morphological and behavioral variables.

Test subjects. Test individuals were selected from hellbenders reared from a single clutch of eggs collected from the Niangua River in 2011. These eggs were hatched at Shepard of the Hills Hatchery and then later brought to SLZ. Maintenance was as described in Bodinof et al. (2012). All individuals were greater than 200 g with total length greater than 25 cm. Hellbenders (n = 40) were transported from the Saint Louis Zoo to the site in coolers with aerated water.

Approximately 2 weeks before testing, a veterinarian at the SLZ performed ultrasounds of the abdominal wall and x-rays of the dorsal and ventral body surfaces of all hellbenders tested. The procedures were repeated one week after the trials.

River Setup. At the study site on the Meramec River, Crawford County, Missouri, I located four rock shelters large enough to provide suitable habitat for hellbenders. The rocks (A–D) were similar in size (L × W × D cm: A—D, 61 × 58 × 13.5; 61 × 60 ×12.5; 74 × 45 × 19; 53 × 51 × 17; respectively). To represent the natural variation of the river, the rocks were positioned at slightly different water depths (A—D: 84.5 cm, 109 cm, 70 cm, 83 cm, respectively) and distances from shore (A—D: 3.5 m, 5.9 m, 5.1 m, 6.0 m, respectively).

A voltage probe was placed in a cavity beneath the rock to record the voltage that the hellbender would experience during exposures. Due to technical limitations, rocks were tested sequentially, with Rock A, the furthest downstream, tested first, and Rock D, the farthest upstream, tested last. Once the voltage probe was safely placed under the rock, I surrounded the rock with gravel, leaving an opening on one end for placement of the hellbender. After all of the exposures were performed at a given rock, the voltage probe was moved to the next rock upstream. Plastic mesh was placed between the voltage probe and the hellbender's side of the compartment to prevent the hellbender from contacting the probe (Fig. 43).

Buoys were set up before the first rock and after the last rock to notify the boat driver when to turn on and off the electroshocker (Fig. 44). Measured water conductivity was used to calculate the target power needed to achieve 250 V and 60 Hz as applied to the water, which are common outputs for electrofishing. Testing occurred during September of 2018.

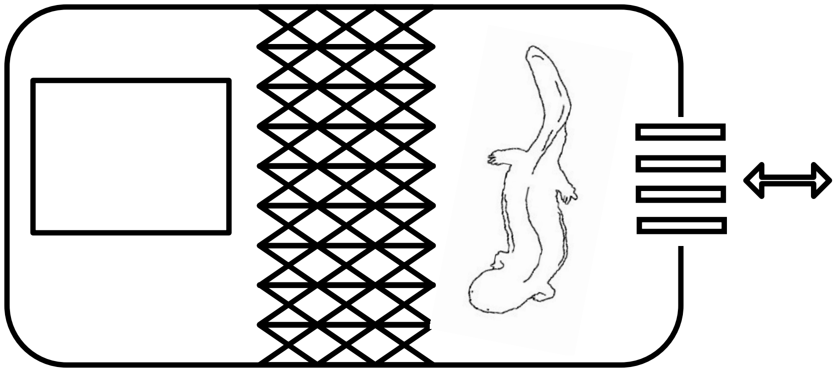

Figure 43. Diagram of the space underneath a test rock in the river. The voltage probe is indicated by the box on the left. Plastic chicken wire is in the middle separating the hellbender from the probe. The four bars represent wooden dowels used to close off the opening to prevent escape. With the exception of the opening caged by the dowels, the space was surrounded by gravel to prevent escape.

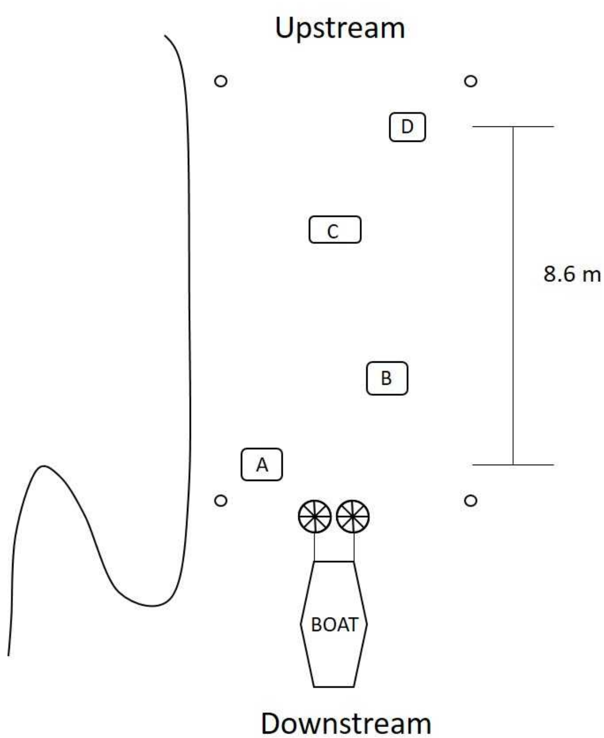

Figure 44. Diagram of the river set up with the boat, buoys (small circles), and rocks (boxes with letters). The shore is depicted on the left of the figure. The boat is depicted with the two booms. The buoys are the small circles before and after the rectangular shaped rocks. The boat moved from downstream to upstream between the buoys for each exposure. During each trial, only one rock (A, B, C or D) contained a hellbender.

Treatments. Individuals were randomly assigned to rock shelter (A–D) and treatment groups (shocked vs not shocked). At each rock, 10 hellbenders were tested, with 5 in the control group and 5 in the electroshocked group per rock (total n = 20 control, 20 electroshocked). During transport, each cooler contained the 10 hellbenders that were assigned to the same rock.

An individual was placed underneath its assigned rock through the opening. The opening was then closed off by wooden dowels, creating a complete enclosure underneath the rock (Fig. 43). Individuals acclimated under the rock for 5 min before the beginning of the trial. A boat with an 80 hp jet motor fitted with standard electrofishing gear drove directly over the rock at a speed of 0.66–1.08 m/s for both the electroshock and control trials so that all individuals experienced a similar level of boat disturbance. For the electroshock treatment, the boat applied electricity at a 25% duty cycle, with the average voltage experienced 0.16–0.34 V/cm. After the boat passed the last buoy and stopped applying electricity, the hellbender was immediately retrieved from the rock and taken to the shore by hand.

Post-exposure data collection. Upon removal from the rock, the hellbender was brought to shore for data collection as soon as possible. All researchers assessing the hellbenders were blind to the treatment (control vs electroshocked) that the individuals had experienced. A researcher with substantial experience with hellbender disturbance secretions (J. Briggler) immediately assessed the hellbender for increased secretions (yes or no) by rubbing his fingers over the surface of the skin along the dorsal portion of the back. Like most amphibians, hellbender skin is slimy due to mucous production, but hellbenders frequently increase their skin secretions following disturbance (Nickerson and Mays 1973, Gall et al. 2010). All trials took place in a plastic arena (61 × 43 × 20 cm) with a 10-cm depth of river water that was collected upstream of the study rocks. Containers were well-rinsed with river water downstream of the

study rocks between each trial. Collection of the following data began 1–3 min following exposures.

Ability to right was assessed using the methods described in the previous experiment. After the righting test, the hellbender was removed from the water and its heart rate was measured using a fetal Doppler heart rate monitor (Habeco) with an 8 MgHz probe. Following the measurement of heart rate tests, cover object use tests were immediately conducted, using the same methods described in the previous experiment.

Minitab v. 17 was used for all statistical analyses, with a type-I error rate of 0.05. Comparisons between treatments for presence/absence data were made by either a Fisher Exact Test or a Chi-squared test. Comparisons of means were made by two-sample t-tests following transformations to correct departures from normality. Latency to move data were logarithmically transformed (log (datum+1)) and latency to touch and latency to cover data were logarithmically transformed (log (datum)).

Results. Despite our efforts to block escape, three individuals, all from the electroshock treatment, escaped from their rock during the exposures; only two of the individuals that escaped were recovered.

The SLZ's veterinarian did not see any obvious differences between the pre- and post-treatment x-rays or ultrasounds. Heartrate also did not differ significantly between treatments (t = 0.24, $p = 0.81$; Fig. 45A). However, hellbenders in the electroshocking treatment had a significantly higher incidence of increased skin secretions than in the control treatment (Fisher Exact test: $p < 0.0005$; Fig. 45B).

In righting reflex trials, almost every hellbender immediately righted itself when placed into water, and latency to right did not differ significantly between the two treatments (t = -0.39,

$p = 0.697$; Fig. 46). None of the behaviors measured during the cover object trials were significantly different between the control and electroshocked treatment groups (Table 12). Whether an individual surfaced or rocked was not associated with treatment group ($\chi^2 = 0.620$, $p = 0.431$). No mortality was observed in either treatment group.

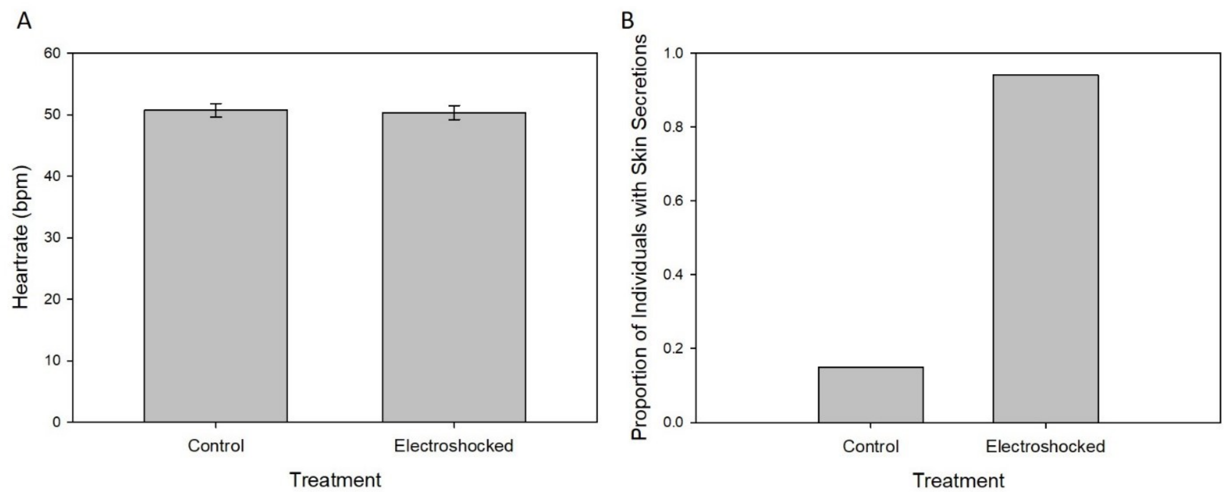

Figure 45. Physiological responses to control and electroshocked treatments by eastern hellbenders. **A.** Heartrate (mean ± 1 SE) of individuals following control and electroshocking exposures. **B.** Proportion of individuals with increased skin secretions following control and electroshocking exposures; n = 20 per treatment

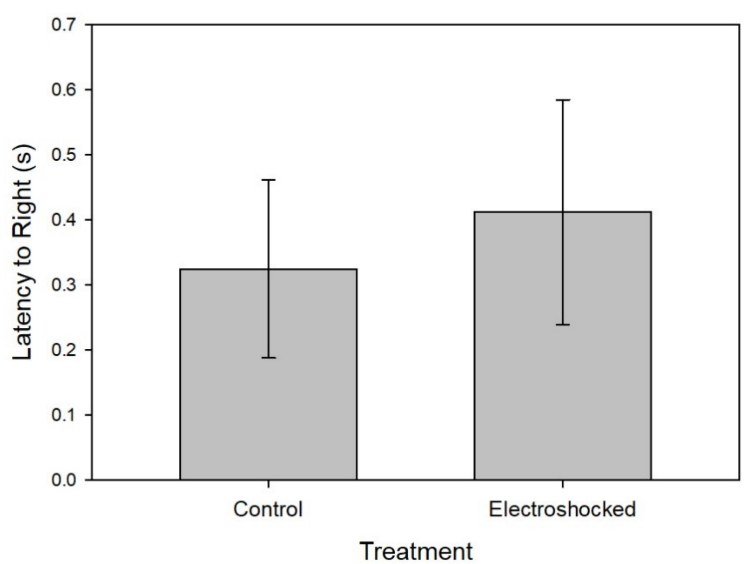

Figure 46. Latency to right (mean ± 1 SE) following control and electroshocking exposures for eastern hellbenders; n = 20 per treatment

Table 12. T-test results for cover use behaviors of 6-year eastern hellbenders immediately after treatment exposure in the field; n = 20 per treatment

Behavior	t	*p*
Movement Rank	2.01	0.053
Latency to cover	-0.73	0.471
Cover Score	0.79	0.433

Discussion

Effects of exposure to electroshocking events for hellbenders ranged from relatively mild indicators of stress to more serious and potentially long-term effects, including, in one case, an individual broken trunk vertebrae in the medium-low voltage treatment group after the second shocking event in the double electrofishing event study. Broken vertebrae and other internal injuries after electroshocking have been observed in some fish species (Schill and Elle, 2000; Clement and Cunjack, 2010; Dagit and Krug, 2016). Some effects were observed in both laboratory and field studies and in both subspecies and age classes.

A particularly consistent effect was an increase in production of skin secretions. In our field study, increased production of secretions was assessed qualitatively by touch; however, the person assessing the skin for secretions was blind to treatments, eliminating the potential for confirmation bias. In the laboratory trials, secretions were readily visible so that production was easy to assess accurately. Hellbenders are known to produce copious secretions under handling stress (Nickerson and Mays, 1973; see Figure 21). The function(s) of the secretions are not known, but Nickerson and Mays (1973) presented anecdotal data suggesting that it is a predator deterrent and hypothesized that it also may have an antipathogenic function. The secretion also has been shown to have an alarm function, warning conspecifics of nearby danger (Crane and Mathis, 2011, 2013). Electrical shock has been used to stimulate secretions in other amphibians, but in those cases the electrode was in direct contact with the skin (e.g., Tyler et al., 1992; Chen

et al., 2003; Cardall et al., 2004). I interpret increased secretions as a symptom of acute stress, but it is not known whether the production of glandular secretions is energetically costly or has other long-term effects. For example, size and number of alarm glands in fathead minnows (*Pimephales promelas*) are affected by body condition, suggesting that the secretions are energetically costly (Wisenden and Smith, 1997). Additionally, if the glands are depleted, regeneration of contents can take substantial time (e.g., for defensive secretions, days: Heethoff, 2012; months: Cardall et al., 2004), leaving the animals in a potentially vulnerable state in the interim. Hellbenders which were in the double electrofishing study took longer to secrete the second time they were shocked. In our laboratory trials, secretions generally did not occur in the lowest voltage treatment, so it appears that a charge somewhere between 0.2 and 0.8 V/cm is required to elicit the secretion response, at least under laboratory conditions. These findings are of importance when considering hellbender conservation because stressed animals tend to perform daily tasks poorly, such as foraging (Watson et al., 2004) and locomotion (Ricciardella et al., 2010).

The most dramatic responses during exposure to the voltage treatments in the lab were twitching and immobility. These responses were absent in the control and rare in the lowest voltage treatments. Most animals in the three highest voltage treatment groups exhibited one or both of these behaviors, with twitching of longer duration in the medium-low voltage treatment and immobility lasting longer in two highest voltage treatments. When twitching occurred, it started at approximately the same time (at about 2–3 s) for all individuals regardless of voltage-level. Hellbenders in the double electrofishing experiment spent more time twitching in the first shocking event than in the second and tended to spend more time immobilized in the second shocking event, although this difference was not significant. Studies of fish have shown that a

multiple pass electrofishing event can be more harmful (Panek and Densmore, 2012). Immobilization is the end goal for electrofishing of fishes, and both twitching and immobilization in hellbenders are qualitatively similar to the responses of fishes (Vibert, 1963).

Whether exposure to voltages used in electrofishing have prolonged effects on hellbenders depends in large part on how quickly they recover. The righting reflex was substantially affected in the laboratory, particularly at higher voltage treatments for Ozark hellbenders (3-year old). At the highest voltage treatment, the average time to right was about a minute and some individuals did not right during the 3-min trial period. Immobilization and prolonged impairment might leave hellbenders vulnerable to being washed downstream, as they occur in high flow rivers (Nickerson and Mays, 1973). This effect could be especially dangerous during the breeding season, as adult males provide paternal care to unhatched eggs (e.g., Nickerson and Mays, 1973; Settle et al., 2018). The righting reflex did not appear to be affected in the field trials, but the voltage experienced by the hellbenders in our field study was similar to the low voltage treatment in the laboratory trials. Preliminary studies by MDC biologists have indicated that hellbenders can experience higher levels of voltage under natural conditions, particularly on gravel substrates that are not beneath rocks (J. Briggler, unpublished data).

Cover object use was affected in several experience, but the pattern was inconsistent. Generally, the strongest effects were for the low-medium level voltage treatments. Whether these differences are meaningful is unclear. An interesting observation is that the three individuals that left their cover objects in the field trials were in the electroshock treatment. Hellbenders are nocturnal (Noeske and Nickerson, 1979) and are usually found under large cover rocks during daylighy (Hillis and Bellis, 1971). Failure to use cover objects appropriately during the day could expose hellbenders to increased predation risk.

Elevated heartrates following shocking were only present for 6-year old eastern hellbenders. In a study measuring the influence of electroshocking on several cardiac variables in rainbow trout (*O. mykiss*) effects on heart rate were also relatively mild (108—132% of resting values), with effects lasting only 40—114 min (Schreer et al., 2004). However, they also found that cardiac arrest occurred during the shocking events and that some variables (notably cardiac output) had larger effects that lasted longer; these variables were not measured in our study.

There were no significant differences in the number of surfaces or rocking behaviors among treatment groups in any experiment. Hellbenders are known to surface for accessory air breathing and gulp in air (Nickerson and Mays, 1973; Settle et al., 2018) or rock back and forth apparently to increase oxygen absorption (Harlan and Wilkinson, 1981) when oxygen content is low or following vigorous activity that may have depleted oxygen levels. Given the high levels of increased secretions following exposure to most voltage treatments, one concern was that the skin secretions would affect the hellbenders' ability to absorb oxygen through their skin. However, the general lack of increased surfaces or rocking behaviors indicated that this was not a problem, at least for the time frame of our observations.

Overall, exposure to the voltages used in our study resulted in some negative effects on 3-year old Ozark and 6-year old eastern hellbenders, with the effects more exaggerated at higher voltages. Increased skin secretions were a highly consistent indicator of acute stress in both laboratory and field trials. During exposures in the laboratory, consistent responses included lower walking/swimming activity and higher twitching and immobilization. Following exposures, hellbenders in laboratory trials also exhibited difficulty with the righting reflex. Exposure to two shocking events did not have significantly stronger responses than a single shocking event, although sample sizes in this experiment were low (n = 5 per treatment). Three

months after shocking, hellbenders that had been maintained in the laboratory showed behavior that was similar to that of control animals. Only one hellbender, exposed to the medium-low voltage treatment in the laboratory, showed morphological injury (a spinal breakage); injuries are generally more common in fishes during electrofishing, although there is some variation among species (Snyder 2003). Potential negative effects to hellbenders and other species of conservation concern should be considered before application of electrofishing to streams containing these species.

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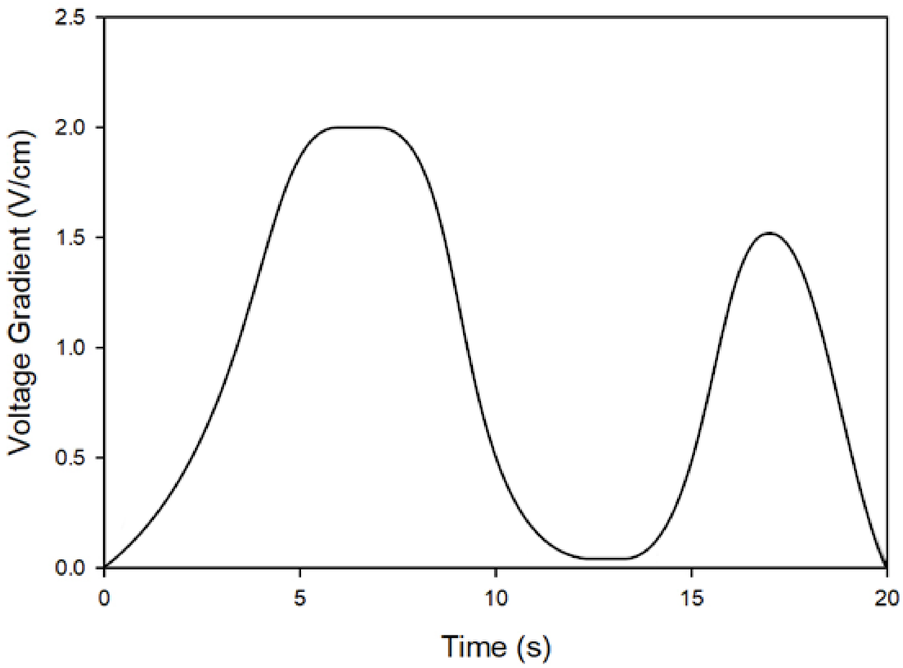
CHAPTER 4: SUMMARY

No previous research has studied the effects of electrofishing on hellbenders, a species of significant conservation concern. The data in this thesis show that hellbenders of all life stages exhibit at least short-term negative effects when exposed to electrofishing. In general, higher voltage gradients create stronger behavioral and physiological responses. Potential negative effects to hellbenders and other species of conservation concern should be considered before application of electrofishing to streams containing these species.

APPENDICES

Appendix A



Depiction of voltage gradient exposure lasting 20 s in the high treatment group.

Appendix B

Correlations of movement behaviors of 3-year old Ozark hellbenders immediately after exposure to control and electroshocking treatments; n = 20 per treatment

Behavior	Pearson Correlation Coefficient	*p*-Value
Latency to Move and Latency to Touch Cover	0.229	0.022
Latency to Move and Latency to Cover	0.031	0.757
Latency to Touch Cover and Latency to Cover	0.459	<0.0005

Correlations of movement behaviors of 3-year old Ozark hellbenders 3 months after exposure to control and electroshocking treatments; n = 20 per treatment

Behavior	Pearson Correlation Coefficient	*p*-Value
Latency to Move and Latency to Touch Cover	0.293	0.003
Latency to Move and Latency to Cover	0.080	0.428
Latency to Touch Cover and Latency to Cover	0.111	0.270

Correlations of movement behaviors of 6-year old eastern hellbenders after exposure to control and electroshocking treatments; n = 5 per treatment

Behavior	Pearson Correlation Coefficient	*p*-Value
Latency to Move and Latency to Touch Cover	0.232	0.265
Latency to Move and Latency to Cover	0.131	0.533
Latency to Touch Cover and Latency to Cover	0.239	0.249

Correlations of movement behaviors of 6-year old eastern hellbenders 3 months after exposure to control and electroshocking treatments; n = 5 per treatment

Behavior	Pearson Correlation Coefficient	*p*-Value
Latency to Move and Latency to Touch Cover	0.472	0.015
Latency to Move and Latency to Cover	-0.188	0.358
Latency to Touch Cover and Latency to Cover	-0.020	0.923

Correlations of movement behaviors of 6-year old eastern hellbenders immediately after the first shocking event of the double electrofishing study; n = 5 per treatment

Behavior	Pearson Correlation Coefficient	*p*-Value
Latency to Move and Latency to Touch Cover	0.089	0.673
Latency to Move and Latency to Cover	0.240	0.248
Latency to Touch Cover and Latency to Cover	0.169	0.419

Correlations of movement behaviors of 6-year old eastern hellbenders immediately after the second shocking event of the double electrofishing study; n = 5 per treatment

Behavior	Pearson Correlation Coefficient	*p*-Value
Latency to Move and Latency to Touch Cover	0.739	<0.0005
Latency to Move and Latency to Cover	-0.108	0.607
Latency to Touch Cover and Latency to Cover	0.050	0.812

Correlations of movement behaviors of 6-year old eastern hellbenders 3 months after exposure to control and electroshock treatments in the double electrofishing study; n = 5 per treatment

Behavior	Pearson Correlation Coefficient	*p*-Value
Latency to Move and Latency to Touch Cover	0.986	<0.0005
Latency to Move and Latency to Cover	0.272	0.188
Latency to Touch Cover and Latency to Cover	0.264	0.203

Correlations of movement behaviors of 6-year old eastern hellbenders immediately after exposure to control and electroshocking treatments in the field; n = 20 per treatment

Behavior	Pearson Correlation Coefficient	*p*-Value
Latency to Move and Latency to Touch Cover	0.547	<0.0005
Latency to Move and Latency to Cover	0.311	0.061
Latency to Touch Cover and Latency to Cover	0.375	0.022