Buffalo State
State University of New York
Department of Biology

Conservation Genetics of New York's Giant Salamander:

The Eastern Hellbender (*Cryptobranchus alleganiensis*)

A Thesis in
Biology

By

Meghan K. Jensen

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

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ABSTRACT OF THESIS

Conservation Genetics of New York's Giant Salamander:

The Eastern Hellbender (*Cryptobranchus alleganiensis*)

The hellbender, *Cryptobranchus alleganiensis*, is North America's only giant salamander and is endemic to the eastern United States. Hellbender populations are declining and management efforts may be essential to their continued survival. The Buffalo Zoo is raising hellbenders collected from the Allegheny drainage by the New York State Department of Environmental Conservation. These animals are being released back into the drainage in an attempt to increase the population size. Little research exists on New York hellbender genetics, yet genetic information would help inform a head-starting program. The main objectives of this study were to 1) survey and collect samples from eight sites in the New York Allegheny drainage, 2) characterize the genetic structure, composition, and diversity of the northern Allegheny watershed and of the head-started cohort, and 3) develop management guidelines for the reintroduction of head-started individuals. Hellbenders were found in four of the eight sites surveyed. Over 200 animals New York and Pennsylvania and the head-started cohort were genotyped at 10 microsatellite loci. Genetic analysis indicated that the upper Allegheny is one ecological management unit, although we discovered evidence of recent genetic drift in the northern-most tributary. The head-started cohort has lower allelic richness compared to wild sites. Reintroducing head-started animals to the northern-most tributary might reverse the effects of genetic drift. However, releasing large numbers of this cohort throughout the New York Allegheny

basin could potentially lower the overall genetic diversity. Future head-starting efforts using targeted egg collection from diverse sites could counteract this potential negative consequence.

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CHAPTER 1: Introduction

Hellbender Natural History

The hellbender, *Cryptobranchus alleganiensis*, is the only extant species of giant salamander (family Cryptobranchidae) native to North America, and endemic to the eastern United States. There are currently two recognized subspecies, the eastern hellbender (*C. a. alleganiensis)* and the Ozark hellbender (*C. a. bishopi*). The latter only occurs in the Ozark Mountains of Missouri, while the eastern subspecies inhabits river drainages from southern New York, to northern Georgia, and west to Missouri (Figure 1). In New York State, the eastern hellbender subspecies can be found in only the Allegheny and Susquehanna River drainages (Figure 2). Like many species of amphibians worldwide (Stuart et al., 2004), hellbender populations are declining at alarming rates throughout their range (Nickerson and Mays, 1973; Williams et al., 1981; Gates et al., 1985; Bothner and Gottlieb, 1991; Trauth et al., 1992; Wheeler et al., 2003; Bauman and Wilson, 2005; Briggler et al., 2007a; Foster et al., 2009).

Several life history attributes of this species make it particularly susceptible to decline. Hellbenders are large, long-lived, and obligately aquatic. Average length of adults is 50 cm (Petranka, 1998) while some individuals are over 70 cm (Nickerson and Mays, 1973; Petranka, 1998). Growth rate data suggest that they can live up to 30 years in the wild (Taber et al., 1975). Long-lived species are particularly sensitive to perturbations due to slow growth, low fecundity, and delayed maturity (Wheeler et al., 2003). Bishop (1941) estimated that hellbenders do not reach sexual maturity until five to six years of age, while others estimate six to seven years (Taber et al., 1975;

Peterson et al., 1988). In addition, hellbenders have a short breeding season that spans from late August through early September (Humphries and Pauley, 2000). Also, recent demographic studies have found a predominance of individuals in older age classes (Bothner and Gottlieb, 1991; Wheeler et al., 2003; Humphries and Pauley, 2005; Foster et al., 2009; Burgmeier et al., 2011), which may suggest that juvenile recruitment is low.

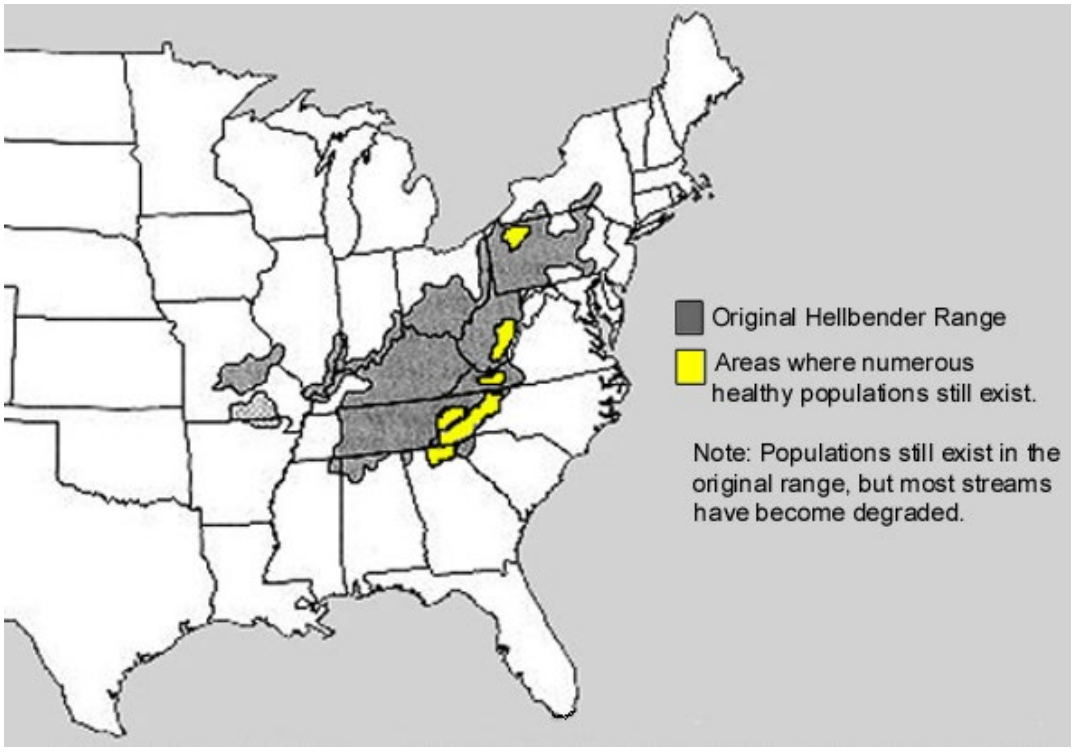


Figure 1: Eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) range map. Map originally developed by Petranka (1998), yellow areas added from survey data by Humphries (2010). Grey represents original hellbender range while yellow represents areas where healthy populations are currently present.

Hellbenders are considered habitat specialists in that they require cool, clean streams, with relatively shallow, fast-flowing waters (Smith, 1907; Hillis and Bellis, 1971; Williams et al., 1981). Although larval hellbenders have external gills, by two years of age gills are lost and replaced by lateral skin folds along the length of the

body (Bishop, 1941). These folds, which increase the surface area of the skin, serve as the primary site of gas exchange via diffusion; this makes these types of pristine stream habitats essential for survival. Hellbenders are cryptic, ambush predators and require substrates composed of large rocks, as well as an abundance of invertebrates such as crayfish (Nickerson and Mays, 1973). Large rocks are essential for hiding and also provide optimal protection for nest sites. Hellbender populations may occur at high densities in some streams (Hillis and Bellis, 1971; Nickerson and Mays, 1973) and as such they are an important component of stream ecosystems as both a predator and prey species (Humphries and Pauley, 2000). Additionally, due to the specific habitat requirements of this species, they can serve as indicators for unaltered, pristine regions of river systems (Peterson et al., 1988).

Summary of Threats/Conservation Status

There are a variety of causes, many of them anthropogenic, contributing to the decline of the hellbender. Habitat alteration and degradation such as siltation, channelization, impoundment, and dredging are widely accepted as the major causes of decline (Nickerson and Mays, 1973; Williams et al., 1981; Gates et al., 1985; Trauth et al., 1992; Wheeler et al., 2003; Mayasich et al., 2003). Other contributing factors may include: poor water quality and pollution (Nickerson and Mays, 1973), over-collection for the illegal pet trade and scientific studies (Nickerson and Mays,

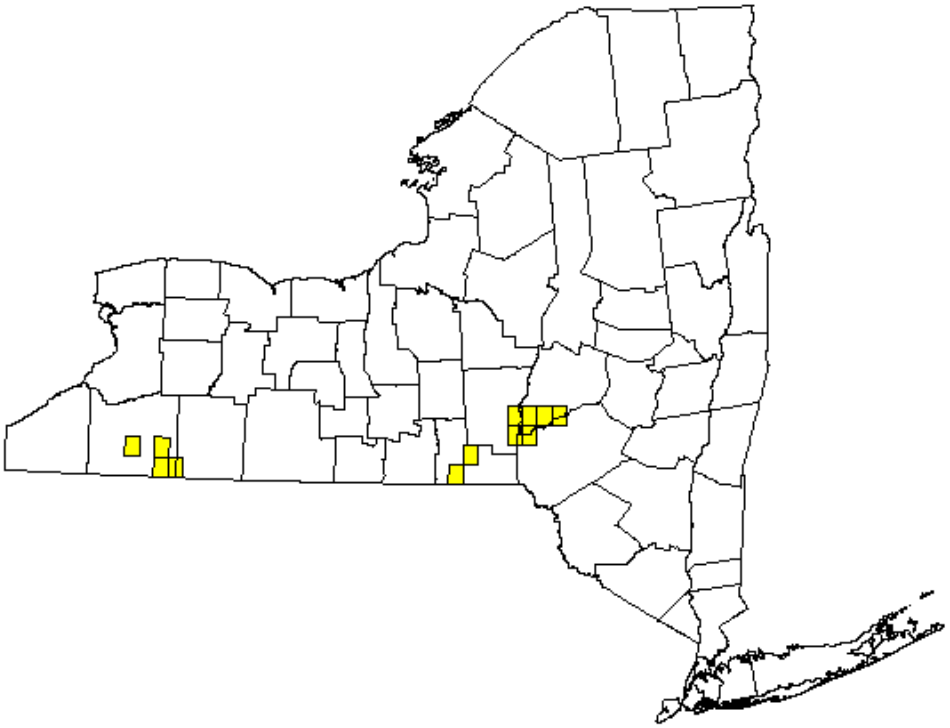


Figure 2: Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) distribution map as reported by the Amphibian and Reptile Atlas Interim Report. Data collection was from 1990-2007 (NYSDEC, 2007).

1973; Humphries and Pauley, 2005; Nickerson and Briggler, 2007), introduced species (Gall, 2008), killing by anglers (Humphries and Pauley, 2005), and pathogens (Mayasich et al., 2003; Briggler et al., 2007b).

Stuart et al. (2004) reported that the major cause of decline for 183 species of amphibians worldwide is habitat loss and degradation. Griffiths and Pavajeau (2008) report that habitat loss and alteration is the most common threat to amphibian species in reintroduction programs and, this is also the most reported threat to hellbenders (Mayasich et al., 2003). In particular, siltation is a major problem as it can reduce the interstitial spaces between coarse substrates which provide refugia for adult and larval

hellbenders (Mayasich et al., 2003). Additionally, reduction in the heterogeneity of substrate via siltation could lead to a decline in the abundance of prey species such as crayfish (Mayasich et al., 2003). Impoundments are also a threat in that they can impede the flow of water, thereby decreasing the oxygen content and reducing the suitability of the habitat, since hellbenders breathe via diffusion (Mayasich et al., 2003). Furthermore, dams create barriers to the immigration and emigration of individuals and reduce the potential for recolonization of extirpated sites from source populations (Mayasich et al., 2003).

Due to their heavy reliance on crayfish as a major food source, hellbenders have been found to accumulate lead and mercury at levels similar to that of fish such as smallmouth bass (*Micropterus dolomieu*) (Huang et al., 2010). Although the effects of mercury on adult amphibians is unknown (Boening, 2000), a toxicology study on Southern leopard frogs (*Rana sphenocephala*) by Unrine et al. (2004) suggested that mercury accumulation may have detrimental effects on amphibian larvae.

Historically, hellbenders have been collected in large numbers for study and for the pet trade. In Missouri, it was estimated that more than 550 individuals were removed from the wild for such purposes in a 20 year span (Nickerson and Briggler, 2007). Invasive species also may play a role in the reduction in hellbender populations. Gall and Mathis (2010) showed that hellbenders lack anti-predatory responses to the chemical cues of non-native salmonids, which may increase the vulnerability of wild individuals to predation. Additionally, natural parasites such as those described by Nickerson and Mays (1973), and diseases like chytrid fungus (*Batrachochytrium dendrobatidis*), which was recently discovered in wild hellbender populations

(Briggler et al., 2007b) , may also contribute to declines. Although these factors may not be significant individually, biological stressors and degraded habitat may have synergistic effects contributing to elevated mortality rates (Mayasich et al., 2003).

As a result of the current declines and present threats to existing hellbender populations, protective status has been assigned to the species in 12 of the 16 states in which it is native (Mayasich et al., 2003). The eastern hellbender is currently listed as a species of special concern in New York State. In addition, the Ozark hellbender has recently been listed as Federally Endangered, and both subspecies have been added to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (USFWS, 2011a; USFWS, 2011b).

Hellbenders in the Allegheny Drainage of New York

As previously stated, hellbenders occur in only two drainages in New York State; the Allegheny and the Susquehanna watersheds (Figure 2). The first published records of the hellbender in the Allegheny drainage appeared in the New York State Museum Bulletin in 1941 (Bishop). Bishop (1941) indicates that there were numerous sightings of hellbenders within the drainage since the 1800's, dating as far back as 1842. Bishop's report indicated that hellbenders may have been relatively abundant; in one case 13 individuals were caught in a single stretch of stream in just one hour (Bishop, 1941).

Since then, two major publications have sought to determine the status and abundance of hellbender populations in the Allegheny watershed. The first was by Bothner and Gottlieb (1991) in which the authors estimated hellbender densities at

eight sights within the drainage, which were sampled between 1981 and 1988. Two decades later, Foster et al. (2009) repeated the sampling protocols from Bothner and Gottlieb (1991) to determine if any changes had occurred in hellbender densities at those same eight sites. Foster et al. (2009) found hellbenders to be extirpated from one site and also found declines in the ecological density of hellbenders in several additional sites. Despite these clear declines in hellbender populations within the New York portion of the Allegheny drainage, the direct cause of these declines remain uncertain (Foster et al., 2009). However, as in many areas throughout their range, it is clear that the hellbender is in need of management in New York State (Williams et al., 1981).

RRT Programs and Genetic Considerations

Over the past few decades, several techniques have been employed to manage threatened and endangered reptile and amphibian populations. Some of those that have been increasing in popularity include reintroductions, repatriations, and translocations (RRT) (Dodd and Seigel, 1991; Germano and Bishop, 2008). RRT programs seek to reduce the probability of extinction of a given species by increasing total number of individuals, genetic diversity, and the number of viable populations (Scott and Carpenter, 1987; Dodd and Seigel, 1991, Vinkey et al. 2006).

A "successful" reintroduction program is one in which there is evidence to suggest a self-sustaining population has been established (Griffith et al., 1989; Dodd and Seigel, 1991). In 1991, a review of such programs by Dodd and Seigel (1991) demonstrated low success rates as a reintroduced population of RRT programs for

reptiles and amphibians compared to other vertebrate taxa (birds and mammals). However, more recent literature has shown much higher success rates. Germano and Bishop (2008) found 36 of 82 (42%) translocation projects to be successful over three decades. Griffiths and Pavajeau (2008) found that 13 of 21 species had successful self-sustaining reintroduced populations. Both studies also mention that a large portion of these programs have unknown outcomes and monitoring of these programs takes several years before success or failure can be determined. As a result, success rates may be underestimated (Germano and Bishop, 2008; Griffiths and Pavajeau, 2008).

RRT programs often incorporate captive-breeding or head-starting. "Head-starting" is a common management technique used to increase recruitment by raising young in captivity until they reach an age or size where predation risk is significantly reduced upon relocation to the wild (Perez-Buitrago et al., 2008). Zoos and aquariums often play an important role in RRT programs as captive-breeding or head-starting locations (Dodd and Seigel, 1991). An added benefit of the participation of these organizations is the promotion of public interest and support of RRT projects (Dodd and Seigel, 1991).

Given the conservation status of the eastern hellbender, the New York Department of Environmental Conservation has implemented a "head-starting" program in an attempt to conserve this rare species in New York. The program entails a collaboration with the Buffalo Zoo where the eggs were hatched and the larvae raised in a hellbender laboratory/exhibit. These animals are being released back into the Allegheny watershed to increase the population size. This program is to work in

conjunction with habitat management programs to prevent further decline while major threats can be identified and addressed (Ken Roblee, NYS DEC, pers. comm.).

Crowhurst et al. (2011) recommended that individuals from genetically differentiated drainages not be translocated, to conserve localized adaptations that may be present in various populations. If individuals from genetically distinct populations are combined through translocations, offspring could potentially suffer from outbreeding depression, since genes from different populations may be incompatible (Sabatino and Routman, 2008). Ultimately, outbreeding depression leads to reduced individual fitness (Hedrick, 2001), which could exacerbate the species decline. In contrast, Tonione et al. (2011) proposed that in some cases intentional mixing of populations by managers may help the species as a whole. Mixing such populations adds to the gene pools of individual subpopulations and subsequently increases the species' adaptive potential. In either case, a full understanding of the genetic structure of a population is essential to properly advise the placement of captive individuals in the wild. This is necessary because understanding genetic patterns within the population can help to predict if mixing populations will increase the adaptive potential of a species by increasing genetic diversity, or reduce the adaptive potential by washing out localized alleles.

Unfortunately, reintroduced and managed populations often suffer a drastic reduction in genetic diversity (Swanson et al., 2006; Pierson et al., 2007). For example, Fitzsimmons et al. (1997) examined allozymes in reintroduced populations of Bighorn sheep (*Ovis canadensis*) and found that the total number of alleles per locus was reduced and allele frequencies significantly differed from the source

population. Robichaux et al. (1997) found a 73% reduction in RAPD polymorphism as a result of a genetic bottleneck in a reintroduced population of the Mauna Kea Silversword (*Argyroxiphium sandwicense* ssp. *sandwicense*). A study by Williams et al. (2002) found that certain reintroduced populations of the fisher (*Martes pennanti*), which were reintroduced 30 years or more prior to the study, had significant differences in allele frequencies compared to their respective source populations. Also, a study using both mtDNA and microsatellite markers found a pronghorn antelope (*Antilocapra americana*) population in Washington had lower genetic diversity compared to its source population. Mock et al. (2004) found evidence of genetic bottlenecks in three translocated populations of Merriam's turkey. Further, a microsatellite study on dice snakes, *Natrix tessellata,* also indicated that reintroduced populations suffer from genetic bottlenecks and that serial reintroductions (subsequently reintroducing animals from already reintroduced populations) can exacerbate this loss of genetic diversity (Gautschi et al., 2002). Jamieson (2010) found that four avian reintroduction programs in New Zealand suffered from severe inbreeding and genetic drift despite having over 20 genetic founders, which is the minimum number of founders recommended for starting captive-breeding programs (Foose et al., 1986; Lacy, 1989; Willis and Wiese, 1993). In addition, a modeling study demonstrated lowered genetic diversity in reintroduced populations, which is primarily a result of inbreeding and genetic drift (Lacy, 1987). Moreover, populations that need reintroductions in order to survive typically already suffer from limited genetic variation (Stockwell et al., 1996, Swanson et al., 2006) from founder effects, small population sizes (O'Brien and Evermann, 1988), and isolation from habitat

fragmentation. These factors contribute to severe genetic drift and increased inbreeding, consequently leading to further loss of genetic diversity (Nei et al., 1975) and adaptive potential (Reed and Frankham, 2003).

However, not all reintroductions ultimately lead to lowered genetic diversity. For example, Pierson et al. (2007) found that a reintroduced American chestnut (*Castanea dentate*) population had higher heterozygosity than the original founders, no alleles were lost, and genetic differentiation was extremely low. Additionally, a study on Rocky Mountain elk found no difference in genetic diversity between five reintroduced populations and two source populations (Hicks et al., 2007). The authors note that these results contrast with reintroductions of elk in the eastern United States (Hicks et al., 2007). They suggest that the maintenance of genetic diversity in the reintroduced Rocky Mountain elk populations was a result of gene flow from other established elk populations in the western US. Similarly, Swanson et al. (2006) propose that the success of the American marten reintroductions in Michigan was a direct result of multiple, subsequent reintroduction events which mimicked natural gene flow in the population.

A modeling study on reintroduction success suggested that the duration of the reintroduction program (and more than one release event) was the most important factor in determining success (Robert, 2009). Jamieson (2010) stresses the importance of developing a long-term strategy for RRT programs to reduce inbreeding and maintain diversity. Therefore, understanding if there is potential for natural gene flow is crucial in deciding on how many releases are necessary for a genetically successful

reintroduction program. If natural gene flow is unlikely, it is important to plan subsequent releases to supplement the population.

An additional concern is that captive populations may already be subject to low genetic diversity given that they are often founded by a small number of wild individuals (Kraaijeveld-Smit et al., 2006). Thus, understanding the genetic composition of captive animals and their potential effect on the populations is crucial for planning management efforts.

Conservation Genetics

Conservation genetics is a relatively new scientific discipline that is of great value to conservationists and wildlife managers. Genetic studies help to explain the ecological and evolutionary forces acting on wildlife populations both spatially and temporally (Crowhurst et al., 2011). In addition, information from these studies can be used to define management units and select the best strategies for conservation (Crowhurst et al., 2011). Previous studies have examined the genetic structure of hellbender populations (Merkle et al., 1977; Routman, 1993; Rayman, 2010; Crowhurst et al. 2011). However, few of these studies included hellbender individuals from New York.

Merkle et al. (1977) found that allozyme diversity of the hellbender was extremely low after sampling twelve sites across the species' range. In fact, only two of 24 loci sampled were polymorphic. Shaffer and Breden (1989) found similar results in a later study, and upon reviewing 102 genetic studies of salamander species, discovered that paedomorphic salamanders generally have lower genetic diversity than metamorphic

species. The authors hypothesized that the obligate aquatic lifestyle of paedomorphic salamanders is responsible for this trend (Shaffer and Breden, 1989). Aquatic habitats are relatively unstable and thus, populations are more subject to extinction, and subsequent re-colonization would reduce overall genetic diversity of the species through the founder effect (Shaffer and Breden, 1989).

Conversely, another range-wide study of hellbenders suggested that there is a relatively high degree of genetic variation in mitochondrial DNA (mtDNA) between river drainages (Routman, 1993). This disparity between markers would best be explained by a recent bottleneck that the relatively quick-evolving mtDNA recovered from, while the slower mutating allozymes had not (Routman, 1993). Routman (1993) also found that while gene flow was relatively high within rivers, different drainages appeared to have minimal gene flow, which suggests that the populations do not experience much mixing. The implication of this result is that female dispersion and migration is limited between river systems since mitochondrial DNA is inherited maternally (Routman, 1993). Furthermore, this could suggest that populations in different river systems are on separate evolutionary paths.

A Missouri study on microsatellites by Crowhurst et al. (2011) further reinforced the findings of Routman (1993). Crowhurst et al. (2011) found gene flow to be high within drainages and minimal between them. These findings suggest that hellbender individuals do not disperse over long distances, and because microsatellites are inherited by both parents, this trait would apply to both sexes (Crowhurst et al., 2011). This is congruent with studies on hellbender movements which suggest that hellbenders are highly sedentary animals (Nickerson and Mays, 1973; Peterson, 1987;

Petranka, 1998; Humphries and Pauley, 2005). Although, in some cases, individuals have been found to move great distances, up to 8 miles, within rivers (Petokas pers. comm., Foster, pers. comm.). This further supports the conclusion that gene flow occurs within drainages but not across them.

An Indiana study, using a different suite of microsatellite loci, found even more genetic diversity within loci than was found in the Missouri population (Unger et al., 2010). This is to be expected because effective population size and gene flow are highest in the central areas of a species' range and therefore marginal populations have less genetic diversity (Eckert et al., 2008). Since Missouri is a peripheral population, founder events and genetic bottlenecking would decrease genetic diversity relative to the Indiana population (Crowhurst et al., 2011). Still, the Missouri population of hellbenders has moderate levels of diversity and did not appear to have "severe genetic erosion" (Crowhurst et al., 2011). However, the continued degradation of riverine habitats could lead to more fragmented populations and ultimately an increase in genetic drift and inbreeding due to reduced population size throughout the range (Sabatino and Routman, 2008; Crowhurst et al., 2011).

Rayman (2010) performed a fine-scale study of New York hellbenders using mtDNA sequences. The results of the study showed much greater variation in sequences than expected, given that New York hellbenders are at the northern-most edge of the range (Rayman, 2010). After the retreat of the glaciers, centrally-located species must recolonize the northern habitats and as a consequence usually experience genetic bottlenecking (Eckert et al., 2008; Rayman, 2010). It is estimated that the most recent ice sheet, the Wisconsonian, receded between 24,000 and 12,000 years before

present (Muller and Calkin, 1993). Based on unique haplotypes, Rayman (2010) found two major genetic groups within the Allegheny River drainage, which may suggest a greater degree of differentiation within a river system than posited by other studies (Routman, 1993; Crowhurst et al. 2011).

Significance

The eastern hellbender is unique to the eastern United States, an intricate part of stream ecosystems (Humphries and Pauley, 2000), and important as an indicator of unaltered streams (Peterson et al., 1988). The potential synergistic effect of many factors has had detrimental impacts on the status of this species across its range (Mayasich et al., 2003). Management efforts are essential to the continued survival of the hellbender. As hellbenders are slow to mature (Bishop, 1941; Taber et al., 1975; Peterson et al., 1988), highly sedentary (Wheeler et al., 2003), and particularly susceptible to perturbations, human-mediated releases of individuals may be necessary to conserve this species (Trenham and Marsh, 2002). The New York State Department of Environmental Conservation (NYSDEC) has already implemented a "head-starting" project where captive-raised hellbenders will be released into the wild in an attempt to increase the overall population size by increasing juvenile recruitment.

When released, the captive-raised individuals could potentially impact the wild Allegheny hellbender population by lowering the overall genetic diversity as is commonly seen in RRT programs (Swanson et al., 2006; Pierson et al., 2007; see Lacy, 1987; Fitzsimmons et al., 1997; Robichaux et al., 1997; Williams et al., 2002; Mock et al., 2004; Stephen et al., 2005; Jamieson 2010). With this in mind, a

comprehensive understanding of the genetic composition of both the wild and head-started individuals could provide insights into any possibility of detrimental effects of the project. If we find there is a possibility of lowered genetic diversity, steps can be taken to develop a long-term management program to maintain genetic diversity in the population. One potential management strategy may include subsequent releases using animals from different source populations to simulate natural gene flow, as this was shown to maintain genetic diversity in a reintroduced population of American marten (*Martes pennanti*) (e.g. Swanson et al., 2006).

Unfortunately, little is known about the genetic diversity and structure of hellbenders in New York. In fact, research on this species is lacking in the northeastern extent of the range. Although studies have been performed recently on the eastern hellbender outside of New York (Merkle et al., 1977; Routman, 1993; Crowhurst et al., 2011), New York and other northeastern populations have the potential to differ strongly from southern populations. This difference could be the result of a variety of factors such as genetic drift, founder events, genetic bottlenecking or localized adaptation to the colder northern climate. Therefore, an analysis using nuclear markers to assess the genetic relationships of hellbender populations in New York would provide information necessary to optimize conservation efforts through best management practices in New York. Furthermore, molecular data can help to identify populations that may be valuable in future captive breeding or rearing of hellbenders for sustaining the species over the long-term (Sabatino and Routman, 2008). Also, this study will provide genetic information on northeastern populations of the hellbender to contribute to the overall genetic

understanding of this species throughout the entire range. Last, the findings of this study may help provide insight into the potential genetic effects of head-starting programs, and guidelines based on the results can be useful when implementing future hellbender head-starting programs.

Study Objectives

There are three main objectives of this study. The first objective is to build upon the existing baseline data on hellbender sites in the Allegheny River of New York State. Field research will be performed to survey new and historic sites to determine if hellbenders are present. Not only will this add to our knowledge of hellbender locations so they can be protected, but genetic samples collected from individuals at these sites will help to increase the connectivity of existing sites for the genetic analysis.

The second objective of this study is to complete a fine-scale genetic analysis of the structure and diversity of hellbenders in the Allegheny drainage of New York and northern Pennsylvania using nuclear microsatellite markers. Additionally, the genetic composition of hellbenders in the head-starting program at the Buffalo Zoo will be analyzed and compared to the wild population so that potential genetic effects of the program can be determined.

The last major objective is to provide management recommendations for the head-starting program in New York based on the genetic analysis. These recommendation will deal with optimal placement of the current head-started cohort individuals in terms of maintaining the current levels of genetic diversity of the species. Further,

general guidelines with a genetic focus will be provided for future head-starting programs throughout the species' range.

CHAPTER II: Building on New York State Baseline Hellbender Data

Introduction

The hellbender (*Cryptobranchus alleganiensis*) is a unique salamander species endemic to the United States. There are currently two recognized subspecies of the hellbender: the Ozark hellbender (*C. a. bishopi*) and the eastern hellbender (*C. a. alleganiensis*). The Ozark hellbender is a federally endangered species (USFWS, 2011a) and native only to the Ozark mountain region of Missouri. The eastern hellbender inhabits watersheds from southern New York, to northern Georgia, and west to Missouri and it is currently under review for the Candidate species list (Mayasich, 2003).

As previously discussed, several studies have found that hellbender populations are declining across the species' range and there are numerous threats that are suspected of contributing to those declines. In addition to the already long list of current threats, there are new potential threats on the horizon. New diseases such as chytrid fungus (*Batrachochytrium dendrobatidis; Bd*) and *Ranavirus* are of growing concern (Bodinof et al., 2011). Climate change and hydrolic fracturing activities also could cause problems for populations in the future (Greg Lipps, pers. comm.).

Given the current declines of hellbender populations and the growing list of threats, monitoring programs are essential for the recovery of the species. Unfortunately, recovery plans are often initiated with an incomplete understanding of the biology and accurate status of threatened and endangered species. Campbell et al. (2002) stress the importance of incorporating monitoring protocols into recovery

programs. Yoccoz et al. (2001) consider monitoring a "necessary step" to improve upon management decisions and establish the conservation status of a species. Thus, it is important to monitor both the Ozark hellbender subspecies to evaluate recovery success and the eastern hellbender to gather data to provide to managers for making a final decision in the listing process. Spatial sampling is a crucial aspect of monitoring to ensure proper management techniques are employed and to aid in the success of recovery programs (Martin et al., 2006). In particular, we must understand the spatial distribution of species that exist across a large landscape (Yoccoz et al., 2001).

The purpose of this study is to add to the body of knowledge on the eastern hellbender subspecies in the northern Allegheny watershed. Our major goals were to determine if hellbenders are still present in historical sites in the drainage and to survey new sites for hellbender presence and locate potential release sites for captive-reared individuals. We also sought to build on existing demographic information and abnormalities of current hellbender populations in New York and collect DNA samples for the genetic portion of this study.

Materials and Methods

Study Sites

We surveyed eight sites in the northern Allegheny watershed for hellbenders. Sites were labeled number 9 through 16 since these sites were surveyed in addition to eight sites (1-8) already surveyed by Foster et al. (2009). Two of these sites had historical records of hellbender presence (NYSDEC); one mainstem site (site 11), and one tributary site (9) (Figure 1). All other sites were new survey sites with no known

historical record. These included one additional site in the mainstem (10) and four tributary sites (10, 14, 15, 16) (Figure 1).

Site Characteristics/Field Captures

Two capture methods described by Foster (2006) were employed when surveying the new study sites; rock turning and trapping. We performed rock turning at all sites except site 11, where trapping was the only feasible method based on depth, flow, and substrate composition. For consistency, protocols from Foster et al. (2008) were followed for each of the aforementioned techniques. However, we added the use of a 16'x4'seine net with ¼" mesh in rock turning to assist in sites with high turbidity or poor visibility. Prior to lifting a rock, the seine net was placed around the perimeter of the rock allowing enough room for one or more researchers to be inside of the net. The wooden poles at the ends of the seine net were then twisted together to ensure there were no openings through which a hellbender could swim. Smaller rocks from the stream bottom were placed on the bottom of the net to weight the bottom and keep it flush with the streambed. The rock was then turned and 16"x16"x12" Baitwell nets with 3/16" mesh (Forestry Suppliers) were used to secure any hellbender escaping from under the rock. After flipping a rock, the rock was gently replaced in the same location and if a hellbender was captured, it was returned to the same rock after processing. We used the distance measurement tool in Google Maps to get approximate stream width (nearest 0.5m) at the center point of each reach surveyed. Percent habitable space, defined by Foster et al. (2009) as the portion of the stream

bottom covered by rocks greater than 30cm in diameter, was estimated visually for each site.

Total length (mm), standard length (mm, tip of the snout to base of the tail), mass (g), and description of unique markings and abnormalities were recorded for all hellbenders captured at each site. Photographs were taken of all individuals for visual documentation. Rock characteristics including depth (m), maximum diameter length (m), and percent embeddedness (visually estimated) were recorded at each rock where a hellbender was captured. Stream temperature (ºC) also was recorded at the time of capture and a GPS location was taken using a Garmin Rino 120 handheld GPS Receiver. In addition, a PIT (passive integrated transponder) tag was inserted subcutaneously in all hellbenders greater than 20cm in length. Using a sterilized pair of dissection scissors, a tail clip, no greater than 10mg, was collected from the end of the tail for all individuals for genetic analysis. Arntzen et al. (1999) demonstrated that tail-clippings do not have a negative effect on amphibian survival or body condition. Additionally, this method was employed by Foster et al. (2009) with no adverse effects observed (Foster, pers. comm.). Tail samples were kept on ice then transferred to a -80ºC freezer where they were stored until DNA could be extracted.

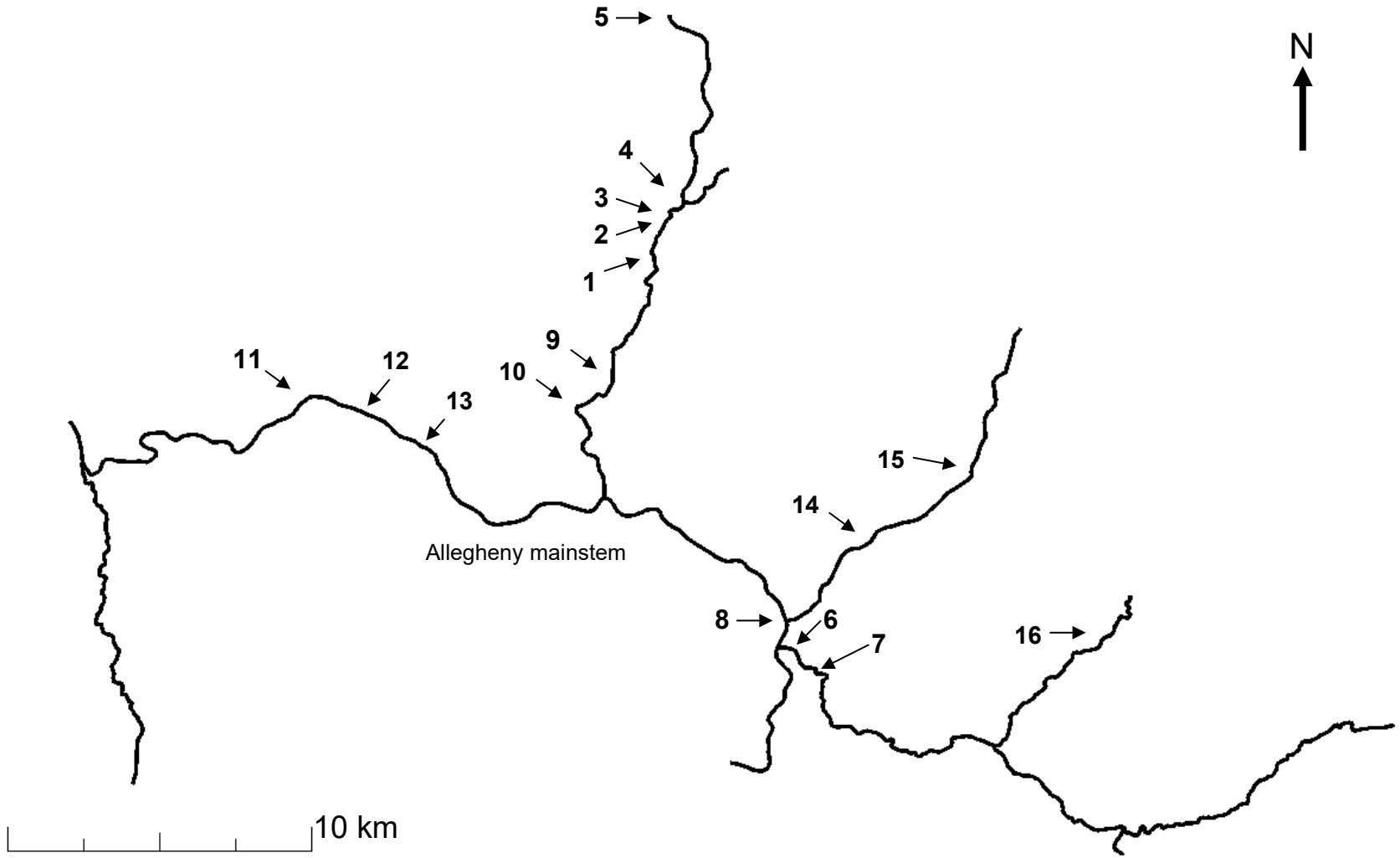


Figure 1: Line map including site locations in the Allegheny River Drainage. Sites 1 through 8 were surveyed in 2004/2005 by Foster (2006). Sites 9 through 16 were surveyed for this study. Sites 9 and 11 had historical records of hellbender presence.

Results

Captures/Site Characteristics

We captured 24 total hellbenders in four of the eight sites surveyed. There also was one additional new capture at Foster's (2006) previously surveyed site 2 during a field training session. All animals were captured using the rock turning method except the one captured at site 11, which was caught in a trap. No animals were found in sites 10, 14, 15, or 16 (Figure 2). Of all sites sampled these four sites had the narrowest stream width. Each of these sites had fewer boulders present compared to the four sites where hellbenders were captured. Additionally, comparatively fewer person hours were spent in these sites. More person hours spent in the field corresponded with more hellbenders captured (R^2=0.81) (Figure 3).

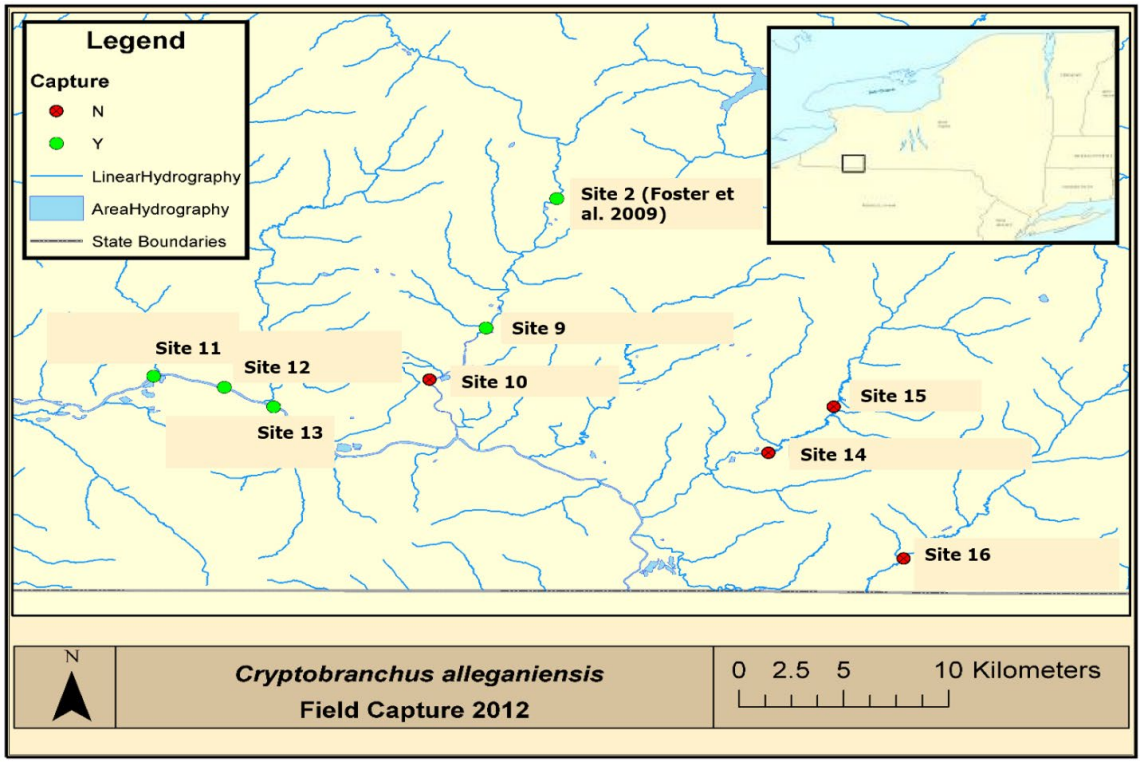


Figure 2: Map of study area. Points in green indicate at least one successful hellbender capture. Hellbenders were not successfully detected in sites shown in red.

Table 1: Descriptive characteristics of sites including width, percent habitable area, and substrate types as well as total person hours spent at each site and total number of hellbenders captured per site. Following the same particle classification as Foster (2006), substrate size was categorized by particle diameter: fines (< 4mm), gravel (4-75mm), cobble (76-300mm), and boulder (> 300mm).

Stream Type	Site #	Width (m)	% Habitable Area	Substrate Description	Person Hours	Number Caught
Tributary	9	35.0	30	Fine bottom, boulders	86.92	7
Tributary	10	13.0	80	Gravel, cobble, boulders	9.67	0
Mainstem	11	67.5	80	Cobble and boulders	84.50	11
Mainstem	12	83.0	10	Fine bottom, boulders	12.50	4
Mainstem	13	82.5	10	Fine bottom, boulders	76 trap nights	1
Tributary	14	16.0	Not determined	Fine bottom, boulders rare	10.50	0
Tributary	15	19.5	Not determined	Fine bottom, boulders rare	13.50	0
Tributary	16	14.5	Not determined	Fine bottom, some cobble, boulders rare	27.83	0

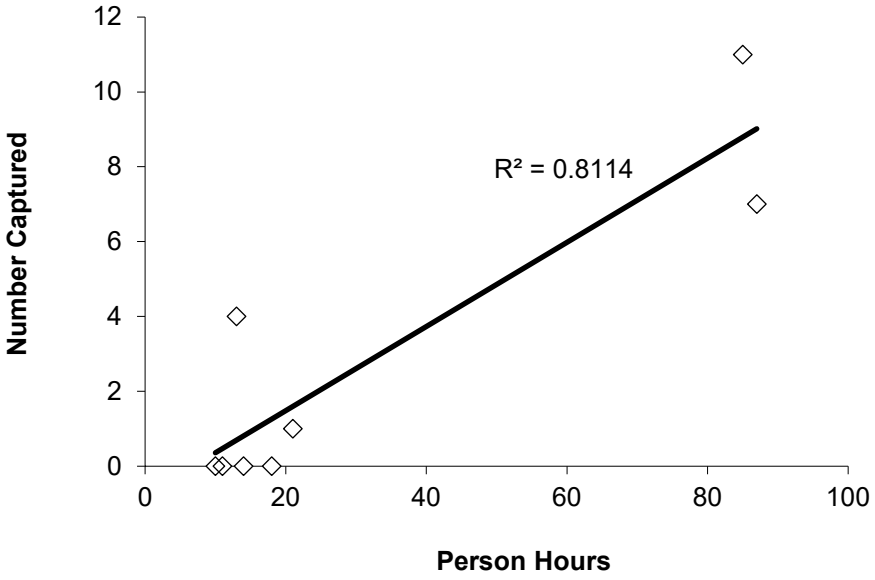


Figure 3: Person hours spent in the field actively searching for hellbenders as a function of total number of hellbenders captured.

Demographics

Out of 24 hellbenders captured, the most common size class was 41 to 50cm (Figure 4). Larger individuals were more commonly captured than smaller individuals. No animals less than 11cm or larger than 60cm were found. Total length ranged from 160mm to 580mm and weights ranged from 30g to 1130g. All animals captured were immature or mature adults (Table 3). There did not appear to be a strong relationship between the size of the hellbender captured and the maximum size of the rock it was under ($R^2 = 0.10$, Figure 5). With the exception of the individual caught in the trap, depth of capture was always ≤1m (Table 2). Rocks ranged from 0% to 95% embedded but 14 out of 23 were 50% embedded or greater (Table 2).

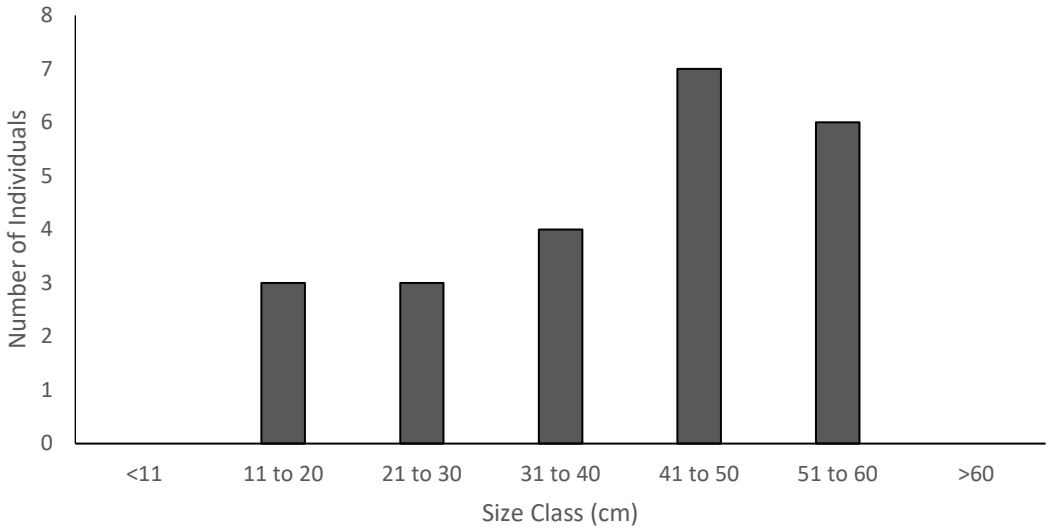


Figure 4: Size class distribution of hellbenders captured in the northern Allegheny watershed from four total sites, summer 2012.

Table 2: Standard length, total length, and weight of each hellbender captured in four sites in the northern Allegheny watershed in summer 2012. Depth, maximum diameter, and embeddedness of hellbender cover rocks are included for all captures except the first animal, which was trapped. For this animal, under the depth category, the first measure is the depth when the trap was set, the second is the depth when the trap was retrieved.

Stream Type	Site #	Std LG (mm)	Total LG (mm)	Weight (g)	Depth (m)	% Embeddedness	Max. rock diameter (m)
Mainstem	11	270	430	490	1.0, 2.0	N/A	N/A
Mainstem	12	165	260	113	0.71	50	1.17
Mainstem	12	353	515	870	0.89	80	1.16
Mainstem	12	360	580	1130	0.65	30	0.83
Mainstem	12	363	560	1080	0.58	10	1.10
Mainstem	12	255	397	500	0.49	95	0.74
Mainstem	12	121	185	56	0.53	70	0.78
Mainstem	12	302	444	650	0.82	30	0.92
Mainstem	12	260	380	410	0.65	40	0.84
Mainstem	12	300	450	590	0.63	50	0.82
Mainstem	12	110	168	40	0.36	80	0.91
Mainstem	12	350	535	1140	0.51	40	0.78
Mainstem	13	280	430	540	0.52	0	1.19
Mainstem	13	332	519	790	0.52	95	1.00
Mainstem	13	235	375	360	0.45	85	1.10
Mainstem	13	342	531	790	0.55	90	0.96
Tributary	2	170	285	178	0.55	Unknown	Unknown
Tributary	9	270	474	Unknown	0.47	50	0.78
Tributary	9	240	385	340	0.47	50	0.78
Tributary	9	334	511	878	0.82	50	1.10
Tributary	9	330	470	708	0.72	50	0.88
Tributary	9	100	160	30	0.25	90	0.66
Tributary	9	275	420	410	0.72	50	0.88
Tributary	9	190	295	130	0.51	0	0.55

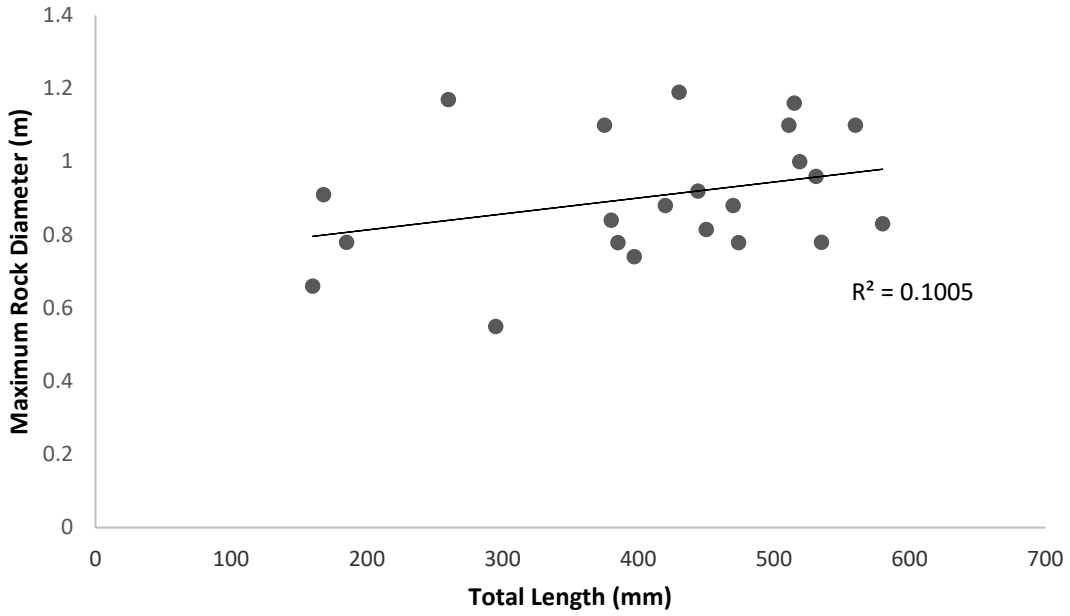


Figure 5: Maximum rock diameter (m) compared to total length (mm) of hellbender from data collected in northern Allegheny River drainage in the summer 2012.

Abnormalities

Of the 24 animals captured, 54% (13) had some type of injury or abnormality (Table 3). Most common injuries included missing toes/limbs and grey splotching on the skin (Table 3). Most injuries/deformities were found in site 12 (Table 4). Four out of the 24 individuals had multiple injuries or deformities. One of the open sores appeared to be a laceration, and this and the lamprey bite were both fresh wounds located on the side of the torso of two different individuals (Figure 6A, B, E). Open sores also were seen on the bottom of both hind feet of one individual (Figure 6C, D). All injuries classified as scars or bite marks had completely healed and were evidence of past injury (Figure 7). Scars were located on either the head (Figure 7A, C, D) or the tail (Figure 7B). Only one abnormal growth was observed and it was located on the foot of an adult (Figure 7E). Another individual had an abnormally shaped tail (Figure 7F). One individual had open sores where toes were missing suggesting it was not born without toes but rather had

recently lost them; these were only classified as missing toes (not included in open sore category) (Figure 8D). We also found grey to blue sections of skin on four different individuals, including one small immature adult hellbender and three mature adults (Figure 9). In the three mature adults, the discoloration was localized; all four feet in one individual (Figure 9C), the dorsal region of the tail in another (Figure 9B), and two small spots in the center of the dorsal region in the last (Figure 9E). The immature adult had extensive discoloration in splotches across the entire dorsal side of the body (Figure 9A). Eleven animals did not appear to have any past injury, open sore, or deformity (Table 3).

Table 3: Total number of hellbenders caught with different types of deformities in the summer or 2012 in the northern Allegheny River drainage. Sum of all individuals does not equal total caught (24) because some individuals had multiple injuries.

Deformity/Injury	Number
Open sores	3
Lamprey bite	1
Scars/bite marks	3
Black marking	3
Abnormal Growth	1
Abnormal tail	2
Missing toes/limbs	4
Grey splotching	4
No deformity/injury	11

Table 4: Total number of injuries/deformities and total number of individuals with injuries/deformities at each site.

Site #	Total Number of Injuries/Deformities	Number Individuals
9	4	3
11	4	1
12	10	8
13	1	1

Figure 6: Lamprey bite and open sores on hellbenders captured in the Allegheny River watershed, summer 2012. A, B. Lamprey bite. C, D. Open sores on hind feet. E. Open laceration on lateral portion of body. Animals were arbitrarily labeled with a number (bottom right) so multiple injuries on the same individual could be displayed in different figures (Figures 6-9).

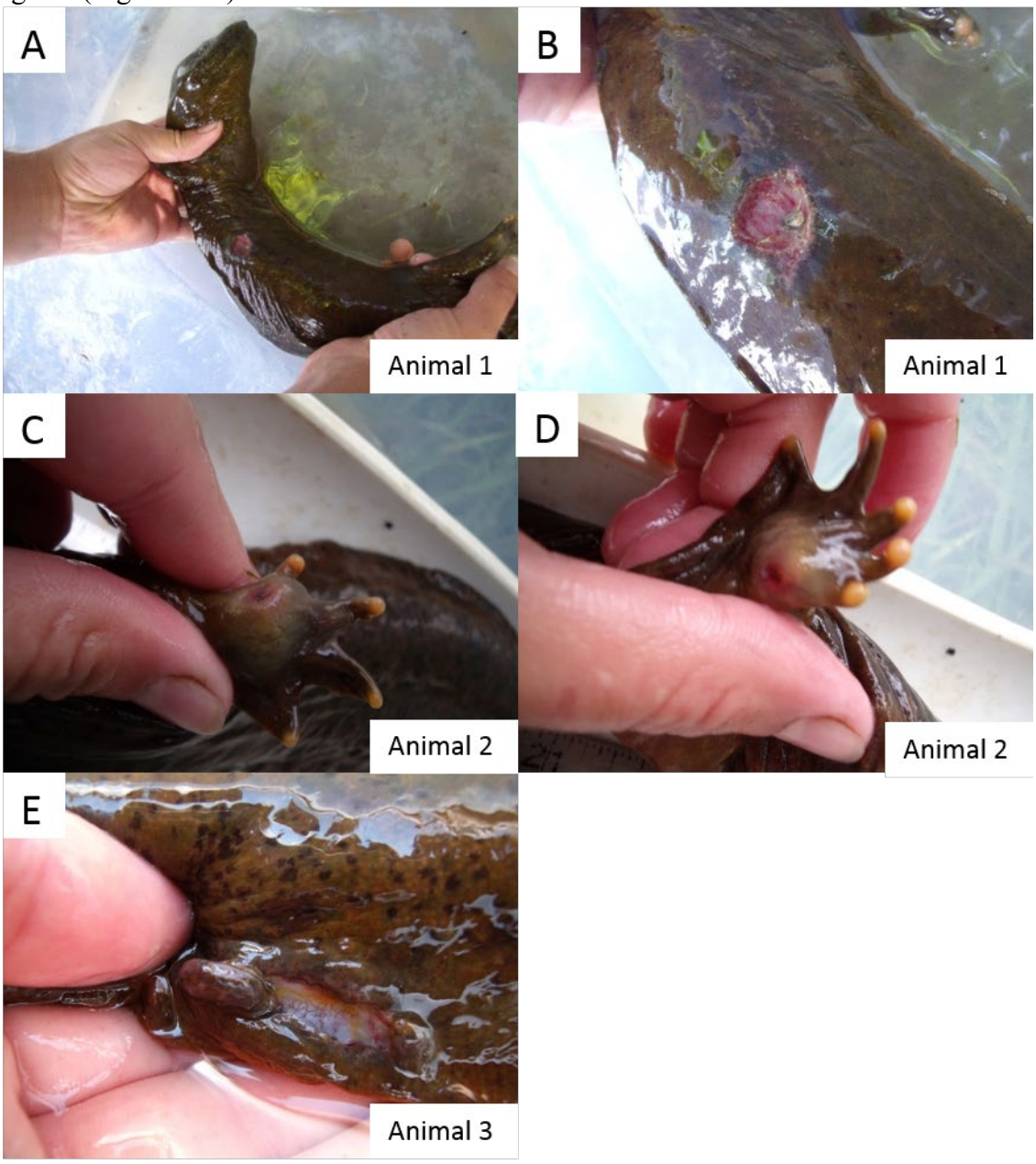


Figure 7: Scars and abnormal growths on hellbenders captured in the Allegheny River watershed, summer 2012. A. Dark bite mark shaped scar on head. B. Light colored scar on end of tail. C, D. Dark black marks on top of head. E. Hard growth on hind foot. F. Abnormal jagged dorsal edge of tail. Animals were arbitrarily labeled with a number (bottom right) so multiple injuries on the same individual could be displayed in different figures (Figures 6-9).

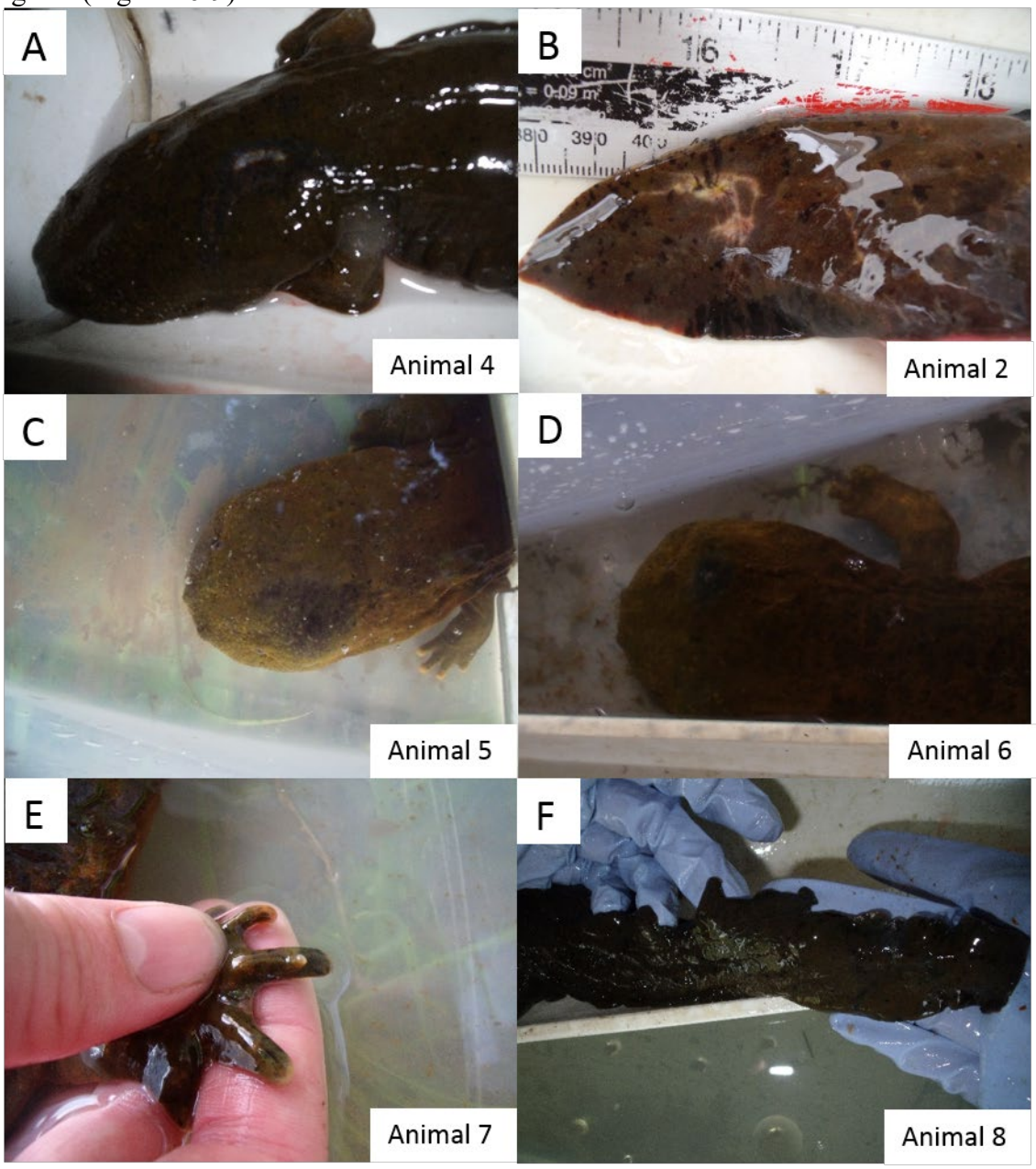


Figure 8: Missing limbs/toes on hellbenders captured in the Allegheny River watershed, summer 2012. A. Missing toe on hind foot. B. Stump foot with under-developed toes. C. Short, flat, swollen toes on hind foot. D. Missing toe resulting in an open sore on front foot. Animals were arbitrarily labeled with a number (bottom right) so multiple injuries on the same individual could be displayed in different figures (Figures 6-9).

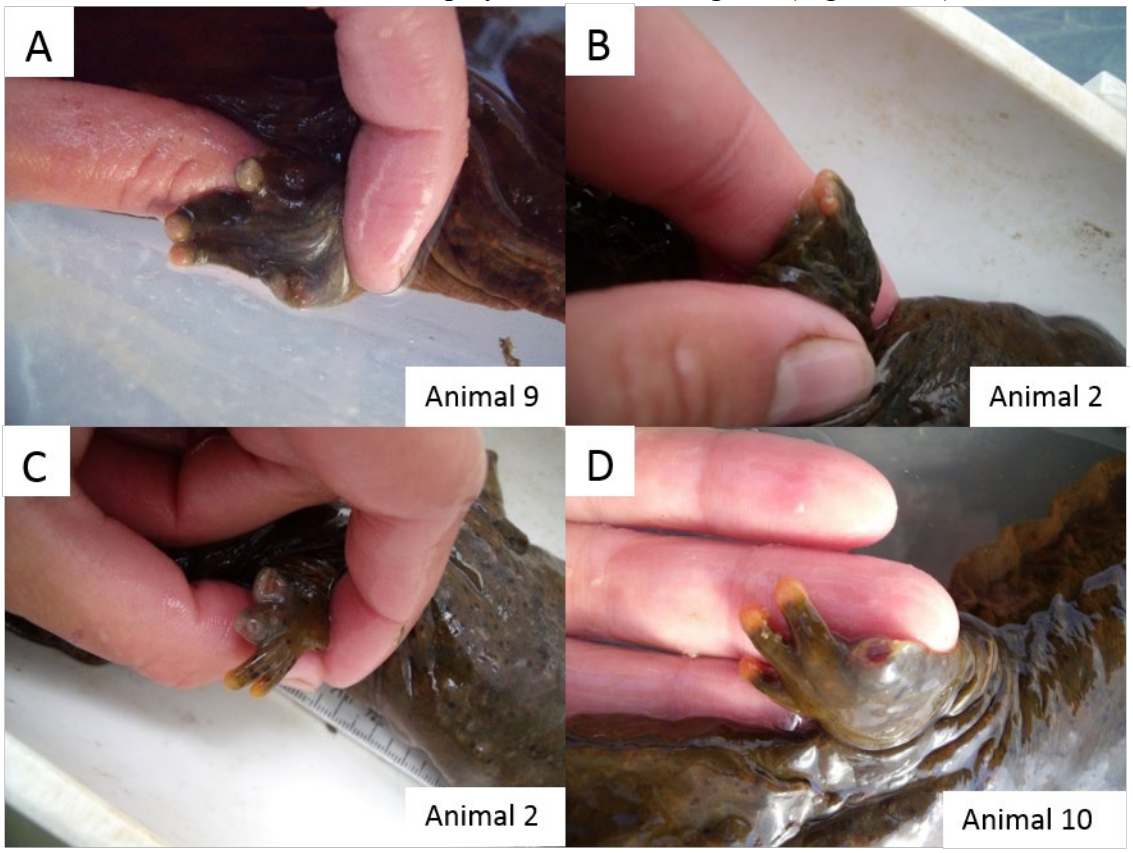
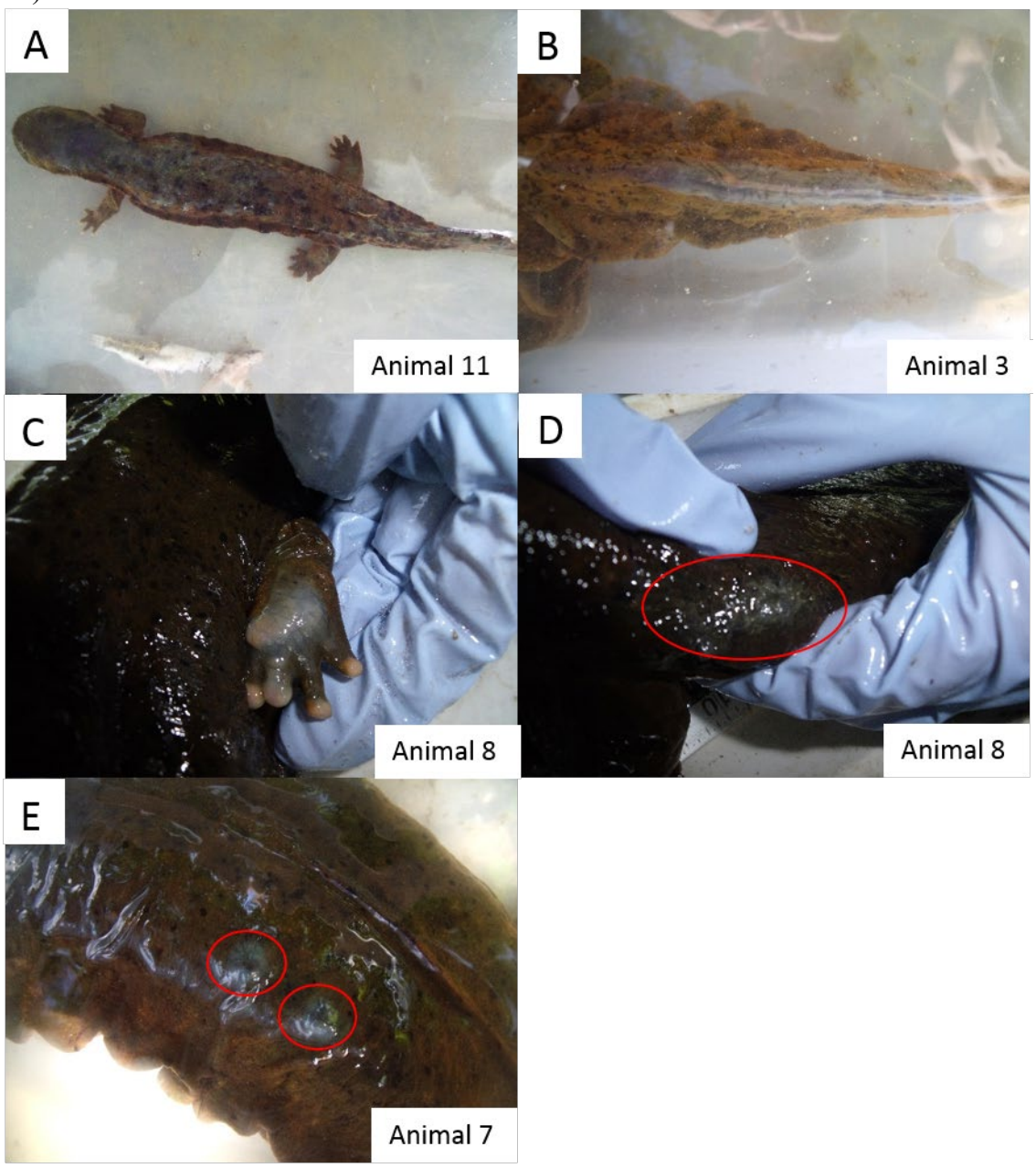


Figure 9: Greyish-blue patches on hellbenders captured in the Allegheny River watershed, summer 2012. A. Splotching across entire dorsal region of immature adult. B. Dorsal section of tail on mature adult. C. Bottom of foot on mature adult. D. Patch on dorsal section behind front foot on mature adult. E. Two spots in the middle of the dorsal section on mature adult. Animals were arbitrarily labeled with a number (bottom right) so multiple injuries on the same individual could be displayed in different figures (Figures 6-9).



Discussion

Captures/Site Characteristics

We found hellbenders in half of the sites we surveyed in the Allegheny drainage. We found more animals in the two historical sites than were incidentally found according to the historical record (NYS DEC Natural Heritage Program, Ken Roblee pers. comm.). From this we can conclude that there are potentially viable populations in these sites. We found two additional sites with hellbender presence in the watershed and these can be monitored in the future.

Moore et al. (2010) posit that from a management perspective, population estimations need to be economically feasible. Yet many amphibians are cryptic species and thus have low detectability making accurate estimations much more difficult and expensive (Wheeler et al., 2003; Bell et al., 2004). Simple detection methods should still be considered for estimating population size (Moore et al., 2010). One such methods is catch-per-unit-effort. In general, more person-hours spent searching for hellbenders in the stream yielded more captures; however, this trend was based on few data points. Data relating to detectability is currently lacking for many cryptic amphibian species such as the hellbender (Wheeler et al., 2003; Moore et al., 2010). Therefore, these results can be compiled with other survey data to determine an optimal number of person-hours to spend per site for detecting hellbenders.

The single hellbender we caught in site 11 by trapping was found in the trap after a large storm event. Foster (pers. comm.) found that hellbenders were often caught in traps after storm events. Unfortunately, due to the rise in water levels and the potential for more serious storms we had to remove traps two nights early (resulting in only 76 trap

nights). The large increase in discharge could have potentially washed traps downstream where they could not be located and thus be a danger to hellbenders trapped inside. It would be useful to optimize a technique to better secure traps in the stream since there may be a relationship between storm events and trap success. Additionally, it would be beneficial to trap this site again in the future to determine if there are more individuals present in this site.

We believed that the incorporation of a seine net into our rock turning methodology improved capture efficiency. It was especially useful in site 9, which was relatively deep and the water was always turbid even during the lowest flow of the season. When using the seine net we were often able to locate escaping hellbenders that we could not see due to poor conditions. Prior to employing the seine netting technique, these animals most likely would have escaped unnoticed. In one instance when using the seine net, a hellbender swam to the surface of the water inside the net making it visible and easy to capture. While the technique seemed very useful it did not ensure we would capture every individual. In three instances, hellbenders were able to escape the seine net in site 13 when conditions were clear and with good visibility. In each case the animals were seen inside the net before it escaped, and only one of these was recovered outside of the net. Presumably the animals were able to find an opening between the stream floor and the bottom of the net. Therefore, when this technique is used it is imperative to ensure the net is flush with the bottom of the stream prior to lifting the rock of interest.

The four sites where no hellbenders were located had the narrowest width of all sites surveyed (Figure 2), which could indicate these sites run dry at certain times and are therefore not suitable for hellbenders. In addition, these sites seemed to lack large

boulders, especially in comparison to the sites where hellbenders were found. Sites 14, 15, and 16 in particular were mostly fine substrate bottom and large boulders were rare. Mudpuppies (*Necturus proteus*) were found in all of these sites, and they tended to be under the few large boulders we found. As a result of the lack of suitable habitat, we spent comparatively less time surveying these sites and tended to survey a much longer reach compared to sites with hellbenders. Still, hellbenders could be present in these sites where we did not detect them, potentially in deeper pools were rock turning was more difficult. Foster (2006) found that it may take several visits to a site to locate hellbenders. It took four visits over a two year span to locate any individuals in one particular site in the Allegheny drainage of New York (Foster, pers. comm.). Additionally, sites 14-16 were surveyed prior to the incorporation of the seine net in our techniques and thus, hellbenders could have escaped capture more easily, especially in poor conditions. Site 10 had a large quantity of cobble and boulders and relatively deep water. We were able to catch two mudpuppies (*Necturus proteus*) in this site but the high density of rock made it nearly impossible to use the seine net technique as we could not get the net flush with the bottom of the stream. However, due to the difficult conditions we spent relatively little time rock turning as we believe trapping would be a better method for capturing hellbenders at this site.

Demographics and Abnormalities

All of the individuals we captured in this study were immature or mature adults. This is congruent with other studies that have found a bias towards captures of individuals in larger size classes (Bothner and Gottlieb, 1991; Blais, 1996; Wheeler et al., 2003;

Humphries and Pauley, 2005). Yet, we did not perform extensive bank searches to locate juveniles. Foster et al. (2008) found that bank searches were the best capture technique for juveniles in the Allegheny drainage in New York. In an impromptu search in site 12 of non-typical bank habitat with pieces of shattered tiles, we captured two small individuals. This result merits future bank searches at this site to search for juvenile or larval hellbenders.

There does not appear to be a strong relationship between the size of the hellbender and the maximum diameter of the rock it is found under. This is surprising given that we would expect that larger individuals need larger rocks to remain concealed. In several cases we found smaller individuals under relatively large rocks. These rocks tended to be thicker and typically were not completely flat on the bottom. As a result, the size of the cavity under the rock was not equal to the size of the rock itself.

Our results suggest a higher prevalence of injuries/deformities than determined in previous hellbender deformity studies. Wheeler and McCallum (2002) compiled data from 12 years of study and found only 8% (17 of 215) of Ozark hellbenders had physical abnormalities. However, the authors note that injuries and deformities were not the main focus of the research and, therefore, this number could be underestimated. In Ohio, 25% (30 of 121) of eastern hellbenders had some type of abnormality (Pfingsten, 1990). Miller and Miller (2005) found that 41% (29 of 70) of adult Ozark hellbenders had abnormalities. Similarly, Hiler et al. (2005) found 40% (36 of 97) of individuals captured in one drainage in Arkansas had injuries. In the northern Allegheny drainage, Foster (2006) reported that 35.4% (56 of 158) of individuals had an injury or deformity. In all of these studies, missing toes or limbs, and scars indicating previous traumatic injury, were

common. In the current study, we found that 54% (13 of 24) of the individuals captured in the northern Allegheny watershed had injuries or deformities. The only other study that found a higher prevalence of abnormalities was Hiler et al. (2005) with 90% (9 of 10). These high percentages of abnormalities in studies with a small sample size could indicate one of three hypotheses: 1) animals with deformities are the easiest and therefore the first to be captured, 2) the population is facing threats that are related to the deformities and thus the population is at risk, or 3) the habitat in the particular location or stream has an inherent problem (such as high debris) causing injuring or abnormalities.

Our study is the first to find evidence of lamprey parasitism on an adult hellbender and the first study to document blue-grey patches on the skin of hellbenders in New York. Sea lamprey are not known to have invaded the Allegheny drainage to date (NYSDEC, Natural Heritage Program, Ken Roblee, pers. comm.) and there were numerous northern brook lamprey (*Ichthyomyzon fossor*) observed in site 12 where the individual with the bite was captured. This suggests that the native northern brook lamprey may feed on adult hellbenders. Additionally, there has been no documentation of animals having blue to grey discolored patches on or throughout the body.

Fungal infections have been documented on hellbenders (Hiler et al., 2005; Foster, 2006), and in both instances the fungus was textured and white to yellow in color. In addition, both studies only documented one individual and indicated that the fungus had grown opportunistically on an existing wound. With increasing concern for the development of chytrid fungus (*Batrachochytrium dendrobatidis*) in wild hellbender populations, it would be important to determine if these sections of discolored skin are the visual manifestation of the fungus. Chytrid fungus or *Bd* is known to infect the

keratinized epidermis of post-metamorphic amphibians and the mouthparts of larval amphibians (Berger et al. 1998). While there has been no documentation of skin discoloration in adult amphibians infected with *Bd*, discoloration is linked to *Bd* infection in the mouths of larval stage frogs (Smith et al., 2007; Symonds et al., 2007). Souza et al. (2012) tested 97 hellbenders for *Bd* and *Ranavirus* in eastern Tennessee and documented all physical lesions on all individuals captured. They found no correlation with the presence of either disease with any type of lesion (Souza et al. 2012). A study similar to the Tennessee study would be beneficial as it could confirm or refute the role of either disease to the discolored skin seen in four individuals found in the Allegheny drainage.

Conclusions

The results of this study show the value of continued monitoring of hellbender populations throughout the Allegheny River watershed and across the species' range. We were able to confirm the continued presence of hellbenders in two historical sites and found two additional sites that can be included in further monitoring, used as release sites for translocation or head-starting programs, or serve as sites for egg collection for future head-starting. In addition, we documented a wound on an adult hellbender from a native lamprey species and found blue-grey discoloration on a few individuals in the watershed. Both of these findings may indicate new potential threats to individual hellbender health and contribute to further declines of the species when added to other threats affecting these populations.

CHAPTER III: Conservation Genetics of the eastern hellbender in the northern Allegheny River Watershed

Introduction

The hellbender, *Cryptobranchus alleganiensis*, is the only extant species of giant salamander (family Cryptobranchidae) native to North America, and endemic to the eastern United States. Like many species of amphibians worldwide (Stuart et al., 2004), hellbender populations are declining at alarming rates throughout their range (Nickerson and Mays, 1973; Williams et al., 1981; Gates et al., 1985; Bothner and Gottlieb, 1991; Trauth et al., 1992; Wheeler et al., 2003; Bauman and Wilson, 2005; Briggler et al., 2007a; Foster et al., 2009). There are numerous threats potentially contributing to hellbender declines across their range including: habitat degradation and fragmentation (Nickerson and Mays, 1973), disease and pathogens (Mayasich et al., 2003; Briggler et al., 2007b), introduced species (Gall, 2008), scientific collecting, illegal collection for the pet trade (Nickerson and Mays, 1973; Humphries and Pauley, 2005; Nickerson and Briggler, 2007), pollution (Nickerson and Mays, 1973), killing by anglers (Gates et al., 1985), climate change (Mayasich et al., 2003), and hydro-fracking (Greg Lipps pers. comm.).

There are many life history attributes that make this species especially susceptible to decline such as slow growth, low fecundity, delayed maturity (Wheeler et al., 2003) and a relatively short breeding season (Humphries and Pauley, 2000). In addition, they are habitat specialists that require fast-flowing, cool, well-oxygenated streams (Smith, 1907; Hillis and Bellis, 1971; Williams et al., 1981) with an abundance of large rocky substrate and crayfish (Nickerson and Mays, 1973). Additionally, some studies suggest juvenile recruitment is low (Bothner and Gottlieb, 1991; Wheeler et al., 2003; Humphries and

Pauley, 2005; Foster et al., 2009; Burgmeier et al., 2011), which is a special concern since models suggest that recovery of this species is dependent on the survival of juveniles to reproduction age (Foster, pers. comm.). Trenham and Marsh (2002) suggest that in cases where habitat fragmentation is extensive, human-mediated releases may be necessary for amphibian species as recolonization of extirpated sites is unlikely to occur naturally.

In the past few decades, relocation, repatriation, and translocation (RRT) programs have been increasing in popularity as a management tool for threatened and endangered amphibian species (Dodd and Siegel, 1991; Germano and Bishop, 2008). As the exact cause of decline is often hard to determine in every portion of the hellbenders range, and given the added effects of their natural history which could make population recovery slow or unlikely, head-starting has become the RRT method of choice to supplement declining populations while threats are identified and addressed. Head-starting programs seek to increase recruitment by raising young in captivity until they reach an age or size where predation risk is significantly reduced upon relocation to the wild (Perez-Buitrago et al., 2008). Hellbender head-starting programs have been implemented in West Virginia, Missouri, Ohio, and New York.

RRT programs are not only used to increase total numbers, but also to increase overall genetic diversity and thus bolster the adaptive potential of the species (Leberg, 1990; Russell at el., 1994; Hogbin and Peakall, 1999). Conservation genetics is a useful tool for determining ecological and evolutionary forces acting on wildlife populations both spatially and temporally (Crowhurst et al., 2011). Recently, researchers have sought to determine the genetic consequences of RRT programs post-implementation (Rhodes et

al., 1995; Fitzsimmons et al., 1997; Williams et al., 2000; Mock et al., 2004; Swanson et al., 2006; Hicks et al., 2007). However, it is rare to have an understanding of the potential genetic effects of captive-raised or head-started individuals prior to release to the wild. The New York State Department of Environmental Conservation is raising hellbenders collected from the wild to supplement populations in the northern Allegheny drainage in New York State. This program gave us a unique opportunity to evaluate genetic considerations prior to the release of the head-started individuals. With the addition of a conservation genetics component to RRT or head-starting programs, management decisions can be made to optimize the genetic and overall success of these programs prior to implementation.

Materials and Methods

Sample collection, DNA Extraction and Quantification

A total of 217 tail clips were taken from hellbenders captured in the field between 2004 and 2012. An additional 49 tail clips were taken from captive head-started individuals. Arntzen et al. (1999) demonstrated that tail-clippings do not have a negative effect on amphibian survival or body condition. Additionally, this method was employed by Foster et al. (2008) with no adverse effects observed (Foster, pers. comm.). Samples were collected from fifteen locations grouped into seven sites throughout the northern Allegheny River watershed in New York and Pennsylvania (Figure 1). Collection locations within a tributary were grouped after a preliminary analysis was performed showing that they were genetically similar. Tail samples were collected, placed in ethanol and stored at -80ºC until further processing. Figure 1 shows the number of samples taken from each site. DNA was extracted from all samples using a modified version of the tissue extraction protocol for the DNeasy Blood and Tissue kit™ by Qiagen (Foster, 2006). Quantitation of DNA was then performed either on the BioRad VersaFluor Fluorometer (Foster, 2006) or the Implen P-Class NanoPhotometer™.

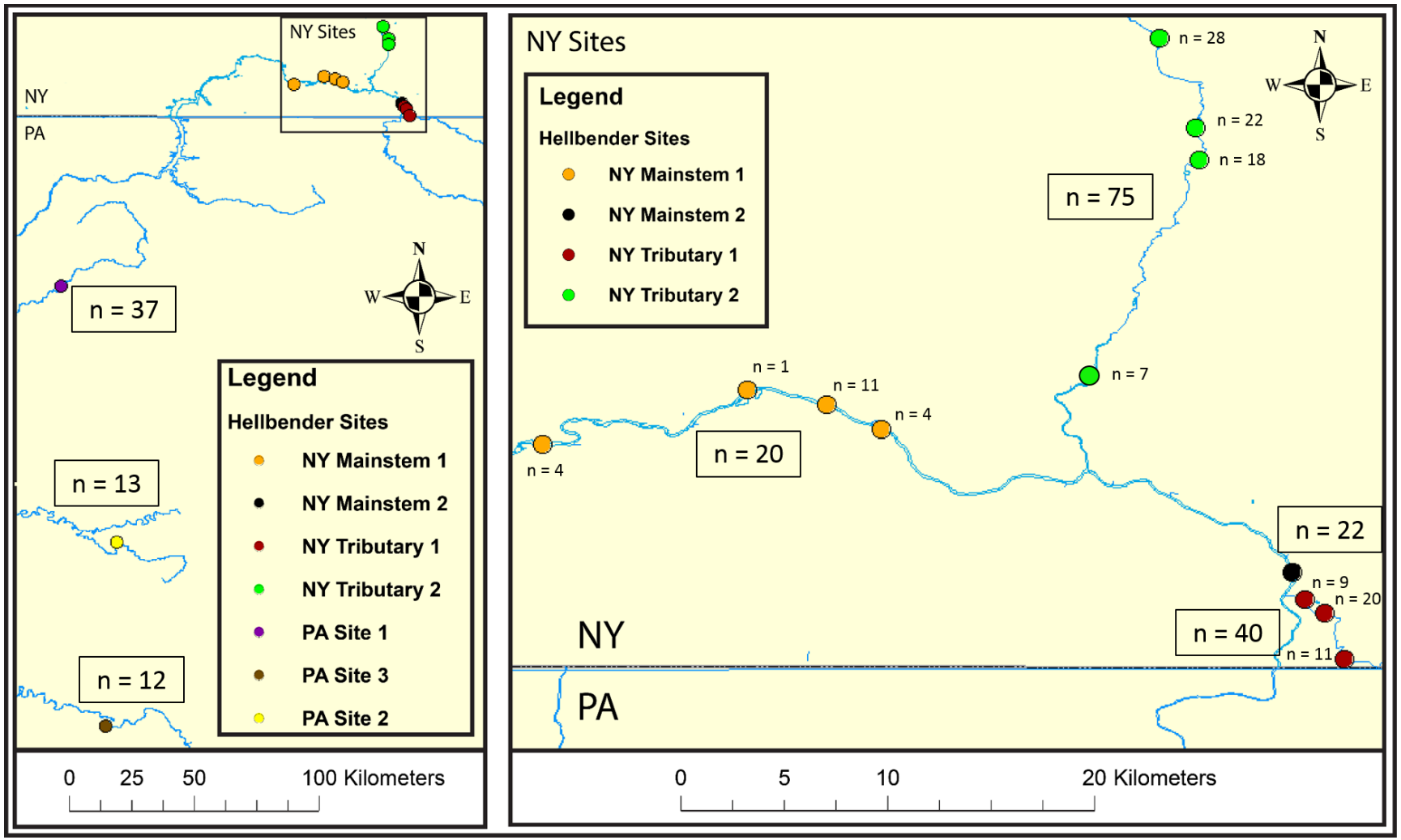


Figure 1: Map of study area in the State of New York and Pennsylvania. Locations are represented by a single colored dot. Locations are grouped into sites of the same color. Sample size for each location (small text) and site (larger text, in box) included.

Microsatellites/Genotyping

Polymerase chain reactions (PCR) were performed to amplify ten microsatellite loci for each individual. For two microsatellite loci (Cral115 and Cral117), primers were developed for Ozark hellbenders by Johnson et al. (2010) (Table 1). For the remaining eight loci (Call26, Call127, Call205, Call232, Call266, Call282, Call341, and Call347), primers were developed for eastern hellbenders by Duvra (summer, 2010, McMillan pers. comm.) and/or Unger et al. (2010) (Table 1). All ten loci contain tetranucleotide repeats, and the allelic range for each locus is presented in Table 2.

PCR reactions were comprised of the following: A PCR buffer (New England Labs or Life Technologies), 0.2mM each of dNTPs, appropriate final concentration of $MgCl_2$ (Table 2), 20µM (Cral115 and Cral117) or 10µM (all other loci) of forward primer, 20µM of reverse primer, 10µM of fluorescent labeled tag, 0.5 U of either standard (New England BioLabs) or Platinum (Invitrogen) *Taq* DNA polymerase (Table 2), and 20ng of template DNA, for a 15µL total reaction. The PCR buffer used depended on the *Taq* DNA polymerase used; New England Labs 10X buffer (1X solution containing 10µM Tris-HCl, 50mM KCl, pH of 8.3) was used with standard *Taq* and Life Technologies 10X buffer (1X solution containing 20mM Tris-HCl (pH8.0), 40mM NaCl, 2mM sodium phosphate, 0.1mM EDTA, and 1mM PTT) was used with Platinum *Taq*.

The tag method originally developed by Schuelke (2000) was used for fluorescent labeling of PCR products. For each locus, an M13 forward (Schuelke, 2000), M13 reverse (Glenn, 2006), or CAG tag (Glenn, 2006) was added to the 5' end of the forward primer for the attachment of a WellRED fluorescent primer (Table 2). WellRED fluorescent dyes are cyanine-based dyes that can be detected on 650nm to 750nm diode

lasers. Since these dyes absorb in the near-infrared region, interference from biological materials is low resulting in high sensitivity detection with background noise (Beckman Coulter, 2002). Thermocycler protocols for Cral115 and Cral117 were as follows: initial denaturation for 5 min at 94ºC, 35 cycles of 94ºC for 30 s, annealing temperature (Table 2) for 30 s, 72ºC for 30 s, and a final elongation at 72ºC for 10 min. For all other loci the thermocycler regime was as follows: initial denaturation for 5 min at 94ºC, 30 cycles of 94ºC for 30 s, annealing temperature (Table 2) for 45 s, 72ºC for 45 s, followed by 8 cycles of 94ºC for 30 s, 53ºC for 45 s, 72ºC for 45 s, and a final elongation at 72ºC for 10 min.

Products were processed on the Beckman Coulter CEQ 8000 or the GeXP, using the appropriate amount of diluted or straight PCR product (Table 2) in 20µL of sample loading solution (SLS) buffer. Loci were co-loaded when possible and dilution schemes of each pair varied depending on dye and signal strength (Table 2). Allele sizes were determined by comparison to a Frag-400 (Beckman Coulter) size standard. Standard settings on the Beckman CEQ 8000/GeXP were used and these are: capillary temperature of 50ºC, denature temperature of 90ºC for 120 seconds, and an inject voltage of 2kv for 30 seconds.

Table 1: Primer sequences with tags (bold) for ten microsatellite loci. The M13 forward tag was used for the following loci: Call26, Call127, Call232, Call266, Call341, Cral115, and Cral117; the M13 reverse tag for Call205, and Call282; and the CAG tag for Call347.

Locus	Primer Sequences including tags (top - forward, bottom - reverse)
Call26	5' - **GAG TTT TCC CAG TCA CGA C**CC CAT AAT GGT AAT AGC TGC AT - 3' 5' - GGA CCC TTG TTC CAG ATT CA - 3'
Call127	5' - **GAG TTT TCC CAG TCA CGA C**GA GGC AGA TGA GAT GCA AGA - 3' 5' - ATG GGT AGT AAC TGC ATG GAA - 3'
Call205	5' - **GGA AAC AGC TAT GAC CAT** TTT GAG CTC TCT TGG CTT ATG - 3' 5' - TGG ACT CCT TCC CTT TCT CC - 3'
Call232	5' - **GAG TTT TCC CAG TCA CGA C**GT ATG CCT GGC ACA TAA CCA - 3' 5' - CCA CCA TAA GAT TCA CAC TGC - 3'
Call266	5' - **GAG TTT TCC CAG TCA CGA C**TC TGC AAG CCA CTA AAT AGC C - 3' 5' - AAC ATT GGG AGG CTG GTA TG - 3'
Call282	5' - **GGA AAC AGC TAT GAC CAT** GGG TGG TTT ATA GGG GCT ACA - 3' 5' - CCC TTG GAG CTA TCT GAG CA - 3'
Call341	5' - **GAG TTT TCC CAG TCA CGA C**GC AAG AAG GTG AGC AAG AGG - 3' 5' - CCA TCT GAA TAT ACC TGC AAT CTG - 3'
Call347	5' - **CAG TCG GGC GTC ATC A**CC AGC AGC AAC CTT ATC TGG - 3' 5' - ACC ATG CAG CCG GTA AGC - 3'
Cral115	5' - **GAG TTT TCC CAG TCA CGA C**TG GGG TTT CAT ACA GGC TTC - 3' 5' - GGTTGAGATTGTGCATGGTG - 3'
Cral117	5' - **GAG TTT TCC CAG TCA CGA C**AT TCC AAG GGG GCT GAA TAC - 3' 5' - CGCCTTGATGTAGCTTTTGG - 3'

Table 2: Microsatellite locus details including basic information on each locus (Forward and reverse primer authors, allelic range in bp); PCR details (final $MgCl_2$ concentration, annealing temperature, and type of *Taq* polymerase used); CEQ/GeXP details (type of fluorescent tag used, fluorescence color, locus paired with in co-load for CEQ or GeXP, SLS dilution scheme, and amount of final PCR product loaded on CEQ/GeXP).

Locus	Forward Primer (Author)	Reverse Primer (Author)	Allelic Range in bp (# total alleles)	Annealing Temp. °C	Final $MgCl_2$ Conc. (mM)	*Taq* Type	Tag	WellRED Dye	Co-Load With	SLS Dilution	Amount to CEQ/GeXP
Call26	Duvra	Duvra	201-329 (20)	60	4.5	Standard	M13 For	D4	Call282	1:1	5uL
Call127	Duvra	Duvra	162-198 (10)	59	4.5	Standard	M13 For	D3	Call347	None	5uL
Call205	Unger	Unger	180-216 (10)	68	3.5	Standard	M13 Rev	D3	None	None	8uL
Call232	Duvra	Unger	171-233 (14)	64	4.0	Standard	M13 For	D4	None	1:1	5uL
Call266	Unger	Duvra	227-267 (12)	65	4.0	Platinum	M13 For	D3	Call341	None	8uL
Call282	Duvra	Duvra	196-236 (11)	65	4.5	Standard	M13 Rev	D3	Call26	None	5uL
Call341	Duvra	Unger	246-282 (10)	65	4.5	Platinum	M13 For	D4	Call266	1:1	5uL
Call347	Duvra	Unger	184-304 (17)	66	4.5	Standard	CAG	D4	Call127	1:1	5uL
Cral115	Johnson	Johnson	139-151 (3)	60	1.5	Standard	M13 For	D4	Cral117	None	5uL
Cral117	Johnson	Johnson	156-188 (9)	50	1.5	Platinum	M13 For	D3	Cral115	None	5uL

Analysis

GENEPOP 4.1 (Raymond and Rousset, 1995; Rousset, 2008) was used to test for linkage disequilibrium between all loci and to test for deviations from Hardy-Weinberg equilibrium using the Fisher's exact test for all loci in all sites. Both these tests (linkage and H-W) use a Markov Chain approximation and default parameters were used (dememorization number = 1000, batches = 100, iterations per batch = 1000). A Bonferroni correction was used to maintain a significance value of 0.05 since numerous comparisons were made. Allele frequencies, genotype frequencies, and expected and observed heterozygosities also were calculated using GENEPOP.

F_{ST} values by locus were calculated using FSTAT (Weir and Cockerham, 1984; Goudet, 1995). F_{ST} determines the degree of difference between populations and F_{ST} values range from 0 to 1 where 0 indicates the two populations are genetically identical and 1 indicates populations are genetically distinct. R_{ST} is a measure similar to F_{ST} (also a scale of 0 to 1) that was developed specifically for microsatellite data because it is based on a step-wise mutation model as opposed to the infinite alleles model. Both pairwise F_{ST} (Weir and Cockerham, 1984) and pairwise R_{ST} (Michalakis and Excoffier, 1996) values were calculated using GENEPOP for all population pairs. We used FSTAT to perform pairwise tests of differentiation between all populations (Goudet, 1995). This test randomizes multi-locus genotypes between two populations for all pairs of populations and uses the G-statistic over all loci to determine a p-value for differentiation. In addition, this test automatically incorporates a Bonferroni correction since comparisons are made between all population pairs.

We used STRUCTURE (Pritchard et al., 2000) to determine if distinct natural genetic groupings occurred within the drainage. The STRUCTURE program is a type of modeling software that uses a Bayesian approach to clustering individuals into groups based on genotype data (unlinked markers). When running STRUCTURE, 10,000 iterations were performed for the program burnin followed by 10,000 iterations for each K (populations) from 1 to 12 assuming the admixture model and correlated allele frequencies. STRUCTURE HARVESTER was used to evaluate STRUCTURE results with the ΔK statistic (Evanno et al., 2005). We performed an AMOVA using GENEALEX6.5 (999 standard permutations) to analyze genetic variation across all loci at three hierarchical levels: within individuals, within populations, and among populations. To perform an AMOVA, the program determines an "observed" F_{ST} based on the genetic groupings provided by the user. Then, the genotypes are randomized and a new F_{ST} is calculated for a given number of permutations. If the observed F_{ST} is greater than the permuted value 95% of the time, there is a significant difference (at the 5% level, $p \leq 0.05$). If there is no difference between the observed F_{ST} and the permuted F_{ST} values, the population is one single panmictic population (genetically similar).

Allelic richness was measured by counting the total alleles that occurred in a given site at each locus, and averaging that number across loci. Private allelic richness was determined by counting the total number of unique alleles in each site and these values also were averaged across loci. To account for variability in sample size, rarefaction was performed with HP-RARE 1.0 (Kalinowski, 2005) to determine allelic richness and private allelic richness for a sample size of 12 individuals (minimum number collected at a site). To determine if sites differed significantly in allelic richness, we performed an ANOVA

using R software. Each locus was used as a replicate for the ANOVA and given the large variation in total number of alleles at each locus, proportions were used to correct for heterogeneous variance within replicates. To do this, the rarified allelic richness for each population was divided by the maximum allelic richness at that locus, so all loci were converted to a value between 0 and 1. Tukey's HSD was used for *post hoc* analysis to determine which sites were significantly different. Unbiased gene diversity between individuals was determined for all loci in all populations using FSTAT. To test for inbreeding, F_{IS} values were calculated across all loci for all populations using GENEPOP. F_{IS} values range from -1 to +1 where values closer to -1 indicates all homozygous individuals and a value of +1 indicates all heterozygotes.

We tested all sites for recent genetic bottlenecks using the program BOTTLENECK (Cornuet and Luikart, 1997). Three of four tests (sign test, Wilcoxon test, and mode shift test) in the program were run for each population assuming each of the three mutation models; infinite allele model (IAM), stepwise mutation model (SMM), and the two-phase model (TPM). Distance (D_A; Nei et al., 1983) was determined for all pairs of populations using POPULATIONS1.2.32. We used these data to construct a phylogenetic tree using the neighbor-joining method in POPULATIONS1.2.32, which was then illustrated in FIGTREE1.4.

For the 49 head-started individuals, we performed the following analysis. Allelic richness was calculated as well as allelic richness using the minimum number of individuals caught at a site (12 individuals) was determined using HP-RARE1.0 for comparison to wild populations. We used GENECLASS2.0 (Piry et al., 2004) to calculate individual assignment probabilities for all head-started individuals to wild populations. This program determines the probability that an individual belongs to (or originates from)

each of the reference populations for all individuals included in the analysis. The output includes the top four populations of possible origin (deemed "ranks") with the associated probability of assignment to each, with rank 1 having the highest probability of assignment and decreasing in probability with each subsequent rank. We set an assignment threshold of $p<0.05$ under a Bayesian framework (Rannala and Mountain, 1997) and used a Monte-Carlo resampling probability computation (Paetkau et al., 2004) using 1000 simulated individuals with an alpha of 0.01. This program was used in a non-traditional method in an attempt to determine if animals in the head-started cohort were genetically similar to other sites in the study, aside from the site of origin.

Results

Wild Populations

We found no evidence of linkage disequilibrium between any microsatellite loci used in this study and none of the loci violated Hardy-Weinberg assumptions in any population. Allele frequencies for each locus are displayed by population in Figure 2. Average expected heterozygosities ranged from 0.76 to 0.80 and average observed heterozygosities ranged from 0.75 to 0.83 (Table 3).

F_{ST} values by locus ranged from -0.012 (Cral115) to 0.018 (Call347). Pairwise F_{ST} and pairwise R_{ST} values were highest between PA Site 3 and all other sites (Table 4). Comparisons between NY Tributary 2 and other sites yielded moderate-level F_{ST} and R_{ST} values and the lowest values occurred in NY Mainstem sites and NY Tributary 1 (Table 4). Results from FSTAT pairwise tests of differentiation showed significant differentiation

between: NY Tributary 1 and PA Site 1; PA Site 3 and NY Mainstem 1, NY Tributary 1, NY Tributary 2; and NY Tributary 2 and all other sites sampled in this study (Table 5). NY Tributary 2 had the third lowest overall allelic richness, and the lowest allelic richness after rarefaction (Table 3). Private alleles occurred in all populations' sampled (range 2 to 5, after rarefaction 0.2 to 0.5) (Table 5). The results of the ANOVA comparing allelic richness between sites was significant ($F_{7,72} = 4.623$, $p = 0.0002$). Tukey's HSD indicated that the head-started cohort had significantly lower allelic richness than all wild sites except NY Tributary 2 (Figure 3). However, the allelic richness in NY Tributary 2 was not significantly different from any of the other sites in the study.

Using the ΔK statistic, STUCTURE results did not indicate more than one natural genetic grouping in the northern Allegheny watershed of New York and Pennsylvania. The AMOVA results show that 96% of the genetic variation is within individuals, 3% among individuals and 1% among populations (Table 8). AMOVA results were significant at all hierarchical levels: among populations (p=0.001), among individuals (p=0.003), and within individuals (p=0.002) (Table 8).

Locus Cral115 had the lowest gene diversity (0.315-0.421) in all sites sampled (Table 6). With the exception of Call341 at NY Mainstem 2 (0.688) all other gene diversity values were greater than or equal to 0.75 (Table 6). Most F_{IS} values were less than 0.15, with the exceptions being locus Call347 (0.153) and Cral 115 (0.593) at PA Site 3 and Call347 (0.204) at PA Site 3 (Table 7). Average F_{IS} across all loci ranged from -0.042 to 0.051 (Table 7).

Table 3: Allelic richness (A), allelic richness after rarefaction (Ar) using HP-RARE software, private allelic richness (Ap), private allelic richness (Arp) after rarefaction using HP-RARE software, expected heterozygosity, and observed heterozygosity for all sites sampled (sample size displayed next to site name) at each locus. Averages of each measure are included in the last row.

	NY Mainstem 1 (n=20)				NY Mainstem 2 (n=22)				NY Tributary 1 (n=40)				NY Tributary 2 (n=75)			
Locus	A (Ap)	Ar (Arp)	He	Ho	A (Ap)	Ar (Arp)	He	Ho	A (Ap)	Ar (Arp)	He	Ho	A (Ap)	Ar (Arp)	He	Ho
Call26	12(1)	10.1(1.0)	0.89	0.90	13(1)	11.5(1.2)	0.93	0.95	14(1)	10.5(0.5)	0.91	0.88	12(0)	8.6(0.0)	0.87	0.87
Call127	10(0)	8.7(0.1)	0.84	0.75	8(0)	7.2(0.1)	0.84	0.91	10(0)	7.5(0.0)	0.84	0.93	9(0)	7.3(0.0)	0.83	0.85
Call205	7(0)	6.5(0.0)	0.82	0.78	9(0)	7.6(0.5)	0.80	0.78	10(0)	7.7(0.1)	0.86	0.86	8(0)	6.6(0.0)	0.81	0.75
Call232	9(0)	7.5(0.0)	0.78	0.80	10(0)	8.6(0.0)	0.86	0.85	11(0)	8.3(0.0)	0.82	0.87	11(1)	7.5(0.2)	0.81	0.79
Call266	10(1)	8.1(0.6)	0.82	0.75	7(0)	6.5(0.0)	0.85	0.95	9(0)	7.1(0.0)	0.84	0.95	7(0)	6.2(0.0)	0.81	0.84
Call282	10(1)	8.4(0.6)	0.87	0.90	9(1)	7.4(0.8)	0.82	0.91	9(0)	7.4(0.0)	0.85	0.90	8(0)	6.8(0.0)	0.82	0.84
Call341	7(0)	6.0(0.0)	0.79	0.80	8(1)	6.2(0.6)	0.69	0.75	7(1)	5.4(0.3)	0.76	0.71	7(0)	5.8(0.0)	0.77	0.80
Call347	10(0)	8.9(0.0)	0.88	0.79	12(1)	9.9(1.3)	0.89	0.86	12(1)	9.0(0.4)	0.88	0.95	11(0)	8.3(0.1)	0.85	0.90
Cral115	3(0)	2.8(0.1)	0.41	0.50	3(0)	2.6(0.0)	0.42	0.45	3(0)	2.5(0.0)	0.38	0.38	2(0)	2.0(0.0)	0.38	0.35
Cral117	7(0)	6.6(0.2)	0.80	0.85	7(0)	6.7(0.1)	0.84	0.86	8(0)	6.2(0.0)	0.81	0.80	8(0)	6.8(0.0)	0.83	0.85
Avg	8.5(0.3)	7.4(0.27)	0.79	0.78	8.6(0.4)	7.4(0.46)	0.79	0.83	9.3(0.3)	7.2 (0.14)	0.80	0.82	8.3(0.1)	6.6(0.04)	0.78	0.78

	PA Site 1 (n=37)				PA Site 2 (n=13)				PA Site 3 (n=12)			
Locus	A (Ap)	Ar (Arp)	He	Ho	A (Ap)	Ar (Arp)	He	Ho	A (Ap)	Ar (Arp)	He	Ho
Call26	13(1)	10.0(0.4)	0.90	0.92	9(0)	8.9(0.0)	0.90	0.92	10(2)	10.0(2.1)	0.90	0.83
Call127	9(0)	7.7(0.0)	0.84	0.81	6(0)	5.9(0.0)	0.75	0.69	8(0)	8.0(0.2)	0.86	1.00
Call205	8(0)	6.3(0.1)	0.79	0.70	8(0)	7.8(0.0)	0.86	0.77	5(0)	5.0(0.0)	0.76	0.92
Call232	11(1)	8.0(0.4)	0.84	0.81	10(0)	10.0(0.1)	0.86	0.92	9(0)	9.0(0.5)	0.86	0.75
Call266	11(2)	8.7(0.7)	0.87	0.76	7(0)	6.8(0.0)	0.84	0.85	7(0)	7.0(0.0)	0.76	0.83
Call282	8(0)	6.4(0.0)	0.77	0.84	8(0)	7.8(0.1)	0.84	0.77	6(0)	6.0(0.0)	0.70	0.75
Call341	7(0)	6.2(0.0)	0.80	0.84	7(0)	6.8(0.0)	0.81	0.69	7(0)	7.0(0.0)	0.82	0.83
Call347	12(1)	9.2(0.7)	0.87	0.83	10(2)	8.8(0.9)	0.90	1.00	8(0)	8.0(0.1)	0.83	0.67
Cral115	3(0)	2.3(0.0)	0.32	0.35	2(0)	2.0(0.0)	0.37	0.15	2(0)	2.0(0.0)	0.34	0.42
Cral117	7(0)	6.4(0.1)	0.80	0.79	6(0)	6.0(0.4)	0.78	0.75	5(0)	5.0(0.0)	0.80	0.83
Avg	8.9(0.5)	7.1(0.24)	0.78	0.76	7.3(0.2)	7.09(0.16)	0.79	0.75	6.7(0.2)	6.7(0.29)	0.76	0.78

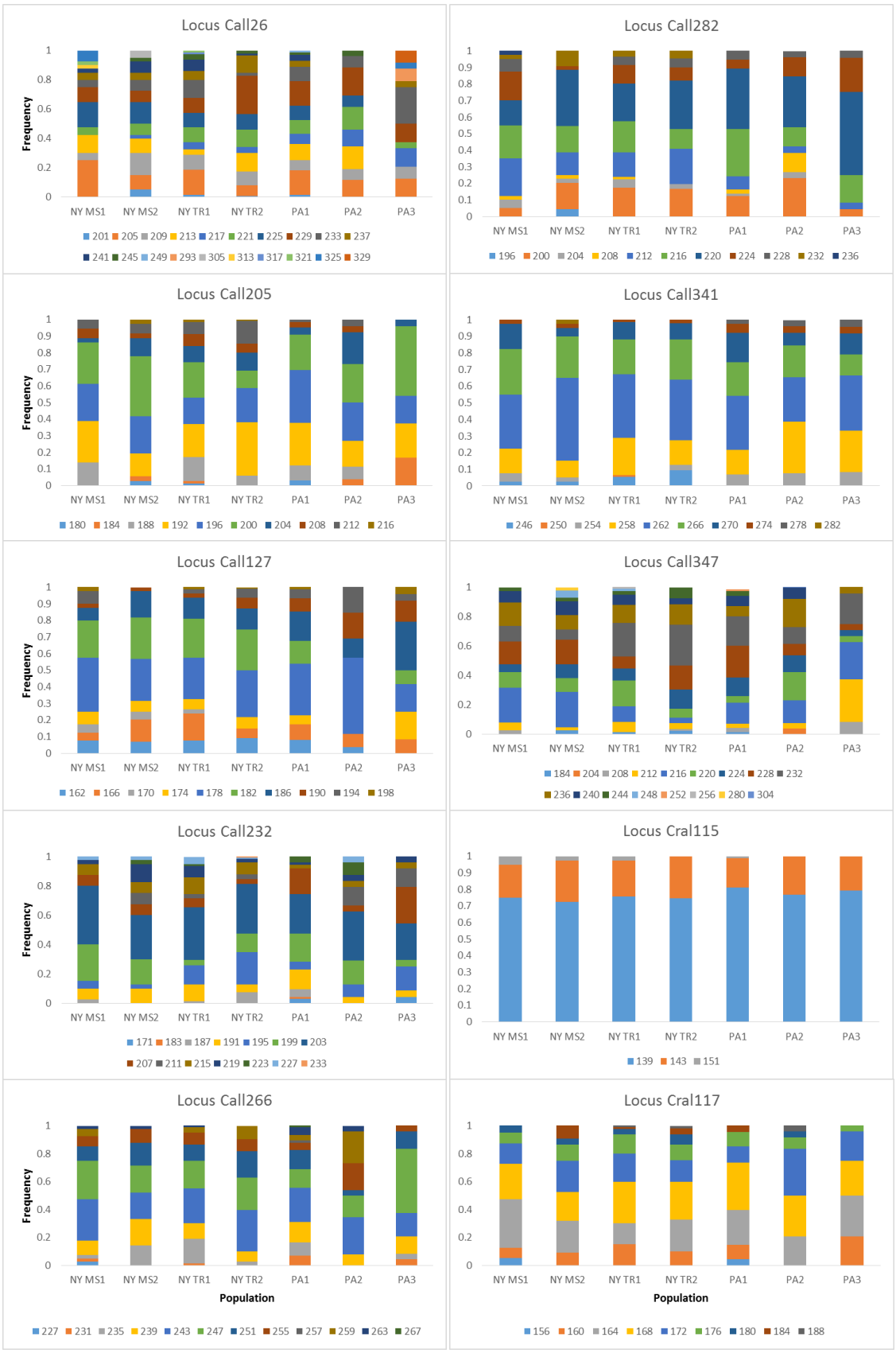


Figure 2: Allele frequencies for all ten microsatellite loci for all sites.

Table 4: Pairwise F_{ST} (below diagonal) and pairwise R_{ST} (above diagonal) results from GENEPOP4.1 for all population (site) pairs.

	NY Mainstem 1	NY Mainstem 2	NY Tributary 1	NY Tributary 2	PA Site 1	PA Site 2	PA Site 3
NY Mainstem 1	–	0.000	0.008	0.043	0.032	0.037	0.005
NY Mainstem 2	0.001	–	-0.017	0.015	0.019	0.009	0.097
NY Tributary 1	0.002	0.000	–	0.014	0.017	0.022	0.122
NY Tributary 2	0.009	0.014	0.007	–	0.011	0.028	0.221
PA Site 1	0.001	0.003	0.006	0.011	–	0.037	0.177
PA Site 2	0.004	0.009	0.006	0.012	0.005	–	0.155
PA Site 3	0.023	0.017	0.019	0.034	0.015	0.021	–

Table 5: P-values from pairwise tests of differentiation based on G-Statistic from the FSTAT program. Values significant ($p \leq 0.05$) after Bonferroni correction denoted by *.

	NY Mainstem 1	NY Mainstem 2	NY Tributary 1	NY Tributary 2	PA Site 1	PA Site 2
NY Mainstem 2	0.1927					
NY Tributary 1	0.3000	0.5405				
NY Tributary 2	0.0024*	0.0024*	0.0024*			
PA Site 1	0.3929	0.0167	0.0024*	0.0024*		
PA Site 2	0.1786	0.0191	0.0238	0.0024*	0.0619	
PA Site 3	0.0095	0.0024*	0.0024*	0.0024*	0.0071	0.0048

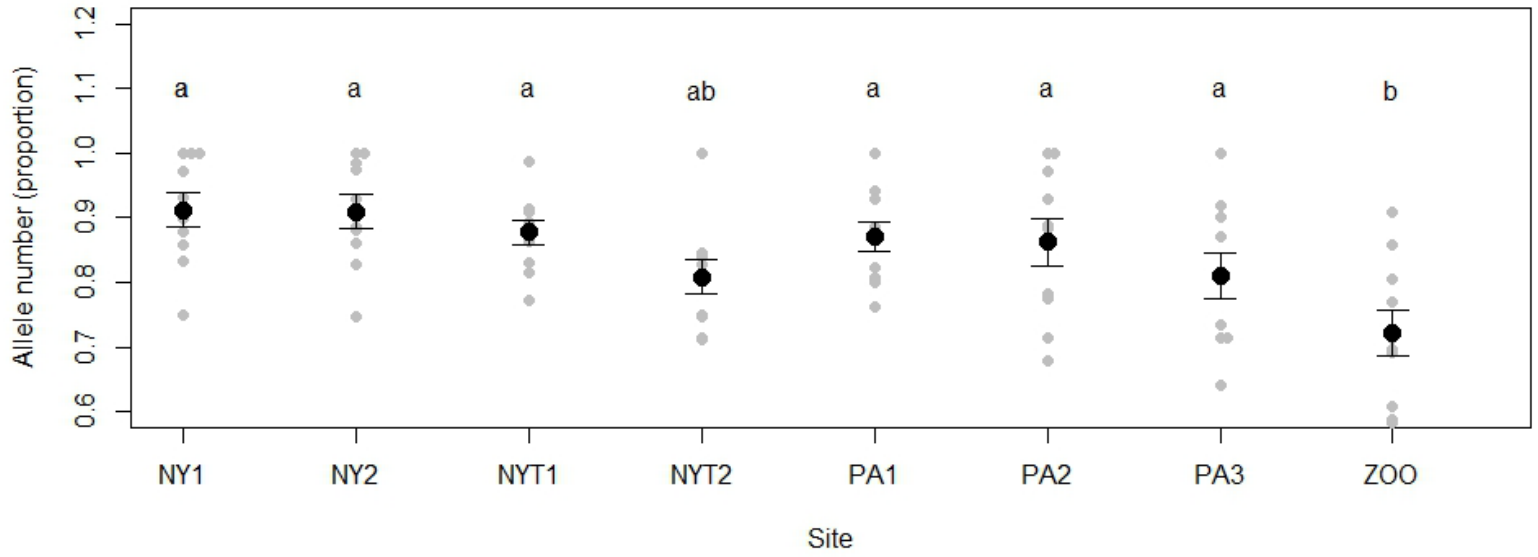


Figure 3: Distribution of allelic richness (in proportion of total alleles) for each site and the head-started cohort. Sites that are significantly different are denoted by different letters above the data points. Each grey dot represents one locus with the average proportional allelic richness and standard error of this mean indicated with a black dot. Site key: NY1 and NY2 = NY Mainstem 1 and 2 respectively; NYT1 and NYT2 = NY Tributary 1 and 2; respectively, PA1, PA2 and PA3 = PA Site 1, 2 and 3; and Zoo = Head-started cohort.

Table 6: FSTAT results of unbiased gene diversity for all loci by site.

	NY Mainstem 1	NY Mainstem 2	NY Tributary 1	NY Tributary 2	PA Site 1	PA Site 2	PA Site 3
Call26	0.886	0.926	0.910	0.865	0.902	0.904	0.905
Call127	0.838	0.837	0.837	0.826	0.838	0.75	0.856
Call205	0.824	0.806	0.861	0.811	0.788	0.859	0.75
Call232	0.775	0.857	0.823	0.808	0.845	0.856	0.864
Call266	0.826	0.851	0.841	0.805	0.874	0.837	0.754
Call282	0.864	0.819	0.852	0.819	0.768	0.843	0.701
Call341	0.789	0.688	0.756	0.771	0.803	0.817	0.818
Call347	0.885	0.892	0.877	0.849	0.875	0.891	0.837
Call115	0.403	0.421	0.385	0.381	0.315	0.378	0.341
Call117	0.795	0.842	0.815	0.825	0.796	0.784	0.795

Table 7: F_{IS} values for all loci in all sites sampled and average F_{IS} across all loci by site.

	NY Mainstem 1	NY Mainstem 2	NY Tributary 1	NY Tributary 2	PA Site 1	PA Site 2	PA Site 3
Call26	-0.016	-0.026	0.039	-0.002	-0.016	-0.021	0.079
Call127	0.105	-0.087	-0.105	-0.023	0.032	0.077	-0.168
Call205	0.056	0.034	0.004	0.075	0.116	0.104	-0.222
Call232	-0.032	0.008	-0.059	0.026	0.04	-0.071	0.132
Call266	0.092	-0.119	-0.129	-0.04	0.134	-0.011	-0.106
Call282	-0.041	-0.11	-0.056	-0.025	-0.091	0.087	-0.07
Call341	-0.013	-0.09	0.061	-0.037	-0.043	0.153	-0.019
Call347	0.107	0.039	-0.078	-0.065	0.053	-0.122	0.204
Cral115	-0.242	-0.069	0	0.09	-0.114	0.593	-0.222
Cral117	-0.07	-0.026	0.018	-0.034	0.002	0.043	-0.048
All loci	0.009	-0.042	-0.033	-0.009	0.021	0.051	-0.028

Table 8: Results of the AMOVA run in GENEALEX6.5 to determine variation among populations, among individuals, and within individuals across all loci for all sites sampled. Significant p-values denoted by *.

Source of Variation	df	Sum of Squares	Est. Var.	Percentage of Variation	Observed value	p-value
Among Populations	6	37.530	0.038	1%	0.009	0.001*
Among Individuals	212	862.630	0.111	3%	0.028	0.003*
Within Individuals	219	842.500	3.847	96%	0.037	0.002*

BOTTLENECK results for determining heterozygosity excess varied depending on model and test. However, all L-shape curves based on the mode shift test were normal for all sites (Table 9). In addition, there was no evidence of significant heterozygosity excess for any site under the step-wise mutation model (Sign test and Wilcoxon test) (Table 9). Under the infinite alleles model, all sites except PA Site 3 showed significant heterozygosity excess in both the sign test and the Wilcoxon test (Table 9). Under the two-phase model, the sign test showed significant heterozygosity excess for NY Tributary 2, PA Site 1, and PA site 2 and the Wilcoxon test showed significant heterozygosity excess for NY Tributary 1, NY Tributary 2, PA Site 1, and PA Site 2 (Table 9).

Table 9: Results from sign test, Wilcoxon test, and mode shift test from the BOTTLENECK program for all sites. Significance values of <0.05 denoted by *, <0.01 by **, and <0.001 by ***.

Site	Sign Test			Wilcoxon Test			MODE SHIFT
	IAM	TPM	SMM	IAM	TPM	SMM	
NY Mainstem 1	*	NS	NS	***	NS	NS	Normal
NY Mainstem 2	*	NS	NS	**	NS	NS	Normal
NY Tributary 1	**	NS	NS	***	**	NS	Normal
NY Tributary 2	**	**	NS	***	***	NS	Normal
PA Site 1	*	*	NS	***	**	NS	Normal
PA Site 2	*	*	NS	***	**	NS	Normal
PA Site 3	NS	NS	NS	**	NS	NS	Normal

Nei et al. (1983) distance measures ranged from 0.046 to 0.166 with the greatest distance values between PA Site 2 and PA Site 3 with all other sites (Table 10). The phylogenetic tree constructed based on D_A calculation defines three groups, and the NY and PA sites were in separate groups based on this analysis (Figure 4).

Table 10: Nei et al. (1983) distance (D_A) measures for all population pairs.

	NY Mainstem 1	NY Mainstem 2	NY Tributary 1	NY Tributary 2	PA Site 1	PA Site 2
NY Mainstem 2	0.094					
NY Tributary 1	0.060	0.055				
NY Tributary 2	0.067	0.084	0.046			
PA Site 1	0.064	0.085	0.064	0.069		
PA Site 2	0.117	0.133	0.103	0.104	0.103	
PA Site 3	0.153	0.159	0.137	0.155	0.123	0.166

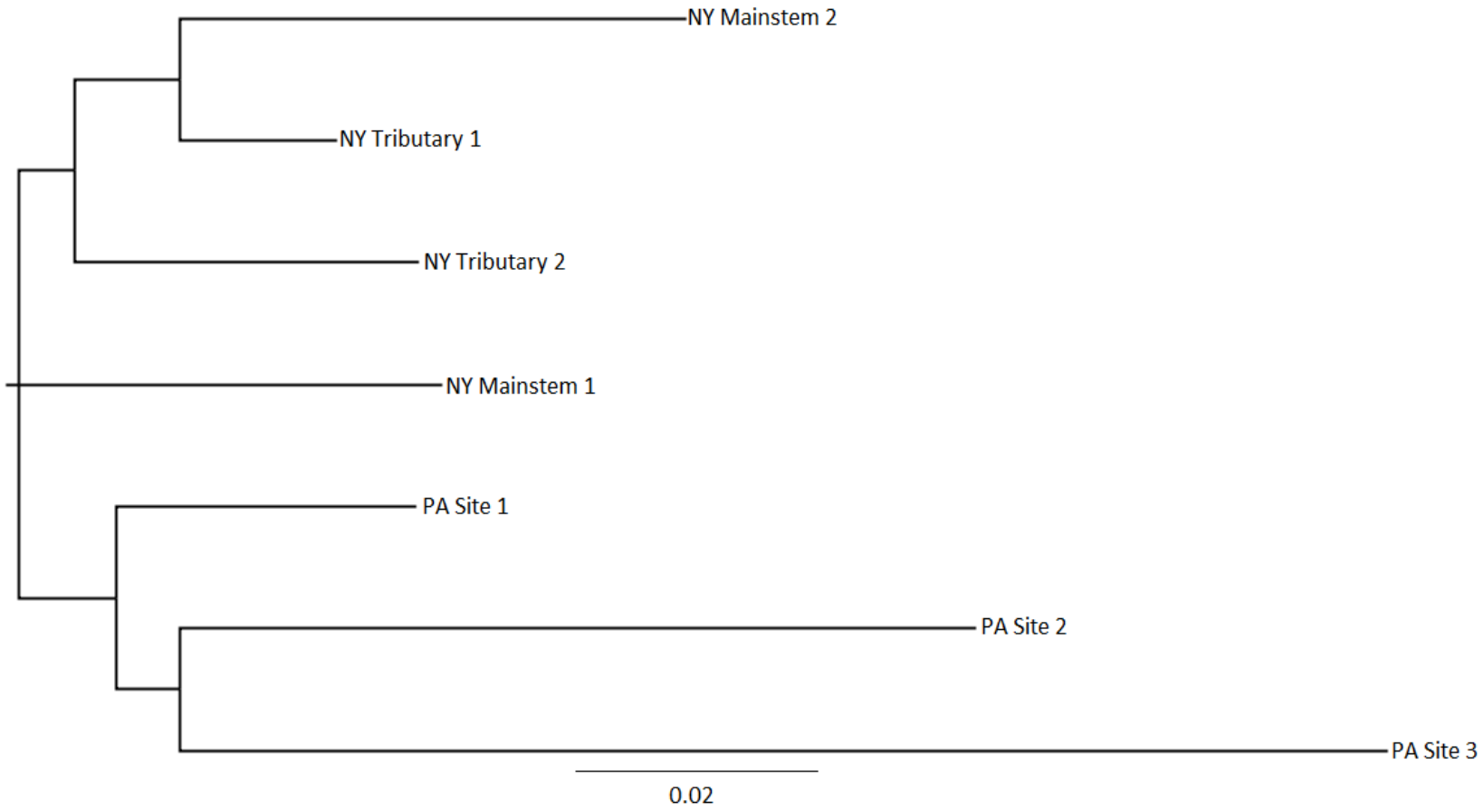


Figure 4: Phylogenetic tree constructed using FIGTREE1.4 based on Nei et al. (1983) distance calculations.

Head-started individuals

According to Tukey's HSD test, the head-started cohort has significantly lower allelic richness than all of the other sites in this study, except NY Tributary 2 (Figure 3). The rarified allelic richness of the head-started individuals for four of ten loci (Call26, Call282, Call347, and Cral117) was lower than the lowest allelic richness that appeared in any site in the wild (Table 11). In five other loci the head-started group had a rarified allelic richness near the middle or lower end of the range that appeared in the wild sites (Table 11).

GENECLASS2 assigned most animals to the correct site of origin (67% of 49 individuals to NY Mainstem 2). In the first rank of assignment, probability ranged from 32.9 to 99.5% likelihood the individual originated from the given site (Table 12). All individuals were assigned to one of the NY Mainstem sites (1 or 2) by the first or second rank of assignment (1^{st} rank indicating the highest probability of the individual originating from a certain site). In the first rank of assignment, animals were assigned to either NY Mainstem 1, NY Mainstem 2, PA Site 2, or PA Site 3 (Table 12). No animals were assigned to NY Tributary 2 in either the first or second rank of likelihood.

Table 11: Allelic richness (A) and rarified allelic richness (Ar) and averages of both of these measures for the head-started hellbender group compared to range present in the wild hellbender sites.

	Allelic Richness (A)		Rarified Allelic Richness (Ar)	
Locus	Range in Wild Sites	Head-started Group	Range in Wild Sites	Head-started Group
Call26	9 - 14	7	8.6 - 11.5	6.2
Call127	6 - 10	8	5.9 - 8.7	6.4
Call205	5 - 10	6	5.0 - 7.8	5.7
Call232	9 - 11	7	7.5 - 10.0	10.0
Call266	7 - 11	7	6.2 - 8.7	9.8
Call282	6 - 10	5	6.0 - 8.4	4.0
Call341	7 - 8	6	5.4 - 7.0	5.5
Call347	8 - 12	7	8.0 - 9.9	5.6
Cral115	2 - 3	2	2.0 - 2.8	2.0
Cral117	5 - 8	4	5.0 - 6.8	3.9
Avg	6.4 – 9.7	5.9	5.96 – 8.16	5.91

Table 12: Number of individuals assigned to each site and probability range of assignment for first and second ranks according to GENECLASS2 results.

	NY Mainstem 1	NY Mainstem 2	NY Tributary 1	PA Site 1	PA Site 2	PA Site 3
Rank #1	10 (50-99.5%)	33 (32.9 - 92.9%)			2 (51.4 - 56%)	4 (46.3 - 85.8%)
Rank #2	9 (11.6 - 37.6%)	19 (0.29 - 43.7%)	5 (0.90 - 15.3%)	4 (1.8 - 15.8%)	4 (4.4 - 22.2%)	8 (2.9 - 46.0%)

Discussion

The results of our fine-scale genetic study suggest that the northern Allegheny watershed is a single ecological management unit. However, there is some evidence that may indicate some populations are starting to suffer from effects of genetic drift. In addition, the head-started cohort in New York has limited genetic diversity and careful management will be necessary to maintain genetic diversity across the drainage. Yet,

releases of the head-started individuals could potentially aid in reversing the effects of drift in sites of interest.

Wild Population Trends

Genetic variation in this study was higher than that found by Crowhurst et al. (2011) in hellbender populations in Missouri. Our observed heterozygosities and allelic richness were more similar per site to those found by Unger et al. (2010) in one site (31 individuals) in Indiana. However, it is important to note that we used a combination of microsatellite markers from both of these studies and the markers used by Crowhurst et al. (2011) had the lowest allelic richness and heterozygosity in our study. Crowhurst et al. (2011) also suggested that differences in variation between the Indiana and Missouri studies could potentially be explained by the central-marginal hypothesis (Eckert et al. 2008). They suggested that the Missouri population is at the edge of the hellbenders range and thus will have lowered genetic diversity compared to central populations (i.e. Indiana) as a result of founder effects during the initial expansion of the species. This idea is further reinforced by a microsatellite study by Tonione et al. (2011) across the species' range in which the Missouri populations tended to have lower observed heterozygosity than other populations. Relatively low observed heterozygosity also was found in one site sampled in the peripheral Susquehanna drainage in Pennsylvania (Tonione et al. 2011). However, this trend does not appear to hold true in the Allegheny drainage based on the relatively high genetic variation found in this study, despite the fact that it is at the northern-most extent of the hellbender range. This result reinforces findings by Rayman (2010), which suggested that mtDNA haplotype diversity in the

Allegheny watershed was higher than anticipated. Results of the AMOVA suggest that most of the genetic variation in this study lies within individuals (96%). Other studies on hellbender have found relatively high microsatellite variability (Crowhurst et al., 2011; Tonione et al., 2011; Unger et al., 2010) especially compared to other markers such as allozymes (Merkle et al., 1977; Shaffer and Breden, 1989). In addition, studies on other species suggest that long-lived species tend to maintain high genetic diversity (e.g. copper redhorse, Lippe et al., 2006; ornate box turtle, Kuo and Janzen, 2004; orang-utan, Goossens et al., 2005).

The AMOVA for genetic differentiation suggested that the F_{ST} calculated with the provided groupings was significant. While the AMOVA shows there is overall differentiation, it does not indicate where those differences occur spatially, and therefore other methods are needed to determine which populations are different from one another. Despite the significant genetic differences indicated by the AMOVA, the STRUCTURE program did not group our data into more than one distinct population and therefore, our data is best described as one ecological management unit. These results are congruent with other studies that have demonstrated high gene flow within drainages (Routman, 1993; Crowhust, 2011. However, according to Pritchard et al. (2000), testing for frequency differences between predefined populations can be more powerful than the STRUCTURE software in certain situations. Given this information, it is important to carefully consider fine-scale differences based on F_{ST} or G_{ST} measures.

Both F_{ST} and R_{ST} calculations indicated PA Site 3 tended to be the most genetically different. However, in contrast to F_{ST} values reported by Crowhurst et al. (2011) (Crowhurst et al. found >0.1, whereas our study was <0.1), this is relatively low

differentiation. Compared to PA Site 3, NY Tributary 2 has moderate pairwise F_{ST}/R_{ST} values, especially when other values between NY sites are very close to zero. Still, based on pairwise tests of differentiation, PA Site 3 is significantly different from all NY sites, and NY Tributary 2 is different from all sites sampled in this study. It is not surprising that PA Site 3 is the most genetically different since this site is the most southern site in the study and geographically furthest from the NY sites. NY Tributary 2, on the other hand, is comparatively close geographically to the other NY sites and so this differentiation is unexpected.

Based on gene diversity measures and F_{IS} values, NY Tributary 2 does not appear to be suffering from inbreeding. Gene diversity measures for each locus are similar to those found in other sites, and overall F_{IS} for NY Tributary 2 suggests a slight heterozygosity excess (-0.009). There is some weak evidence to suggest that NY Tributary 2 is genetically different from all other sites due to genetic drift effects. First, while no locus deviated from H-W equilibrium after Bonferroni correction, two loci (Call205 and Call282) did have low p-values (<0.05) in the NY Tributary 2 site.

Also, the BOTTLENECK program provides some evidence of significant heterozygosity excess in NY Tributary 2, which is an indicator of a recent genetic bottleneck. This is because a population under-going genetic drift will maintain high heterozygosity levels while the number of available alleles in the populations will decrease (Cornuet and Luikart, 1997). Theoretically, microsatellites follow a step-wise model of mutation. However, there is evidence to suggest that all microsatellites this mutation model (Cornuet and Luikart, 1997). Thus, Cornuet and Luikart (1997) recommend the two-phase mutation model in the BOTTLENECK program for microsatellite studies, which is a

model that is an intermediate model between step-wise and the infinite alleles model. Under this model, NY Tributary 2 significantly shows an excess heterozygosity for the Wilcoxon test ($p<0.001$) and the Sign test ($p<0.01$) but not the Mode shift test. PA Site 1 and PA Site 2 also had low p-values for the Wilcoxon and Sign test. Tributary 1 also shows heterozygosity excess in the Wilcoxon test but not for the sign test. PA Site 2 has a low sample size, though, and it cannot be determined with certainty if this result is real or a result of sample size. Still, NY Tributary 2 is shows a highly significant heterozygosity excess in two of the three tests performed, under the TPM model. No site shows any evidence under the Step-wise mutation model. It is also important to mention that this is not an artifact of low sample size since NY Tributary 2 had the largest sample size of any site sampled ($n=75$).

Additionally, basic genetic measures show the retention of heterozygosity and the reduction in alleles in the NY Tributary 2 site. This site has an overall heterozygosity measure comparable to other sites in this study as well as other studies (Unger et al. 2010, Tonione et al. 2011). However, the overall allelic richness after rarefaction is the lowest of all sites sampled in NY and PA (Table 3). In six of ten loci, the allelic richness is the lowest or second lowest of all sites sampled and this pattern holds true before rarefaction despite that fact that NY Tributary 2 has the largest sample size. Therefore, there is further evidence that this site is beginning to exhibit genetic drift. Some authors posit that only one migrant per generation is needed to counter the effects of drift (Wright, 1951). Thus, it is possible that this site is somehow isolated from other populations in the drainage, whether it is a physical or behavioral. An alternate hypothesis that may explain

this trend is rapid decline in population size at this site as a result of severe habitat degradation (Ken Roblee, pers. comm.).

While Tukey's HSD test did not indicate that the low allelic richness in NY Tributary 2 was significant, the allelic richness NY Tributary 2 was also not significantly different from the head-started cohort. The head-started cohort was, however, had significantly lower allelic richness compared to all other sites in the study. One locus, Cral117, appears to be an outlier in allelic richness in NY Tributary 2. In fact, Cral117 retains high allelic richness across all sites. This could indicate that this locus is linked to an area of the genome which has naturally high variability (e.g. major histocompatibility complex). In contrast, the other nine markers tend to have comparatively low allelic richness in NY Tributary 2 which further suggests genetic drift in this site. Since microsatellite loci are neutral markers, we presume that they do not exhibit selection pressure. So, if all of these markers are in fact neutral (and not linked to alleles which do undergo selection pressure), genetic drift would be responsible for the lowering of genetic diversity at these loci. If natural selection was responsible for this lowered genetic diversity, we would not expect to see a trend towards lower allelic richness across all of the natural markers sampled in this study.

Distance (D_A) measures (Nei et al. 1983) indicate that the sites in this study are closely related. Again, this supports the previous finding that populations or sites within a drainage are genetically similar (Routman, 1993; Crowhurst et al., 2011). This reinforces the idea that genetic drift in NY Tributary 2 is weak and most likely very recent because if drift has been occurring for a long period of time we would expect to see greater genetic distance from other sites. The phylogenetic tree constructed based on D_A shows

three clusters; one containing NY Mainstem 1, one containing all other NY sites, and the last containing all PA sites. These relationships coincide with the geographical locations of each cluster. It is important to note, however, that neighbor-joining based phylogenetic trees do not produce one single "correct" tree (Hall, 2001); therefore, this tree is simply one possible illustration of the basic relationships within the northern Allegheny drainage. Additionally, the time scale produced by this method may not be entirely accurate as D_A is a better measure for topology relationships and does not produce reliable branch lengths (Takezaki and Nei, 1996). Furthermore, given the close proximity geographically and genetically of the sites in this study, an accurate time scale may be rather difficult to produce.

Head-started Hellbenders

Crowhurst et al. (2011) posited that individual probability assignments using GENECLASS2 or similar programs could help to identify source populations of captive-raised individuals to evaluate their potential use in breeding programs. However, we used this program in a non-traditional manner to evaluate potential release sites based on probability assignments since we knew the population of origin of the head-started cohort (NY Tributary 2). The results of the program could indicate other sites, other than the source population, to which some of the head-started individuals have a similar genetic composition. While the majority of the head-started animals were correctly assigned to the NY Mainstem 2 population of origin by GENECLASS2, several animals were misassigned to alternate sites. In particular, several animals were misassigned to NY Mainstem 1 and a few animals were also misassigned to PA sites 2 and 3 in the first rank.

Based on the assumption that neutral markers can be used as indicators for alleles with selection pressure, the GENECLASS2 results suggests that these sites may be used as release sites for head-started animals based on genetic similarity. In the second rank, a subset of individuals were assigned to all populations with the exception of NY Tributary 2. This provides further evidence that NY Tributary 2 is genetically different from the other sites in the drainage and from the head-started individuals since no animals were misassigned to this population in either the first or second rank.

The head-started hellbenders have significantly lower allelic richness when compared to wild sites in the northern Allegheny drainage. Although preliminary results of a parentage analysis suggest that more than one female and more than one male contributed gametes to the head-started egg mass, all individuals in the cohort are siblings or half-siblings (Chudyk, 2013). There are ~600 animals that will be returned to the wild from the head-starting program and given their relatively low genetic diversity, the overall diversity of the wild population could be lowered, assuming high survival and breeding success. This could result from common alleles in the head-starting group increasing in frequency and thus reducing or eliminating rare alleles in certain sites. In most cases genetic the consequences of RRT programs are realized after the program has already been implemented. This study provides a rare opportunity for managers to understand potential genetic consequences prior to the release of individuals, which can be used to make informed management decisions.

Implications for Conservation and Management

There are two major contrasting ideas and/or approaches when considering releases of captive-raised or head-started individuals into a wild population. The first is to release individuals into sites where the native population contains animals that are genetically different from the head-started animals in an attempt to raise overall genetic diversity (Tallmon et al., 2004; Crowhurst et al., 2011. The second is to release animals into sites where the native population contains individuals that are genetically similar to the released animals. Tonione et al. (2011) suggested this latter strategy as a method to conserve specialized adaptations specific to certain sites or populations. Furthermore, Storfer (1999) suggests that mixing of populations to increase genetic diversity could lower the short-term population fitness and should not be executed until gene flow rates and ecological differences between sites are fully understood.

Our results suggest that, in general, the sites in the northern Allegheny watershed are genetically similar to each other and there are no natural genetically distinct groups. However, there is some evidence to suggest that NY Tributary 2 is differentiated as a result of weak or recent genetic drift, potentially due to isolation from other sites within the drainage. Given this information, the head-started individuals could be placed in most locations that have similar genetic composition (all but NY Tributary 2) to increase overall numbers, and presumably, localized adaptation would be conserved. This is only presumed because microsatellites are neutral markers (no selection pressure) and therefore, we assume that these markers are indicators of genetic patterns occurring on genes with selection pressure. In the case of NY Tributary 2, it may be beneficial to release head-started individuals there, to increase genetic diversity by simulating gene

flow from the lower portion of the drainage. According to Wright (1931), only one migrant per generation is needed to counter genetic divergence of populations. However, the success of mixing head-started animals into Tributary 2 to reverse drift is based on the assumption that our neutral microsatellite markers accurately reflect the genetic trends in areas of the genome that under-go selection pressure. In general, mixing of individuals may be useful in instances were inbreeding or genetic drift are creating genetic differences between populations since both can potentially lead to dramatic lowering of genetic diversity (Keller et al., 2012). In contrast, mixing of individuals may not be advisable in situations where there is evidence that natural selection is the driver of genetic differentiation (Storfer, 1999). On a cautionary note, since this site is the northern-most site in this study and we evaluated the sites using neutral markers, it is difficult to determine if natural selection is playing an important role in creating differentiation. Therefore, physical and ecological attributes of NY Tributary 2 should be compared to other sites to determine if there are any significant differences between these sites and any animals released there should be monitored.

Given the decline in hellbender populations throughout the range (Nickerson and Mays, 1973; Williams et al., 1981; Gates et al., 1985; Bothner and Gottlieb, 1991; Trauth et al., 1992; Wheeler et al., 2003; Bauman and Wilson, 2005; Briggler et al., 2007a; Foster et al., 2009) and the numerous potential and realized threats to hellbender populations (Mayasich et al., 2003), human-mediated releases may be necessary to restore hellbender populations (Trenham and Marsh, 2002). While the head-starting program in New York has the potential to substantially increase the population in the Allegheny watershed, it may also have genetic consequences such as the lowering of

overall genetic diversity. Without sufficient genetic diversity, a species loses its evolutionary potential, which is its ability to adapt to a changing environment (Reed and Frankham, 2003; Day et al., 2003; Willi et al., 2006), because natural selection acts upon genetic variability (Hedrick, 2001). This is a fundamental idea and since eventual environmental change is inevitable, mangers should attempt to maintain high evolutionary potential in any reintroduced species (Groombridge et al., 2012; Jamieson and Lacy, 2012).

Lowered genetic diversity is a common result of reintroduction programs because threatened or endangered species are often already depauperate of genetic variability, and reintroduced cohorts are typically small so inbreeding and genetic drift further reduce the genetic diversity (Swanson et al., 2006; Pierson et al., 2007; see Lacy, 1987; Fitzsimmons et al., 1997; Robichaux et al., 1997; Williams et al., 2002; Gautschi et al., 2002; Mock et al., 2004; Stephen et al., 2005; Jamieson, 2010). However, in some cases, the new or supplemented population maintains high genetic diversity as a result of high gene flow due to either a natural history trait of the species or management actions (see Pierson et al., 2007; Hicks et al., 2007; Swanson et al., 2006).

Certain natural history traits of the eastern hellbender could potentially slow reduction in genetic diversity after the release of the head-started cohort. Hellbenders can live up to 30 years in the wild (Taber et al., 1975) and thus, adults can contribute to the gene pool for many generations after sexual maturity is reached three to four years according to Bishop (1941) or six to seven years according to Taber et al. (1975) and Peterson et al. (1988). Studies suggest that in long-lived species where older individuals do not suffer from lowered reproductive success (via selection or physiology), there is an inherent

genetic rescue effect (a genetic buffer against inbreeding and drift) and relatively high genetic diversity is maintained despite small population size (e.g. copper redhorse, Lippe et al., 2006; ornate box turtle, Kuo and Janzen, 2004; orang-utan, Goossens et al., 2005). Therefore, if resident adult hellbenders successfully breed with head-started individuals for many generations, the potential negative genetic effect of lowered genetic diversity could be prevented naturally.

Conversely, the potential for gene flow, which is often an important factor in maintaining genetic diversity (Storfer, 1999), may be limited due to other natural history traits of the hellbender. Adult hellbenders are highly sedentary animals (Nickerson and Mays, 1973; Peterson, 1987; Humphries and Pauley, 2005) and therefore, gene flow by adult migration may be limited. Still there is clear evidence to suggest high gene flow between hellbender populations within drainages (Routman, 1993; Crowhurst et al., 2011). Juvenile dispersal may be a major mode of gene flow for hellbender populations; however, little is known about the dispersal habits of juveniles. In many populations throughout the range juveniles are under-represented in surveys, suggesting low juvenile recruitment (Bothner and Gottlieb, 1991; Wheeler et al., 2003; Humphries and Pauley, 2005; Foster et al., 2009; Burgmeier et al., 2011). In the New York Allegheny, however, Foster et al. (2009) did not find a reduction in juveniles in the Allegheny watershed compared to the 1980's study by Bothner and Gottlieb (1991). So, in the worst case scenario, juvenile recruitment may be low and therefore the greatest potential for gene flow between sites would be limited. This could result in lowered genetic diversity after the release of head-started individuals, as the gene flow that aided in maintaining genetic diversity in the past or in other long-lived species would not be as likely in this scenario.

Although there are natural history traits of the hellbender that may prevent the lowering of genetic diversity, current population status could increase the probability of lowered genetic variability. Further research is needed to determine the extent that these contrasting factors will have on the overall effects of the head-starting program. Thus, we recommend taking a conservative approach when managing this species and assume the worst; that is assume the head-starting program will have negative genetic consequences, and therefore take actions to prevent this effect. Specific management recommendations and head-started animal reintroduction guidelines based on these results are discussed in Chapter IV.

Chapter IV: Hellbender RRT Recommendations with a Conservation Genetics Focus

Background

What are RRT programs?

Reintroduction, repatriation, and translocation (RRT) programs are management strategies, used for threatened and endangered species, which have been employed for numerous species in the past few decades. RRT programs can have several purposes: to reverse declines in distribution and abundance due anthropogenic activities (Robichaux et al., 1997), re-establish populations where they have become extirpated (Griffith et al., 1989; Dodd and Seigel, 1991), supplement existing populations to reduce the chance of demographic or genetic collapse (Keller et al., 2012), curb the loss of biodiversity (Griffith et al., 1989), and ultimately save species from extinction (Robert, 2009). Head-starting is a specific type of RRT program that often incorporates the assistance of zoological parks (Dodd and Seigel, 1991), and these programs can be used for either reintroduction to extirpated areas or translocation to areas that need augmentation. In head-starting programs, wild harvested eggs or young are raised in captivity until they reach an age or size at which they are less vulnerable to predation (Perez-Buitrago et al., 2008; Ewen et al., 2012). This particular technique is used to increase the survival of juveniles (Perez-Buitrago et al., 2008) and can be useful for species suffering from low juvenile recruitment, such as the hellbender (*Cryptobranchus alleganiensis*) (Bothner and Gottlieb, 1991; Wheeler et al., 2003; Humphries and Pauley, 2005; Foster et al., 2009; Burgmeier et al., 2011). Head-starting programs have already been implemented for this species in Missouri, West Virginia, and New York.

The Main Objectives of RRT programs

RRT programs often have two primary goals. The first is to increase the total number of individuals in existing populations undergoing decline, or in areas that have been extirpated, such that the end result is a viable, free-ranging, self-sustaining population (Scott and Carpenter, 1987; Griffith et al., 1989; IUCN, 1998). The second goal is to increase or retain genetic diversity so that the species can adapt to environmental change (Guerrant, 1996).

Why use RRT for management?

Success stories of species such as the Arabian oryx (*Oryx leucoryx*) (Stanley Price, 1989) and peregrine falcon (*Falco peregrinus*) (Cade and Burnham, 2003) highlight the worth of RRT programs in management of threatened and endangered species. Yet, RRT programs are expensive in terms of money, time, and effort, and are not always successful (Griffith et al., 1989; Dodd and Seigel, 1991; Wolf et al., 1996; IUCN, 1998). Historically, studies evaluating success of RRT programs have reported low success rates (Griffith et al., 1989; Beck et al., 1994; Wolf et al., 1996; Dodd and Seigel, 1991). The first review of amphibian and reptile RRT programs suggested only 19% of 26 programs were considered successful (Dodd and Seigel, 1991). However, more recent studies have found encouraging results suggesting that the number of successful reptile and amphibian RRT programs is increasing. For example, Germano and Bishop (2008) and Griffiths and Pavajeau (2008) found recent amphibian and reptile RRT programs to be 42% and 62% successful, respectively. RRT programs have improved greatly as a result of research on genetics, disease, habitat requirements, and demographic modeling (Parker et al., 2012).

Furthermore, RRT programs may be the best option for managing rare species while threats are determined and addressed. Griffiths and Pavajeau (2008) stress that RRT programs are useful tools for conservation that "we cannot afford to exclude from the toolbox", as long as they do not distract managers from efforts to achieve natural success such as threat mitigation and habitat restoration and management. RRT programs may be especially useful for species such as the hellbender that exhibit life history traits that make them susceptible to declines such as slow growth, low fecundity, delayed maturity (Wheeler et al., 2003), and habitat specialization (Smith, 1907; Hillis and Bellis, 1971; Williams et al., 1981).

The bad news: genetic consequences of RRT programs

Griffith et al. (1989) postulate that RRT programs can be an effective tool for managing wildlife, but it is imperative that managers understand that moving individuals between populations may have genetic consequences (Vinkey et al., 2006). Unfortunately, RRT programs often result in a lowering of overall genetic diversity of a population (Lacy, 1987; Robichaux et al., 1997; Gautschi et al., 2002; Williams et al., 2002; Stephen et al., 2005; Jamieson, 2010).

In the case of reintroductions, lowered genetic diversity is a result of a combination of factors including founder effects (O'Brien and Evermann, 1988; Frankham et al., 2002), a reduced effective population size (Hicks et al., 2007), and habitat fragmentation (Slough, 1994). Founder effects include inbreeding depression, genetic drift, and outbreeding depression. Since there are a small number of "founders" used in reintroduction programs (Vinkey et al., 2006), which inherently have limited genetic diversity compared to the

original (source) population, inbreeding and genetic drift are often the consequence (Leberg, 1993; Vinkey et al., 2006; Hicks et al., 2007; Pierson et al., 2007). Inbreeding increases the number of homozygotes in a population, which could lower individual fitness due to the accumulation of recessive deleterious alleles that are identical by decent (Hamilton, 2009). Ultimately, inbreeding depression can also lead to an overall reduction in genetic diversity in a population (Lacy, 1987). Inbreeding depression also has been linked to lowered viability and fecundity in some species (Falconer, 1981; Ralls and Ballou, 1983).

Genetic drift is the random loss of alleles in a population over time and the effects of genetic drift are more pronounced in smaller populations. RRT programs are typically used for rare, threatened, and endangered species that have experienced a drastic reduction in population size and thus, a genetic bottleneck (Groombridge et al., 2012). So the "stock" for the founders of RRT programs are often already depauperate of genetic variability (Swanson et al., 2006). Genetic drift then, further reduces the already reduced genetic diversity of the population after reintroduction (Keller et al., 2012).

On the other hand, reintroduced populations suffer from a reduced effective population size, which can lead to different problems associated with outbreeding depression (Tallmon et al., 2004). This is especially an issue for programs occurring on a long temporal scale. In outbreeding depression, alleles that are less "fit" are introduced into a population and as a result locally adapted alleles are reduced in that population (Vinkey et al., 2006). This results in lowered overall fitness of the population as less "fit" alleles are mixed into the population (Storfer, 1999). Further, when a large number of individuals are translocated, the high amount of human-mediated gene flow can

completely swamp out locally adapted alleles (Storfer, 1999). Storfer (1999) suggests that in some cases this trend can completely contradict the initial purpose of the translocation program and ultimately lead to population decline.

Without sufficient genetic diversity, a species loses its evolutionary potential, which is its ability to adapt to a changing environment (Frankham et al., 1999; Day et al., 2003; Reed and Frankham, 2003; Willi et al., 2006). Since natural selection acts upon genetic variability, evolutionary adaptations can only occur if genetic variability exists in the population (Hedrick, 2001). Since eventual environmental change is inevitable, mangers should attempt to maintain high evolutionary potential in any reintroduced species by maximizing genetic diversity (Groombridge et al., 2012; Jamieson and Lacy, 2012).

Given the known negative repercussions of lowered genetic variability in reintroduced populations and of washing out localized adaptation by translocations, it is important to evaluate the genetic variation of a population after the program is implemented (Swanson et al., 2006). More importantly, it may be even more advantageous to assess the genetic composition of the wild population and of the founders used in the program prior to the releases of individuals. This is crucial because this way, managers can attempt to avoid negative genetic consequences rather than need to correct genetic problems after the program has already been completed.

General considerations for RRT programs

There are many important considerations when starting and executing an RRT program. Pre-release modeling such as population viability analysis (PVA; Armstrong et al., 2006) should be incorporated into any RRT program, as they use population modeling

to help set goals for RRT programs (Armstrong et al., 2002; Schaub et al., 2009). Pre-release models are beneficial for estimating survival and reproduction of reintroduced individuals and the entire population (Armstrong et al., 2002). Other factors estimated by modeling can include number of individuals to use for founding the reintroduced population, total number of individuals to release across the entire duration of the program, and how many releases to perform (Seddon et al., 2012). These types of models also can be used to determine the potential effectiveness of RRT programs given the presence of current threats (e.g. exotic predators, see Armstrong et al., 2006). Modeling potential outcomes based on different management regimes can assist decision making for optimal management of populations (Armstrong et al., 2006; Schaub et al., 2009).

Care also must be taken when selecting habitats for releases. Osborne and Seddon (2012) note several factors to consider when examining habitats for releasing animals. First, historically suitable habitats may no longer be suitable and thus may not be a good choice for releases. Next, species absence from a location does not necessarily indicate that the habitat is unsuitable. Also, habitats that are suitable presently may not remain suitable in the future. Last, individuals from different areas of the range may not be suited to all potential release sites. It is also important to note that in some cases, a habitat may need to be engineered and managed to maintain suitability for the reintroduced individuals (Osborne and Seddon, 2012).

Monitoring is an essential component of species management. Monitoring can occur before, during, and after the implementation of a management technique. Pre-release monitoring of habitat, predators, and potential competitors can be used where appropriate to optimize RRT programs prior to implementation (Nichols and Armstrong, 2012;

Osborne and Seddon, 2012). Further, many authors stress the importance of post-release monitoring after the execution of an RRT program (Yoccoz et al., 2001; Armstrong et al., 2002; Campbell et al., 2002; Nichols and Armstrong, 2012). Managers must be cognizant of the extensive requirements for monitoring any RRT program that is put in place. In fact, it may take several years of monitoring before the success or failure of a program can be determined (Germano and Bishop, 2008; Griffiths and Pavajeau, 2008). Unfortunately, a large proportion of RRT programs evaluated in reviews are categorized as "success undetermined" due to a lack of sufficient or any data from post-monitoring efforts (Dodd and Seigel, 1991; Germano and Bishop, 2008; Griffiths and Pavajeau, 2008). Still, monitoring is essential for the proper evaluation of an RRT program as it can provide insight into the cause of failure or success (Seddon et al., 2007; Germano and Bishop, 2008). Monitoring also can assist in the refinement of pre-release modeling by validating or refuting model predictions (Armstrong and Davidson, 2006; Armstrong et al., 2007; Wakamiya and Roy, 2009).

Arguably, one of the most important considerations for RRT programs is that of addressing the threats that lead to the decline of the species in the first place (Seigel and Dodd, 2002; Dodd, 2005). Too often, RRT programs are initiated for reptile or amphibian populations before threats are properly addressed (Seigel and Dodd, 2002; Dodd, 2005). Since many declining reptile and amphibian populations suffer from numerous threats, it is imperative that some of these are dealt with prior to implementing RRT programs (Griffiths and Pavajeau, 2008). Griffiths and Pavajeau (2008) suggest that in particular, threats that are relatively easy to address on a local scale such as some human-mediated

issues including killing, collection, and introduced species should be the focus, as large scale problems like global climate change and disease may not be eliminated as easily.

While all of these considerations are important and crucial for the overall success of any RRT program, the objective of this report is to discuss considerations specific to maintaining genetic diversity and evolutionary potential when using RRT programs for the both subspecies of hellbender salamander (*Cryptobranchus alleganiensis*); the Ozark hellbender (*C. a. bishopi*) and the eastern hellbender (*C. a. alleganiensis*).

The Case of the Hellbender

When implementing a hellbender head-starting program, there are two major contrasting genetic considerations that must be kept in mind. First, Crowhurst et al. (2011) recommend that animals from genetically differentiated drainages should not be mixed so as to conserve localized adaptations unique to various populations. In contrast, Tonione et al. (2011) propose that, in some cases, intentional mixing of individuals from different populations may be advisable to increase the species' genetic diversity across the range. Keeping these two ideas in mind, following are suggestions about the genetic analysis, collection of eggs, release of animals, and post-release monitoring of hellbenders in a head-starting program.

Genetic analysis

First and foremost, a genetic analysis of the drainage of interest should be performed before starting a head-start program. There are a few reasons why collecting this

information may be beneficial. One benefit is that this will help to establish a historical level of genetic diversity. Unfortunately, RRT programs are usually implemented for species that have already suffered from a reduction in genetic diversity (Groombridge et al., 2012). This is because rare, threatened, and endangered species have typically experienced a genetic bottleneck due to a reduction in population size (Groombridge et al., 2012). So, the current population may not have the same level of genetic diversity as the historical population (Groombridge et al., 2012). Still, obtaining this information as soon as possible and prior to releasing animals provides some sort of baseline for comparison in the monitoring phase (Groombridge et al., 2012). Another reason to collect genetic information prior to starting the head-starting program is to help identify sites for both egg collection and for releases. More information will be discussed on egg collection and releases in the next two sections of this paper.

To complete a genetic analysis, genetic samples (e.g. tail clips) should be collected from as many sites as possible. According to Hale et al. (2012), 25 to 30 samples per "population" is sufficient for a robust genetic analysis using microsatellite loci. Defining a population may be difficult, but many authors have found that hellbender populations within drainages are genetically similar (Routman, 1993; Crowhurst et al., 2011). Thus, in a fine-scale study, such as the New York case study discussed later, each individual stream may be considered a population. However, sites along the same waterway that are separated by physical barriers, such as dams or culverts, should also be considered separately. In cases where 25 to 30 samples cannot be collected, as many samples as possible are still useful for analysis. If smaller sample sizes are collected, more genetic markers can be examined to reduce errors in genetic analysis that result from the under-

detection of rare alleles (Hale et al., 2012). Hale et al. (2012) also suggest that under-detection of alleles as a result of low sample size does not apply to populations that are already small (because you are sampling presumably nearly all individuals). So, if detection probability is high and few animals are captured, the site may simply have few individuals or low densities and reliable genetic information can still be obtained with fewer than 25 to 30 samples (Hale et al., 2012).

The purpose of a genetic analysis is to determine if any of five factors are creating differences between subpopulations within a drainage. These five factors include mutation, migration, inbreeding, genetic drift, and natural selection. Population geneticists first look for inbreeding and genetic drift because these two factors typically result in lowered genetic diversity of a population. Also, there are relatively straight-forward genetic measures that indicate if a population is undergoing either inbreeding or genetic drift. In general, mutation is not a strong factor since it takes a relatively long time for mutations to accumulate in a population (Hamilton, 2009). Based on current hellbender data, which indicates that hellbenders are very sedentary (Nickerson and Mays, 1973; Peterson, 1987; Humphries and Pauley, 2005), a lack of immigration in this species may increase the potential for genetic drift. Since adults are sedentary, immigration may be primarily driven by juvenile dispersal, but some research suggests that populations may be declining in part due to low juvenile recruitment (Bothner and Gottlieb, 1991; Wheeler et al., 2003; Humphries and Pauley, 2005; Foster et al., 2009; Burgmeier et al., 2011). However, this is still an area of active investigation in hellbender biology. Still, when there is not a clear indication of inbreeding or genetic drift, differences seen between populations that are in close proximity to one another is may be

a result of natural selection. This is only an assumption because we use neural markers for genetic analysis (e.g. microsatellites). Neutral markers do not experience selection pressure so it is difficult or impossible to determine if natural selection is driving genetic differentiation using only neutral markers. If natural selection is suspected, however, further genetic analysis of markers that do undergo selection may be used, such as the major histocompatibility complex (MHC).

Egg collection

After a genetic analysis has been completed, the next step in implementing a head-starting program is to collect eggs. Sites with high genetic diversity should be top priority for egg collection. Recent genetic research suggests that animals should not be taken from one major drainage and subsequently released into a different drainage because separate drainages have been shown to be genetically distinct (Routman, 1993; Sabatino and Routman, 2008). For example, Crowhurst et al. (2011) found that for the eastern hellbender, the Meramec, Niangua, and Gasconade/Big Piney river drainages were all genetically significantly different from one another. Only the Gasconade and Big Piney drainages were not significantly different, however these river systems were connected (Crowhurst et al., 2011). Mixing individuals between drainages then could result in the loss of localized adaptations for a variety of reasons such as; the drainage may have different habitat characteristics (e.g. substrate, climate, physical/chemical conditions of water) or the population may exhibit specialized behaviors (e.g. breeding or feeding). The only exception would be if an extensive genetic analysis was completed beforehand that showed two distinct drainages contained genetically similar populations or if the drainage

to be re-populated has been shown to be completely extirpated. Vinkey et al. (2006) stress the importance of demonstrating extirpation as opposed to simply positing it before introducing individuals from a genetically distinct population. In their study, they found that genetically distinct fishers (*Martes pennanti*) were introduced to an area where extirpation had not actually occurred, and as a result, a certain source population has left a genetic legacy across the species range (Vinkey et al., 2006). This over-representation of a single source population could potentially swamp out local adaptations (Leberg, 1990; Tallmon et al., 2004; Storfer, 1999) and reduce the evolutionary potential (Day et al., 2003; Reed and Frankham, 2003; Willi et al., 2006) of this species across the range. To be conservative, when collecting animals to be released in a given drainage, ensure that egg collection sites are also within that drainage.

In general, for maximizing genetic diversity, collecting animals from as many sites as possible may be best. Individuals collected from many places across the drainage will increase the amount of genetic variation represented in the head-started cohort. Given the frequent money and space constraints associated with collecting and rearing animals, the total number of individuals will need to be identified prior to egg collections. The total number to be reared and released will depend on numerous factors such as; facility space limitations, how many sites will be supplemented/re-populated, the desired density/total number per population to be achieved, how many releases will be performed, at what age the animals will be released, survival estimates based on modeling, and other site- and species-specific considerations. Each head-starting program will differ in its specific goals. Regardless of how many total animals will be reared, we suggest collecting from as many nests as possible to reach the total number of animals desired.

At least 20 individuals is recommended by zoo biologists to maintain genetic diversity when creating captive stock (Foose et al., 1986; Lacy, 1989; Willis and Wiese, 1993). In general, this idea may be applied to large-scale head-starting programs and ten nests could be a minimum target number from which to collect eggs. By conservatively assuming only two parents contributed eggs to each nest (especially when collecting only a subset of eggs from a nest), ten nests would satisfy the 20 founder rule recommended by zoo biologists (Foose et al., 1986; Lacy, 1989; Willis and Wiese, 1993). Further, collecting from sites with the greatest genetic diversity (based on prior genetic analysis) will increase the overall genetic diversity of the cohort and lessen the potential for negative effects of inbreeding. In the New York head-started cohort, for example, over 600 individuals were collected from one nest. All the individuals in this cohort were either full or half-siblings, despite the large number of eggs in the nest. So, eggs should be collected from more than one nest to prevent the potential for inbreeding depression. When collecting eggs from many nests, a tool can be used to extract eggs from under nest rocks to prevent physical damage to the nest or remaining eggs (Briggler, pers. comm.). This technique was used by Briggler to gently pull a strand of eggs out from under a rock and then sterile scissors were used to cut off a limited number of eggs from the nest. The number of individuals taken from each nest will depend on the target number of animals determined necessary for the particular head-starting program.

Releases – Where and how many?

After eggs have been collected, the next consideration of a head-starting program is the selection of release sites. There are three potential outcomes of the genetic analysis

that will result in different management strategies for releasing individuals. The first would be if populations are strongly genetically differentiated with genetic structuring, and geographically distant from one another. In this case, animals from these populations should not be mixed, to avoid the loss of locally adapted alleles. An example of this would be populations in different drainages, if these separate drainages have been shown to be genetically distinct. By avoiding the mixing of these populations, local adaptations can be preserved, as natural mixing of these populations is unlikely, and these populations may be on separate evolutionary paths.

The second potential outcome would be populations that are in close proximity to one another that do not appear to have genetic structuring, yet have some indication of genetic differentiation. By examining the five genetic factors discussed earlier (mutation, immigration, genetic drift, inbreeding, and natural selection), there are certain trends that can be identified that may influence the ultimate management decision. If genetic drift or inbreeding are apparent, intentional mixing of individuals from different populations may be beneficial. This is because inbreeding and genetic drift can negatively affect a population by lowering the genetic diversity. Thus, mixing individuals that are somewhat genetically different into the population can reverse the effects of genetic drift and lower the potential for further inbreeding. In contrast, if there is no indication of inbreeding or drift, it may be best to avoid mixing individuals between populations. In this case, natural selection may be the most likely reason for genetic differences. This means there may be locally adapted genes in these populations that should be conserved. Although, when using neutral markers there is no way to verify these differences are natural selection.

Still, the cautious and conservative approach would be to avoid mixing populations if inbreeding or genetic drift are not creating differences.

The third potential outcome is that there is no evidence of structuring or genetic differentiation between sites. If this is the case, mixing populations is unlikely to swamp out local adaptations. Also, mixing individuals from different sites is not necessary to increase diversity since inbreeding and drift are not an issue. Therefore, in this case, management decisions can be guided by demographic needs rather than genetic needs. For example, areas that are in need of re-population or are suffering from extremely low numbers may be the primary focus for releases. In the long term, this may be beneficial genetically for populations that are especially small since genetic drift tends to have a greater effect on smaller populations as opposed to larger ones. By increasing total numbers in sites with the lowest numbers/densities, potential for genetic drift and inbreeding is reduced.

Modeling studies can be used to determine the optimal number of individuals to be released to maintain rare alleles in a population (e.g. Tracy et al., 2011). Unfortunately, no modeling has been done for any amphibian species. However, a modeling study similar to the one designed by Tracy et al. (2011) would be useful to determine an ideal number of individuals to release per site. Yet, this number also will rely on other factors. First, constraints of space will determine how many individuals are head-started and the number released per site will depend on the goals of the specific program. Also, this number will vary depending on the survival of the head-started individuals. If mortality post-release is high, more individuals will need to be released to maintain the genetic diversity desired. This uncertainty in survival further suggests the need for post-release

monitoring to determine the survival rate of released individuals and to determine how many are surviving to reproductive maturity. To determine how many released animals are capable of reproducing, monitoring will need to continue for several years as it may take anywhere from five to seven years for hellbenders to become reproductively active (Bishop, 1941; Taber et al., 1975; Peterson et al., 1988). Scott and Carpenter (1987) stressed that given the high cost of programs like head-starting programs, we cannot afford to release animals if there is a low probability that they will survive or, more importantly, contribute genetically to the population. Gathering further information on survival and reproductive success and developing models specific to hellbenders may better inform the question of how many individuals to release, in a genetic context. However, while this type of information is gathered, the best strategy may be to release a group of animals that includes individuals from a variety of source nests to each site. By doing this, it will ensure the genetic variability obtained when collecting eggs is represented when releasing animals. In other words, all of the siblings from one nest should not be released into a single site unless there is a genetic basis (such as an indication of locally adapted alleles in that particular source site) for making this decision.

Swanson et al. (2006) suggest that several reintroductions of different founders of American marten (*Martes americana*) was the major factor contributing to the maintenance of genetic diversity in a reintroduced population in Michigan. The authors posit that this management technique mimics natural gene flow in the population, thus reversing the potential for loss of alleles (Swanson et al., 2006). In cases where locally adapted traits are unlikely, (no genetic differentiation or genetic differentiation caused by

inbreeding or drift) using multiple releases over time may be ideal for maximizing the genetic diversity of the reintroduced and the supplemented populations.

Continued Monitoring and Adaptive Management

Maintaining the genetic diversity and evolutionary potential of a species is a multi-faceted issue with numerous factors contributing to the outcome of an RRT program. Many authors stress the importance of monitoring programs post-release and this is true in all aspects of the program including genetics (Yoccoz et al., 2001; Armstrong et al., 2002; Campbell et al., 2002; Nichols and Armstrong, 2012). In most cases, genetic data is collected after the implementation of these types of programs (Groombridge et al., 2012) and therefore, genetic consequences are addressed post-release. Since hellbenders are long-lived species that do not reach sexual maturity for several years, a complete genetic re-analysis may not be informative for many years. Therefore, it will be a long time before the ultimate effects of the head-starting program can be determined. As such, following basic guidelines to inform the collection and placement of individuals may help to reduce the chance of negative genetic consequences. By pre-planning the legacy of hellbender head-starting programs we can leave the species in the best possible condition to deal with current and future threats as well as inevitable environmental change.

In addition, planning to monitor and make changes to a head-starting program based on new information could be an effective way to increase the chance of success. Adaptive management is a way of dealing with uncertainty in management situations (Rout et al., 2009) and helps to improve future performance of management practices (Walters, 1986; Holling, 1978). Adaptive management is essentially *a priori* plan to use monitoring to

influence changes in management (Walters, 1986; Holling, 1978). This requires deciding what monitoring will be done and how management will change in the event of several possible outcomes (McCarthy et al., 2012). This way management actions can change quickly and smoothly based on the data collected during monitoring (McCarthy et al., 2012). Adaptive management may be one of the most useful strategies for novel RRT programs since these programs contain a high number of uncertainties (McCarthy et al., 2012). This may indeed be true of hellbenders given the current lack of knowledge on much of the biology of the species, the ultimate effects of head-starting programs, and the ultimate consequences of head-starting both demographically and genetically on many species including the hellbender.

A New York State case study

The New York hellbender head-starting program can provide some insight into the application of a genetic analysis for providing management recommendations. In 1991, Bothner and Gottlieb sampled eight sites within the Allegheny River drainage of New York State. Almost two decades later, Foster et al. (2009) resampled those same sites and determined the population was declining in this region of the watershed. As a result, the New York State Department of Environmental Conservation (NYS DEC) began a head-starting program and collected ~600 eggs from one nest at one site which had a few contributing parents (Chudyk, 2013). We performed a genetic analysis of over 200 wild hellbenders from eight sites within the northern Allegheny watershed and of 50 individuals from the head-started cohort. The main findings of our study were that 1) all sites sampled within the northern Allegheny River watershed can be deemed one single

ecological management unit; 2) there was some evidence that one tributary was suffering from weak or recent genetic drift, and 3) the head-started hellbender cohort had less overall genetic diversity compared to genetic diversity in the wild sites.

Given the results of our study, we surmised that releasing the head-started animals into any of the sites is unlikely to cause outbreeding depression or reduce the fitness of the site by washing out locally adapted alleles, since the northern section of the drainage is one ecological management unit. Therefore, we would suggest following Tonione's management recommendation and use the head-starting program to increase the genetic diversity and the adaptive potential of the population. So, we recommend that animals from the head-started program are released throughout the New York portion of the drainage to avoid flooding any one site or only a few sites with numerous siblings (which increases the potential for inbreeding).

We also determined that releasing head-started individuals into the tributary potentially experiencing genetic drift could help to rescue that population and reverse loss of alleles inevitable with genetic drift. Only a few animals will be necessary to reverse the drift pattern based on Wright's (1931) one-migrant per generation rule. We recommend that only a few animals be released into this site and carefully monitored in case natural selection is the driver of the genetic differences in this site. Further, future research should examine genetic markers that under-go selection pressure in the sites sampled in this study to determine if natural selection is a factor.

The low diversity of the head-started cohort suggests that releasing these animals into sites throughout the region could lower the genetic diversity of the northern Allegheny population, as is commonly seen in RRT programs (Lacy, 1987; Robichaux et al., 1997;

Gautschi et al., 2002; Williams et al., 2002; Stephen et al., 2005; Jamieson, 2010). Yet, given some of the natural history traits of the hellbender, such as their long life-span, this lowering of genetic diversity may not be as pronounced as with other species. For example, research has shown an unexpectedly high level of genetic diversity and no evidence of inbreeding or drift in long-lived species suffering from population declines (e.g. ornate box turtle, Kuo and Janzen, 2004; orang-utan, Goossens et al., 2005; copper redhorse, Lippe et al., 2006). This suggests that long-lived species have an inherent genetic rescue effect. This may be because they can breed for many generations, older individuals breed with younger individuals, and reproductive success does not decrease with age (Lippe et al., 2006). Conversely, since hellbenders are relatively sedentary (Nickerson and Mays, 1973; Peterson, 1987; Petranka, 1998; Humphries and Pauley, 2005), and juvenile recruitment may be low (Bothner and Gottlieb, 1991; Wheeler et al., 2003; Humphries and Pauley, 2005; Foster et al., 2009; Burgmeier et al., 2011), a lack of immigration between populations may exacerbate the lowering of genetic diversity. Therefore, to ensure the optimal outcome of this program and to prevent the potential for any lowering of overall genetic diversity, we recommend that head-starting efforts continue in this region using eggs collected from several other sites within the drainage.

Conclusions

RRT programs can be effective management tools but there are numerous considerations when implementing an RRT program, including maintaining genetic diversity and stability. It is difficult to determine specific strict genetic guidelines for all RRT programs since these programs can be used for a multitude of species (Parker et al,

2012). Also, our knowledge the ultimate genetic effects of RRT programs is limited to generalizations from a few specific studies (Groombridge et al., 2012). It can even be difficult to define specific genetic guidelines for one species that exists across a large landscape; however the general guidelines described in this paper may assist in the genetic planning specifically for hellbender head-starting programs. Based on our current knowledge, steps can be taken to avoid negative genetic consequences such as inbreeding, loss of genetic diversity, and loss of localized adaptations. While most genetic studies of reintroduction programs are performed after they are implemented, I would argue that a genetic analysis prior to collecting or releasing animals is the best was to avoid negative genetic consequences. This is especially true when considering long-lived species that are slow to mature since it will take many years before the ultimate genetic effects of an RRT program can be determined. It is also still important to build on our current knowledge by evaluating and monitoring programs to determine the efficacy of the program and fine-tune guidelines for each species and for specific regions of the species range. Following generic conservative guidelines coupled with adaptive management can help to ensure optimal genetic results to increase the overall success of a head-starting program for hellbenders.

Suggested Reading

Reintroduction Biology Conservation Science and Practice no. 9. John G. Ewen, Doug P. Armstrong, Kevin A. Parker, and Philip J. Seddon. Wiley-Blackwell 2012

Selkoe, K.A., and R.J. Toonen. 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecology Letters 9:615-629

CHAPTER V: Conclusions

The eastern hellbender salamander (*Cryptobranchus alleganiensis)* is a species suffering from population declines throughout its range. Since there are numerous threats that are potentially triggering these declines, human-mediated releases may be necessary to supplement populations while threats are identified and addressed. Supplementing these populations may be especially important given the natural history traits of hellbenders such as slow growth, low fecundity and a short breeding season that reduce the potential for the natural recovery of populations.

Reintroduction, repatriation, and translocation (RRT) programs are useful conservation tools for rare, threatened, and endangered species. The efficacy of these types of programs can be improved by incorporating a genetic analysis of wild populations and of captive-reared individuals to optimize management decisions to maintain genetic variability in the species. Further, a full understanding how individuals or populations are presently distributed in the landscape can be used to help determine how and where an RRT program should be implemented. Understanding the spatial distribution can also provide insight into the genetic structuring of populations within the drainage of interest to guide management decisions for head-starting programs.

The field portion of this study confirmed hellbender presence in four of eight sites that had not been surveyed previously. In the 24 individuals captured, we found a higher prevalence of injuries and abnormalities in the northern Allegheny watershed then previously found by Foster (2006). In the list of injuries and abnormalities was the first evidence of a lamprey parasitism on an adult hellbender and also the first documentation of blue-grey patches on the skin of hellbenders in New York. For a species suffering from

numerous threats, native lamprey parasitism on hellbenders could negatively affect an already declining New York Allegheny watershed population. Also, further research into the blue-grey skin patches observed in this study may provide insight into current threats affecting the population as they may be related to disease adding to the decline of the species. The identification of these additional potential threats reinforce the need for management of this species in New York.

DNA samples were collected from the individuals captured in this study and these were included in a full genetic analysis of the northern Allegheny drainage (including sites from both New York and Pennsylvania). Individuals from the head-starting program implemented by the New York State Department of Environmental Conservation were also included in this analysis. The results of the genetic analysis suggested that the northern Allegheny is one ecological management unit. However, the northern-most tributary within the drainage may be suffering from recent genetic drift. This is important because genetic drift can lower the genetic diversity of the population and it suggests that this tributary is isolated from the rest of the drainage or this population in this site is declining rapidly.

Supplementing the New York population with head-started individuals may benefit the population by reversing the effects of drift in certain populations. In contrast, the head-started cohort may lower the overall genetic diversity of the drainage by swamping out rare alleles in the population. Certain natural history traits of the hellbender may counter this potential loss of genetic variability. For example, long-lived species appear to have an inherent maintenance of genetic diversity, despite extensive reductions in population size (e.g. ornate box turtle, Kuo and Janzen, 2004; orang-utan, Goossens et al.,

2005; copper redhorse, Lippe et al., 2006). In contrast, other natural history traits of the hellbender may exacerbate this effect such as limited gene flow due to the sedentary nature of adults (Nickerson and Mays, 1973; Peterson, 1987; Humphries and Pauley, 2005) and low juvenile recruitment (Bothner and Gottlieb, 1991; Wheeler et al., 2003; Humphries and Pauley, 2005; Foster et al., 2009; Burgmeier et al., 2011). Yet, careful and conservative management based on our understanding of the genetic structure and diversity of the wild hellbender population may help to ensure the maintenance of genetic diversity in this species.

When implementing a head-starting program for eastern hellbenders, basic genetic guidelines should be followed to optimize the outcome of the program. These guidelines include 1) performing a genetic analysis prior to egg collection, 2) collecting eggs from many nests primarily in locations with the highest genetic diversity, 3) using the results of the genetic analysis to choose release sites to either maximize genetic diversity or reduce the potential for loss of locally adapted alleles and, 4) monitor and adaptively manage all aspects of the program, including genetic outcomes. Following genetic guidelines such as these, or other guidelines developed specifically for a particular region or species, are useful since RRT programs should not only be used to increase total numbers but also to increase the evolutionary potential of the species for which they are implemented.

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