University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
May 2019

Non-invasive Population Survey of *Cryptobranchus alleganiensis* Using Nest Boxes and eDNA in Western North Carolina

Katherine Tyrlik
Biology Major
The University of North Carolina Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty Advisor: Dr. R. Graham Reynolds

Abstract

The purpose of this project was to use non-invasive surveying techniques in order to determine the presence of *Cryptobranchus alleganiensis* at Bent Creek and at the Davidson River. At Bent Creek we took abiotic measurements (pH, temperature, and conductivity) and implanted 12 nest boxes into the creek. We also employed the use of eDNA, we took 6 eDNA samples from Bent Creek, 1 from the hellbender tank at the WNC Nature Center and 5 from the Davidson. We analysed the eDNA using PCR and qPCR. The abiotic data showed a pH gradient, transitioning from more basic to more acidic. Over the course of this project period (July 2018-April 2019), there was no hellbender occupancy in any of the nest boxes. The lids on boxes 1,9, and 10 were either destroyed during a storm event or washed away to the point that we could not relocate them. The PCR and gel electrophoresis were negative for any amplified DNA. The qPCR presence/absence test showed that there was not enough amplified DNA in order to confirm the presence of eDNA. These results showed that further research should be conducted in order to improve on the nest box model specifically for North Carolina rivers. Researchers should further compare nest boxes sites and hellbender habitats across states in order to understand the environmental differences that may affect nest box implantation in North Carolina. The eDNA extraction and qPCR protocol should be optimized in future hellbender eDNA studies.

1. Introduction

Cryptobranchus alleganiensis, or hellbenders, are a cryptic species of fully aquatic, giant salamanders endemic to the Eastern United States. These salamanders live in highly oxygenated, fast-moving water[7] and primarily eat a diet of crawfish and fish[6]. Hellbenders have become a major concern of many conservationists. Over the years this species has seen a large population decline which has resulted in hellbenders becoming a state-listed endangered species. There are many factors that are contributing to the decline of hellbenders including: human harvest, predation by non-native fish, disease, and deterioration of water quality[2]. However, one of the main factors in hellbender population decline is habitat degradation. Increased siltation in rivers and streams from industrial dumping seals the rock crevasses in which hellbenders nest.

In order to focus our current conservation effort, it is imperative that we understand the locations of hellbender populations. This means that researchers need to employ surveying tools to monitor hellbender populations. One possible surveying technique is the use of nest boxes. Nest boxes are artificial habitats constructed from non-toxic cement which are implanted into a riverbed. These boxes are designed to mimic the rock crevasses in which hellbenders live. The nest boxes have a lid with a PVC cap which can be removed in order to monitor nest box activity. The advantage in this method is that seeing live members of the population could help conservationists determine the population demographics which is vital to understanding the mechanisms of population decline[11].

Another surveying method, which is useful for hellbender monitoring, is the use of environmental DNA (eDNA). This method is based on the principle that DNA is deposited into the environment from the everyday activity of an individual[1]. During this surveying process, water samples are taken from sites of interest. After DNA extraction, qPCR is used to amplify hellbender DNA. This method can detect low levels of DNA, as low as 0.00002 ng/microliter[9]. Previous studies have shown that when hellbenders are mating there is a high amount of eDNA detected. Therefore, eDNA is a useful tool when determining a reproductively viable population[10]. Data from eDNA studies can be compared to historical records to identify hellbender decline[8].

Figure 1. Hellbender salamander.

Figure 1. Hellbender salamander (*Cryptobranchus alleganiensis*) from the Hiwassee River, TN. Photo by R. Graham Reynolds.

Bent Creek has been a site of interest for hellbender conservation for a number of years. In 2013, an UNCA student surveyed Bent Creek and confirmed the presence of a few adult specimens[4]. Since Bent Creek flows from Lake Powhatan to the French Broad River, the hellbender population in Bent Creek would remain isolated from any existing in the French Broad River. Bent Creek runs through the NC Arboretum and therefore benefits from its' protection. The Davidson River has many historical sites of hellbender populations. During this project, we employed nest boxes and eDNA as part of our survey of Bent Creek. We also used eDNA to monitor historical hellbender populations in the Davidson River. This study will compare the advantages and disadvantages of each surveying method. In addition, all the data collected will contribute to larger data collective in order to better understand these hellbender populations.

2. Methods

2.1 Nest Boxes

The hellbender nest boxes were assembled using the coffin model. We assembled the nest boxes by inserting a wire fame into a wooden mold and filling the mold with cement. The lids of the nest boxes were also constructed in this fashion. In addition, the PVC cap was spray-painted black in order to try to better camouflage the nest boxes. We added cement dye while mixing the cement to camouflage the nest boxes in the water. While securing the lid to the nest box, we used large zip ties and attached signs designating the nest boxes as a surveying tool. We constructed 12 nest boxes which were then placed downstream of Lake Powhatan in Bent Creek.

Implantation took place in July 2018 in order to make sure the boxes were established in the creek before the hellbender mating season (late August to early October), when they are most active[5].We chose the nest box location based on visual clues of a favorable hellbender habitat (low siltation, some bedrock, and canopy cover). We placed

large rocks on top of the nest boxes to camouflage and secure the boxes and placed a GPS marker on each nest box to catalog their locations. We assigned a number, 1-12, to each nest boxes in order to distinguish them from one another. We monitored the nest boxes biweekly by holding a net next to the entrance of the nest box, removing the PVC cap and peering into the box. While checking the boxes for any activity we also performed maintenance on the boxes. This maintenance included emptying silt out of the box and replacing lost covering rocks. During the month of November, 2018, we took pH, and temperature measurements every 2-3 meters along the length of the Bent Creek research site.

2.2 eDNA

We collected 6 water samples from Bent Creek and 5 water samples from the Davidson to test for eDNA. We collected a water sample from the hellbender tank at the Western North Carolina Nature Center to function as our positive control, since this is a relatively new method, eDNA sampling is prone to false positives[11] . We collected the samples using sterile 1000 ml Nalgene bottles and wore gloves during sample collection to prevent contamination.. We used Nalgene Rapid-Flow filters which we cut in half. We stored one half of the filter in 95% ethanol and cut the other half into small pieces for DNA extraction. We followed the Wizard SV Genomic DNA Purification System Protocol for the extraction. For the qPCR, we selected a mitochondrial cytochrome b sequence using the following primers: forward primer 5' GTTTGCATGAGTATTRCGGATT 3'; reverse primer 5' TCGCTATRCATTATACAGCAGATACA 3'; and a qPCR probe 5' 6FAM – CATCTCGGCAGATATG – MGB-NFQ 3' containing a fluorophore on the 5' end (6FAM).
We ran qPCR using: 2.5 µL of $MgCl_2$, 10.4 µL of water, 5 µL of flexibuffer, 1.25 µL of dNTP, 0.125 µL of Taq polymerase, 2.5 µL of each primer, 1.25 µL of the qPCR probe, and 3 µL of the sample extract. We ran the qPCR, presence/ absence test, at 95 °C for 15 minutes. We then ran 50 cycles at 94 °C and at 60 °C for 60 seconds each[9].

Figure 2. Nest box.

Figure 2 One of 12 nest boxes constructed for Bent Creek surveys. Photo by author.

3. Results

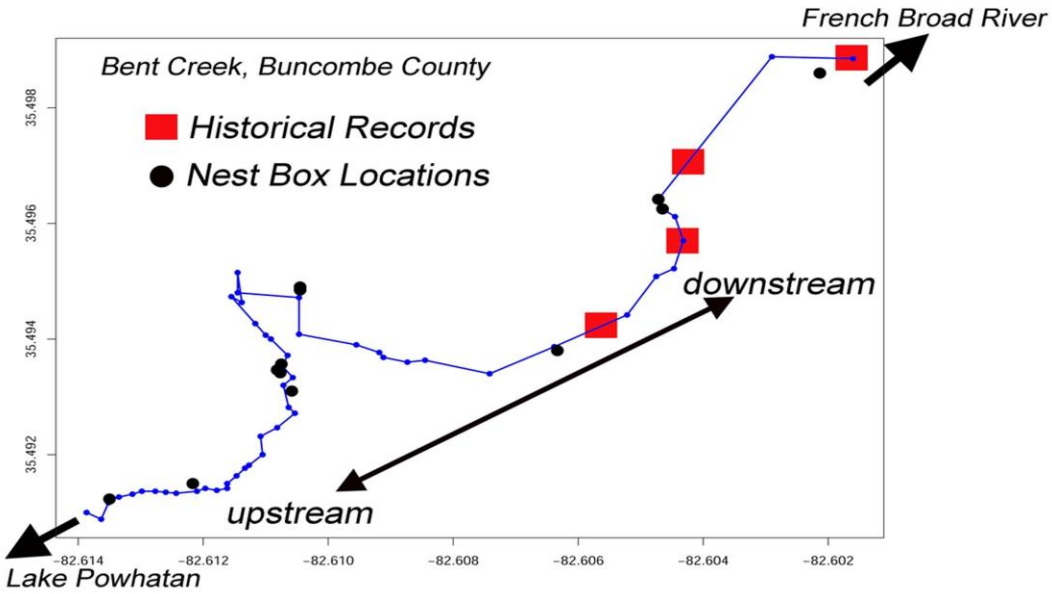

Figure 3. Map of study site.

Figure 3 Map showing the historical hellbender records and the nest boxes locations in Bent Creek.

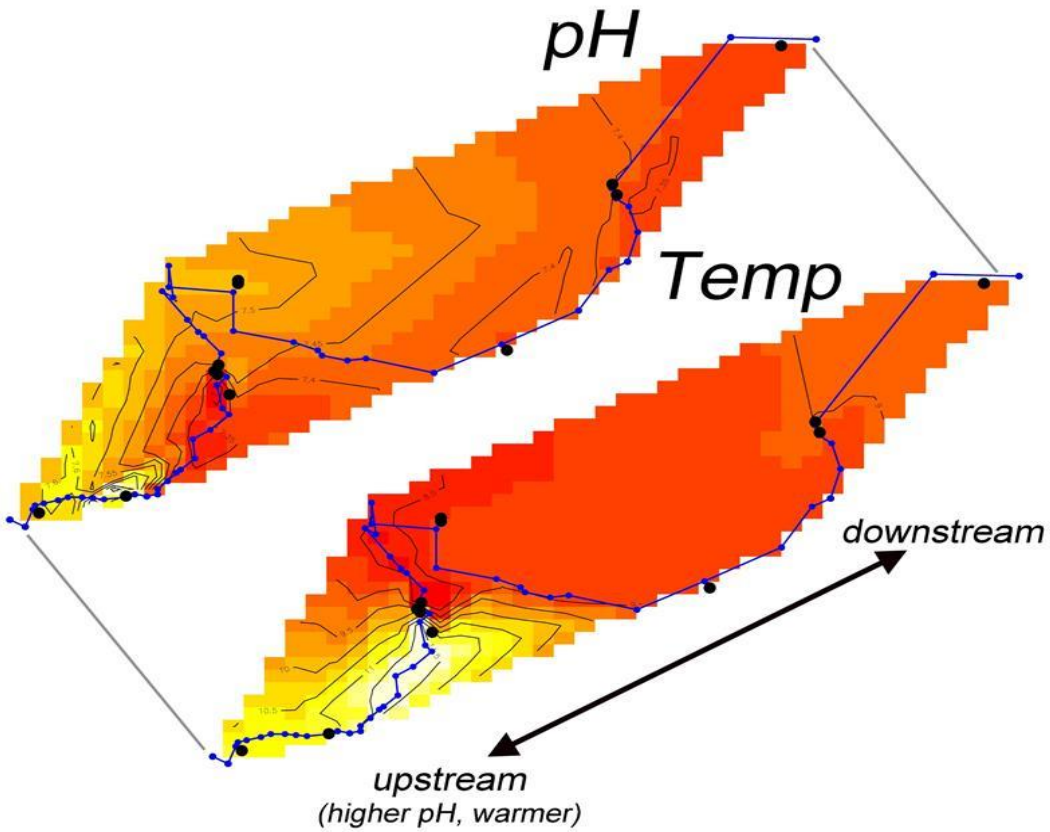

Figure 4. Temperature and PH results

.

Figure 4 The pH and temperature measurements superimposed on the map of Bent Creek from Figure 3.

Figure 5. Nest box destruction.

Figure 5 The result of heavy rainfall on nest box 1, the lid is missing and the nest box is rotated and washed out of the hellbender habitat.

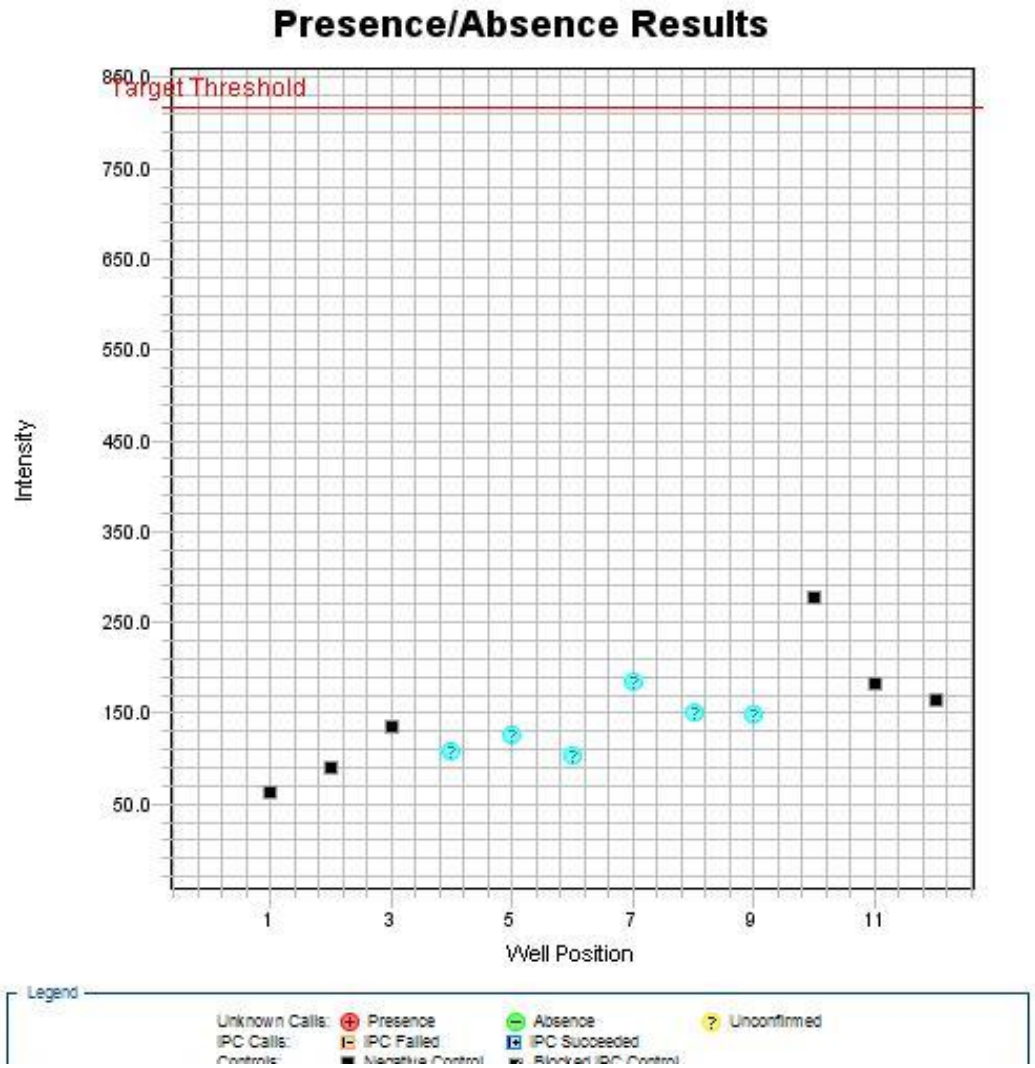

Figure 6. eDNA results.

Figure 6 Graph generated by the qPCR machine, the black dots are the negative controls and the blue dots are the samples.

3.1 Nest Boxes

The pH measurements at Bent Creek showed a gradient becoming relatively more acidic upstream relative to downstream, with an overall range of 7.32 to 7.88. The temperature of the creek increased further downstream, with a range of 8.2 to 11.8 ° C (Figure 4). There was no hellbender nest box occupancy during the course of the study. The only organism that was spotted in the nest boxes was a trout of an unidentified species. Nest boxes 1, 9, and 10 had their lids destroyed during a storm event (Figure 5). About half the nest boxes stayed relatively stationary, while the other half were translocated by the current at some point during the study. This included some of the boxes being rotated. In Bent Creek, some areas in the creek had a high amount of silt in the water. This meant that the nest box entrances would become blocked and in some cases the boxes were almost buried in silt.

3.2 eDNA

The PCR and gel electrophoresis did not show any amplified eDNA. For qPCR, the amplified DNA did not reach the target threshold resulting in a negative result (Figure 6). This showed that there was no quantifiable DNA in the extracted samples.

4. Discussion

Due to the cryptic nature of hellbenders, conservationists are pushing to find effective non-invasive surveying techniques. In states outside of North Carolina, researchers have found success in using hellbender nest boxes with confirmed cases of hellbender occupancy[3]. However, the state of North Carolina has faced unique difficulties in trying to establish consistent hellbender nest box occupancy. Future research should compare nest box sites in different states to determine possible abiotic factors that could influence hellbender nest box occupancy. When continuing this project, we will use rope or cords to secure the lids to the nest box. We will replace the lids that were lost during the summer of 2019. In future studies, other researchers should investigate alternative ways to better secure the nest boxes into the substrate. The high amount of silt in the water of Bent Creek could have been caused by the Powhatan Dam and the small bridges along the creek. These two factors can disrupt the natural flow of the river causing a buildup of sediment.

Since the positive control did not yield any eDNA, it is unlikely that the cause of the negative results was the collection method. This means the eDNA extraction protocol and the qPCR master mix recipe should continued to be modified in future studies. With an optimized protocol, Reynolds lab should also continue to collect samples from Bent Creek during and after the hellbender mating season. These data could help determine if a hellbender population existing in Bent Creek is reproducing.

5. Acknowledgements

Thanks to the staff at the WNC Nature Center for permission to sample water from the hellbender tank. I further wish to thank Wild South (Morgan Harris) and Tangled Bank Conservation (JJ Apodaca and Hope Smith). I thank the University of North Carolina Asheville for funding, as well as the McCullough Fellowship and Sonia Marcus at UNC Asheville for tremendous support. I am grateful to the NC Wildlife Resources Commission and the US Forest Service for permits (to Dr. Reynolds).

6. References

1. Bohmann K, Evans A, Gilbert MT, Carvalho GR, Creer S, Knapp M, Yu DW, de Bruyn M. Environmental DNA for wildlife biology and biodiversity monitoring. Trends Ecol Evol. 2014 Jun 29 [accessed 2019 Jan 19]:358–367. doi: 10.1016/j.tree.2014.04.003.

2. Ettling J, Wanner MD, Schuette CD. Captive Reproduction and Husbandry of Adult Ozark Hellbenders, Cryptobranchus alleganiensis bishopi. Herpetology Review. 2013 [accessed 2017 Mar 2]:605–6010.

3. Jachowski CM. Effects of Land Use on Hellbenders (Cryptobranchus alleganiensis) at Multiple Levels and Efficacy of Artificial Shelters as a Monitoring Tool. Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy In Fish and Wildlife Conservation. 2016 May 9 [accessed 2018 Apr 3]:1–216.

4. Levine A. Determining the Presence of Eastern Hellbender (Cryptobranchus alleganiensis alleganiensis) and Differentiators of Occupied vs. Unoccupied Habitats in Bent Creek, Buncombe County, North Carolina. UNCA Undergraduate Research. 2013 Dec [accessed 2017 Mar 1]:152–158.

5. Mayasich J, Grandmaison D, Phillips C. EASTERN HELLBENDER STATUS ASSESSMENT REPORT. National Regulatory Research Institute. 2003 Jun [accessed 2017 Sep 17]:1–40.M

6. Messerman A. The Use of Nest Boxes by the Hellbender Salamanders in Western North Carolina. 2014 Apr [accessed 2017 Mar 1]:1–28.

7. Nickerson M, Briggler J. Harvesting as a factor in population decline of a long-lived salamander; the Ozark hellbender, Cryptobranchus alleganiensis bishopi Grobman. Applied Herpetology. 2007;4(3):207–216. doi:10.1163/157075407781268354

8. Pitt AL, Shinskie JL, Tavano JJ, Hartzell SM, Delahunty T, Spear SF. Decline of a giant salamander assessed with historical records, environmental DNA and multi-scale habitat data. Freshwater Biology. 2017 [accessed 2019 Jan 19];62(6):967–976. doi:10.1111/fwb.12917

9. Spear SF, Groves JD, Williams LA, Waits LP. Using environmental DNA methods to improve detectability in a hellbender (Cryptobranchus alleganiensis) monitoring program. Biological Conservation. 2015 [accessed 2018 Nov 20];183:38–45. doi:10.1016/j.biocon.2014.11.016

10. Takahashi MK, Meyer MJ, Mcphee C, Gaston JR, Venesky MD, Case BF. Seasonal and diel signature of eastern hellbender environmental DNA. The Journal of Wildlife Management. 2017 [accessed 2019 Jan 20];82(1):217–225. doi:10.1002/jwmg.21349

11. Wineland SM, Welch SM, Pauley TK, Apodaca JJ, Olszack M, Mosher JJ, Holmes JN, Waldron JL. Using environmental DNA and occupancy modelling to identify drivers of eastern hellbender (Cryptobranchus alleganiensis alleganiensis) extirpation. Freshwater Biology. 2018 [accessed 2019 Jan 18];64(1):208–221. doi:10.1111/fwb.13210