HELLBENDER (*CRYPTOBRANCHUS ALLEGANIENSIS*) GENE FLOW WITHIN RIVERS OF THE MISSOURI OZARK HIGHLANDS

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Master of Arts

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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a candidate for the degree of Master of Arts in Biological Sciences,

and hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

For my children, Brooklyn and Isaiah. I cannot imagine going through this journey without you. You are my motivation and my world. Please know that your smiles and laughter inspired me every step of the way. Remember to always chase your dreams because you deserve the world and all the success it has to offer. I love you.

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ABSTRACT

Theory and empirical evidence suggest that small and/or endangered populations suffer from genetic erosion as a consequence of their susceptibility to genetic drift and inbreeding. Thus, for species of conservation concern, effective management includes maintaining robust population sizes while monitoring and promoting genetic variability. Because dispersal can contribute significantly to both population stability and genetic variation, management decisions can benefit from a thorough understanding of population connectivity. For hellbenders *Cryptobranchus alleganiensis*), an aquatic salamander species experiencing dramatic declines in population size, little is known about genetic variation at the within-river scale and whether habitat patches within rivers are genetically and/or demographically connected. Given that suitable habitat patches are isolated, and that hellbenders exhibit extreme site fidelity and low vagility, gene flow may be restricted among these discrete habitat patches. Using several polymorphic microsatellite loci, I assessed the fine-scale genetic relationships between hellbenders occupying various habitat patches within a river. My results indicate that a substantial amount of gene flow is occurring between habitat patches, with no evidence to support genetic differentiation between patches. Since dispersal is the mechanism driving gene flow, it can be inferred from this data that—contrary to popular knowledge—hellbenders do disperse, with dispersal hypothesized to occur during the

larval and/or juvenile phase. Dispersal can occur in both males and females. However, the propensity for one sex to disperse more frequently than the other is driven by evolutionary forces and mating strategies. Using molecular techniques, I investigated differential dispersal between males and females in order to evaluate sex-biased dispersal in hellbenders. My results suggest that male and female hellbenders disperse, and that both sexes contribute to the observed levels of gene flow. Because hellbender populations are experiencing low juvenile recruitment in addition to the declines in population size, I compared heterozygosity levels in survived and dead offspring to evaluate how genetic diversity influences offspring survival. My results suggest that heterozygosity-fitness correlations would be an intriguing area of research to pursue in future studies, and may give further insight into causes for hellbender declines. The results presented in my study are valuable for hellbender conservation, and have important implications for management. In particular, my results are informative for restorative release efforts and indicate that propagated hellbenders should be released back into the river of origin. Although release into a specific habitat patch is not warranted (given the lack of observable population genetic structure), attention should be paid to those habitat variables necessary for hellbender survival and settlement as described in previous studies.

CHAPTER 1

General introduction

Having evolved from the first land-dwelling tetrapods (circa 400MYA), amphibians have survived centuries of selective pressures and several mass extinction events (Wells 2007). However, approximately one-third of all amphibian species are considered threatened, with a number of species having already succumbed to extinction during the last few decades (Stuart et al. 2004). The damaging influences of global climate change and anthropogenic disturbance (exploitation, habitat alteration/degradation, etc.), as well as the emergence of fatally infectious diseases—particularly amphibian chytrid fungus (*Batrachochytrium dendrobatidis*)—have taken a devastating toll on anurans, caecilians, and salamanders alike (Collins & Storfer 2003).

Hellbenders (*Cryptobranchus alleganiensis*), representing the most primitive suborder of salamanders (Cryptobranchoidea), are among the threatened. This species is found throughout a large portion of the eastern United States within rocky streams characterized by clear, fast flowing currents and cool temperatures (Smith 1907; Nickerson & Mays 1973; Fobes 1995). Two subspecies, the eastern hellbender (*C. a. alleganiensis*) and the Ozark hellbender (*C. a. bishopi*), are distinguished from one another by both biogeographical and morphological differences (Grobman 1943; Nickerson & Mays 1973), as well as genetic disparity (Sabatino & Routman 2009; Crowhurst et al. 2011). Although the distribution is quite large, Missouri is the only

state in which both hellbender subspecies can be found. Over the last twenty years, these populations, and others across the range (Gates et al. 1985; Pfingsten 1989; Trauth et al. 1992), have experienced dramatic declines (>75%, Wheeler et al. 2003). Suspected causes include habitat degradation (Nickerson & Mays 1973; Williams et al. 1981), disease (Briggler et al. 2008), introduction of novel predators (Gall & Mathis 2010), and overharvest (Nickerson & Briggler 2007). As a result, the Ozark hellbender has been federally listed as endangered (USFWS 2011a) and has received protection under CITES Appendix III (USFWS 2011b) alongside the Eastern hellbender.

For species of conservation concern, an effective management strategy involves monitoring and maintaining genetic variability (Lande & Barrowclough 1987; Schwartz et al. 2007). Theory suggests these populations are more susceptible to the negative consequences of drift and inbreeding due to small population size (Lande & Barrowclough 1987; Frankham 1995). The earliest investigations using allozymes suggested that hellbenders and nonmetamorphosing salamanders, in general, have lower levels of genetic variation than has been observed in other vertebrate species (Merkle et al. 1977; Shaffer & Breden 1989). However, more recent studies using data from mitochondrial DNA (mtDNA) and/or microsatellite loci, have revealed moderate to high levels of polymorphism and heterozygosity in a variety of paedogenic salamanders (Karlin & Means 1994; Murphy et al. 2000; Parra-Olea et al. 2007; Steele et al. 2008), including hellbenders (Templeton et al. 1990; Routman et al. 1994; Sabatino & Routman 2009; Crowhurst et al. 2011).

Using variable regions of the mitochondrial genome (i.e. cytochrome-*b*, cytochrome oxidase I, NADH dehydrogenase subunit 4) and samples from 16 locations across the species distribution, scientists have identified 32 unique hellbender haplotypes and between 0.7 and 5.4% sequence divergence among populations (Sabatino & Routman 2009). Although these numbers are lower than what is typically observed in other taxa, the data demonstrate that genetic variation within hellbenders is greater than initially believed. Microsatellite data further supports moderate levels of genetic variability in hellbenders and suggests that even geographically close populations can exhibit significant levels of differentiation (Crowhurst et al. 2011). Such progression in molecular data demonstrates that as technology advances so does our ability to detect differentiation and delineate the hierarchical levels of intraspecific diversity, including the structuring that occurs at the among-, between-, and within-population levels.

While previous studies have focused on broad-scale patterns that give a coarse perspective of genetic relationships among hellbenders, I analyzed the fine-scale genetic patterns occurring within Missouri streams. I was able to investigate how gene flow and dispersal influence habitat patch connectivity, and how movement between habitat patches shapes within-stream genetic patterns. In addition to evaluating fine-scale genetic relationships between hellbenders at the habitat patch level, I also evaluated differential dispersal in sexes (males/females) to assess whether one sex contributes more to gene flow than the other. Lastly, I conducted a preliminary investigation into heterozygosity fitness correlations (HFC) in order to evaluate the role of genetic

diversity in offspring survival and provide insight about potential causes of low juvenile recruitment. The resulting information is beneficial to the Missouri Department of Conservation and the St. Louis Zoo, who in an effort to augment hellbender numbers, have invested in a captive breeding program in conjunction with the U.S. Fish and Wildlife Services, U.S. Forest Service, and National Park Service. Findings from my study aid in hellbender recovery by serving to identify appropriate locations for the release of propagated hellbenders.

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CHAPTER 2

Gene flow in the hellbender (*Cryptobranchus alleganiensis*) and the implications for restorative release

Abstract

Understanding how populations are genetically and demographically connected is beneficial for species management, since gene flow and dispersal contribute to genetic diversity and population persistence. For hellbenders (*Cryptobranchus alleganiensis*), an aquatic salamander species experiencing dramatic declines in population size, fine-scale (i.e. within river) patterns of genetic diversity and gene flow are not well understood. Given that hellbenders are habitat specialists that exhibit extreme site fidelity and low vagility, it can be hypothesized that gene flow is restricted among the several, discrete habitat patches within a river. Using 15 polymorphic microsatellite loci and 497 hellbenders from four Missouri rivers, I assessed fine-scale patterns of genetic diversity in order to infer population connectivity and aid in population management. Results indicate moderate levels of genetic variation (H_O = 0.66—0.78) with little differentiation among habitat patches (avg. F_{ST} = 0.002) and no genetic isolation by distance. My data suggests that populations within rivers are panmictic with substantial amounts of gene flow occurring between even the most distantly located habitat patches (> 100km). Such data is useful for hellbender management, especially in terms of making informed decisions regarding restorative releases of captively propagated individuals.

Introduction

Demographic stability and genetic diversity are two important factors to consider when making management decisions for species of conservation concern. In addition to being characterized by small numbers, endangered populations are expected to exhibit diminished genetic variation as a result of inbreeding and genetic drift (Frankham 1995). The deterioration of genetic diversity is further exacerbated when the species habitat is marked by discontinuity where movement between isolated habitat patches is restricted (Wright 1943). Once isolated populations begin to differentiate, separate and individualized management plans may be required (reviewed in, Fraser & Bernatchez 2001), complicating conservation efforts.

The mechanism that prevents isolation, and drives population (demographic and genetic) connectivity is dispersal. Dispersal, as long as it is followed by successful reproduction, provides a buffer against population differentiation by facilitating the movement of alleles between populations (i.e. gene flow; Slatkin 1985). Gene flow, by contributing to population connectivity, helps to maintain genetic diversity, and thus, promotes the adaptive potential of species (Reed & Frankham 2003).

The hellbender (*Cryptobranchus alleganiensis*), an elusive, long-lived and fully aquatic salamander species unique in its behaviors and life history, was once abundant throughout large portions of the mid-eastern and eastern United States. However, in the past few decades, hellbender populations have undergone precipitous declines throughout the distribution (Gates et al. 1985a; Pfingsten 1989; Trauth et al. 1992;

Wheeler et al. 2003; Foster et al. 2009; Burgmeier et al. 2011b). In the state of Missouri, the only location where both hellbender subspecies (eastern hellbender, *C. a. alleganiensis*, and Ozark hellbender, *C. a. bishopi*) can be found, population sizes have decreased by greater than 75% (Wheeler et al. 2003). In an effort to augment hellbender numbers, the Missouri Department of Conservation and the St. Louis Zoo have invested in a captive breeding program in conjunction with the U.S. Fish and Wildlife Services, U.S. Forest Service, and the National Park Service. Before propagated individuals can be released back into the wild, a more detailed understanding of population connectivity, and population genetic structure, is needed as current knowledge regarding dispersal and gene flow is lacking.

From recent studies, we know that clear genetic structuring occurs at two hierarchical levels, with genetic differentiation identified between the eastern and Ozark subspecies and between river basins within subspecies (Templeton et al. 1990; Sabatino & Routman 2009; Crowhurst et al. 2011). However, no assessments regarding genetic structure at the population-level (i.e. within river) have been attempted. Based on broad-scale genetic observations, populations within rivers appear to be panmictic (Templeton et al. 1990; Routman 1993). Yet, observations based on movement in adult hellbenders would conflict with an assertion of panmixia, and would instead indicate that additional genetic structuring is present.

Telemetry and mark-recapture studies have found that adult hellbenders are rather sedentary, exhibiting extreme fidelity to a small home range (Nickerson & Mays 1973; Peterson & Wilkinson 1996). Home range sizes in Missouri are especially small,

with an average of 28m^2 for females and 81m^2 for males (Peterson & Wilkinson 1996). In other regions, however, home range sizes have been found to be substantially larger (1545m^2, Burgmeier et al. 2011a). Additionally, hellbenders are habitat specialists that require cool, swift flowing streams with ample rock cover (Smith 1907; Fobes 1995; Bodinof et al. 2012b). In Missouri streams, these riffle habitats are often isolated from one another (> 1 km), with distances between habitat patches being typically greater than maximum distances observed in hellbender movement (Nickerson & Mays 1973; Gates et al. 1985b; Burgmeier et al. 2011a; Bodinof et al. 2012a). Essentially no data is available regarding larval and or young juvenile dispersal.

Therefore, a fine-scale assessment of genetic structuring is needed to elucidate how population decline, life history, and local environments impact connectivity between hellbender habitat patches within a single stream. To gain a more thorough knowledge of population connectivity, I will address the following questions: (1) Do Missouri hellbender populations exhibit fine-scale structuring? In other words, are within-stream populations genetically homogenous or are they differentiated? (2) Do levels of diversity differ between habitat patches within a single stream? (3) What do these results suggest about population connectivity and what implications does connectivity have for species management?

Due to the patchiness of hellbender habitat and low vagility of adults, I expect to observe significant differentiation between spatially discrete habitat patches. In addition, if a stream is divided into discrete subpopulations which possess their own distinct gene pools, diversity would appear to be high when these subpopulations are

pooled together even if diversity within subpopulations is low. Therefore, I anticipate that genetic diversity will be high within streams but low within patches.

Methods

Collection and laboratory procedures. Hellbender tail clippings (n = 497) were collected over a 7 year period (2002—2011) by the Missouri Department of Conservation. Upon capture, hellbenders were categorized into an estimated age class (either adult or juvenile) based upon size (i.e. snout-vent length measurements). Samples were collected from four Missouri rivers in total, with each subspecies represented by two rivers (Table 1). For the eastern hellbender, samples originated from the Niangua and Big Piney Rivers; Ozark hellbender samples were obtained from the North Fork of the White River and the Current River (Figure 1). The total length of habitat (measured as the stream distance between the most upstream site and the most downstream site), and the number of habitat patches along this length, varied among rivers (Table 2). Total habitat lengths ranged from 29 to 169 km, and lengths between habitat patches within rivers ranged from 1.7 to 6 km.

Samples were stored frozen in ethanol. Extractions were carried out using Qiagen DNeasy kits (Qiagen, Valencia, CA) following the manufacturer's instructions. Extracted DNA was amplified using the polymerase chain reaction (PCR) and four microsatellite multiplex arrangements (m1a, m1b, m2, and m3). In total, I used 15 polymorphic primer pairs (Johnson et al. 2009; Unger et al. 2010), each of which had been fluorescently labeled on the forward primer (Table 3). Reactions were carried out

in 8µl volumes according to the protocol outlined in the Qiagen multiplex kit (adjusted for a reduced volume), incorporating approximately 7.5ng DNA and 0.65µl bovine serum albumin (BSA). Amplifications were performed on Eppendorf ep (Eppendorf, Hamburg, Germany) thermocyclers and were initiated at 95°C for 15 min, followed by 35 cycles of: 94°C for 30 sec, multiplex specific T_A (Table 3) for 90 sec, 72°C for 60 sec. A final extension of 60°C was carried out for 30 min. Positive and negative controls were included in all reactions to ensure allele scoring consistency and absence of contamination, respectively.

Fragment analysis was performed at the University of Missouri DNA Core facility on an ABI 3730xl DNA analyzer (Applied Biosystems), where fragment peak size was standardized by the addition of LIZ 600 (Genescan). Resulting chromatograms were visualized and genotyped in GENEMARKER software, version 1.95 (Softgenetics). After all samples were genotyped, a genotyping error rate was calculated by repeating approx 8% of samples, all of which were chosen randomly.

Statistical analyses. Because Crowhurst et al. (2011) identified genetic differentiation among all rivers included in this study, and because the goal of this study was to investigate fine-scale (or within river) patterns of genetic diversity, analyses for all four rivers were conducted separately, unless otherwise noted.

To test if the loci have sufficient power to differentiate individuals, a probability of identity, or $P_{(ID)}$ test (Paetkau et al. 1995) was performed in GENALEX v. 6.41 (Peakall & Smouse 2006). Significance for $P_{(ID)}$ was set at $p \leq 0.001$ following the suggested

threshold proposed by Waits et al. (2001). Resulting values determine the minimum number of loci each sample must be successfully genotyped at to reliably identify individuals even among siblings. Samples genotyped at less than 9 loci were excluded from analyses.

Prior to pooling samples across years, I needed to ensure that allelic diversity remained constant across years. To do so, I performed an Analysis of Molecular Variance (AMOVA) in ARLEQUIN v. 3.5 (locus by locus, 1,000 permutations; Excoffier et al. 2005). No significant temporal genetic variation was observed (< 0%, p-value = 1.00), and thus samples from all years were pooled for subsequent analyses.

I tested for deviations from genotype frequencies expected under Hardy-Weinberg equilibrium and for linkage disequilibrium in GENEPOP v. 4.0.10 (Rousset 2008). Because these tests involve multiple comparisons, a Bonferroni correction (Rice 1989) was applied to decrease the chance of Type I error. I tested for the presence of null alleles using MICROCHECKER v. 2.2.3 (Van Oosterhout et al. 2004). The program LOSITAN (Antao et al. 2008) was used to test for evidence that the loci conform to the general expectation of neutrality, where the presence of an F_{ST} outlier would indicate nonconformity, suggesting the influence of selection. Analyzing all four rivers at once with each river representing a single population, I had the program estimate a forced F_{ST} and an F_{ST} under neutral expectations using 100,000 simulations and both the Infinite Allele Model (IAM) and Stepwise Mutation Model (SMM). The IAM (Kimura & Crow 1964) assumes that all mutations introduce completely novel alleles into a population—

each of which resulted from a unique (potentially an "infinite") number of repeat units. The SMM (Kimura & Ohta 1978) assumes that mutations result from the addition or subtraction of a single repeat unit, such that mutated alleles shift slightly from the original allele in a "step-wise" fashion. I considered a locus to be under the influence of selection only if it was observed to be a potential candidate for selection in both the SMM and IAM models at the 95% confidence level.

To address potential concerns involving sampling bias—specifically, whether or not samples represent highly related individuals —I used COANCESTRY (Wang 2011) to identify both population mean relatedness (*R*) and relatedness between all possible pairs of hellbenders within river. A number of *R* metrics exist; determining which measure performs best for a given data set is oftentimes difficult and depends on how well the data fit the assumptions of the specific measure, as well as the number of microsatellite markers utilized and number of samples to be compared. I used COANCESTRY's simulation option to circumvent this difficulty by creating 100 individuals for whom the program randomly assigned genotypes according to known allele frequencies (obtained in GENALEX; Peakall & Smouse 2006). This allowed me to compare true relatedness values for these simulated individuals with estimated relatedness values provided by the differing *R* metrics available in COANCESTRY, which included two maximum likelihood estimators proposed by Wang (2007) and Milligan (2003), as well as five moment estimators proposed by Wang (2002), Lynch and Ritland (1999), Ritland (1996), Li et al. (1993), and Queller and Goodnight (1989). The best *R* metric exhibits the lowest variance, lowest standard errors, and highest correlation with

the true (simulated) relatedness value (Wang 2011). The estimator that matched this criteria for my data was Wang's (2007) triadic likelihood estimator (Var = 0.05—0.06, SE = 0.01—0.03, correlation coefficient r = 0.80—0.88) abbreviated as TrioML hereafter. TrioML is a relatedness coefficient that improves upon a pairwise relatedness calculation by incorporating a third individual. This third individual acts as a reference, helping to differentiate alleles identical by descent and alleles identical by state (Wang 2007).

Within river genetic diversity. I used GENALEX (Peakall & Smouse 2006) to calculate heterozygosity and allelic richness. I further investigated allelic richness by adjusting for differences in sample size (i.e. rarified allelic richness) among rivers and among habitat patches within a river, and looking for differences in private alleles with HPrare (Kalinowski 2005).

Within river population genetic structure. Using AMOVA, I evaluated genetic partitioning at the following fine-scale hierarchical levels: among rivers supporting the same subspecies, among habitat patches within rivers, among individuals within habitat patches, and within individuals. The AMOVA was performed in ARLEQUIN using the locus-by-locus calculation and 1,000 permutations.

To estimate genetic distances between habitat patches, I compared differences in allelic frequencies between patches using the permutation calculation for population pairwise F_{ST} and 10,000 permutations in ARLEQUIN (Excoffier et al. 2005). Because sampling was uneven among patches within a stream, I limited the pairwise F_{ST}

calculation to only those patches that supplied ≥ 5 samples to ensure a more accurate comparison. The resulting F_{ST} matrix was utilized in a Mantel test, in conjunction with associated stream distances, so that the correlation between estimates of genetic distance and geographical stream distance (i.e. isolation by distance) could be evaluated in GENALEX (Peakall & Smouse 2006).

An additional Mantel test was performed in the R package ECODIST (Goslee & Urban 2007). This test plotted relatedness values, obtained in COANCESTRY, between pairs of hellbenders against the pair's associated stream distance (km) to illustrate how related individuals are distributed in space.

The program STRUCTURE (Pritchard et al. 2000) is commonly used for detecting genetic structuring among subpopulations. Although the program works well for many empirical data sets, evidence suggest that it is less robust when differentiation between "subpopulations" is low (F_{ST} values below ~ 0.03, Latch et al. 2006). Preliminary F_{ST} results from ARLEQUIN indicate that STRUCTURE would be inappropriate for this data set. As a result I chose to use an alternate program, ADEGENET (Jombart 2008) implemented in R v. 2.15.1 (R Core Team 2012), to evaluate the number of distinct genetic clusters (*k*) within rivers. ADEGENET applies a discriminant analysis of principal components (DAPC) approach with a Bayesian Information Criterion (BIC) to partition existing genetic structure. The initial step in this method (principal component) transforms the data so that it is "perfectly uncorrelated", thus ensuring that *k* is evaluated without any constraints imposed by linkage disequilibrium (if present). In

addition to performing an ADEGENET analysis for each of the four rivers individually, I performed a broader-scale analysis of structure by including all four rivers in a single analysis. I did this to ensure that the ADEGENET approach is robust for the analyzed populations, as evidence for genetic structure in hellbenders at the Missouri scale (between subspecies and between rivers) were summarized in Crowhurst et al. (2011) and could offer a point of comparison for efficacy.

Genetic consequences of population decline. Documented declines in hellbender population size suggest that Missouri populations may have experienced a genetic bottleneck. Populations that have undergone a recent reduction in effective population size (N_e) will exhibit a reduction in allelic diversity, but because heterozygosity levels decrease at slower rate than the loss of alleles (Nei et al. 1975), heterozygosity levels appear excessive when compared to the number of alleles present (Cornuet & Luikart 1996; Luikart & Cornuet 1998). I analyzed the data for heterozygosity excess, which would be indicative of a relatively recent (within ~ 2-4N_e generations) bottleneck event, using the program BOTTLENECK (Piry et al. 1999). I adjusted the parameters to 10,000 replications and utilized the infinite allele model (IAM, Kimura & Crow 1964), the strict stepwise mutation model (SMM, Kimura & Ohta 1978), and the two-phase model (TPM, Di Rienzo et al. 1994). While the SMM model is recognized as the most conservative parameter, IAM is prone to Type I error—often detecting false bottlenecks (Luikart & Cornuet 1998). The more flexible TPM model is similar to the SMM but differs by following empirically observed mutation patterns and allowing for occasional multistep mutations (Di Rienzo et al. 1994). Although all three models were implemented, I

believe TPM provides the most reliable assessment of a recent bottleneck as it is proposed to be the most realistic model (Di Rienzo et al. 1994; Piry et al. 1999). I set the parameters for TPM according to the author's suggestion with 95% adherence to SMM and the variance of multistep mutations as 12.

Results

The observed error rate in genotyping across all loci equaled 1.7%. Three of the four rivers deviated from Hardy-Weinberg equilibrium after Bonferroni correction due to a significant deficit in heterozygosity at a single locus: Big Piney River, Call 347 ($p < 0.0001$); Current River, Call 266* ($p < 0.0001$); North Fork of the White River, Cral 4 ($p = 0.0018$). Although heterozygosity values for the Niangua River appeared to conform to expectations under Hardy-Weinberg equilibrium, Call 266* was out of equilibrium in this river ($p = 0.0014$), exhibiting heterozygosity deficit ($p < 0.0001$).

Several pairs of loci exhibited signs of linkage disequilibrium after Bonferroni correction. However, pairs varied between rivers: Big Piney River, Cral 13 and Cral 9 ($p = 0.0014$); Current River, Call 127 and Call 204 ($p < 0.001$); North Fork of the White River, Cral 4 and Call 26 ($p = 0.0017$), Cral 17 and Call 347 ($p = 0.0032$), and Call 127 and Call 341 ($p = 0.0032$). Because of the inconsistency across rivers, no loci were removed from analyses as a result of linkage disequilibrium.

Four of the 15 genotyped microsatellite loci presented F_{ST} outliers at the 95% confidence level in LOSITAN, thus indicating a potential influence of natural selection. These "non-neutral" loci identified were some of the least polymorphic, typically

possessing less than 3 alleles in each river—most of which were unique to the river and/or subspecies. Inspection of the published sequences for these loci revealed no high similarity to any described genes in the National Center for Biotechnology Information (NCBI) GenBank database. Although it is unlikely that microsatellite loci themselves would be the target of selection, I removed Cral 4, Cral 9, Cral 13, and Call 351 from additional analyses as their inclusion may have introduced bias.

MICROCHECKER identified a number of loci with null alleles at relatively high frequencies. Call 266* was removed from the Current and Niangua River analyses (null alleles at 13.76% and 7.36%, respectively). Call 347 was removed from the Big Piney River analyses (null alleles at 28.44%). Had Cral 4 remained in the analysis, it would have required removal in the North Fork of the White River due to null alleles (10.77%).

Mean relatedness values (*R*), based upon the TrioML estimator in COANCESTRY, indicated that sampling was likely random with regards to kinship and not biased towards highly related individuals. In fact, relatedness was low in all rivers and only a few instances of parent-offspring, full-sib, and half-sib relationships were identified. The Current River had the fewest number of related individuals. Furthermore, relatedness at the within river level (R = 0.058—0.039) was comparable to relatedness within habitat patches, where $R \leq 0.056$ (Table 4).

Genetic comparisons between rivers. Because a different set of loci remained in the analyses for each river, comparisons of genetic diversity between rivers were based on the entire set of loci genotyped (Table 5). Rarified allelic richness averaged across these 15 loci was similar for North Fork of the White, Big Piney, and Niangua Rivers at 7.0 $\pm$

0.23 alleles per locus. The Current River had higher rarified allelic richness with an average of 10.2 alleles per locus. The Current River also had the highest observed heterozygosity (averaged across all 15 loci) at 0.64. Observed heterozygosity (averaged across all 15 loci) for the other three rivers was again similar (0.58—0.63). The observed heterozygosity was slightly lower than the expected heterozygosity for all rivers except for the Niangua River where the observed heterozygosity matched expected heterozygosity.

Within river genetic diversity and population structure. Using the pruned set of loci (10 for all rivers except the North Fork of the White River which had 11), and making comparisons between habitat patches within a river, HPrare indicated that no habitat patch was more genetically diverse than another. All habitat patches shared similar, if not equal, levels of allelic richness with no particular stream segment (i.e. upstream, midstream, downstream) containing more private alleles.

In agreement with HPrare, AMOVA reveals no significant genetic variation between habitat patches in a single river (EHB: $p = 0.73$; OHB: $p = 0.56$; Table 6). Instead significant amounts of variation are observed among individuals within habitat patches (EHB: 2.29%, $p < 0.01$; OHB: 2.89%, $p = 0.001$), with the greatest amount of variation harbored within individuals (EHB: 81.8%, $p < 0.001$; OHB: 71.43%, $p < 0.001$), as expected.

If dispersal and gene flow are restricted over historical time, I would expect to see differentiation between habitat patches as a result of genetic drift. Results from ARLEQUIN reveal no significant differences in genetic distance (F_{ST}) between any pair of

habitat patches in the Current, North Fork of the White, and Niangua Rivers. One habitat patch within the Big Piney River was significantly different from two of the 7 other habitat patches after Bonferroni correction (p-values ≤ 0.0036). The most distant habitat patches within a river were not more differentiated than habitat patches in close proximity (Table 7).

No significant correlation between genetic distance (F_{ST}) and geographic distance was observed in any river using the Mantel tests performed in GENALEX ($r = 0.12—0.27$, $p > 0.05$), indicating no statistical support for isolation by distance. Although not statistically significant, inspection of the Mantel trend lines do show a slight trend for increasing dissimilarity with increasing distance in all rivers, except the North Fork of the White River (Figure 2).

Similarly, no significant correlation was observed between relatedness and geographic distance in the ECODIST Mantel test ($r = -0.03$ — -0.01, $p > 0.05$; Figure 3). A variety of relatedness values are presented at each distance interval—including at the smallest distance interval (0 km)—indicating that some highly related individuals as well as completely unrelated individuals are occupying the same habitat patch. A general lack of a perceptible pattern in kinship across the stream length is well illustrated in the associated graphs (Figure 3).

ADEGENET proved to be useful in delineating genetic structure among the four rivers analyzed (Figure 4). In agreement with the Crowhurst et al. (2011) findings, four distinct populations were identified—each of which represented one of the four rivers analyzed with 100% of individuals assigning to the river of origin. However, support for

fine-scale (or within river) population substructure was not found in any river. Results from ADEGENET, like all other results, suggest that each river contains a single genetic cluster, and that no habitat patch represents a unique subpopulation or a differentiated genetic pool.

Genetic consequences of population decline. No river exhibits the signatures of a recent genetic bottleneck, except under the IAM model where all rivers show significant heterozygosity excess at p-values ≤ 0.05 with the Wilcoxon one-tailed test. Because IAM is prone to erroneous detection of bottlenecks, and no support for a genetic bottleneck was detected with the SMM or TPM models, I do not consider this result to be informative.

Discussion

The goal of my study was to examine the fine-scale patterns of genetic diversity in endangered populations of Missouri hellbenders to infer population connectivity and aid in management decisions. I was particularly interested in determining if each river represents a single, patchily distributed population or, if alternatively, the river contained one homogenous population with no genetic sub-structuring. Given the observed limited dispersal abilities of the species and the relatively large distances between suitable habitats, I hypothesized that each river would contain several unique subpopulations. My results do not support this hypothesis.

Gene flow and inferred dispersal. There appears to be a significant amount of gene flow occurring within all rivers, such that there is no detectable genetic differentiation and/or isolation between even the most distant habitat patches in a river. In fact, no genetic population sub-structuring was observed within any of the four rivers analyzed, suggesting that each river is a panmictic population—a conclusion that aligns well with the observations of Templeton et al. (1990) and Routman (1993), but contradicts known hellbender movement tendencies based upon behavioral data and direct observation. However, a few points of discussion are worthy of acknowledgement here. First, it is important to note that movement can be categorized into dispersal and migration, where dispersal indicates movement from the natal habitat patch to future reproductive patches and migration is any movement from one habitat patch to another patch following natal dispersal and first reproductive event (i.e. movement between breeding sites). Additionally, discrepancies between observational measures of dispersal and those measures inferred from estimates of gene flow are not uncommon, as these direct and indirect methods actually measure differing components of population connectivity (demographic vs. genetic connectivity) at differing time scales ("instantaneous" vs. ecological timescales) (Slatkin 1987; Lowe & Allendorf 2010). Because these hellbender populations have experienced declines in population size, connectivity may be overestimated, as temporal and very recent shifts in the rate of gene flow may not have yet manifested in the observed genetic structure due to associated lag times (Nei & Chakravarti 1977).

Given the data presented in my study, hellbender movement appears to be more complex than previously thought, suggesting that our current knowledge of dispersal is poor. Previous studies investigating hellbender movement patterns have utilized larger (i.e. older) individuals which exhibit extreme site fidelity, garnering the hellbender recognition as a "sedentary" species. However, dispersal often occurs during the juvenile stage in many salamander species (Semlitsch 2008). Thus, significant amounts of movement may be occurring in either larval or juvenile hellbenders at a time when size and detectability would make direct observation difficult. If dispersal occurs during one of these younger life stages, studies utilizing older hellbenders cannot address dispersal as the time frame for such an event has already passed.

Given the scope of my study, the mechanisms facilitating dispersal and/or movement can only be speculated. Habitat patch connectivity may be largely promoted via environmental characteristics, and the interaction between those characteristics and hellbender movement behavior. As obligate stream species, hellbenders occupy highly dynamic, aquatic environments that are susceptible to both change and degradation. Within a hellbender lifetime, several perturbations have likely occurred within the river and as a result, these disturbances have subsequently altered not only the quality of habitat patches, but also the distribution of individuals (e.g. floods). Changes in stream depth, velocity, and discharge may affect hellbender settlement and movement, and may facilitate 1) permanent dispersal from the natal habitat patch to future reproductive patches and/or 2) infrequent migrations after initial settlement.

Evidence suggests that larval stream salamanders are susceptible to downstream drift and current-mediated advection (Johnson & Goldberg 1975; Stoneburner 1978; Bruce 1985, 1986; Petranka & Sih 1986; Petranka et al. 1987; Thiesmeier & Schuhmacher 1990). While evidence would suggest that hellbender dispersal is also commonly aided by the direction of stream flow (Gates et al. 1985b; Bodinof et al. 2012a), movement solely in the downstream direction should result in higher genetic diversity at downstream sites and lower diversity at upstream sites. Yet, all habitat patches in this study—regardless of position—exhibited similar levels of variation. Differentiation, however, may be buffered if passive dispersal is subsequently accompanied by a more active movement with individuals choosing when and where to enter or exit the current (Johnson & Goldberg 1975; Stoneburner 1978; Thiesmeier & Schuhmacher 1990), and demonstrating purposeful upstream orientations (Bruce 1986; Ferguson 1998). For hellbenders, research demonstrates that juvenile and adult hellbenders orient downstream but are also capable of moving against the current towards upstream habitats (Gates et al. 1985b; Peterson 1987; Bodinof et al. 2012a).

Genetic diversity. While the hellbender populations analyzed in my study are the same populations analyzed in Crowhurst et al. (2011)—where broad-scale genetic patterns were evaluated between the Ozark and Eastern subspecies, using fewer samples—greater estimates of genetic diversity are observed in my study. The disparity likely results from the differing numbers and sets of microsatellite markers utilized; Crowhurst et al. (2011) used only the Johnson et al. (2009) primers which proved to be, on average,

less variable than the Unger et al. (2010) primers which were incorporated into my study.

Of the four rivers analyzed, the Current River exhibited the greatest amount of genetic diversity. Habitat attributes of the Current River may provide potential explanations for this observation. When compared to the North Fork of the White, Big Piney, and Niangua Rivers, the Current River contains the longest stretch of hellbender habitat (Table 2), is the most spring-fed (Vineyard & Feder 1974), and has the highest stream discharge (http://mdc.mo.gov/landwater-care/stream-and-watershed-management/missouri-watersheds). These characteristics may influence gene flow and genetic diversity, especially if stream flows help facilitate dispersal and/or movement, and if there is local adaptation occurring at some of the Current River spring locations.

Given that Missouri populations have undergone dramatic declines in population size, the presented levels of diversity are still quite extraordinary and no indication of a genetic bottleneck was detected. Although demographic instability often results in a reduction of genetic variation, evidence suggests that this is not necessarily true for all populations as species longevity may help slow the process of genetic erosion. Results from my study are congruent with the findings of Kuo and Janzen (2004), Pittman et al. (2011), and Lippe et al. (2006) who suggest that in declining populations of long-lived species, the retention of genetic variability is attributable to the high proportion of mature individuals remaining in the population, as they represent multiple generations and various decades of reproduction. The majority (93%) of hellbenders sampled in this study were presumed to be adults given their size and/or number of times recaptured.

Adult hellbenders are theoretically 5 to 30 years of age, based on average age of reproductive maturity and known longevity (Taber et al. 1975; Peterson et al. 1983).

Larval samples are absent in this study, making it difficult to draw conclusions about current rates of gene flow. Genetic patterns based solely upon adult genotypes may be reflective of patterns from several decades past, and are not likely to help us understand how standing genetic variability is being reallocated in present-day progeny, if at all. Nevertheless, I assert that in the populations studied, newer generations of hellbenders would still exhibit robust variation as the observed levels of heterozygosity, allelic richness, as well as low relatedness—even at the habitat patch level—would indicate that matings should be occurring between genetically diverse individuals. Thus, I believe nonrandom mating, and inbreeding depression, are not yet major problems in these populations.

Management/conservation implications. Results indicate that Missouri hellbender populations are currently doing remarkably well from a genetic perspective. However, in populations of long lived species with overlapping generations, a disproportionate amount of the genetic diversity may be harbored in the oldest cohorts. If this is true for the populations analyzed here, much of the observed genetic diversity could be lost as these individuals cease reproduction and eventually die-off. This is especially problematic given the lack of reproduction and/or juvenile recruitment. My investigations into relatedness reveal only a small proportion of highly related individuals (i.e. parent-offspring, full-sibs, half-sibs). At this point it is unclear whether

the lack of relatedness is due to a lull in reproduction or if some factor is restricting the successful recruitment of juveniles into the population.

To maintain the genetic integrity of hellbenders, and the high levels of diversity observed in my study, multiple individuals—that represent the full spectrum of genetic variation—should be used for propagation (Frankham 1995). If offspring result from a limited set of mating pairs, the genetic potential of the species will be restricted—not only in terms of genetic diversity, but also in their capacity to adapt to future conditions. Because captive reproduction has been so successful, hellbender propagation should transition from being numbers-driven to being genetically informed and diversity-driven. Such a shift would help to avoid the deleterious consequences associated with an unintentional founder effect, which may be introduced into our genetically healthy population upon the mass release of propagated individuals.

Furthermore, propagated individuals should be returned to the river of origin (i.e. the broodstock's river of origin) following the recommendations of Bodinof et al. (2012b). Given the genetic homogeneity demonstrated within rivers, releases do not have to be restricted to a particular habitat patch as long as the strict recommendation for river origin is met. If adequate resources (i.e. cover, den sites, etc.) are available to support additional individuals, attention should be placed on augmenting the number of hellbenders present in less-populated habitat patches so as to encourage future mating opportunities.

The continued genetic health of the population will be largely dependent on how well the species is managed. The success of the captive reproduction program at the

Saint Louis Zoo is promising. With a carefully chosen, genetically diverse broodstock, and effective translocations of headstarted offspring (i.e. restorative releases), it appears that this endangered species may be able to recover to a robust population size. However, to maintain a stable future trajectory, issues in the environment that have contributed to the observed population decline must be identified, addressed, and resolved.

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Table 1. Number of Ozark hellbender (*C. a. bishopi*) and Eastern hellbender (*C. a. alleganiensis*) samples collected from each of four rivers in Missouri**.**

Population	N
Ozark hellbenders	
Current River	92
North Fork of the White River	159
Eastern hellbenders	
Big Piney River	131
Niangua River	115

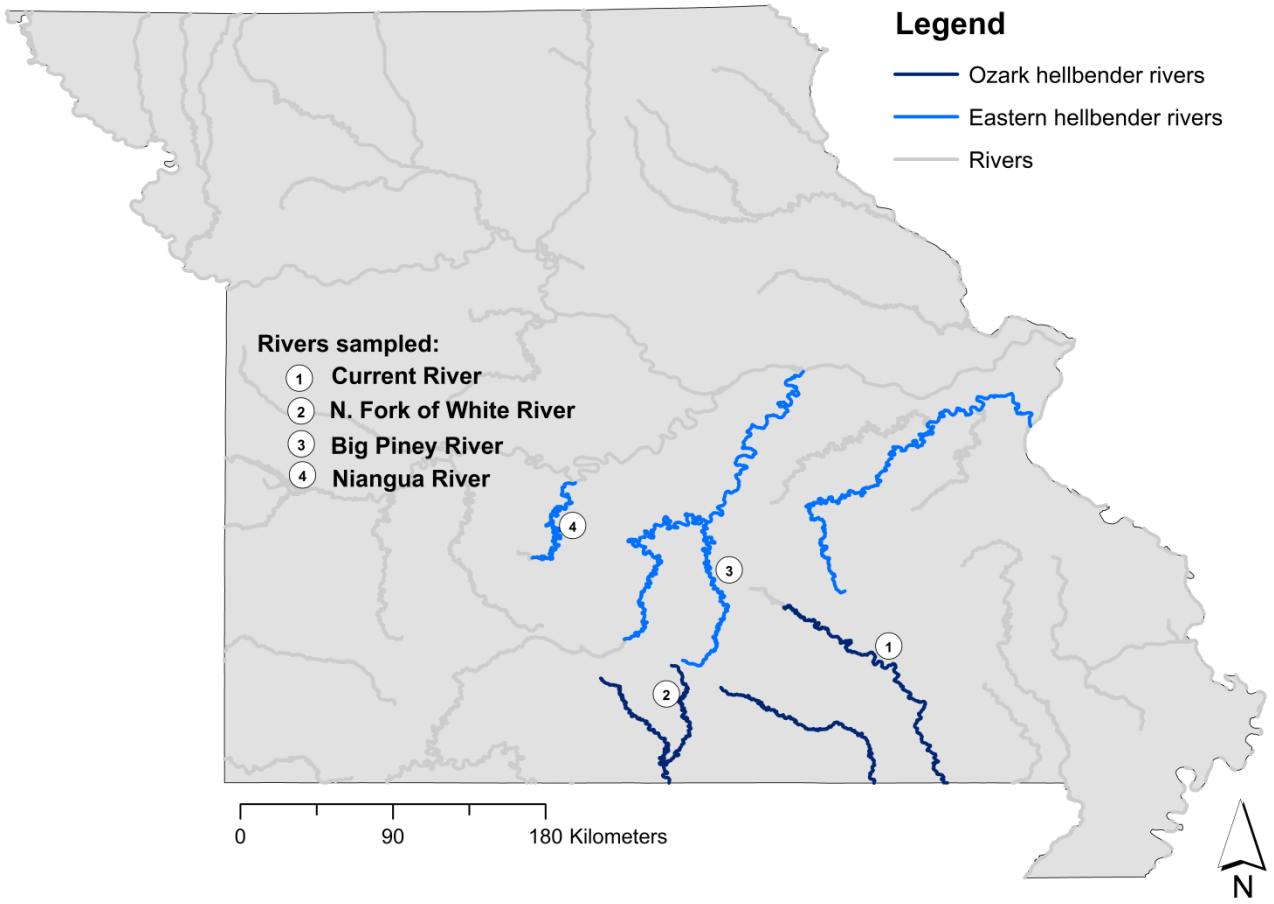


Figure 1. Map indicating hellbender (*Cryptobranchus alleganiensis*) distribution in Missouri, including the four rivers where samples for my study were collected from Ozark hellbenders (*C. a. bishopi*) and eastern hellbenders (*C. a. alleganiensis*).

Table 2. Extent and characteristics of hellbender (*C. alleganiensis*) habitat within the four rivers analyzed.

River	Total habitat length* (km)	Average distance between habitat patches (km)	No. of habitat patches	No. of patches with ≥ 5 hellbenders
Ozark hellbender				
Current	169	6	25	6
North Fork of White	29	1.7	21	7
Eastern hellbender				
Big Piney	108	4.5	28	7
Niangua	49	2.4	18	6

*total habitat length = distance from the most upstream site to the most downstream site, including the intervening non-suitable habitat in between

Table 3. Microsatellites (n= 15) used for genotyping hellbender populations (*C. a. bishopi & C. a. alleganiensis*). Microsatellites beginning with Cral were designed by Johnson et al. (2009) and those beginning with Call were designed by Unger et al. (2010).

Locus	Repeat motif	Primer sequence	Label	Base pair range	No. alleles observed
Multiplex 1a, T_A = 60°C					
Cral 13	$(GT)_{12}$	F: TCAACGTATAAAGTAACATAAAACCAA R: GGCTCAGAATGTCTAGGTGGTC	FAM	118-134	7
Call 26	$(GATA)_{18}$	F: CATAATGGTAATAGCTGCATGG R: CCTTGTTCCAGATTCACACC	FAM	176-252	20
Call 127	$(TATC)_{14}$	F: TGAACTGTGGAGTTGGATCG R: CACATATGGGTAGTAACTGCATGG	FAM	336-376	13
Call 351	$(TATC)_{20}$	F: ACATTAATTCTCCTTCCTGTCACC R: CAATCCTAAGGAGGAATTGAAGC	VIC	218-274	15
Call 204	$(GATA)_{21}$	F: TTCGCGAGCATTACTCTACG R: ACGGTCCAGACATTGTTGC	NED	213-281	17
Call 282	$(GATA)_{15}$	F: ACCCGAAAGGGTGGTTTATAG R: TAATGAGCCGTTAGCCCTTG	PET	195-243	13
Multiplex 1b, T_A = 58.5°C					
Cral 17	$(CTAT)_{24}$	F: ATTCCAAGGGGGCTGAATAC R: CGCCTTGATGTAGCTTTTGG	FAM	133-217	22
Cral 10	$(AC)_{14}$	F: GCTCGGATGACAGAGGTTTC R: TGGCAAATTTCATTCTGCTTC	FAM	237-267	14
Cral 4	$(CA)_{10}$	F: AGGGCACCACACAAACAAA R: AAACCCAGAGACATGCTTCC	VIC	161-175	7
Multiplex 2, T_A = 65°C					
Call 232	$(TGTC)_{18}$	F: CGTATGCCTGGCACATAACC R: CCACCATAAGATTCACACTGC	FAM	168-248	20
Call 205	$(GATA)_{20}$	F: TTTGAGCTCTCTTGGCTTATG R: TGGACTCCTTCCCTTTCTCC	VIC	165-209	12
Call 341	$(GATA)_{15}$	F: GCAAGAAGGTGAGCAAGAGG R: CCATCTGAATATACCTGCAATCTG	NED	210-282	18
Call 266*	$(GATA)_{11}$	F: GCATTCTGCAAGCCACTAAA R: AACATTGGGAGGCTGGTATG	PET	211-289	30
Multiplex 3, T_A = 64°C					
Cral 9	$(CA)_{12}$	F: CCCACCCCTAGAGAAGAAGG R: AAGGGACTGTGTGTGTACCTTAGA	VIC	125-135	6
Call 347	$(GATA)_{20}$	F: ACCAGCAGCAACCTTATCTGG R: ACCATGCAGCCGGTAAGC	FAM	181-225	12

T_A = PCR amplification annealing temperature

*indicates that the primer was redesigned from the author's sequences using Primer3 (Rozen & Skaletsky 2000) software to accommodate multiplex arrangement

Table 4. Mean triadic likelihood (TrioML) relatedness values for hellbender (*Cryptobranchus alleganiensis*) populations.

	Within river	**Within habitat patches**		
	Mean relatedness	Mean relatedness	Mean relatedness between females	Mean relatedness between males
Current	0.039	0.038	0.0304	0.0284
North Fork of White	0.056	0.048	0.0394	0.0435
Big Piney	0.058	0.056	0.0508	0.0573
Niangua	0.055	0.041	0.0447	0.0379

Table 5. Genetic summary statistics by locus for each hellbender (*Cryptobranchus alleganiensis*) river where A, A_E, H_O, and H_E represent number of alleles, number of effective alleles, heterozygosity observed, and heterozygosity expected (respectively).

	Current River				North Fork of the White River				Big Piney River				Niangua River			
Locus	A	A_E	H_O	H_E	A	A_E	H_O	H_E	A	A_E	H_O	H_E	A	A_E	H_O	H_E
Cral 13^{S}	4^{S}	1.22^{S}	0.18^{S}	0.18^{S}	2^{S}	1.04^{S}	0.04^{S}	0.04^{S}	3^{S}	1.64^{S}	0.39^{S}	0.39^{S}	2^{S}	1.03^{S}	0.03^{S}	0.03^{S}
Call 26	13	9.81	0.89	0.90	11	4.99	0.83	0.80	10	6.38	0.79	0.84	12	8.39	0.88	0.88
Call 127	9	3.72	0.68	0.73	4	2.27	0.55	0.56	8	5.93	0.76	0.83	6	3.90	0.77	0.74
Call 351^{S}	14^{S}	10.03^{S}	0.92^{S}	0.90^{S}	10^{S}	4.98^{S}	0.82^{S}	0.80^{S}	12^{S}	6.38^{S}	0.79^{S}	0.84^{S}	10^{S}	6.07^{S}	0.85^{S}	0.84^{S}
Call 204	11	7.09	0.81	0.86	11	2.97	0.61	0.66	11	3.90	0.75	0.74	11	7.22	0.85	0.86
Call 282	9	6.11	0.78	0.84	7	3.18	0.70	0.69	6	2.38	0.55	0.58	8	4.18	0.78	0.76
Cral 17	19	11.07	0.91	0.91	7	3.20	0.67	0.69	9	3.27	0.64	0.69	7	5.29	0.77	0.81
Cral 10	10	2.96	0.64	0.66	4	2.04	0.50	0.51	3	1.37	0.29	0.27	3	2.11	0.51	0.53
Cral 4^{S}	3^{S}	1.03^{S}	0.03^{S}	0.03^{S}	**3**^{N,S}	**1.53**^{N,S}	**0.26**^{N,S}	**0.35**^{N,S}	3^{S}	1.77^{S}	0.45^{S}	0.44^{S}	3^{S}	2.12^{S}	0.52^{S}	0.53^{S}
Call 232	14	7.75	0.86	0.87	6	3.01	0.69	0.67	7	5.74	0.85	0.83	6	1.34	0.29	0.27
Call 205	9	4.62	0.73	0.78	12	4.54	0.74	0.78	7	3.17	0.70	0.69	7	4.43	0.78	0.77
Call 341	15	7.53	0.85	0.87	8	4.05	0.75	0.75	8	2.13	0.54	0.53	9	2.04	0.49	0.51
Call 266*	**13**^{N}	**8.19**^{N}	**0.63**^{N}	**0.88**^{N}	13	4.89	0.77	0.80	6	3.63	0.73	0.72	**8**^{N}	**6.61**^{N}	**0.72**^{N}	**0.85**^{N}
Cral 9^{S}	3^{S}	1.02^{S}	0.02^{S}	0.02^{S}	8^{S}	1.00^{S}	0.00^{S}	0.00^{S}	10^{S}	1.38^{S}	0.28^{S}	0.28^{S}	7^{S}	1.48^{S}	0.35^{S}	0.32^{S}
Call 347	7	2.54	0.60	0.61	1	5.20	0.78	0.81	**4**^{N}	**5.90**^{N}	**0.34**^{N}	**0.83**^{N}	2	4.46	0.81	0.78
Average^{A}	10.20	5.65	0.64	0.67	7.13	3.26	0.58	0.59	7.13	3.66	0.59	0.63	6.73	4.05	0.63	0.63
Average^{R}	11.6	6.32	0.78	0.80	8.27	3.67	0.69	0.70	7.50	3.79	0.66	0.67	7.60	4.34	0.69	0.69

Parameters in bold indicate significant deviation from HWE with a bonferroni correction ($p \leq 0.0033$)

^{S} indicates that the locus was removed because it was a candidate for selection ($p \leq 0.05$)

^{N} indicates that the locus was removed due to null alleles observed at a frequency > 7%

^{A} averaged across all 15 loci

^{R} averaged across a reduced number of loci (only those remaining after HWE/null alleles, selection tests)

Table 6. AMOVA revealing hierarchical amounts of genetic variation in hellbender (*C. a. bishopi* & *C. a. alleganiensis*) populations.

Source of variation	Fixation indices	Percent variation	P-value
Ozark hellbenders			
Among rivers (ϕ_{IS})	0.26	25.46	< 0.001
Among habitat patches within rivers (ϕ_{SC})	0.003	0.22	0.559
Within habitat patches (ϕ_{CT})	0.39	2.89	0.001
Within individuals (ϕ_{IT})	0.29	71.43	< 0.001
Eastern hellbenders			
Among rivers (ϕ_{IS})	0.16	16.08	< 0.001
Among habitat patches within rivers (ϕ_{SC})	-0.002	-0.17	0.727
Within habitat patches (ϕ_{CT})	0.03	2.29	0.004
Within individuals (ϕ_{IT})	0.18	81.80	< 0.001

Table 7. Genetic differentiation (F_{ST}) between hellbender habitat patches within the A) Current (above the diagonal) and Niangua (below the diagonal) Rivers, and within the B) North Fork of the White (above the diagonal) and Big Piney (below the diagonal) Rivers. F_{ST} values significant after Bonferroni correction are indicated in bold.

A)

CU / NI	Habitat 1	Habitat 2	Habitat 3	Habitat 4	Habitat 5	Habitat 6
Habitat 1	--	0.002	0.003	0.001	0.008	-0.004
Habitat 2	-0.011	--	0.030	0.009	0.007	0.006
Habitat 3	-0.029	-0.017	--	0.016	-0.005	-0.009
Habitat 4	-0.013	0.000	-0.005	--	-0.009	-0.019
Habitat 5	-0.013	-0.005	-0.020	0.004	--	-0.016
Habitat 6	-0.001	0.007	-0.007	0.001	-0.012	--

B)

NF / BP	Habitat 1	Habitat 2	Habitat 3	Habitat 4	Habitat 5	Habitat 6	Habitat 7
Habitat 1	--	-0.006	-0.025	0.007	-0.021	-0.007	0.002
Habitat 2	**0.059**	--	-0.031	-0.019	-0.003	-0.003	-0.000
Habitat 3	0.007	0.030	--	0.010	-0.016	-0.011	-0.001
Habitat 4	0.004	**0.045**	-0.008	--	-0.003	0.015	0.019
Habitat 5	-0.013	0.033	-0.019	-0.013	--	-0.012	-0.003
Habitat 6	0.007	0.047	0.007	0.007	-0.011	--	0.004
Habitat 7	-0.006	0.038	0.003	-0.003	-0.003	0.016	--

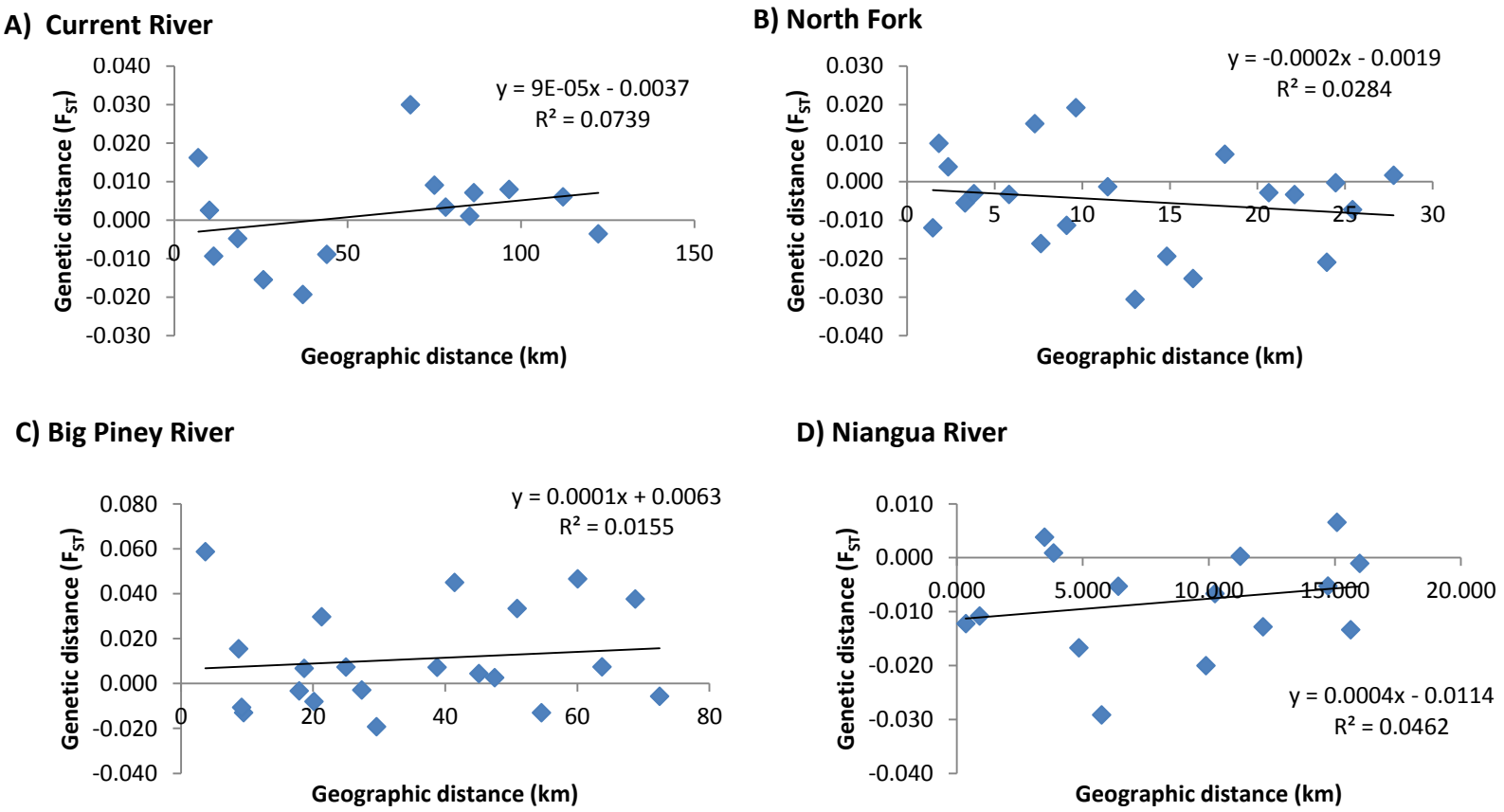
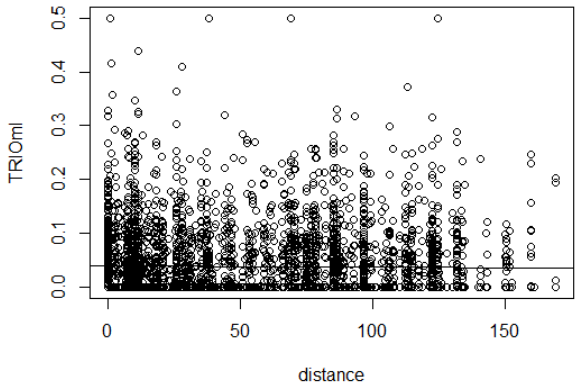
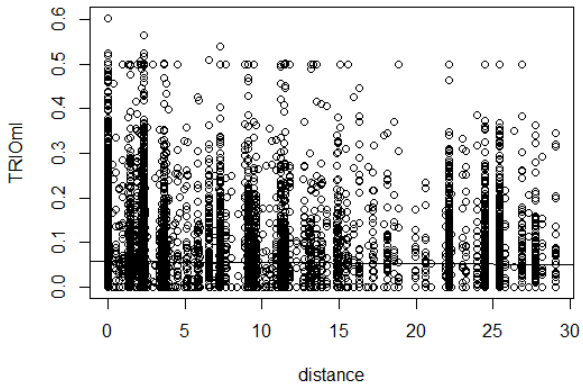


Figure 2. Isolation by distance results for all four rivers, where the relationship between genetic distance (F_{ST}) and geographic distance (stream distance between habitat patches in km) was evaluated using Mantel tests.

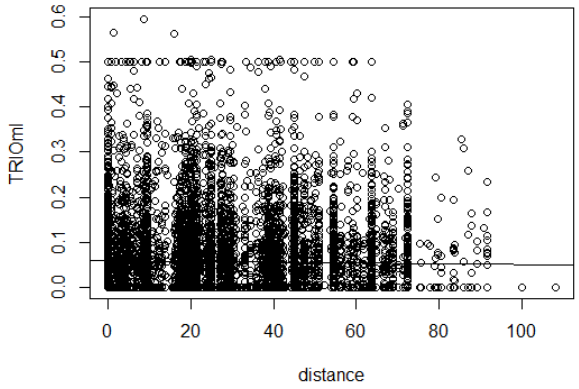
Current River (r = -0.0187, p-value= 0.24)



North Fork River (r = -0.0271, p-value= 0.10)



Big Piney River (r= -0.0205, p-value= 0.10)



Niangua River (r = -0.0125, p-value= 0.43)

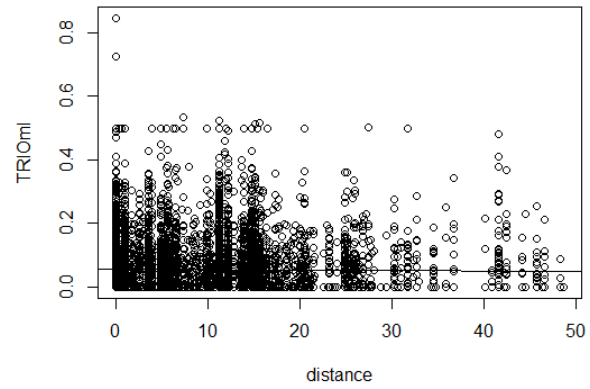


Figure 3. Relationship between hellbender (*Cryptobranchus alleganiensis*) relatedness (TrioML) and spatial proximity (stream distance in km).

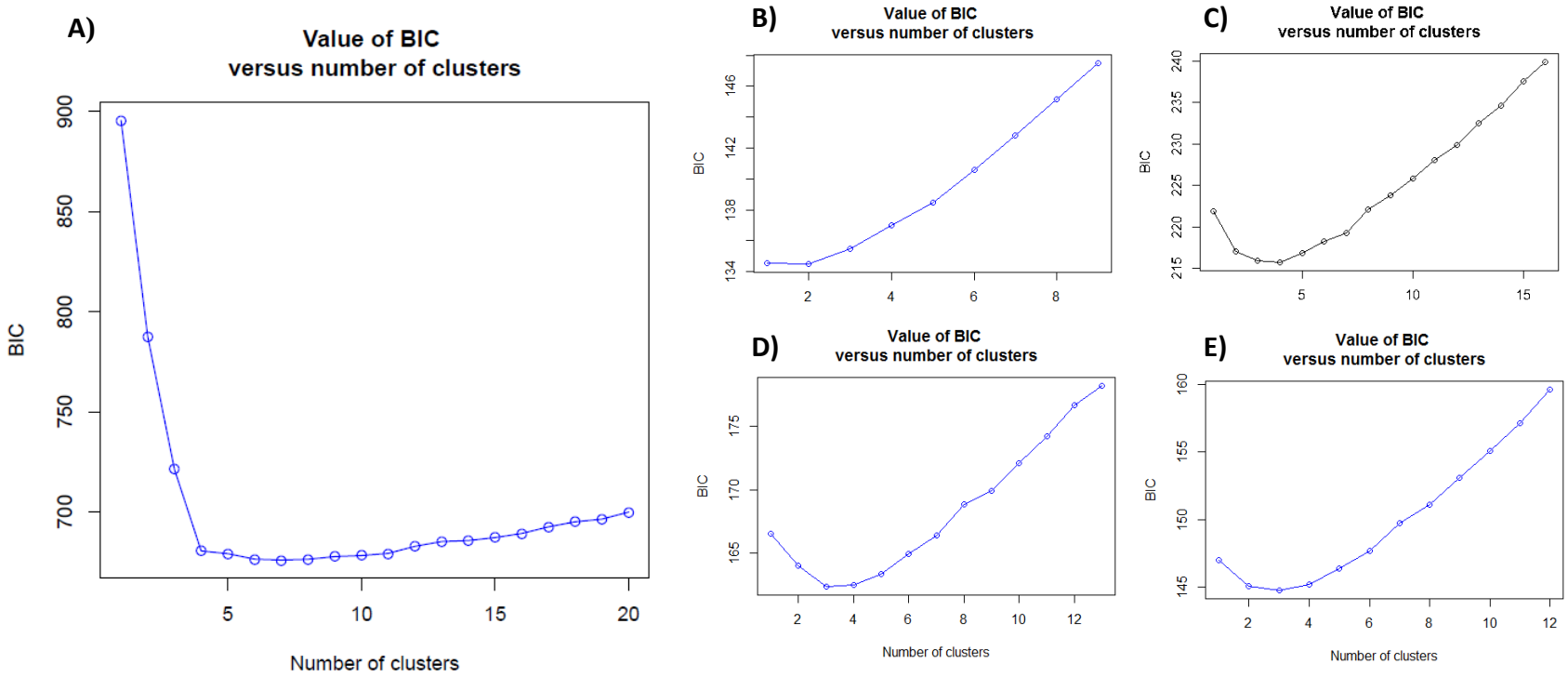


Figure 4. Results from ADEGENET revealing clear population genetic structure among all four hellbender (*Cryptobranchus alleganiensis*) rivers (graph A), but no population genetic structure within any river (Current, B; North Fork of the White River, C; Big Piney, D; Niangua, E). Although a slight bend is noticeable in the B—E graphs, note the small changes in BIC (< 5) which indicates that additional clusters do not add significantly to the model.

CHAPTER 3

Dispersal in the hellbender (*Cryptobranchus alleganiensis*): Is it sex-biased?

Abstract

Sex-biased dispersal has evolved to decrease inbreeding. Theory suggests that both mating strategies and competition for resources determine which sex will remain philopatric and which will disperse. Although dispersal patterns are fairly consistent in birds and mammals, with female birds and male mammals dispersing, patterns in amphibians are much more variable. In this study, I use molecular techniques to evaluate sex-biased dispersal in the hellbender (*Cryptobranchus alleganiensis*), a promiscuous amphibian species exhibiting territoriality. Data from 253 hellbenders (160 males, 93 females) suggests that dispersal occurs in both males and females, as no mitochondrial structuring could be observed and no significant differences in microsatellite parameters (F_{IS}, F_{ST}, *r*, mAIc, vAIc) could be detected between sexes. From this evidence, I conclude that both sexes contribute to gene flow. However, additional investigation is needed to determine if dispersal distances, directions, and/or timing differ between sexes, and whether density impacts dispersal and/or settlement.

Introduction

When an organism disperses from its natal population to another population where it will later reproduce, that organism is facilitating gene flow and, thus, helping to maintain genetic variation and population connectivity. Within a given species,

dispersal can occur in both sexes, or can be predominantly mediated by either males or females. Theory suggests that sex-biased dispersal has evolved to decrease the incidence of inbreeding (Pusey 1987), where mating strategies (e.g. resource-competition, mate-competition, monogamy, polygamy) drive differential movement between sexes (Greenwood 1980). Although exceptions exist, a uniform pattern is observed in mammals and birds, with class-specific mating strategies that lead to male-biases in mammals and female-biases in birds (Greenwood 1980). For amphibians with variable mating strategies, sex-biases in dispersal do not appear to be consistent. Rather, a growing body of knowledge suggests that dispersal patterns differ, with some species exhibiting a female-bias (bullfrog, *Rana catesbeiana*, Austin et al. 2003; common frog, *Rana temporaria*, Palo et al. 2004), and others a male-bias (tungara frog, *Physalaemus pustulosus*, Lampert et al. 2003; Cascades frog, *Rana cascadae*, Monsen & Blouin 2003; red-backed salamander, *Plethodon cinereus*, Liebgold et al. 2011, but see Cabe et al. 2007; alpine salamander, *Salamandra atra*, Helfer et al. 2012). In some amphibian species, no bias has been detected (wood frog, *Rana sylvatica*, Berven & Grudzien 1990; Fowler's toads, *Bufo fowleri*, Smith & Green 2006; moor frog, *Rana arvalis*, Knopp & Merila 2009).

Hellbenders (*Cryptobranchus alleganiensis*) are cryptic, paedomorphic salamanders that inhabit cool, fast flowing streams where they can be found hiding under large rock cover. Despite being adept swimmers, adult hellbenders exhibit little movement and extreme site fidelity (for example, see Nickerson & Mays 1973b; Burgmeier et al. 2011a). Such sedentary behavior would suggest that connectivity

between hellbender habitat patches is restricted. Yet genetic investigations suggest that within-river populations are panmictic and that significant amounts of gene flow occur between disjunct, and distantly located (> than 100km) habitat patches—a potential consequence of dispersal during the larval and/or juvenile phase (Chapter 1). This lack of genetic structure may result from two potential dispersing scenarios: both sexes disperse, or one sex disperses.

If dispersal is driven by competition for resources, females should disperse while males remain philopatric (Greenwood 1980). For hellbenders, which are solitary and promiscuous animals, this would suggest that males refrain from dispersing, as they are territorial and guard preferential nesting sites against conspecifics (J. Briggler, personal communication; Smith 1907). Yet, if dispersal is not a sex-specific behavior, but is instead a result of passive movement facilitated via stream flow (Chapter 1), dispersal should be equal among sexes as passive dispersal is not likely to act differentially upon males and females.

I use both bi-parentally inherited microsatellite genotypes, as well as sequence data from maternally inherited mtDNA to infer biases in hellbender dispersal. The inclusion of both types of genetic information is important in detecting a sex-bias as differences in the mode of inheritance may lead to differing conclusions if interpreted separately (Monsen & Blouin 2003). Given the information presented in Chapter 1, where I discuss the potential influence of passive dispersal in larval/juvenile hellbenders, I hypothesize that all hellbenders disperse, regardless of sex.

Methods

Sampling. Tail clippings were collected from 253 hellbenders of known sex (Males = 160, Females = 93) from four Missouri Rivers, representing both Eastern (*C. a. allegeniensis*) and Ozark (*C. a. bishopi*) hellbender populations (Table 1). These rivers have been previously shown to be genetically distinct from one another (Crowhurst et al. 2011). Thus, analyses will focus on within-river dispersal patterns. Clippings were stored in ethanol at -20°C. DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA).

Microsatellite analysis. Samples were genotyped using 10 polymorphic microsatellite loci (Johnson et al. 2009; Unger et al. 2010) which were fluorescently labeled, and grouped into four multiplex arrangements (Table 2). The polymerase chain reaction (PCR) was carried out in 8μl volumes according to an adjusted Multiplex PCR Kit (Qiagen, Valencia, CA) protocol, incorporating approximately 7.5ng DNA and 0.65μl bovine serum albumin (BSA). Amplifications were performed on Eppendorf ep (Eppendorf, Hauppauge, NY) thermocyclers using the following profile: 95°C for 15 min, 35 cycles of denaturing at 94°C for 30 sec, annealing at multiplex specific temperatures (Table 2) for 90 sec, primer extension at 72°C for 60 sec, and a final extension at 60°C for 30 min. A positive and a negative control was included in each PCR to ensure that genotype scoring was consistent and to detect contamination of reagents.

The resulting microsatellite fragments were analyzed on an ABI 3730xl (Life Technologies, Grand Isle, NY) automated sequencer at the University of Missouri DNA

Core Facility, where fragment peak size was standardized by the addition of LIZ 600 (Genescan, Life Technologies). Genotypes were determined in GENEMARKER (v. 1.95, Softgenetics, State College, PA). Genetic summary statistics (allelic richness, observed and expected heterozygosities) were calculated in GENALEX (Peakall & Smouse 2006).

To detect biases in hellbender dispersal, I used the randomization method available in FSTAT (Goudet 2002) which evaluates the differences in mean assignment indices (mAI_C), the variance in these indices (vAI_C), relatedness (r), genetic differentiation (F_{ST}), and inbreeding coefficients (F_{IS}) between males and females. Significance is calculated by comparing values obtained from the empirical data set with values obtained from 10,000 generated samples where sex is randomly assigned according to observed sex-ratios. Under differential dispersal rates, the parameters are expected to be significantly different between males and females. Specifically, the philopatric sex is expected to exhibit higher (i.e. positive) *mAIc v*alues, higher relatedness, and higher genetic differentiation, but lower *vAIc* and lower F_{IS}. A discussion of how these differences arise can be found in Goudet et al. (2002) and Prugnolle and de Meeus (2002).

Mitochondrial (mtDNA) analysis. Because polymorphisms are needed to detect biases in dispersal, I identified the most variable and most informative mtDNA sequences by screening a minimum of 8 hellbenders per river using the following mtDNA regions: 787 bp of cytochrome *b* (Cyt*b*-CA, Sabatino & Routman 2009), approx. 750 bp of cytochrome oxidase I (COI-CA, Sabatino & Routman 2009), and 264 bp of control region.

Primers for the control region (FWD: CGCATCAAGAAAAAAGGGATT, RVS: GGGCAGACTCAGTTATGCGTT) were designed in PRIMER 3 (Rozen & Skaletsky 2000) using sequences resulting from the universal primers L15926 and 12SAR-H (Kocher et al. 1989; Palumbi et al. 1991; Crowhurst et al. 2009). PCR reactions were carried out in 25μl volumes, including 0.2mM dNTP, 0.4mM forward primer, 0.4mM reverse primer, 2mM $MgCl_2$, 1x PCR buffer (Applied Biosystems), 0.5u TAQ gold DNA polymerase (Applied Biosystems), and 2μl extracted DNA (approx 30—40 ng). Amplification occurred on Eppendorf ep (Eppendorf, Hauppauge, NY) thermocyclers according to the following PCR profile: 95°C 10 min, 45 cycles consisting of denaturing at 94°C for 30 sec, annealing at primer specific temperature (T_A = Cytb-CA: 61°C, COI: 58.5°C, Control region: 55.5°C, NADH-4: 60°C) for 30 sec, extension at 72°C for 60 sec, followed by a final extension at 72°C for 4 min. After purification, using Exonuclease I and FastAP thermosensitive Alkaline Phosphatas (Fermentas), PCR products were directly sequenced on an ABI 3730xl (Life Technologies, Grand Isle, NY) automated sequencer using BigDye™ Terminator cycling chemistry (Applied Biosystems) at the University of Missouri DNA Core Facility.

In addition to screening the three previously mentioned primer sets, I also screened the Current and North Fork of the White River samples for polymorphisms using 547 bp of NADH-4. Primers for NADH-4 (FWD: CAATTGCCGGTTCAATAGTTT, RVS: GCATAGGTATAAGGTGCAGAACA, T_A = 60°) were designed in PRIMER 3 (Rozen & Skaletsky 2000) using hellbender mitochondrial sequences located on GenBank

(Chippindale et al. 2004, Accession #AY691763; Zhang & Wake 2009, Accession #GQ368662).

During the screening process, I had varying success in amplification across rivers with relatively few polymorphic regions. To compensate for low mtDNA variation, I assigned haplotypes according to a single concatenated sequence that was comprised of the two most informative primer sets. For the Big Piney and Niangua Rivers (*C. a. alleganiensis*), concatenated sequences totaled 1051 bp and consisted of the Cyt*b*-CA and control region sequences. NADH-4 and Cyt*b*-CA sequences were concatenated for the North Fork of the White River (*C. a. bishopi*), with a length totaling 1334 bp. Amplification and diversity was particularly poor in the Current River (*C. a. bishopi*) for all screened primer sets, except NADH-4. Thus, the Current River could only be analyzed using NADH-4 (547 bp).

Sequences were aligned in GENEIOUS PRO (Biomatters, http://www.geneious.com). The number of haplotypes, and the observed frequencies of haplotypes, were calculated using the collapse tool in FaBOX (Villesen 2007). I used ARLEQUIN (Excoffier et al. 2005) to measure population variation in terms of both haplotype (*h*) and nucleotide (π) diversity, where *h* is the probability of two randomly sampled hellbenders having different haplotypes and π represents the number of pairwise differences. Because strong genetic structuring is indicative of sex-biased dispersal, I also used ARLEQUIN to hierarchically partition mtDNA genetic variability

(Analysis of Molecular Variance, or AMOVA) at the within population (i.e. river) and among habitat patch levels.

Results

Microsatellite analysis. Summary statistics are presented in Table 3. No significant differences were observed between male and female hellbenders in the parameters investigated in FSTAT (Table 4). For the Big Piney River, differences in relatedness and F_{ST} approached significance ($p = 0.068$ and $p = 0.057$, respectively) with females exhibiting lower relatedness and less differentiation than males. Although not statistically significant, opposite trends were observed in the Niangua, Current, and North Fork of the White Rivers where females were more related and more differentiated than males.

Mitochondrial (mtDNA) analysis. The Current River was withdrawn from mtDNA analysis; in 34 samples and approx. 3,000 base pairs of sequence, only a single haplotype was detected. The remaining three rivers were polymorphic (Table 5; Figure 1A-1E), with four to six haplotypes observed within each river. All but one haplotype was unique to the river sampled and not found in other rivers; one individual in the Big Piney River exhibited a haplotype only found in the Niangua River (Figure 1A). Overall, there were no obvious clusterings of haplotypes within habitat patches, and no common haplotype appeared to be more frequent in one sex or the other (Figure 1A-E). However, the Big Piney River again demonstrates a somewhat differing pattern, which is only evident in Big Piney River habitat patch B where all males within the patch exhibit

the same haplotype—a haplotype that differs from those represented by females within the patch (Figure 1C).

Rare haplotypes, or haplotypes observed in only one hellbender, were represented by both males and females. Across rivers, rare haplotypes were more frequently carried by females (55%). However, at the within river scale, only the Big Piney River exhibited a higher proportion of rarity within females (67%), whereas in the Niangua River, males were the more frequent carrier (67%). In the North Fork of the White River, rare haplotypes were represented in equal proportions between sexes (50%).

The greatest amount of genetic variation is found within habitat patches, and not among habitat patches within a river (Table 6). Variation at the river level (i.e. among habitat patches) was not significant, indicating a lack of mtDNA structuring.

Discussion

If dispersal is biased in hellbenders, with one sex dispersing more frequently than the other, such a bias was not detected in my study. Microsatellite and mtDNA data indicate that dispersal occurs in both male and female hellbenders. Because larval hellbenders are not likely to be differentially influenced by stream flow based upon sex, equal movement between males and females aligns with the hypothesis that hellbender dispersal occurs passively during the larval stage via current advection (see Chapter 1). Dispersal driven by crowding or a tendency to colonize new habitat patches would be an alternate, potential mechanism driving dispersal in both sexes (Goudet et al. 2002). Additionally, habitat degradation could drive both males and females to disperse.

While I am confident in the results presented here, improvements to these analyses could be made in the future: Individuals sampled were reproductively mature, and were assumed to be settled within post-dispersal habitat patches. However, age could not be estimated. Given that hellbenders are a long-lived species (Taber et al. 1975), the sampling pool may contain multiple generations. Because FSTAT assumes non-overlapping generations, some degree of error may have been introduced into the microsatellite analysis. The use of a single generation helps to account for the fact that, in bi-parentally inherited DNA, alleles introduced by the dispersing individual get redistributed in subsequent generations and the signal of dispersal becomes lost. However, the results from microsatellite data are congruent with the results from mtDNA, where patterns of dispersal are still detectable. Thus, any bias introduced by overlapping generations may be negligible.

In my study, males represent a larger proportion of individuals sampled. Although male-biased sex ratios are commonly reported in hellbenders (Foster et al. 2009; Burgmeier et al. 2011c), future studies investigating sex-biases should aim to correct for such skewing to increase resolution. If skewing is to be corrected, a more effective technique for determining sex in hellbenders must be developed. Although an established method exists, this method is based upon external traits that are only discernible between sexes during the breeding season (Nickerson & Mays 1973a). An alternate, vitellogenin-based method has been proposed by Burgmeier et al. (2011b), but its use is also confined to the breeding season. Molecular sex determination, which would prove useful throughout the year, may be a promising technique to pursue in the

near future. Although no such technique is currently available for hellbenders, the approach has been successful in other species (for an amphibian example, see Berset-Brändli et al. 2006). It should be noted, however, that molecular sexing can only be useful when sex is determined genetically. Because no evidence, to date, indicate that hellbender sex is determined environmentally, establishing a molecular sexing technique is highly desirable.

The data indicates that both male and female hellbenders disperse from their natal habitat to other habitat patches. Yet, factors driving equal movement in the sexes remain a question of interest for future studies. Despite this new information, hellbender movement behavior continues to be poorly understood. With continued investigation, we can only improve our knowledge. An ideal approach would combine evidence from molecular techniques with direct observation. Other components worthy of investigation include whether or not dispersal distances, directions, and/or timing differ between sexes, with attention paid to determining whether dispersal and/or settlement are impacted by density.

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Table 1. Number of hellbenders (*Cryptobranchus alleganiensis*) utilized in the microsatellite and mitochondrial (mtDNA) analysis for sex-biased dispersal.

	Number of hellbender samples	
Population	Microsatellite analysis	mtDNA analysis
Eastern hellbenders		
Big Piney River		
No. habitat patches	10	2
Male	41	7
Female	14	4
Total	55	11
Niangua River		
No. habitat patches	7	3
Male	42	11
Female	21	7
Total	63	18
Ozark hellbenders		
Current River		
No. habitat patches	10	4
Male	29	14*
Female	26	18*
Total	55	32*
North Fork of the White River		
No. habitat patches	8	2
Male	48	8
Female	32	8
Total	80	16

*removed from mtDNA analysis as only one haplotype was observed

Table 2. Microsatellites used to infer sex-biased dispersal in hellbenders (*Cryptobranchus alleganiensis*). Microsatellites beginning with Cral were designed by Johnson et al. (2009) and those beginning with Call designed by Unger et al. (2010).

Locus	Repeat motif	Primer sequence	Label	No. alleles Observed
Multiplex 1a, T_A = 60°C				
Call 26	$(GATA)_{18}$	F: CATAATGGTAATAGCTGCATGG	FAM	20
		R: CCTTGTTCCAGATTCACACC		
Call 127	$(TATC)_{14}$	F: TGAACTGTGGAGTTGGATCG	FAM	13
		R: CACATATGGGTAGTAACTGCATGG		
Call 204	$(GATA)_{21}$	F: TTCGCGAGCATTACTCTACG	NED	17
		R: ACGGTCCAGACATTGTTGC		
Call 282	$(GATA)_{15}$	F: ACCCGAAAGGGTGGTTTATAG	PET	13
		R: TAATGAGCCGTTAGCCCTTG		
Multiplex 1b, T_A = 58.5°C				
Cral 17	$(CTAT)_{24}$	F: ATTCCAAGGGGGCTGAATAC	FAM	22
		R: CGCCTTGATGTAGCTTTTGG		
Cral 10	$(AC)_{14}$	F: GCTCGGATGACAGAGGTTTC	FAM	14
		R: TGGCAAATTTCATTCTGCTTC		
Multiplex 2, T_A = 65°C				
Call 232	$(TGTC)_{18}$	F: CGTATGCCTGGCACATAACC	FAM	20
		R: CCACCATAAGATTCACACTGC		
Call 205	$(GATA)_{20}$	F: TTTGAGCTCTCTTGGCTTATG	VIC	12
		R: TGGACTCCTTCCCTTTCTCC		
Call 341	$(GATA)_{15}$	F: GCAAGAAGGTGAGCAAGAGG	NED	18
		R: CCATCTGAATATACCTGCAATCTG		
Multiplex 3, T_A = 64°C				
Call 347	$(GATA)_{20}$	F: ACCAGCAGCAACCTTATCTGG	FAM	12
		R: ACCATGCAGCCGGTAAGC		

T_A = PCR amplification annealing temperature

Table 3. Hellbender (*Cryptobranchus alleganiensis*) genetic summary statistics averaged across 10 microsatellite loci, where A_R, H_O, and H_E represent the average number of alleles, average observed heterozygosity, and average expected heterozygosity (respectively).

Population	A_R	H_O	H_E
Big Piney River			
Males	6.70	0.65	0.67
Females	5.20	0.59	0.62
Overall	5.95	0.62	0.64
Niangua River			
Males	6.90	0.68	0.68
Females	6.10	0.70	0.68
Overall	6.50	0.69	0.68
Current River			
Males	10.10	0.81	0.80
Females	10.30	0.74	0.78
Overall	10.20	0.77	0.79
North Fork of the White River			
Males	6.9	0.67	0.68
Females	6.4	0.71	0.69
Overall	6.65	0.69	0.68

Table 4. Results from FSTAT, indicating no significant differences in the molecular parameters derived from microsatellite data between male and female hellbenders (*Cryptobranchus alleganiensis*). Significant p-values are indicated in bold (none present).

	Big Piney River		**Niangua River**		**Current River**		**North Fork of the White River**	
Molecular parameters	Males	Females	Males	Females	Males	Females	Males	Females
F_{IS}	0.02	0.16	0.03	-0.02	0.04	0.06	0.04	-0.03
F_{ST}	0.01	-0.10	-0.02	0.01	-0.003	0.01	-0.003	0.03
Relatedness	0.01	-0.18	-0.03	0.02	-0.01	0.01	-0.01	0.06
Mean AIc	0.02	-0.06	-0.07	0.15	-0.35	0.40	-0.09	0.13
Variance mean AIc	4.47	3.55	4.00	4.56	3.51	2.51	14.04	8.60

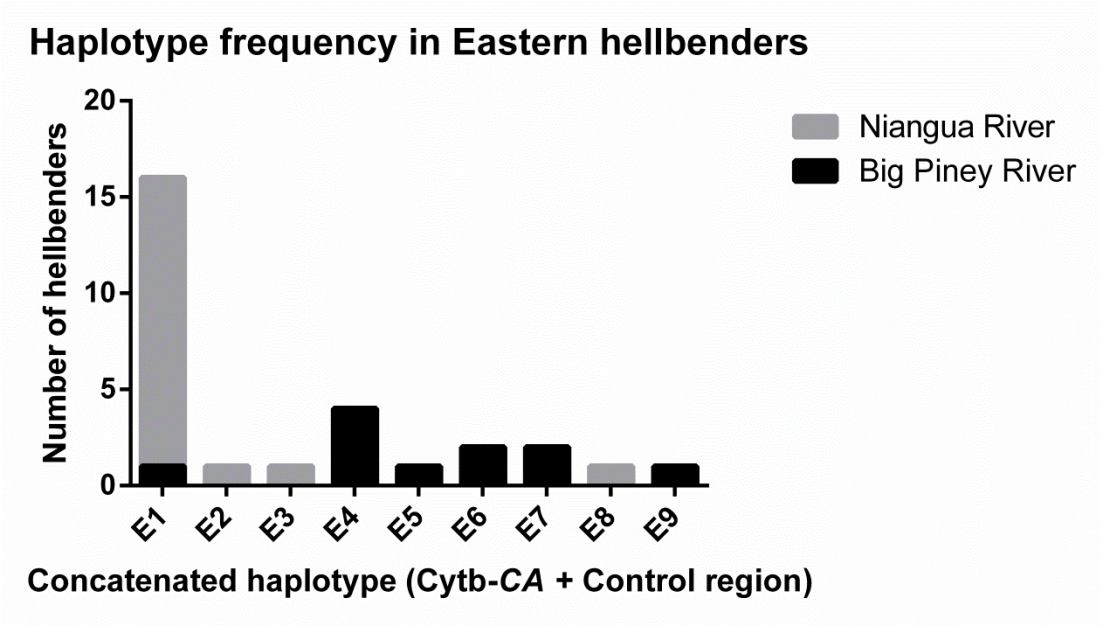

Figure 1A. Mitochondrial (mtDNA) haplotype frequencies observed in Eastern hellbenders (*C. a. alleganiensis*).

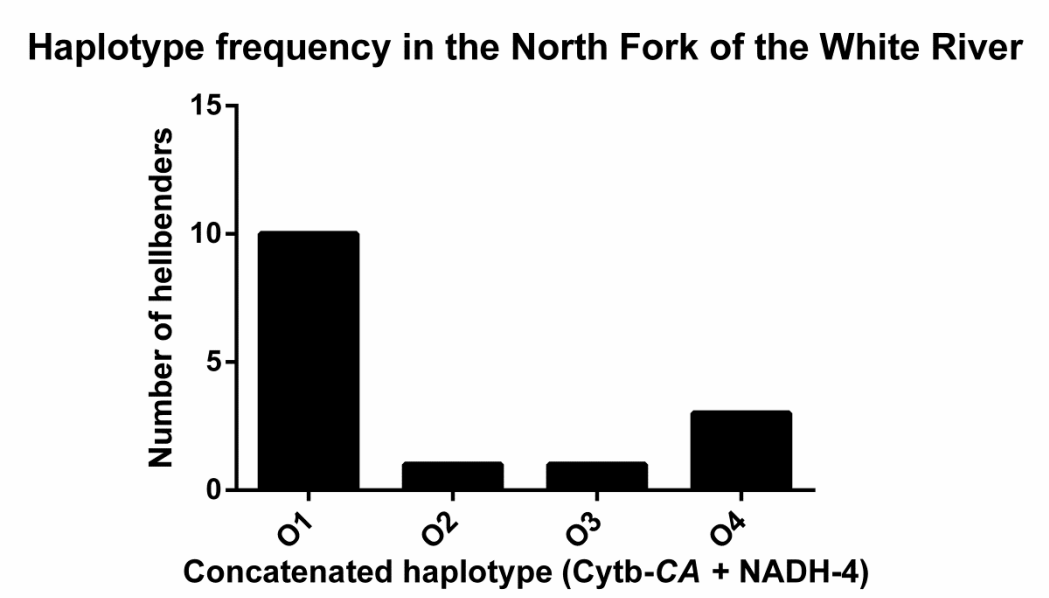

Figure 1B. Mitochondrial (mtDNA) haplotype frequencies observed in Ozark hellbenders (*C. a. bishopi*) within the North Fork of the White River.

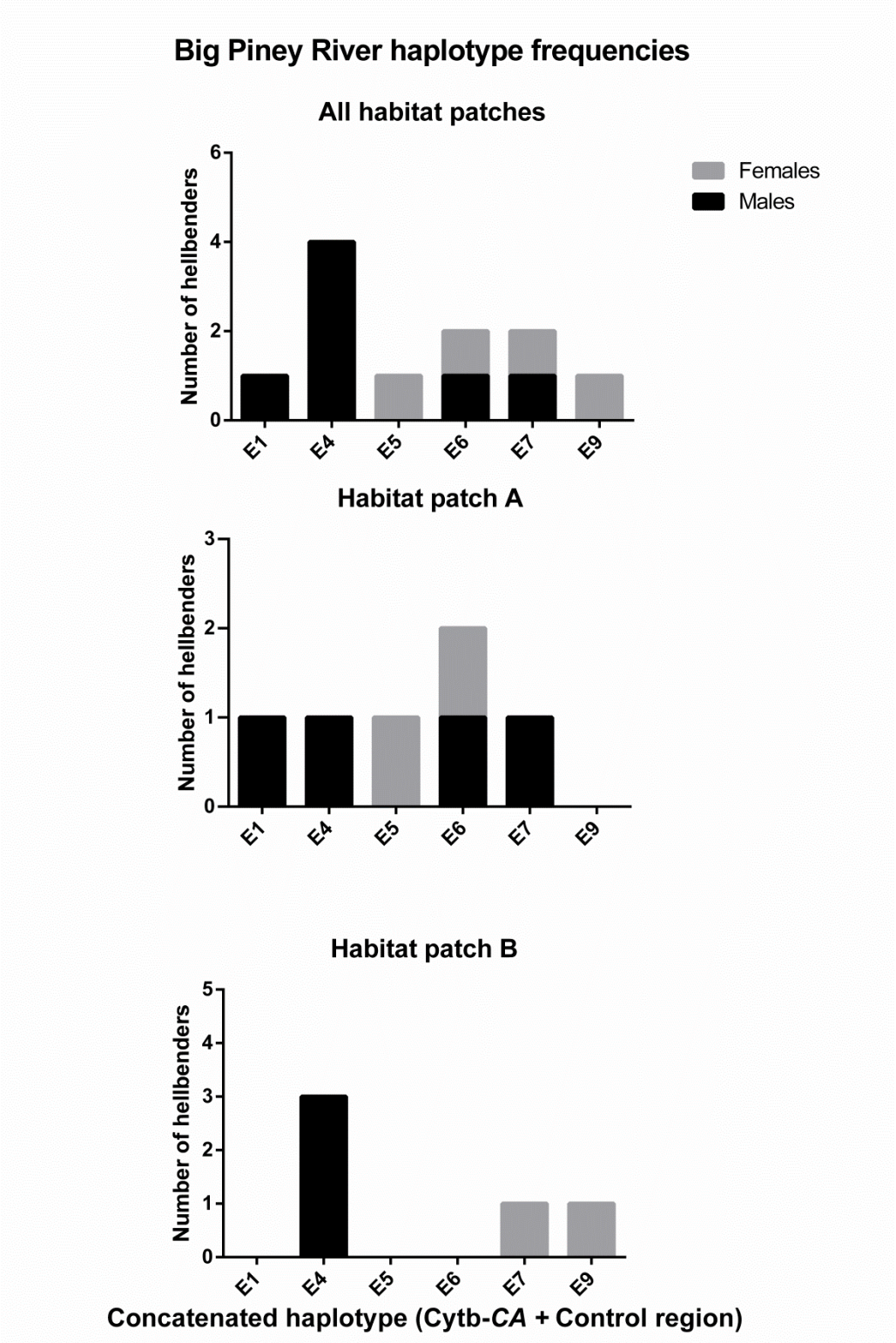

Figure 1C. Mitochondrial (mtDNA) haplotype frequencies observed in the Big Piney River with emphasis on differences among sexes and habitat patches.

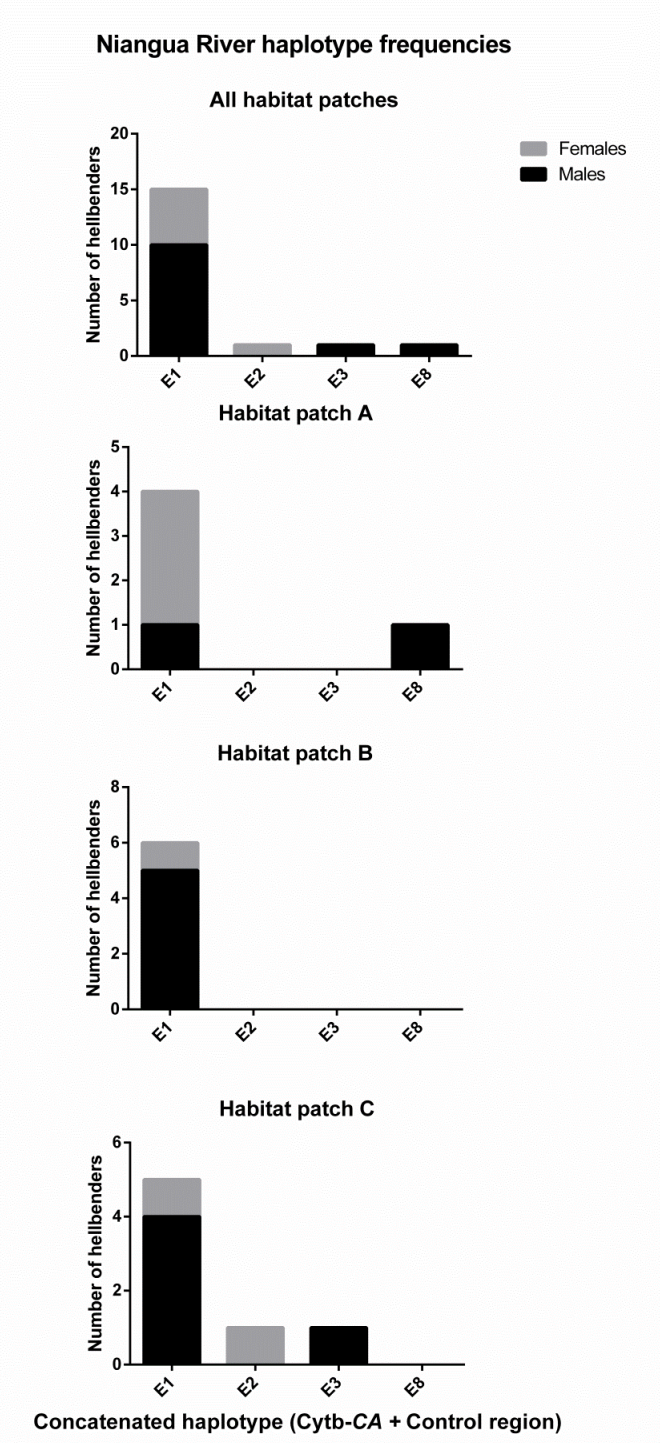

Figure 1D. Mitochondrial (mtDNA) haplotype frequencies observed in the Niangua River with emphasis on differences among sexes and habitat patches.

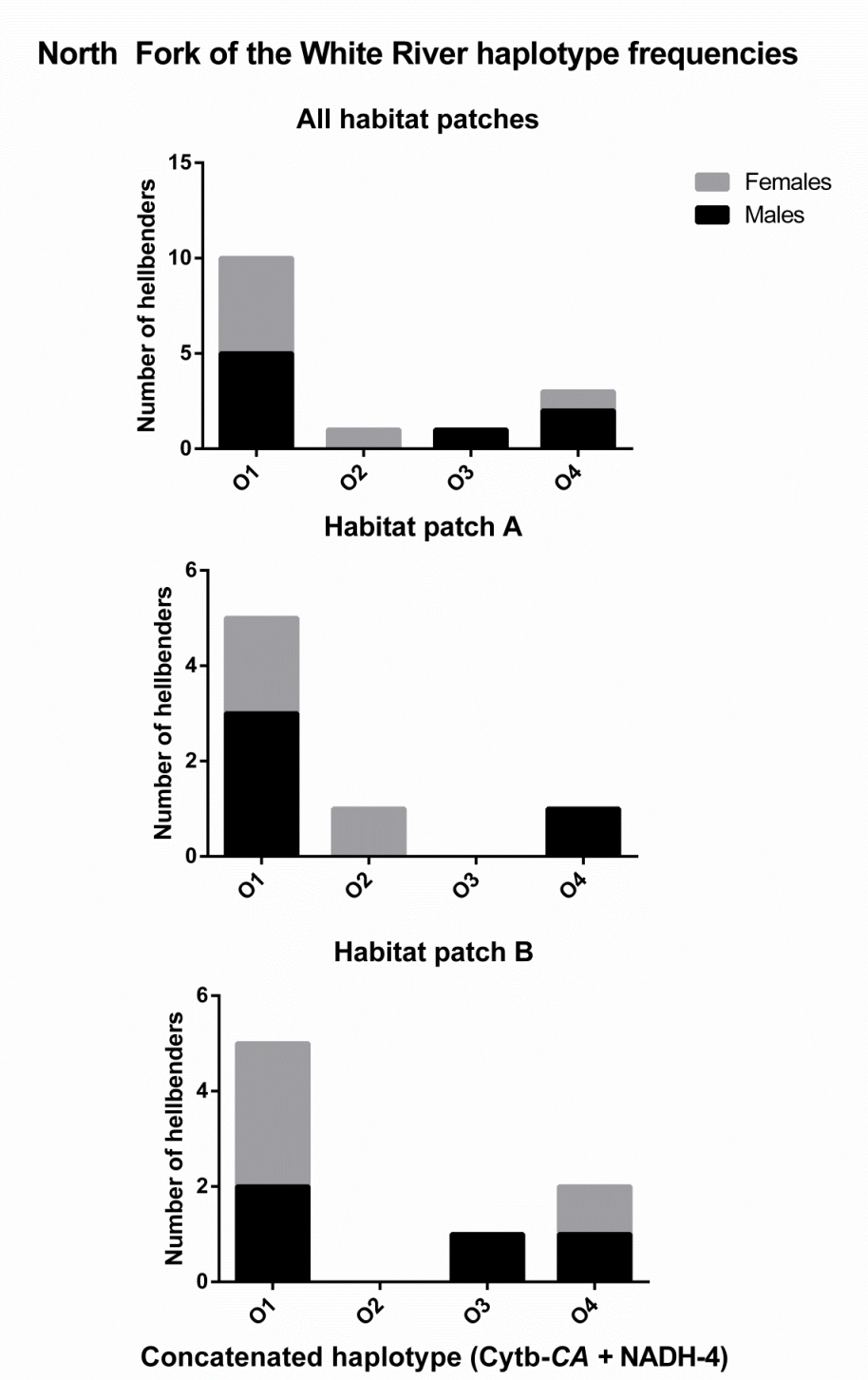

Figure 1E. Mitochondrial (mtDNA) haplotype frequencies observed in the North Fork of the White River with emphasis on differences among sexes and habitat patches.

Table 5. Haplotypic (*h*) and nucleotide (π) diversities observed in the concatenated hellbender (*Cryptobranchus alleganiensis*) mtDNA sequences within three Missouri rivers.

River	Concatenated sequence	Total length of sequence (bp)	*h*	π
Big Piney	Cytb-*CA* + Control region	1051	0.85 ± 0.09	1.96 ± 1.20
Niangua	Cytb-*CA* + Control region	1051	0.31 ± 0.14	0.33 ± 0.35
North Fork of the White	Cytb-*CA* + NADH-4	1334	0.54 ± 0.13	0.61 ± 0.51

Table 6. AMOVA revealing hierarchical genetic variation in hellbender (*Cryptobranchus alleganiensis*) mtDNA, at the among population and within population levels, where population represents "habitat patch" within a river.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Big Piney River				
Among pops	1	2.25	0.26	23.53
Within pops	9	7.57	0.84	76.47
Total	10	9.82	1.10	
ϕ_{ST}	0.24			
p-value	0.07			
Niangua River				
Among pops	2	0.32	-0.001	-0.82
Within pops	15	2.51	0.17	100.82
Total	17	2.83	0.17	
ϕ_{ST}	-0.01			
p-value	0.68			
North Fork of the White River				
Among pops	1	0.18	-0.02	-6.20
Within pops	13	4.09	0.31	106.20
Total	14	4.27	0.30	
ϕ_{ST}	-0.06			
p-value	0.91			

CHAPTER 4

Heterozygosity Fitness Correlations (HFC) and the influence of genetic diversity on hellbender (*Cryptobranchus alleganiensis*) juvenile recruitment

Abstract

The relationship between genetic diversity and fitness is often evaluated using heterozygosity-fitness correlations (HFC), where fitness can include any number of parameters relevant to survival, growth, and/or reproductive success. In a variety of species, studies have found weak but significant associations between HFC and offspring survival. I conducted a preliminary investigation into HFC in hellbenders (*Cryptobranchus alleganiensis*), which are reported to have low juvenile recruitment. In order to assess the potential influence of genetic diversity on offspring survival, I genotyped 240 hellbender offspring from two clutches. Using homozygosity by locus (*HL*), I observed differences in the average *HL* between those offspring that survived (avg. *HL* = 0.256 and 0.208, clutch A and clutch B, respectively) and those offspring that died (avg. *HL* = 0.186 and 0.169, clutch A and clutch B, respectively) offspring, indicating that HFC is an interesting area of hellbender research needing further investigation.

Introduction

Genetic variation provides the material for evolutionary change. Genetic variation is commonly defined according to observed levels of heterozygosity; thus, heterozygosity-fitness correlations (HFC) can be used to better understand the association between molecular diversity and differing measures of individual fitness. Studies of HFC have

shown that a number of fitness parameters—including growth and other morphological traits, resistance to parasites and disease, and fecundity—can be influenced by an individual's genotype (reviewed in David 1998; Coltman & Slate 2003; Chapman et al. 2009). Yet, skepticism about the reliability of HFC—particularly whether HFC is universal or not—and debates as to the mechanisms driving HFC, are prominent (reviewed in Szulkin et al. 2010).

Criticism further revolves around publication bias where a disproportionate amount of attention has been placed on positive correlations (i.e. heterozygosity increases fitness), instigating questions as to whether positive correlations are truly more common than nonsignificant and/or negative correlations. In their review of published HFC literature, Chapman et al. (2009) address this concern and suggest that relationships between fitness and genetic diversity remain significant, although admittedly weak, even with the inclusion of those underrepresented nonsignificant and/or negative correlations. Such data suggest that HFC can shed light on the interplay between molecular variation and fitness in small and/or endangered populations whose demographic histories and population characteristics dramatically shape genetic variation at both the population level and the individual level.

The hellbender (*Cryptobranchus alleganiensis*) is a unique, long-lived, aquatic salamander species experiencing pronounced population declines throughout its range (Gates et al. 1985; Pfingsten 1989; Trauth et al. 1992; Wheeler et al. 2003; Foster et al. 2009; Burgmeier et al. 2011). Because populations exhibit significant age structuring, where adult individuals are abundant but juvenile and/or larvae are nearly absent,

concerns regarding juvenile recruitment have been raised (Wheeler et al. 2003; Humphries & Pauley 2005; Foster et al. 2009; Burgmeier et al. 2011). Here I evaluate the role of genetic diversity in hellbender juvenile recruitment by presenting a preliminary analysis of HFC in two eastern hellbender (*C. a. alleganiensis*) clutches originating from the Big Piney River, Missouri, which exhibits population decline (Wheeler et al. 2003) but robust genetic variation (avg H_O across 15 microsatellite loci = 0.59, see Chapter 1). Although my study is limited and statistical significance cannot be evaluated, I aim to investigate the following questions to stimulate further discussion and additional research: 1) Does survival differ between offspring with differing levels of heterozygosity? 2) Is genetic diversity, in terms of heterozygosity, predictive of offspring survival? 3) Do parental genotypes influence offspring survival?

Methods

Collection and laboratory procedures. Fertilization occurs externally in hellbenders, with the male providing offspring brooding (Nickerson & Mays 1973). During the breeding season of fall 2007, two hellbender egg clutches were found by the Missouri Department of Conservation in the Big Piney River. These clutches were found in a single nest site, where they were being guarded by a male hellbender (HB465, PIT tag # AVID099278604). Due to concerns about the observed low juvenile recruitment, the clutches were collected and transferred to Missouri Department of Conservation Shepherd of the Hills hatchery to be reared in captivity as a part of long-term propagation efforts.

In 2011, tail clippings were collected from these offspring, some of whom were dead (frozen) and some of whom were alive (Table 1). Clippings were immediately placed in Longmire buffer (Longmire et al. 1988) and stored at -20°C. For DNA extraction, I used the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Thirteen polymorphic microsatellite loci (Johnson et al. 2009; Unger et al. 2010) were grouped into three multiplex arrangements for genotyping using fluorescently labeled forward primers (Table 2). The polymerase chain reaction (PCR) was carried out in 8µl volumes according to an adjusted protocol for the Multiplex PCR Kit (Qiagen, Valencia, CA), incorporating approximately 7.5ng DNA and 0.65µl bovine serum albumin (BSA). Amplifications were performed on Eppendorf ep (Eppendorf, Hauppauge, NY) thermocyclers using the following profile: 95°C for 15 min, 35 cycles of denaturing at 94°C for 30 sec, annealing at multiplex specific temperatures (Table 2) for 90 sec, primer extension at 72°C for 60 sec, and a final extension at 60°C for 30 min. I included a positive control in each PCR to ensure consistency of allele scoring across reactions, and a negative control to aid in the detection of contamination.

The resulting microsatellite fragments were analyzed at the University of Missouri's DNA Core facility on an ABI 3730xl (Life Technologies, Grand Isle, NY) automated sequencer, where fragment peak size was standardized by the addition of LIZ 600 (Genescan, Life Technologies). Alleles were assigned after visual inspection of chromatograms using the program GENEMARKER (v. 1.95, Softgenetics, State College, PA).

All 13 loci were utilized in the parentage analysis. A subset of 8 loci (clutch A) and 9 loci (clutch B) were utilized in the heterozygosity-fitness correlation, as some loci were monomorphic within a clutch and/or exhibited signs of selection in the hellbender fine-scale population structure analysis (Chapter 1).

Parentage Analysis. Because embryo development was more advanced in one clutch, it was hypothesized that two female hellbenders contributed to the nest. This was verified by Crowhurst et al. (2009), who found that each clutch exhibited a different mitochondrial DNA haplotype. However, no direct assessment of parentage was made, either in regards to maternity or to confirm that the guarding male was the sire. To assign parentage to these clutches, I used a likelihood-based approach with the program CERVUS (Kalinowski et al. 2007), incorporating all known hellbenders of reproductive age (n_{male} = 54, n_{female} = 16, $n_{unknown}$= 61) from the Big Piney River that were genotyped using identical microsatellite loci in Chapter 1. For each candidate parent, CERVUS calculates a LOD (log of the odds ratio) score, which evaluates the likelihood of that parent being the true parent. In addition to calculating each parent's LOD score, a LOD score for the mother-father-pair is also calculated. When calculating the LOD score, CERVUS takes into consideration the proportion of the population sampled, proportion of mistyped loci, and proportion of loci typed. These parameters are unique to each study. For my study, detection and sampling of all hellbenders in the population is likely incomplete. Although thorough population surveys were conducted for 7 continuous years, hellbenders are elusive by nature and their aquatic habitat makes censusing difficult. Thus, the proportion of the population sampled was estimated at 90%,

allowing for the possibility that the true parent may not be included in the candidate pool. The genotyping error rate was set to 2%, based upon the rate calculated in Chapter 1.

Critical values of these LOD scores are established with simulation, where 10,000 offspring are simulated from population genotype data (allele frequencies), and observed success in parental assignment is compared to expected success in those simulated individuals. CERVUS employs both a strict confidence level and a relaxed confidence level. For my analyses, I used strict confidence and trusted parental assignment only if confidence was at least 95% with no more than one mismatched locus.

Once parentage was confirmed and/or identified, relatedness (r) between parents was determined using Wang's (2007) triadic likelihood estimate in COANCESTRY (Wang 2011).

Heterozygosity-fitness correlations. I used IRmacroN4 (Amos et al. 2001) to calculate both internal relatedness (*IR*) and homozygosity weighted by locus (*HL*). Both *IR* and *HL* estimate an individual-based "inbreeding coefficient", or an individual's genetic diversity. *IR* (Amos et al. 2001) estimates the extent of parental relatedness and is calculated according to the frequency of alleles, placing more weight on rare alleles and less weight on more common alleles. *HL* (Aparicio et al. 2006), on the other hand, considers the contribution of each locus according to its allelic richness, where loci with

higher diversity and more evenly-distributed allele frequencies are assigned greater weight.

Despite these differences in calculation, *IR* and *HL* are expected to be highly correlated. Thus, I tested for correlation using linear regression (Excel data analysis package). Because strong correlations were found (clutch A, $R^2 = 0.967$; clutch B, $R^2 = 0.944$), further analyses were conducted with the *HL* values only, as *HL* is suggested to outperform *IR* when microsatellite variation is high (mean heterozygosity per locus > 0.5; Aparicio et al. 2006) which was demonstrated for the Big Piney River hellbender population in Chapter 1.

HL has an upper limit of 1, for which an individual would be homozygous at all loci, and a lower limit of 0, indicating heterozygosity at all loci. The average *HL* of survived offspring was compared to the average *HL* of dead offspring to determine which category (survived vs. dead) exhibited the higher level of heterozygosity. I also wanted to evaluate the usefulness of *HL* to predict the probability of offspring survival, which would potentially be indicative of a heterozygosity-fitness correlation. Thus a logistic regression was performed in R, where the outcome for each offspring—survival or death (coded as 1 and 0, respectively)—was regressed against the offspring's *HL* value.

Results

Parentage analysis. CERVUS confirmed, with at least 95% confidence, that the guarding male (HB465, PIT tag no. AVID099278604) was indeed the sire for clutch A and that the contributing female was HB469 (PIT tag no. AVID075552542)—a female that was not

related to the male ($r < 0.001$, $p = 0.05$) and was sampled from the same habitat patch as the male. The guarding male was also the sire for clutch B (>95% confidence), but maternity could not be identified as none of the potential female or unsexed candidates could be assigned to the clutch at even the 80% confidence level. The male was relatively homozygous ($H_O = 0.46$) with 7 of 13 loci exhibiting homozygosity.

Heterozygosity-fitness correlations. HL varied among offspring (Figure 1). In the clutches analyzed, average *HL* for surviving offspring differed from the average *HL* of deceased offspring, with surviving offspring exhibiting greater homozygosity and higher *HL* values (Table 3). Logistic regression (Figure 2) suggests that *HL* would be helpful in predicting survival, similarly indicating higher homozygosity is associated with increased odds of survival.

Discussion

Results from my study indicate that, within the two hellbender clutches analyzed, more homozygous offspring have greater survival than heterozygous offspring. It is important to reiterate that my samples were taken from juvenile hellbenders (approx. 5 years old). Due to the age of these hellbenders, I may have missed a critical fitness threshold if that threshold occurs at a young developmental stage. Perhaps my results would be more informative if sampling had included unhatched versus hatched embryos or if survival had been evaluated in larval hellbenders (< 18 months old). Additionally, it should be reiterated that the hellbenders utilized in my study were raised in captivity. Although conditions at the hatchery have been designed to mimic stream environments and natural conditions, selective pressures may be different or eased in the captive

environment such that survival may have been observed differently had the offspring been raised in the wild.

Regardless, a negative HFC is not unheard-of. While positive HFC dominate the literature (Chapman et al. 2009), a number of studies have also revealed negative relationships where higher homozygosity confers greater fitness (Lee et al. 2002; Lieutenant-Gosselin & Bernatchez 2006; Blanchet et al. 2009; Kupper et al. 2010).

Evidence suggests that HFC can arise from either linkage disequilibrium (local effects hypothesis) or identity disequilibirium (general effects hypothesis) which may be introduced into a population via demographic events (i.e. founder effects, genetic bottlenecks) and/or mating patterns (i.e. full or partial inbreeding, admixture) (Hansson & Westerberg 2002; Szulkin et al. 2010). Negative HFC may indicate that although microsatellite loci may be selectively neutral, they may exhibit associative underdominance with functional loci as a result of linkage disequilibrium imposed by matings between genetically dissimilar individuals, as in the case of admixture, and may, therefore, signify an outbreeding depression (LeBas 2002; Lee et al. 2002; Neff 2004; Chapman et al. 2009; Kupper et al. 2010).

Relatedness coefficients indicate that parental hellbenders were not related. Thus, the observed negative HFC is likely in agreement with the local effect hypothesis which is suggested to be driven by admixture-induced linkage disequilibrium (whereas inbreeding would generate identity disequilibrium and, thus, the general effect hypothesis). The genotypes of the parental hellbenders further support this conclusion, where paternal genotype and maternal genotypes mimic admixture with the relatively

homozygous male representing a “resident” individual and the female of each clutch representing a more heterozygous “migrant”.

While I was unable to test statistical significance for the logistic regression and differences in average *HL*, the data provides a look at the role of genetic variation in hellbender juvenile recruitment and suggests that HFC in hellbenders would be an interesting avenue to pursue in future research. Additional studies should aim to compare levels of heterozygosity across several independent clutches with different parents, utilizing randomly chosen dyads from each clutch, with one surviving offspring and one dead offspring per clutch (for example, see the methods in Kupper et al. 2010). Because full-siblings share identical parentage, this method would allow for inbreeding coefficients to be held constant so as to focus on the influence of additive genetic variation rather than those effects generated through inbreeding, and in this fashion, may help to discern whether HFC patterns indicate a general or local effect as a result of multiple loci or a single locus in accordance with the differing hypotheses (Hansson & Westerberg 2002; Lieutenant-Gosselin & Bernatchez 2006; Chapman et al. 2009).

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Table 1. Clutch sizes for the two analyzed hellbender (*Cryptobranchus alleganiensis*) nests, indicating sample sizes for those offspring that survived and those that did not survive.

Clutch	Total offspring (n)	Survived (n)	Dead (n)
A	74	35	39
B	166	78	88

Table 2. List of polymorphic microsatellites used for genotyping hellbender (*Cryptobranchus alleganiensis*) offspring and potential parental hellbenders.

Locus	Repeat motif	Primers	Authors
Multiplex 1a, T_A 60°			
Cral 13	$(GT)_{12}$	F: TCAACGTATAAAGTAACATAAAACCAA R: GGCTCAGAATGTCTAGGTGGTC	Johnson et al (2009)
Call 26	$(GATA)_{18}$	F: CATAATGGTAATAGCTGCATGG R: CCTTGTTCCAGATTCACACC	Unger et al (2010)
Call 127	$(TATC)_{14}$	F: TGAACTGTGGAGTTGGATCG R: CACATATGGGTAGTAACTGCATGG	Unger et al (2010)
Call 351	$(TATC)_{20}$	F: ACATTAATTCTCCTTCCTGTCACC R: CAATCCTAAGGAGGAATTGAAGC	Unger et al (2010)
Call 204	$(GATA)_{21}$	F: TTCGCGAGCATTACTCTACG R: ACGGTCCAGACATTGTTGC	Unger et al (2010)
Call 282	$(GATA)_{15}$	F: ACCCGAAAGGGTGGTTTATAG R: TAATGAGCCGTTAGCCCTTG	Unger et al (2010)
Multiplex 1b, T_A 58.5°			
Cral 17	$(CTAT)_{24}$	F: ATTCCAAGGGGGCTGAATAC R: CGCCTTGATGTAGCTTTTGG	Johnson et al (2009)
Cral 10	$(AC)_{14}$	F: GCTCGGATGACAGAGGTTTC R: TGGCAAATTTCATTCTGCTTC	Johnson et al (2009)
Cral 4	$(CA)_{10}$	F: AGGGCACCACACAAACAAA R: AAACCCAGAGACATGCTTCC	Johnson et al (2009)
Multiplex 2, T_A 65°			
Call 232	$(TGTC)_{18}$	F: CGTATGCCTGGCACATAACC R: CCACCATAAGATTCACACTGC	Unger et al (2010)
Call 205	$(GATA)_{20}$	F: TTTGAGCTCTCTTGGCTTATG R: TGGACTCCTTCCCTTTCTCC	Unger et al (2010)
Call 341	$(GATA)_{15}$	F: GCAAGAAGGTGAGCAAGAGG R: CCATCTGAATATACCTGCAATCTG	Unger et al (2010)
Call 266*	$(GATA)_{11}$	F: GCATTCTGCAAGCCACTAAA R: AACATTGGGAGGCTGGTATG	Unger et al (2010)

*Forward and reverse primer sequence was redesigned using Primer 3 software

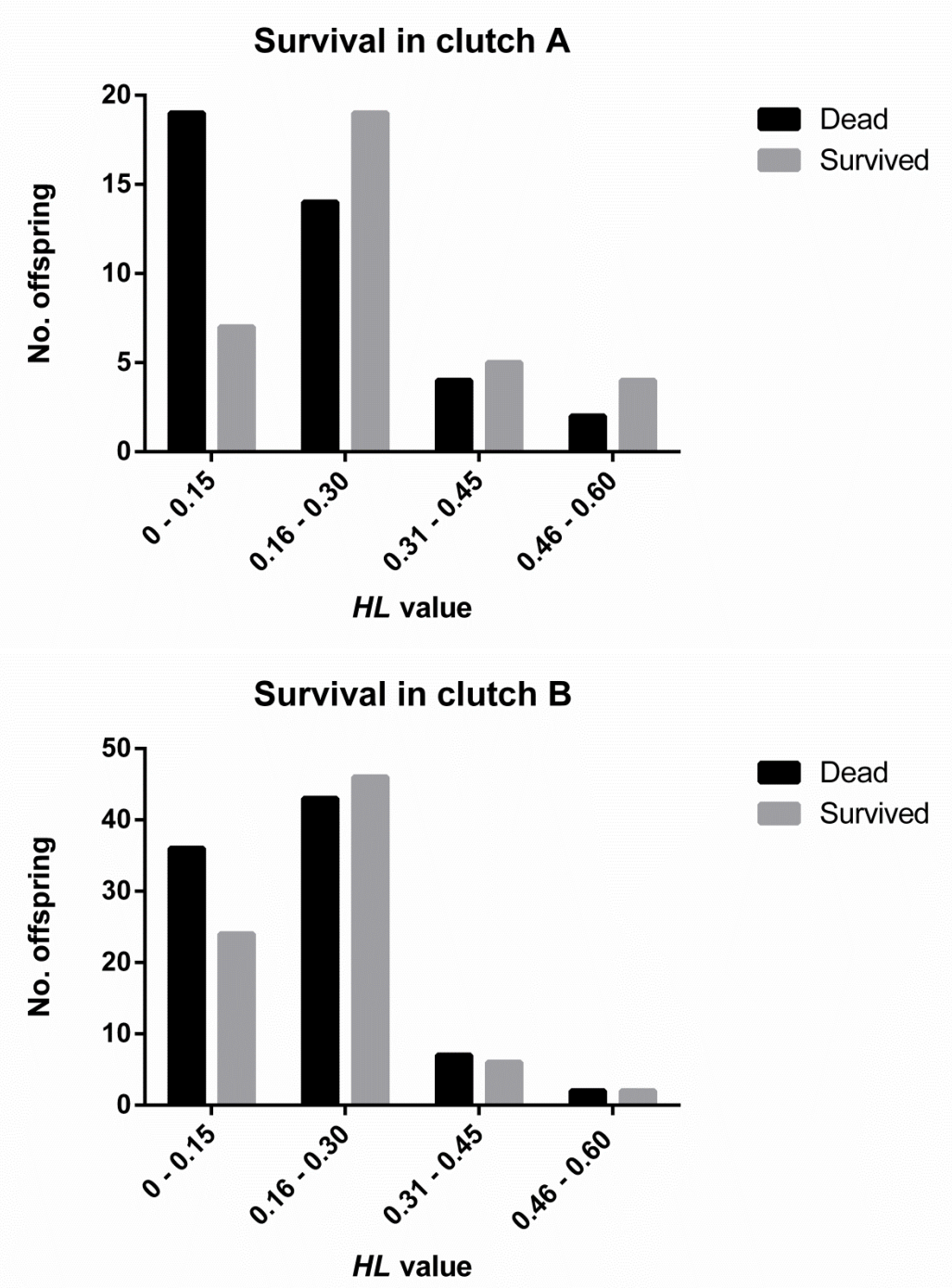
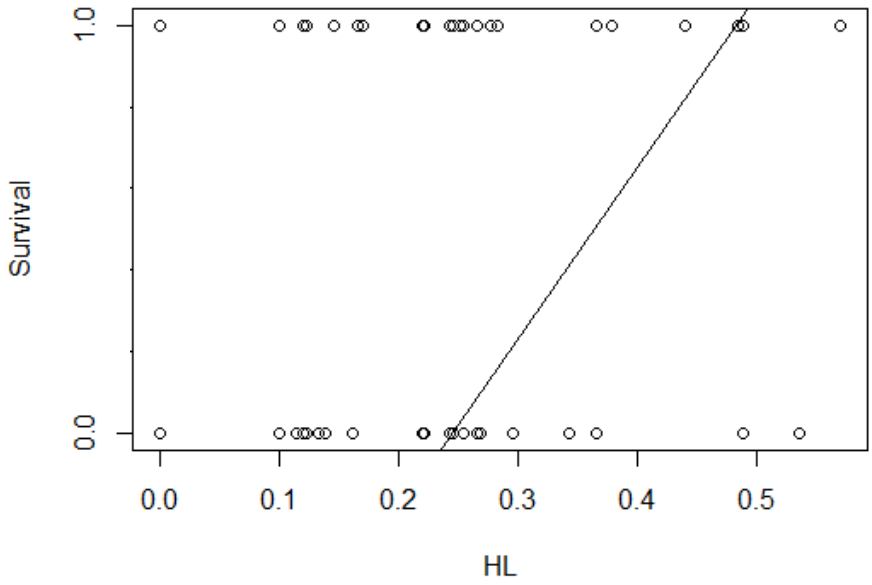


Figure 1. Numbers of hellbender (*Cryptobranchus alleganiensis*) offspring with differing survival but similar *HL* values, where more homozygous offspring exhibit higher *HL* values.

Table 3. Differing levels of heterozygosity exhibited by survived and dead offspring for hellbender (*Cryptobranchus alleganiensis*) clutch A and clutch B.

	Avg. *HL* for survived	**Avg. *HL* for dead**
Clutch A	0.256	0.186
Clutch B	0.208	0.169

Clutch A)



Clutch B)

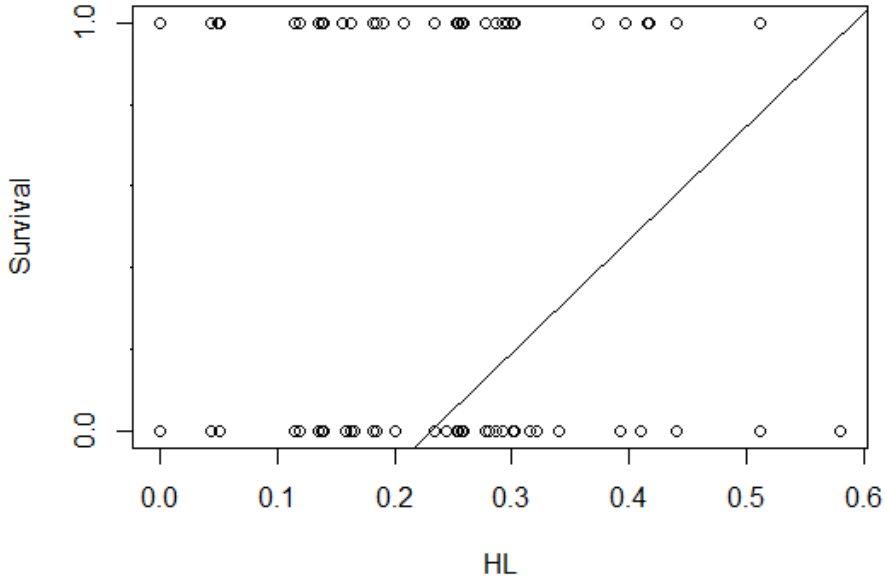


Figure 2. Regression of hellbender (*Cryptobranchus alleganiensis*) offspring survival, where 1 indicates survival and 0 indicates died, against individual levels of heterozygosity (*HL*) where higher values indicate higher homozygosity.