Examining genetic diversity, outbreeding depression, and local adaptation in a native fish reintroduction program

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Abstract

Reintroductions are a common approach for preserving intraspecific biodiversity in fragmented landscapes; however, reintroduced populations are often smaller and more geographically isolated than native populations. Reintroductions may therefore exacerbate the reduction in genetic variability initially caused by population fragmentation due to the small effective population size of the reintroduced populations. Mixing genetically divergent sources is assumed to alleviate this issue by increasing genetic diversity, but the effects on genetic diversity are often not monitored and there are potential negative tradeoffs for mixing genetically distinct sources.

I examined the consequences of mixed-source reintroductions on the ancestral composition, genetic variation and fitness of a small stream fish, the slimy sculpin (*Cottus cognatus*), from three source populations at nine reintroduction sites in southeast Minnesota. I used microsatellite markers to evaluate allelic richness and heterozygosity in the reintroduced populations relative to computer simulated expectations. I then inferred the fitness of each crosstype in the reintroduced populations by comparing their overall persistence, growth rates, and relative body conditions. Finally, I modeled the response of fitness related variables in the reintroduced populations to variation in habitat using a combination of regression and ordination methods.

Ancestry analysis revealed that one of the three sources had more ancestors than the other two sources at most reintroduction sites. Sculpins in reintroduced

populations exhibited higher levels of heterozygosity and allelic richness than the sources, but only slightly higher than the most genetically diverse source population. Simulations of maximum genetic variation indicated only a modest expected increase over the most diverse source. Growth rate, body size, and relative body condition suggest significantly reduced fitness in second generation hybrids. I detected evidence of local adaptation in the source populations based on greater predicted fitness for each source in its respective habitat. This local adaptation is strongly associated with a gradient in winter water temperatures. My study indicates that using more than one source for reintroductions may not substantially enhance genetic diversity. Furthermore, using multiple sources risks disruption of important adaptations and may cause outbreeding depression. Future reintroductions may be improved by evaluating the potential for local adaptation in ongoing reintroduction programs.

Table of Contents

List of Tables

List of Figures

Prologue

Reintroductions aim to reestablish a species to its former range, but this often must occur in environments that are modified by humans. The goal is not only to ensure species viability and enable long-term persistence in the face of environmental change, but also to preserve the evolutionary processes that sustain genetic diversity. It is a challenge for conservation biologists to decide whether the existing populations that they choose as a source of reintroduced animals should be matched to a set of local conditions at the reintroduction site or be mixed to provide greater genetically-based adaptive potential for novel (disturbed) environments. Mixed-source reintroductions are thought to be advantageous in disturbed environments, but they have drawbacks because unique evolutionary lineages should be preserved as much as possible to preserve genetic diversity. Mixing sources may also disrupt lineages that have distinct adaptations to local conditions. My research investigates the success and persistence of reintroduced populations of a stream fish, the slimy sculpin, in the driftless region of southeast Minnesota. Populations of slimy sculpin were present in this region historically, but many were extirpated as a result of poor land use practices.

Fish have often been stocked to reintroduce populations of extirpated species, yet we still know little about the factors that lead to success or failure of this approach. My research will be one of the first studies in which multiple reintroductions will be carried out simultaneously in close proximity to one another. This degree of "field replication" is unusual and is a necessary next step to advance this kind of research.

Because these populations are both isolated and geographically close, they provide an ideal model to address general questions relevant to other reintroductions of freshwater fish throughout the world. Although there are currently greater than 50 peer-reviewed papers per year related to reintroductions, only 4% of them focus on fish species (Seddon et al. 2007). My research will lead to a better understanding of the biology of sculpins and provide guidance for managers to improve reintroductions as a tool for conserving imperiled species. By addressing key questions in the emerging field of reintroduction biology we can improve this species' recovery in the region and produce research that will advance the field.

I wrote each chapter of this dissertation in the form of a manuscript to be submitted to a peer-reviewed journal for publication. Bruce Vondracek and Loren Miller will be included as co-authors on all chapters and Christopher Chizinski will be included as a co-author on Chapter 2 and 3. I, therefore, use the plural pronoun "we" rather than the singular "I" in each of the chapters. As of the time of this writing, Chapter 1 has been accepted for publication in the journal, Conservation Genetics, pending revisions.

Chapter 1

Patterns of ancestry and genetic diversity in reintroduced populations of the slimy sculpin: Implications for conservation

Introduction

Reintroductions that attempt to re-establish a species within its historical range (IUCN 1998) are a common approach for preserving intraspecific biodiversity in fragmented landscapes (Fischer and Lindenmayer 2000, Seddon et al. 2007). However, reintroduced populations are generally smaller and more isolated than native populations. Reintroductions may therefore exacerbate the genetic erosion initially caused by population fragmentation by reducing the effective population size (Lande and Barrowclough 1987) of both the source and reintroduced populations (Griffith et al. 1989, Wolf et al. 1996). Several studies have documented significant reductions in the genetic variability of reintroduced populations relative to their source (Fitzsimmons et al. 1997, Mock et al. 2004). Reduced genetic variation may decrease evolutionary potential, reduce ability to fight off disease, and increase other harmful effects of inbreeding (Keller and Waller 2002).

A reintroduction method employed to increase genetic variability is to mix genetically divergent source populations (Tallmon et al. 2004, McClelland and Naish 2007). Intraspecific hybridization is thought to impart a high degree of adaptive potential for the novel ecological situations that often occur at reintroduction sites (Lesica and Allendorf 1999, Jones 2003). There are at least two potential disadvantages associated with mixed sources. First, hybridization between genetically disparate individuals may result in a decline in fitness of the offspring, referred to as outbreeding depression (Edmands 2007, McClelland and Naish 2007). Mechanisms implicated in

outbreeding depression may be “intrinsic,” due to the breakdown of co-adapted gene complexes in hybrid progeny that result in the loss of adaptive traits that significantly affect survival (Templeton et al. 1986). “Extrinsic,” mechanisms for outbreeding depression occur when each parent is locally adapted to different ecological conditions. The offspring have an intermediate phenotype, rendering them less fit in either parental environment. Second, mixing source populations may disrupt unique genetic identities (Moritz 1999, Jones 2003). Preserving the intraspecific variation present across a species’ range is widely accepted as a critical conservation priority, and the loss of genetically distinct populations is considered by some to be as significant as the loss of entire species (Ehrlich 1988, Foster et al. 2003). From a restoration standpoint, preserving genetic identity is desirable to protect the ecological and genetic processes in neighboring communities and in remnant conspecific populations that may be influenced by gene flow from the reintroduced population.

There is a troubling decline of indigenous freshwater fish populations in North America (Miller et al. 1989, Minckley and Deacon 1991, Jelks et al. 2008) and reintroductions have been included as part of more than 80% of the recovery plans for threatened and endangered species (Williams et al. 1988). Although most endangered and rare species exist as small, isolated populations (Holsinger and Gottlieb 1989), populations of common species are becoming fragmented and isolated as well. The loss of genetic diversity within species that have small, fragmented populations due to habitat destruction is a major concern in species conservation (Lande 1988, 1998). Our

study organism, the slimy sculpin (*Cottus cognatus*), tends to be locally abundant (Bond 1963, Petrosky and Waters 1975), but is a poor disperser (Schmetterling and Adams 2004). Contrary to the biased view of rare species extirpation as a result of habitat destruction, abundant species can be among the first to be locally extirpated. Although common species tend to be superior competitors in a given habitat, they may be vulnerable because of poor dispersal abilities (Tilman et al. 1997). Furthermore, common species have been shown to be at least as susceptible to the genetic consequences of habitat fragmentation as rare species (Honnay and Jacquemyn 2007).

It is important to assess the founder effects of reintroduction practices on genetic variation and evaluate alternatives that could alleviate undesired consequences, yet there are relatively few studies of the effects of reintroductions on genetic diversity or the number and identity of founders that contributed to the newly established populations (Latch and Rhodes 2005). Our study documents the ancestral composition and genetic variation resulting from mixed source reintroductions of slimy sculpin at nine distinct stream sites that are in close geographic proximity. We completed genetic analyses from the source and reintroduced populations using eight microsatellite loci to achieve three objectives: 1) to compare the levels of heterozygosity and allelic richness relative to modeled expectations; 2) to quantify the proportional source ancestry of the populations, as well as the ancestry of individual fish and compare these to the originally stocked proportions; and 3) to characterize the genetic identity of the reintroduced populations relative to the source populations. Because a number of

isolated and easily sampled populations are available, this reintroduction program provided a unique opportunity to understand post-reintroduction processes and evaluate options for future reintroductions that may improve conservation practices for native fishes.

Methods

Study organism

The slimy sculpin is a small (< 130 mm), cryptic, freshwater fish that occupies benthic habitats in lakes, rivers, and small streams. The range of slimy sculpin extends from Virginia, USA to Labrador in eastern Canada and northwest across Canada to eastern Siberia (Scott and Crossman 1979). *Cottus* spp. are often locally abundant and are frequently a prominent constituent of ecosystems suited to trout and other cold-water fish (Petrosky and Waters 1975, Goyke and Hershey 1992). Slimy sculpins in the study region spawn once per year during the early spring beginning at age II, or rarely at age I, and live up to 6 years (Petrosky and Waters 1975).

Slimy sculpin populations in southeastern Minnesota occur at the warmer southern limits of their range. They are limited by sparse thermal habitat in the small reaches of stream near cold perennial groundwater inputs. As a result, these populations are completely isolated from other populations making them susceptible to local extinction through risks such as catastrophic events or inbreeding depression (Saccheri et al. 1998). Anthropogenically triggered shifts in hydrologic regimes could cause slimy sculpins, which require a constant source of cold water, to disappear or struggle to survive at the peripheries of their range where clinal thermal variation is clearly identifiable (Meisner 1990a, Magnuson and Destasio 1997).

Study area and reintroduction project

The study area (Fig. 1) is located in the Driftless Region of southeast Minnesota, USA. This region is characterized by steep-sided ridges, caves, limestone and sandstone bluffs, sinkholes, and 5,800 kilometers of spring creeks fed by limestone and sandstone aquifers that eventually drain into the Mississippi River (Tester and Keirstead 1995). The area remained free of ice during the most recent glacial maxima, despite the expansion of the Laurentide ice sheet much farther to the south (Holliday et al. 2002).

Prior to major settlement by European immigrants beginning in 1850, nearly all of the spring-fed streams in the region held trout and presumably slimy sculpins. In subsequent years, slimy sculpin and other cold-water fish abundance declined because of overexploitation and severe habitat degradation (Waters 1977, Leopold and Sewell 2001). Since the 1940s, the Minnesota Department of Natural Resources (MNDNR) and other organizations have completed hundreds of in-stream and watershed improvement projects. These projects stabilized eroding banks, narrowed streams to increase velocity and depth, improved instream substrates, increased fish cover, and increased riparian tree abundance to help maintain cool water temperatures (Waters 1977, Thorn et al. 1997, MNDNR 2003).

In locations where the habitat has been restored sufficiently, MNDNR personnel recently reintroduced slimy sculpins. The goal was to reestablish viable, self-sustaining populations where native populations were likely present historically, but were extirpated. Beginning in 2003, nine streams in the study area (Table 1; Fig. 1) were stocked with a mixture of sculpins from three donor streams. The source streams are

small tributaries within separate sub-drainages that enter the Mississippi River along approximately 40 river kilometers. Stocked fish were randomly selected from each of the source streams for translocation, but young-of-the-year fish were generally avoided. Prior to stocking each year, sculpins were collected from the source streams and subsequently examined by MNDNR fish pathology lab personnel for evidence of diseases to verify that translocated sculpins would not transmit pathogens to fish living in recipient streams.

Sampling

Sampling for the genetic analysis was conducted in autumn 2007 at all source and recipient sites except Pickwick Creek, which was sampled in autumn 2008. Fish were collected using a Wisconsin™ Abp-3 pulsed DC backpack electrofisher with power output settings adjusted to minimize negative effects on the reintroduced fish (Cowx and Lamarque 1990). Each fish was lightly anesthetized using tricaine methanosulfate (Summerfelt and Smith 1990), weighed, and measured for standard length. A small amount of tissue was clipped from the left pelvic fin of each fish and preserved in 95% ethanol for genetic analysis. After processing, fish were returned to a recovery bucket with fresh water and eventually returned to the stream. . None of the originally stocked fish, which were marked with a fin clip upon their release, were re-sampled.

DNA extraction and amplification

We used eight microsatellite loci developed for other *Cottus* species that resolved genetic variation in *C. cognatus*: Cgo18, Cgo42, Cgo310, and Cgo1033 (Englbrecht et al.

1999); Cott290, Cott686, and CottES1 (Nolte et al. 2005b); and Cba14 (Fiumera et al. 2002). We prepared samples for polymerase chain reaction (PCR) amplification using a simple DNA extraction procedure. A small sliver of fin tissue (approximately 1 mm^2) was placed in a 1.5 ml tube with 250 ml of a 5% solution of a chelating resin (Chelex®, Sigma Chemical, St. Louis, MO). Samples were incubated overnight in a 56°C water bath and boiled 8 min. Microsatellite amplification was performed in 15 µL reactions containing 1x polymerase buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton® X-100), 1.5 mM MgCl2, 0.2 mM each dNTP, 0.5 µM of the forward and reverse primers, with the forward primer labeled with a fluorescent dye 6FAM, VIC, NED or PET, and 0.5 units Taq DNA polymerase (Promega, Madison, WI). Loci were combined into three multiplexed reactions: Cba14, CottES1, Cott290 and Cott686; Cgo18 and Cgo310; and Cgo42 and Cgo1033. Each set of samples included a water blank as a negative control to detect possible contamination of PCR solutions. Amplification was carried out in a thermocycler (Hybaid Omn-E, Thermo-Hybaid U.S., Franklin, MA) with 35 cycles at the following temperature profile: 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min; followed by a 20 min extension at 72°C. We submitted PCR products to the Biomedical Genomics Center (University of Minnesota, St. Paul) for electrophoresis on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). We scored alleles using the software program GENOTYPER 2.5 (Applied Biosystems 2001).

Genetic diversity analysis

We evaluated several aspects of the genetic diversity within each sample, including allelic richness, the inbreeding coefficient (F_{IS}), which measures the degree of inbreeding due to the mating system, and the observed (H_O) and expected (H_E) heterozygosities. Allelic richness, a measure of allele counts adjusted to a common sample size, was calculated using rarefaction techniques in HP-Rare (Kalinowski 2005). F-statistics were calculated and conformance with Hardy-Weinberg expectations was tested for each locus in each sample using the exact test (Guo and Thompson 1992), as implemented in GENEPOP v4.0.4 (Raymond and Rousset 1995) using 10,000 dememorizations, 20 batches, and 500 iterations per batch. GENEPOP was also used to test for linkage disequilibrium between pairs of loci. Significance values for both tests were adjusted using sequential Bonferroni procedures (Rice 1989). Data from the three source populations were evaluated in MICROCHECKER v2.2.3 to detect evidence of null alleles or scoring errors due to large allele drop-off (Van Oosterhout et al. 2004). We used transformed fixation index (F_{ST}) estimates ($-\ln [F_{ST} - 1]$) (Reynolds et al. 1983) among pairs of reintroduced and source populations to generate a cluster dendrogram using the flexible-β algorithm with $\beta = -0.7$ implemented in PC-ORD (Legendre and Legendre 1998, McCune and Mefford 1999).

We modeled the genetic diversity that the reintroduced populations might obtain after one generation, assuming random mating and reproductive success, using a computer program written as function scripts implemented in the R language (Ihaka and

Gentleman 1996). Allele frequencies at each of the eight microsatellite loci were calculated using baseline data from the three source populations. Alleles were then chosen randomly for each locus in proportion to their occurrence in the source populations to artificially generate multilocus genotypes for the total number of diploid individuals that were stocked at a given site from each source population (Table 1). These individuals were pooled and a 1:1 ratio of males to females was designated randomly in this population. A male and a female were randomly drawn without replacement and haploid gametes were then extracted from these by randomly selecting one of the two copies at each locus. Each artificial "sperm" and "egg" was combined randomly until enough offspring were created to replace all of the parents. The family size of each pairing was chosen randomly from a Poisson distribution with a mean of two. Fifty of these offspring were randomly chosen and used to quantify allelic richness and heterozygosity values to simulate the rarefaction method used to calculate genetic variation statistics from our actual data. This entire process was looped 500 times for each reintroduced population to produce means and error estimates. These function scripts may be obtained from the senior author upon request.

Ancestry analysis

The contribution of each source population to the recipient populations was estimated using the Bayesian clustering algorithm implemented in the program STRUCTURE (V. 2.2.3; (Pritchard et al. 2000); also refer to http://pritch.bsd.uchicago.edu). The number of populations (K) was set to 3, the known number of genetically distinct source

populations, with an admixture model and correlated allele frequencies. The program was run with a 50,000 burn-in period followed by 100,000 Monte Carlo simulations. Baseline individuals were included in the runs without population identification to assist resolution of genetically differentiated clusters and determine the ability of STRUCTURE to correctly determine the ancestry of known fish. The contribution of each source population was estimated by the proportion of membership assigned to each of the K populations across all individuals in a recipient site sample, and using the known baseline fish to determine the correspondence of the STRUCTURE-determined populations and the known sources. Expected overall ancestry values were the proportions of each source population stocked at the recipient sites.

We also determined ancestry for individual fish and categorized them into nine crosstypes: pure strain (Beaver, Garvin, and Cold Spring), F_1 generation hybrids (Beaver x Garvin, Beaver x Cold Spring, Cold Spring x Garvin) and advanced generation crosses, which include F_2 hybrids and back-crosses (Beaver x Garvin, Beaver x Cold Spring, Beaver x Garvin x Cold Spring, Garvin x Cold Spring). Because reintroduced populations were sampled within a few years of stocking, the software NewHybrids (Anderson and Thompson 2002) was used to classify individual fish according to pure strain or hybrid categories assuming that only first- or second-generation descendents of founders were present. Individual classification required a two-step process because NewHybrids allows only two parental populations. First, for each source population individuals were removed from the dataset if STRUCTURE indicated they had a low probability of

ancestry (<0.125 for populations with second generation descendents and <0.25 for Pickwick and Little Pickwick sites, that only had first generation descendents) from that source. If all three sources contributed ancestry, the individual was classified as BxGxC and not included in a NewHybrids dataset. Then, for each dataset containing fish from only two source populations, the probability that each fish was either from one source or a hybrid cross (F_1, F_2 or backcross) was determined using NewHybrids. Baseline individuals from the two appropriate sources were included in the analyses. Each run had a 50,000 burn-in period followed by 150,000 simulations, using Jeffrey's priors for allele frequencies and mixing proportions. Runs were repeated using different seeds to verify that consistent solutions were found. Individuals were classified into a parental or hybrid category if their probability of membership was greater than or equal to 0.70; otherwise the specific classification of the individual was considered uncertain. Because second-generation hybrids were difficult to distinguish, probabilities of membership to the F_2 and backcross categories were combined into one category called "advanced-generation mixtures."

Expected cross type proportions were estimated using a multinomial distribution based on the proportions of each strain that were stocked assuming equal survival and reproduction with individual replacement (i.e. no population growth in proceeding generations) for two generations beyond stocking. The first generation only consisted of pure strain and F_1 hybrids and the second generation included the advanced-generation mixtures in addition to the first generation crosstypes. Expected proportions were based

on equal numbers of individuals from both generations. The two Pickwick sites only included first generation crosstypes in the expected estimate because of the timing of stocking relative to sampling. Statistical assessment of the difference between expected and observed values for each category were made using the Median test (Zar 1999), a version of the Kruskal-Wallis ANOVA that frames the computation in terms of a contingency table.

Results

Genetic diversity

No source population deviated from Hardy-Weinberg Equilibrium (HWE) at any locus, and MICROCHECKER provided no evidence for null alleles or allele drop-off. In contrast, all reintroduced populations showed significant deviation from HWE (heterozygote excess or deficiency) at one or more loci (Table 2). Inbreeding coefficients (F_{IS}) ranged from -0.25 to 0.25 across all loci, but there were no clear patterns in heterozygote excesses or deficits among reintroduced populations. Overall F_{IS} values at the source streams (Table 3) were all slightly negative.

There were significant differences among the populations in both H_O (ANOVA; main effects: population, locus; $F(11, 77) = 7.62$, $p << 0.001$) and allelic richness (ANOVA; main effects: population, locus; $F(11, 77) = 10.94$, $p << 0.001$) (Fig. 2). Allelic richness (Table 2) among loci ranged from 1 to 15.1, and was higher for the reintroduced populations (mean = 6.4) than for the source populations (mean = 4.1). Of the source sites, Cold Spring Brook had the lowest heterozygosity and allelic richness, whereas Beaver had the highest (Table 3). Overall, heterozygosity and allelic richness at the reintroduction sites was only slightly higher than for the Beaver source population (Fig. 2). Simulated H_O was generally similar to H_O in the reintroduction populations, while simulated allelic richness was higher than all nine reintroduced populations (Table 3).

Pairwise F_{ST} values indicate substantial genetic differentiation among the three source populations (Garvin vs. Cold Spring, F_{ST} =0.56; Beaver vs. Cold Spring, F_{ST} =0.48;

Beaver vs. Garvin, F_{ST} =0.32). The dendrogram, generated from pairwise F_{ST} estimates, indicated distinct divisions that corresponded with the three source populations (Fig.3). The reintroduced populations all clustered with Beaver and were relatively similar, although F_{ST} estimates among all pairwise comparisons were significant ($P<0.05$).

Patterns of ancestry

Overall ancestry determined using STRUCTURE (Table 4) indicated that a higher than expected proportion of ancestry from the Beaver population occurred in all but one of the reintroduction sites. Cold Spring and Garvin ancestry generally occurred in lower proportions than expected, but each considerably exceeded expectations in a few locations. For example, Cold Spring ancestry was 17% greater than expected at the Hay Creek reintroduction site and Garvin ancestry was 14% and 11% greater at Pickwick Creek and Rock Creek reintroduction sites, respectively. These large deviations were apparent even though bias tended to equalize ancestry estimates among sources. The known source samples analyzed using STRUCTURE each had 2-4% assignment error (ancestry was assigned to one of the other two sources). Because these errors were approximately symmetrical among sources, ancestry estimates were biased toward less prevalent ancestry, such as Cold Spring. Indeed, at Latsch Creek and Rock Creek reintroduction sites overall estimates indicated 4% Cold Spring ancestry, but no individuals appeared to be Cold Spring descendants based on low levels of assignment to Cold Spring ancestry and a lack of Cold Spring specific alleles.

Individual ancestry analysis (Table 5, Fig. 4) revealed that there were more sculpins of pure Beaver ancestry at the reintroduction sites (mean=27%) than any other cross type. The number of Beaver individuals was significantly greater than expected based on the number that were stocked (Median test; median=0.111, Chi-Square=14.40, p=0.0001), whereas Cold Spring (Median test; median=0.014, Chi-Square=3.60, p=0.0578) and Garvin x Cold Spring advanced-generation mixtures (Median test; median=0.216, Chi-Square=5.56, p=0.0184) occurred at significantly lower frequencies than expected. Garvin pure strain fish were on average less abundant than Beaver (mean=8%) and there were relatively few fish of pure Cold Spring ancestry (mean=1%). Beaver x Garvin was the most abundant F_1-hybrid type (mean=13%) and Beaver x Cold Spring and Garvin x Cold Spring were similar in abundance (mean=3-4%). All of the advanced generation mixtures accounted for a total of 32% of the fish and 12% of the fish were of uncertain origin. Although fish of pure strain Cold Spring ancestry were rare at the reintroduction sites, overall Cold Spring ancestry was represented in roughly the same proportion as Garvin ancestry (Table 4) because F_1 and advanced generation mixtures that contained Cold Spring ancestry were abundant.

Errors in assigning individual cross types may have occurred at two occasions, when the dataset was reduced to include only two source ancestries at a time and when the remaining individuals were classified into cross types. Simulated genotypes run in STRUCTURE indicated low rates of erroneous inclusion (< 1%) in reduced two-ancestry datasets for all crosstypes except advanced generation cross types involving all three

ancestries. F_1, F_2 and advanced generation crosstypes involving only two ancestries were incorrectly designated as advanced Beaver x Garvin x Cold Spring at rates of 0.1-5% while 11-15% of simulated advanced Beaver x Garvin x Cold Spring was incorrectly included in reduced two-ancestry datasets. Simulations for each pair of sources (Beaver and Garvin, Beaver and Cold Spring, Garvin and Cold Spring) in NewHybrids estimated error rates of 0-6% for Beaver, Garvin, Cold Spring and F_1 crosstypes; all errors assigned to advanced generation crosstypes. For advanced generation cross types, 1-4% of Garvin x Cold Spring and 4-12% of Beaver x Garvin and Beaver x Cold Spring assigned to Beaver, Garvin, Cold Spring or F_1.

Discussion

Reintroductions and genetic diversity

The few reports that describe levels of genetic diversity in slimy sculpin populations (Zimmerman and Wooten 1981, Strauss 1986) indicate that it generally tends to be low. This is presumably the result of isolation, small population sizes, and low vagility, among other potential causes (Fiumera et al. 2002). Genetic diversity potentially affects a variety of population and community processes. Our understanding of its effects depends on the relationship between measured variation in putatively neutral markers and the magnitude of variation in phenotypic traits. In some cases, phenotypic characteristics within populations that correspond to ecologically relevant traits and neutral genetic variation are correlated (Reed and Frankham 2001). More genetically diverse mixtures of reintroduced animals will have a higher probability of including a genotype that is more productive in a novel environment, such as a reintroduction site (Barrett and Schluter 2008). Mixed stock reintroductions in our study, however, resulted in mean heterozygosity and allelic richness levels across all sites that were only slightly higher than those found at the Beaver source site. Simulated heterozygosities were comparable to the observed values while simulated allelic richness values were only moderately higher (mean = +12%). Our expectation was that simulated values would be much higher than the observed values because our simulations were designed to mimic ideal circumstances for high genetic variation: random mating, equal survival, random reproductive success, and measurement of

variation in the first generation after stocking. Our simulations also indicated that, at best, we would only expect a moderate increase in heterozygosity and allelic richness beyond levels in Beaver, the highest diversity source population. A similar result was reported for reintroductions using the ibex, *Capra ibex* as a model species (Maudet et al. 2002). The fact that observed diversity levels are as high as they are is a bit surprising because it is clear that some combination of differential fitness, assortative mating, or other factors led to asymmetrical ancestral proportions that favored the Beaver strain. The enhanced diversity came especially from the relatively abundant admixed individuals at many sites. Although Garvin and Cold Spring populations had lower diversity overall, they had multiple unique alleles. Interpopulation crosses would have introduced new alleles and produced many more heterozygotes compared with the Beaver population.

The two sites stocked most recently, Pickwick Creek and Little Pickwick Creek, had the highest allelic richness. A reasonable explanation is that these populations, with only first-generation descendents of stocked fish, lost fewer rare alleles due to drift. The other populations with second-generation descendents would have been susceptible to additional drift losses. After a few generations, the decrease in allelic richness should slow as there are fewer rare alleles left to be lost (Nei et al. 1975). Except for higher richness in the Pickwick populations, there were no other strong trends in genetic diversity measures versus the number of successive stocking events.

Although all of the reintroduced populations indicated significant deviations from HWE, the lack of a clear pattern across markers and sites in heterozygote deficiency or excess is likely due to the complexity of effects that are interacting at the sites. For example, heterozygote excesses could arise from disassortative mating among strains or differences in allele frequencies among males and females in populations with small effective size (Allendorf and Luikart 2007). Heterozygote deficits could arise from within-strain mate choice or demic structure that corresponds to ancestral origins because of heritable differences among stocks in characteristics such as spawning habitat preference and timing of spawning (Ryman et al. 1979). Furthermore, selection acting on homozygous or heterozygous genotypes associated with strain crosses could also cause excess or deficits. Although departures from HWE are difficult to explain, they may suggest strain differences in mating systems, behavior, and survival and therefore warrant further investigation. Many microsatellite loci deviated from HWE in the reintroduced populations, whereas there were no significant deviations from HWE in the source populations. These deviations from HWE up to two generations after reintroduction suggests barriers to admixture, as HWE should be achieved in the first generation of random mating and a heterozygote advantage would produce heterozygote excesses broadly (Allendorf and Luikart 2007). Locus-specific effects on fitness at the reintroduction sites (Nolte et al. 2009), in addition to previously mentioned dynamics, could obscure a broad pattern of heterozygote deficits.

Reintroductions and patterns of ancestry

Even minor differences in the relative fitness of introgressant lineages can lead to drastically different admixture rates (Epifanio and Philipp 2000). Beaver ancestry was over-represented at eight of nine populations, often by a substantial margin (Table 4), whereas both Cold Spring and Garvin ancestry was under-represented with a few exceptions. Possible explanations that could operate alone or simultaneously to explain the differential success of the Beaver strain include an adaptive advantage in the Beaver strain, or inbreeding depression in the Garvin and Cold Spring strains. Simultaneous inbreeding and outbreeding depression has been demonstrated in animal populations (Marshall and Spalton 2000) and may be especially common in fish species that exhibit philopatry or low vagility (Neff 2004). The first possibility is that an adaptation to local conditions in Beaver Creek is advantageous at a majority of the reintroduction sites, thereby conferring a selective advantage upon sculpins with Beaver ancestry. In this case, a significantly higher proportion of pure strain Beaver fish would be expected across all or most reintroduction sites. The other two strains may reduce the fitness of the Beaver strain through introgression by disrupting beneficial gene complexes or local adaptations. This explanation is consistent with our analysis because pure strain Beaver sculpins were persistent in high proportions at nearly all of the reintroduction sites even though nearly complete admixture with the other two strains after a few generations would have been expected (Epifanio and Philipp 2000).

An alternative to adaptive differences among strains is that there are disadvantages in the Cold Spring and Garvin strains as a result of inbreeding depression. This scenario predicts that pure strain Cold Spring and Garvin individuals should perform poorly across all sites and that heterosis could occur in F_1 hybrids and advanced generation mixtures. There were relatively few pure strain Cold Spring and Garvin individuals that persisted, but overall ancestry was similar or only slightly lower than one would expect given the number of these fish that were originally stocked. Mixed-ancestry descendents could have benefited from increased heterozygosity that alleviated inbreeding depression. However, pure Garvin and Cold Spring individuals occurred in relatively high numbers at one or more reintroduction sites and are abundant at the source streams (D. D. Huff, unpublished), which indicates a lack of widespread, severe inbreeding depression in these strains.

Classification errors in determining crosstypes were unlikely to alter our major findings and conclusions. The strong differentiation among source populations provided high power to distinguish crosstypes. Simulations indicated some potential bias toward reduced estimates of advanced Beaver x Garvin x Cold Spring crosstypes when reducing datasets to two source ancestries and toward reduced estimates of Advanced Beaver x Garvin and Beaver x Cold Spring and conversely increased estimates of Beaver, Garvin, Cold Spring and F_1 when assigning crosstypes. Contrary to the potential biases, estimates of Garvin, Cold Spring and F_1 crosstypes were all less than expected.

Genetic Identity

Summary of genetic divergence among populations using F_{ST} values or genetic distance measures (Pasko and Maslak 2003, Mock et al. 2004, Latch and Rhodes 2005) should be used cautiously in assessing the genetic identity of mixed source reintroductions. The mean value represented by F_{ST} can be misleading because a presumed population may remain a conglomeration of distinct syntopic populations for many generations. Our dendrogram (Fig. 3) indicated that the reintroduced populations are most similar to, or retained the "genetic signature," of the Beaver source population. In reality, the genetic composition of the reintroduced sites (Fig 4) is a mixture of various pure strain and interpopulation hybrids unlike that found at Beaver. Mixtures of distinct strains are of concern when relying on population assignment techniques to describe the composition of populations. Assigning individuals to a single source population based on the highest proportion of ancestry alone oversimplifies the situation and ignores the possibility of hybridization (e.g. Latch and Rhodes 2005) and hybrids can have very different fitness than their parental strains (Edmands and Timmerman 2003). By determining the ancestry of individuals with an admixture model, we avoided these issues and were able to represent the complexity of the ancestral composition in the reintroduction populations.

Knowledge regarding the genetic distinctiveness of source populations is essential for making decisions about reintroduction practices. When genetic distinctiveness among populations is historically based, it could be preserved because it

is irreplaceable; whereas adaptive divergence is potentially reproducible, provided that the evolutionary processes that created it and raw genetic diversity are intact (Moritz 1999). The question of how best to preserve genetic identity is a matter of scale. Slimy sculpins occur at the warmer southern limits of their range in the Driftless Region. Given the precarious long-term persistence of cold water habitat globally (Meisner 1990b, Magnuson and Destasio 1997), the choice to preserve individual populations in the Driftless Region may be considered by some managers as subordinate to the necessity to maximize genetic diversity within the region. Should the goal be to preserve the genetic identity of slimy sculpins within the Driftless Region as a whole? If yes, then mixing distinct populations from within the region would appear to be the best decision. This option is more likely to provide adaptive potential for reintroduced populations, yet retain regional, historically based genetic identity. If source populations only have adaptive differences, then the selective pressures present in the reintroduction environment may act upon mixed populations again and restore their unique adaptive trajectory. On the other hand, if differences among populations within the region are historical and deep, then these populations should not be mixed unless there is evidence that genetic rescue (Tallmon et al. 2004) is necessary.

Reintroductions: One versus multiple sources

Thus, the decision whether or not to mix source populations for reintroduction depends on knowledge of the degree of distinction among the candidate source populations. Some conservation biologists have suggested that such a distinction is signified by

reciprocal monophyly as determined by mtDNA haplotypes, in addition to divergence of allele frequencies at nuclear loci (Moritz 1999). Others argue that evidence of ecological divergence is sufficient to warrant actions to preserve genetic identity (Crandall et al. 2000). These issues continue to be actively debated (Zink 2007).

The time and expense involved in determining whether populations are ecologically or genetically divergent often require that a decision be made without adequate knowledge. Clearly there are tradeoffs associated with using one versus multiple sources in reintroductions. Intraspecific variation could be utilized to enhance the success of reintroductions in which complete habitat restoration is beyond the reach of natural resource managers. Organisms used as a source for reintroductions have a complex history of selective pressures that maximized their survival and can be difficult to quantify. Deviation from these conditions will likely reduce fitness. Therefore, the best choice of reintroduction source and whether to use a single source or to mix sources depends not only on the characteristics of the populations but also on the condition of the reintroduction environment (Lesica and Allendorf 1999, Jones 2003). Hybrid mixtures among populations could be suitable for relatively disturbed sites that are unlikely to have a compatible “pre-adapted” source population, or if there is evidence of maladaptation (Crespi 2000), no local adaptation, or high phenotypic plasticity (Mittelbach et al. 1999).

Because of variation in habitat and degree of isolation, sculpin populations in the Driftless Region may not only have a unique evolutionary history, but also be

ecologically inexchangeable with one another (Crandall et al. 2000, Rader et al. 2005). The choice to mix sources may have the disadvantage of disrupting different adaptations among populations in the region and lead to decreased fitness. This raises the concern for potential outbreeding depression, which has been shown to occur in a variety of fish species wherein unrelated populations were crossed (McClelland and Naish 2007). Unfortunately, the consequences of outbreeding are difficult to predict. An interesting outcome of outbreeding has recently been documented for a closely related species, the European sculpin (*Cottus gobio*), in the River Rhine drainage in Germany (Nolte et al. 2005a). In this instance, anthropogenic removal of natural barriers between long separated lineages generated invasive intraspecific hybrids that were adapted to conditions unlike those of either parental strain.

Conclusions

Mixing distinct genetic sources produced only modest increases over the most genetically diverse source in heterozygosity and allelic richness in the reintroduced populations. Although the increases were modest, our simulations indicated that they were not greatly lower than that expected under an ideal scenario for enhancing diversity. There were no clear patterns in moderate departures from HWE at reintroduction sites. Although these departures are difficult to explain, they may provide insights into among-strain differences in mating systems, behavior, and survival. They also indicate potential barriers to admixture among the source strains at the recipient sites.

Beaver ancestry was substantially over-represented at the reintroduction sites, whereas both Cold Spring and Garvin ancestry was under-represented. However, Cold Spring and Garvin ancestries persisted widely through hybridization with Beaver descendants. Our analyses are consistent with the hypothesis that an adaptation to local conditions in the Beaver population is advantageous at a majority of the reintroduction sites, thereby conferring a selective advantage upon sculpins with Beaver ancestry.

Given the absence of information regarding deep phylogenetic separation among populations, the potential for disrupting beneficial adaptations, and the lack of evidence that genetic rescue is necessary, the most conservative option available for future reintroductions of slimy sculpin in our study area would be to use a single source, such as Beaver. This strain is relatively diverse according to neutral genetic markers and evidence from our study indicates better persistance than other sources in its pure form. Single source reintroductions may also be considered in other parts of the Driftless Region on a trial basis using local strains to maximize genetic and ecological similarity to inhabitants of the surrounding area. Managers should seriously consider the risks of outbreeding before intentionally hybridizing populations (Epifanio and Philipp 2000, Edmands 2007). Careful monitoring of reintroduced populations can identify the need for genetic rescue or to select alternative source populations.

Figure Captions

Fig. 1 - Source (•) and recipient (▲) sites in southeast Minnesota. The Driftless Region where study sites are located is shown in the inset superimposed on a map of the United States and indicated by the cross-hatched area covering portions of Minnesota, Wisconsin, Iowa, and Illinois.

Fig. 2 – Least-squares mean observed and simulated potential heterozygosity and allelic richness across eight microsatellite loci. Source populations, indicated with an asterisk, do not have simulated values. Error bars represent 95% confidence intervals about the least-squares site-based error component (Weisberg 2005).

Fig. 3 - Summary of genetic divergence among populations derived from agglomerative cluster analysis, using flexible beta linkage, of transformed pairwise F_{ST} estimates (i.e. – ln [F_{ST} – 1]) based on eight microsatellite loci. Branch lengths are proportional to the degree of divergence among populations and are indicated by the F_{ST} distance scale bar. Source populations are italicized and indicated with an asterisk.

Fig. 4 - Persistence of sculpin crosstypes at recipient sites (B=Beaver Creek, G=Garvin Brook, C=Cold Spring Brook). Bars indicate the mean number of individuals. Black bars indicate pure strain individuals; grey bars indicate F_1 generation hybrid individuals; white bars indicate advanced generation mixtures such as F_2 or back-cross individuals. Error bars represent 95% confidence intervals about the mean.

Figure 1

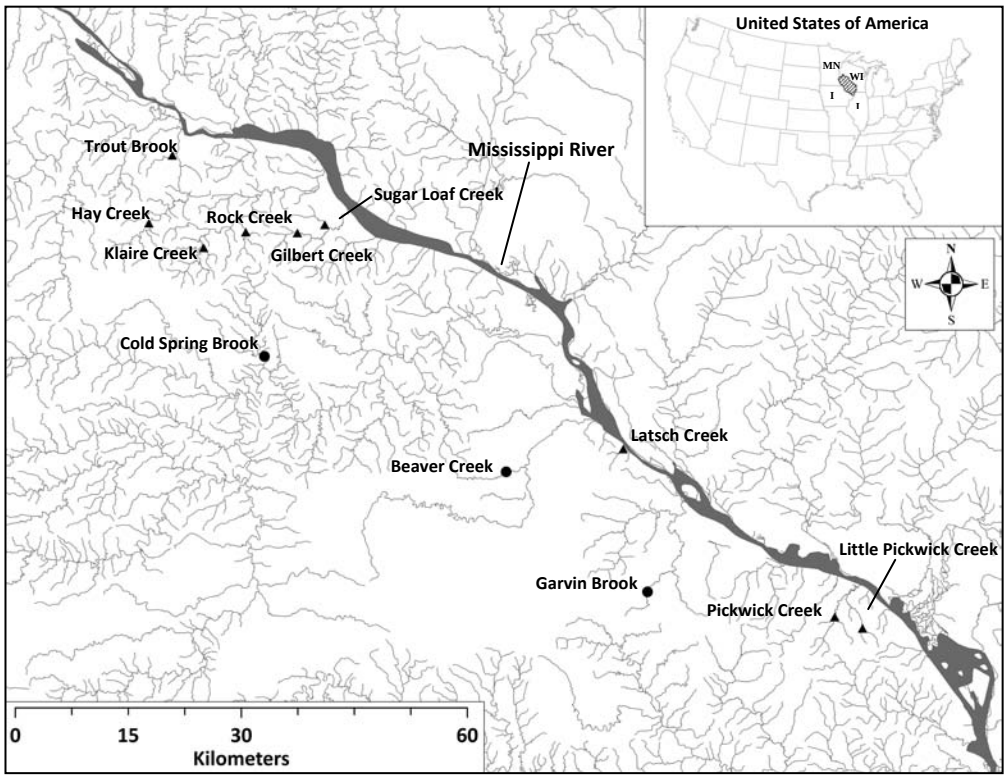

United States of America
MN
WI
Trout Brook
Mississippi River
Sugar Loaf Creek
Hay Creek
Rock Creek
Klaire Creek
Gilbert Creek
N
W
E
S
Cold Spring Brook
Latsch Creek
Beaver Creek
Little Pickwick Creek
Garvin Brook
Pickwick Creek
0
15
30
60
Kilometers

Figure 2

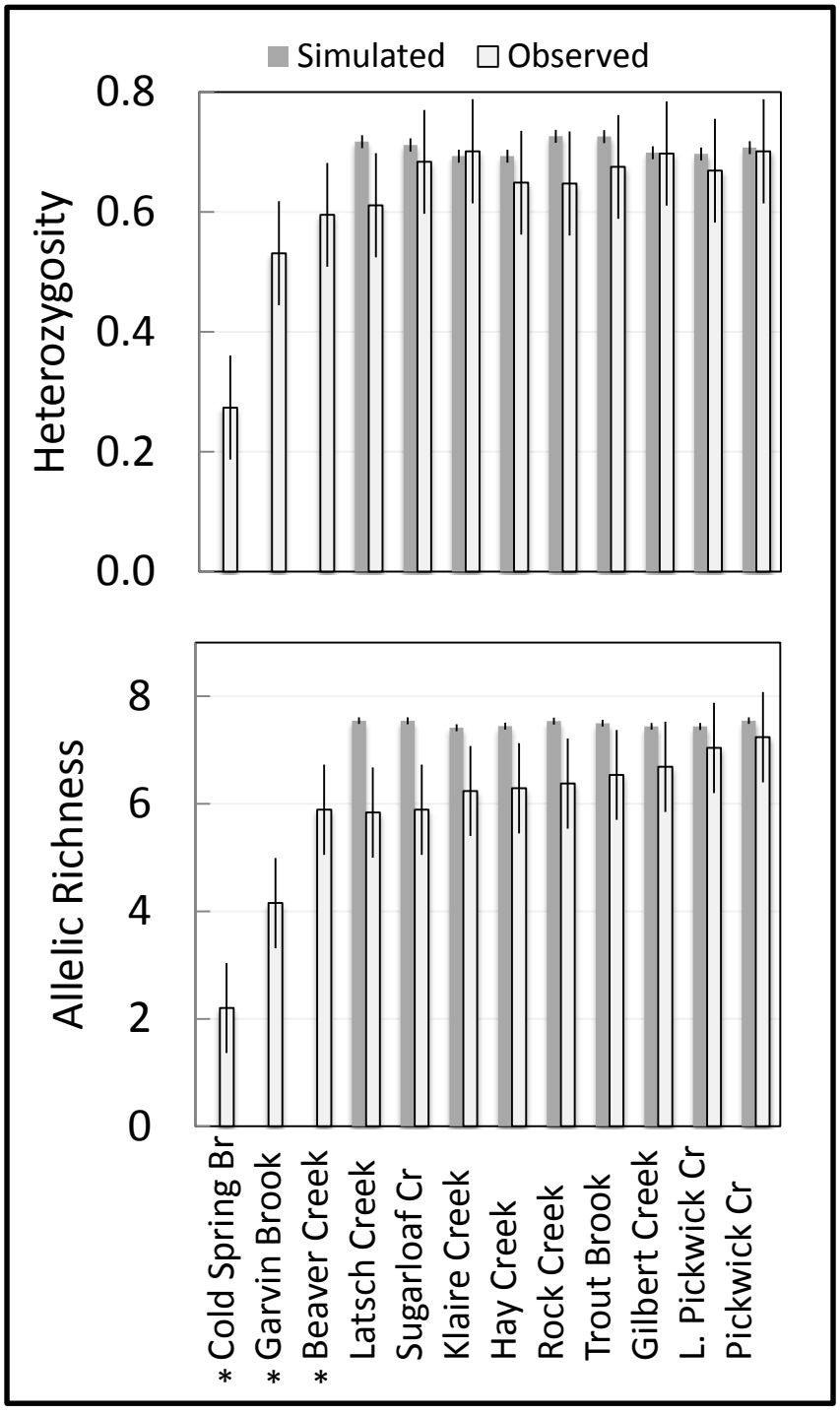

Simulated
Observed
Heterozygosity
0.8
0.6
0.4
0.2
0.0
Allelic Richness
8
6
4
2
0
* Cold Spring Br
* Garvin Brook
* Beaver Creek
Latsch Creek
Sugarloaf Cr
Klaire Creek
Hay Creek
Rock Creek
Trout Brook
Gilbert Creek
L. Pickwick Cr
Pickwick Cr

Figure 3

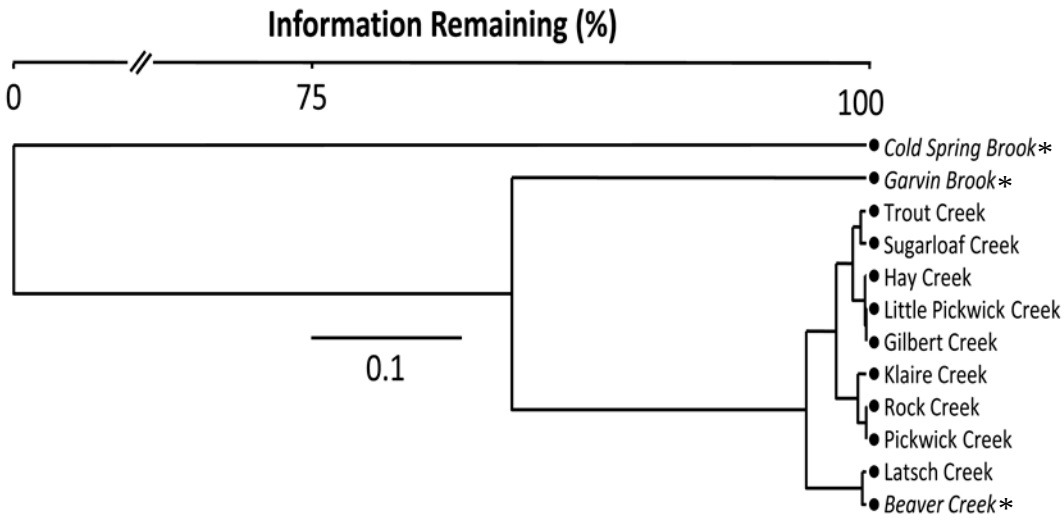

Information Remaining (%)
0
75
100
Cold Spring Brook*
Garvin Brook*
Trout Creek
Sugarloaf Creek
Hay Creek
Little Pickwick Creek
Gilbert Creek
Klaire Creek
Rock Creek
Pickwick Creek
Latsch Creek
Beaver Creek*
0.1

Figure 4

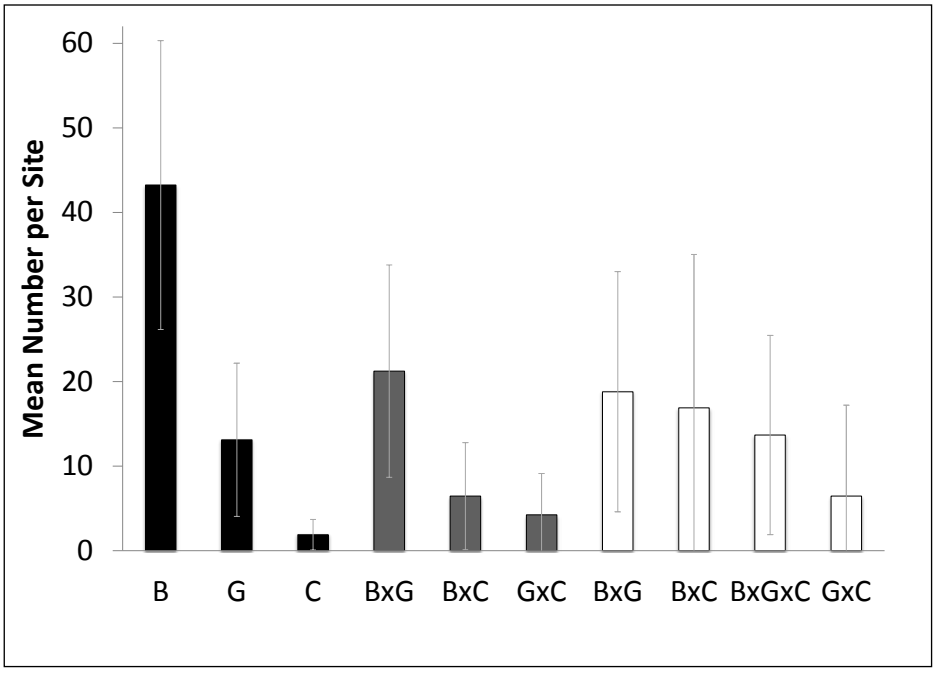

Mean Number per Site
60
50
40
30
20
10
0
B
G
C
BxG
BxC
GxC
BxG
BxC
BxGxC
GxC

Table 1 – Stocking history for slimy sculpin reintroductions: total number of individual fish stocked from Beaver Creek (B), Garvin Brook (G) and Cold Spring Brook (C) from 2003-2005. In 2003 and 2005, 50 fish were stocked from each source. In 2004, few sculpins were stocked from Cold Spring Brook; the number from each source is in parentheses.

Recipient Population	2003	2004	2005
Gilbert Creek	150	165 (70B, 75G, 20C)	150
Hay Creek	150	155 (80B, 75G)	150
Trout Brook	150	140 (76B, 64G)	150
Klaire Creek	-	160 (76B, 84G)	150
Rock Creek	-	149 (75B, 74G)	150
Latsch Creek	-	150 (75B, 75G)	150
Sugar Loaf Creek	-	150 (75B, 75G)	150
Pickwick Creek	-	-	150
Little Pickwick Creek	-	-	150

Table 2 – Sample sizes (n), expected (H_E) and observed (H_O) heterozygosity the inbreeding coefficient (F_{IS}), and allelic richness at eight microsatellite loci.

Population	Locus	n	H_E	H_O	F_{IS}	Richness
Sources:	Cba14	60	0.00	0.00	-	1.0
Cold Spring Brook	Cgo1033	90	0.13	0.13	-0.07	2.0
	Cgo18	86	0.69	0.73	-0.06	4.0
	Cgo310	71	0.00	0.00	-	1.0
	Cgo42	67	0.67	0.66	0.02	4.0
	Cott290	40	0.31	0.33	-0.05	2.0
	Cott686	91	0.00	0.00	-	1.0
	ES1	90	0.29	0.34	-0.20	2.6
Garvin Brook	Cba14	58	0.60	0.60	0.00	4.0
	Cgo1033	98	0.24	0.27	-0.10	4.0
	Cgo18	86	0.75	0.69	0.08	7.7
	Cgo310	69	0.52	0.52	0.01	3.0
	Cgo42	68	0.55	0.59	-0.07	5.0
	Cott290	36	0.51	0.56	-0.09	3.0
	Cott686	97	0.41	0.46	-0.14	2.0
	ES1	98	0.61	0.56	0.08	4.5
Beaver Creek	Cba14	100	0.68	0.75	-0.10	5.9
	Cgo1033	100	0.74	0.81	-0.09	6.7
	Cgo18	85	0.85	0.85	0.01	15.1
	Cgo310	77	0.44	0.47	-0.07	3.0
	Cgo42	91	0.79	0.81	-0.03	6.8
	Cott290	97	0.00	0.00	-	1.0
	Cott686	98	0.51	0.47	0.07	2.8
	ES1	95	0.63	0.60	0.06	5.8
Recipients:	Cba14	149	0.75	0.73	0.02	6.7
Latsch Creek	Cgo1033	149	0.62	0.67	-0.09	5.5
	Cgo18*	149	0.86	0.86	0.00	11.8
	Cgo310	149	0.66	0.63	0.04	4.0
	Cgo42*	139	0.81	0.77	0.05	8.7
	Cott290	128	0.18	0.20	-0.10	2.0
	Cott686	138	0.32	0.37	-0.15	2.0
	ES1*	149	0.70	0.66	0.06	6.0
Rock Creek	Cba14	135	0.76	0.73	0.04	5.9
	Cgo1033	135	0.70	0.70	0.00	7.0
	Cgo18*	126	0.88	0.89	-0.01	11.8
	Cgo310	134	0.72	0.73	-0.01	4.9
	Cgo42*	106	0.84	0.80	0.04	10.7
	Cott290	132	0.23	0.20	0.11	2.0
	Cott686	134	0.42	0.43	-0.01	2.0
	ES1*	135	0.74	0.70	0.05	6.7
Hay Creek	Cba14	147	0.70	0.71	-0.01	5.7
	Cgo1033	146	0.82	0.78	0.05	6.3
	Cgo18	62	0.89	0.84	0.06	14.6
	Cgo310*	129	0.65	0.49	0.25	3.9
	Cgo42	142	0.83	0.81	0.02	8.9
	Cott290	139	0.57	0.56	0.01	3.0
	Cott686	148	0.26	0.26	-0.02	2.0
	ES1	148	0.73	0.74	0.00	5.9

Asterisks indicate loci that show significant ($P<0.05$, Bonferroni corrected) deviation from Hardy-Weinberg equilibrium based on the exact test (Guo and Thompson 1992).

Table 2 continued – Sample sizes (n), expected (H_E) and observed (H_O) heterozygosity the inbreeding coefficient (F_{IS}), and allelic richness at eight microsatellite loci.

Population	Locus	n	H_E	H_O	F_{IS}	Richness
Little Pickwick	Cba14*	190	0.82	0.76	0.07	8.0
	Cgo1033*	186	0.83	0.83	0.08	8.2
	Cgo18*	189	0.90	0.90	-0.01	13.4
	Cgo310*	190	0.69	0.57	0.18	4.5
	Cgo42	179	0.86	0.85	0.00	10.6
	Cott290	188	0.46	0.46	0.01	3.8
	Cott686	190	0.39	0.39	0.00	2.0
	ES1*	190	0.68	0.59	0.13	5.8
Trout Creek	Cba14*	172	0.73	0.82	-0.13	6.7
	Cgo1033*	172	0.81	0.83	-0.02	7.0
	Cgo18*	159	0.84	0.82	0.02	11.9
	Cgo310	171	0.61	0.58	0.05	4.8
	Cgo42*	162	0.82	0.79	0.04	9.7
	Cott290	122	0.46	0.43	0.05	3.7
	Cott686	164	0.49	0.40	0.18	2.0
	ES1	169	0.64	0.73	-0.13	6.5
Sugar Loaf Creek	Cba14*	168	0.70	0.64	0.09	5.5
	Cgo1033*	162	0.69	0.62	0.10	7.2
	Cgo18*	167	0.85	0.93	-0.10	11.4
	Cgo310*	167	0.61	0.76	-0.24	4.0
	Cgo42*	163	0.84	0.83	0.00	8.0
	Cott290	120	0.43	0.38	0.11	4.0
	Cott686*	164	0.48	0.60	-0.25	2.0
	ES1*	168	0.72	0.71	0.02	5.0
Gilbert Creek	Cba14*	183	0.79	0.78	0.01	6.0
	Cgo1033	180	0.82	0.81	0.02	7.7
	Cgo18*	174	0.90	0.93	-0.02	14.3
	Cgo310	182	0.69	0.70	-0.03	4.0
	Cgo42	160	0.87	0.91	-0.04	9.8
	Cott290	182	0.29	0.29	0.00	3.8
	Cott686	183	0.35	0.37	-0.07	2.0
	ES1	182	0.75	0.79	-0.05	5.9
Klaire Creek	Cba14	117	0.72	0.74	-0.02	7.0
	Cgo1033	113	0.72	0.77	-0.06	6.0
	Cgo18*	115	0.87	0.87	0.01	12.6
	Cgo310*	117	0.65	0.64	0.01	4.0
	Cgo42*	93	0.85	0.92	-0.09	8.8
	Cott290	104	0.46	0.50	-0.10	3.5
	Cott686	116	0.43	0.47	-0.10	2.0
	ES1*	117	0.69	0.70	-0.02	6.0
Pickwick Creek	Cba14*	91	0.75	0.66	0.13	7.0
	Cgo1033*	90	0.73	0.73	-0.09	8.1
	Cgo18*	92	0.91	0.93	-0.03	15.0
	Cgo310	91	0.69	0.68	0.01	4.0
	Cgo42	82	0.84	0.90	-0.08	10.0
	Cott290	92	0.42	0.42	-0.01	4.0
	Cott686*	92	0.43	0.42	0.01	2.0
	ES1	92	0.80	0.87	-0.09	7.8

Table 3 – Observed and potential genetic diversity across eight microsatellite loci for source (s) and reintroduced (r) populations. Sample sizes (n) and mean values across all loci for the overall inbreeding coefficient (F_{IS}), expected (H_E) and observed (H_O) heterozygosity, simulated heterozygosity, allelic richness and simulated allelic richness. Populations are sorted from lowest to highest richness.

Population	n	Overall F_{IS}	H_E	H_O	Simulated H	Richness	Simulated Richness
Cold Spring Brook (s)	91	-0.05	0.26	0.27	•	2.2	•
Garvin Brook (s)	98	-0.01	0.52	0.53	•	4.1	•
Beaver Creek (s)	100	-0.02	0.58	0.60	•	5.9	•
Latsch Creek (r)	149	0.00	0.61	0.61	0.67	5.8	7.1
Sugar Loaf Creek (r)	168	-0.03	0.67	0.68	0.68	5.9	7.1
Klaire Creek (r)	117	-0.04	0.68	0.70	0.67	6.2	7.1
Hay Creek (r)	150	0.05	0.68	0.65	0.69	6.3	7.2
Rock Creek (r)	135	0.02	0.66	0.65	0.68	6.4	7.1
Trout Brook (r)	172	0.00	0.68	0.68	0.69	6.5	7.2
Gilbert Creek (r)	183	-0.02	0.68	0.70	0.70	6.7	7.2
Little Pickwick (r)	190	0.06	0.70	0.67	0.70	7.0	7.2
Pickwick Creek (r)	92	-0.02	0.70	0.70	0.70	7.2	7.2

Table 4 – Observed versus expected average proportions of source ancestry in each of the recipient streams. Observed values are based on results from STRUCTURE population assignment. Expected ancestry assumes an equal contribution from each source that is proportional to the number of individuals from each source population that were stocked in a given reintroduction site (see Table 1). Populations are sorted from highest to lowest proportion of Beaver Creek ancestry.

Population	B_{OBS}	B_{EXP}	O-E	C_{OBS}	C_{EXP}	O-E	G_{OBS}	G_{EXP}	O-E
Latsch Creek	73	42	+31	4	17	-12	23	42	-19
Sugar Loaf Creek	61	42	+20	10	17	-7	29	42	-13
Gilbert Creek	55	36	+19	20	26	-6	25	37	-13
Little Pickwick Creek	51	33	+18	15	33	-18	34	33	+1
Trout Brook	56	40	+15	27	22	+5	17	37	-21
Klaire Creek	53	40	+13	13	17	-4	34	43	-9
Pickwick Creek	44	33	+11	8	33	-25	47	33	+14
Rock Creek	44	42	+2	4	17	-13	52	41	+11
Hay Creek	32	39	-8	39	22	+17	29	38	-10
Overall	**52**	**39**	**+14**	**16**	**23**	**-7**	**32**	**39**	**-6**

B=Beaver Creek, G=Garvin Brook, C=Cold Spring Brook. The third column in each group (O-E) is the expected subtracted from the observed ancestry for each population. Table values are percentages.

Table 5 – Individual ancestry by cross type at reintroduction sites. Populations are sorted from highest to lowest proportion of Beaver Creek ancestry. "Adv" designates a combination of advanced generation mixtures including F_2 hybrids and both back-crosses. Unknown values are due to low probability of assignment to any crosstype. Pickwick and Little Pickwick sites had no advanced crosstypes because of the recent timing of stocking.

Population	B	G	C	F_1: BxG	F_1: BxC	F_1: GxC	Adv: BxG	Adv: BxC	Adv: BxGxC	Adv: GxC	U
Latsch Creek	54	1	0	4	0	0	22	0	0	0	19
Pickwick Creek	37	13	1	34	13	3	0	0	0	0	0
Sugar Loaf Creek	35	2	1	4	9	0	37	0	0	0	12
Klaire Creek	28	3	0	3	0	14	21	2	10	0	19
Gilbert Creek	23	1	1	16	2	0	4	17	15	4	18
Little Pickwick Cr.	21	20	3	32	15	8	0	0	0	0	0
Rock Creek	19	25	0	19	0	0	14	0	0	0	23
Trout Brook	17	3	1	8	1	0	3	42	18	1	7
Hay Creek	5	5	4	1	1	0	1	22	26	28	9
Mean	**27**	**8**	**1**	**13**	**4**	**3**	**11**	**9**	**8**	**4**	**12**

B=Beaver Creek, G=Garvin Brook, C=Cold Spring Brook, U=Unknown. Table values are percentages of individuals per crosstype.

Chapter 2

Mixed-source reintroductions lead to outbreeding depression in the second generation descendents of a native North American fish

Introduction

Reintroductions, which are intended to re-establish a species within its former range (IUCN 1998), are a common practice (Fischer and Lindenmayer 2000, Seddon et al. 2007) and are integral to a high proportion of recovery plans for imperiled species in North America (Williams et al. 1988). As habitats continue to be degraded (Jenkins 2003), there is concern that fragmented wild animal populations are vulnerable to inbreeding depression (Keller and Waller 2002) and reduced evolutionary potential. However, reintroduction practices may hasten a decline in genetic variability by reducing the effective population size (Lande and Barrowclough 1987) of the source and reintroduced populations (Griffith et al. 1989, Wolf et al. 1996). Intentional hybridization of genetically divergent source populations is a reintroduction approach that may alleviate inbreeding depression (e. g. Pimm et al. 2006) and provide novel genetic combinations required for rapid evolutionary change (Lewontin and Birch 1966, Stockwell et al. 2003).

There are risks associated with intentional hybridization of distinct populations (Lesica and Allendorf 1999, Jones 2003); among these is a decline in fitness caused by hybridization between genetically disparate individuals known as outbreeding depression (Lynch 1991). Outbreeding depression is thought to occur by two general mechanisms: 1) chromosomal rearrangements that result in underdominance for fitness, and 2) epistasis among different loci (Dobzhansky 1951). Hybridization may consequently decrease fitness because introduced non-local alleles cause a population

to become less suited to local environmental conditions by producing intermediate phenotypes (Hatfield and Schluter 1999). Reduced fitness by the first mechanism would be apparent in the F_1 generation, but outbreeding depression that occurs by the second mechanism (i.e. through disrupted co-adapted gene complexes) would only be detectable beyond the first generation because of recombination and segregation during meiosis in the F_1 generation. The resulting F_2 genomes may contain genes with different evolutionary histories that have not undergone co-adaptive selection as a group (Brncic 1954, Templeton et al. 1986).

Although there is debate about the relevance of inbreeding depression to wild populations (Pusey and Wolf 1996), there is extensive evidence to support its incidence (Keller and Waller 2002). Conversely, while recent literature has highlighted research on outbreeding depression, there is a relative paucity of reports on this topic (Edmands 2007, McClelland and Naish 2007). Most outbreeding studies rely on controlled crosses conducted in a laboratory, whereas studies in natural environments are rare, especially those in wild fish populations (Edmands 2007, McClelland and Naish 2007). While laboratory studies focus on purely genetic components that require no environmental context, field studies can provide opportunities to measure the genetic and ecological constituents of hybrid fitness acting in concert. The environment-dependent reduction in hybrid fitness is relevant to both conservation efforts and our understanding of the role of divergent evolution as the basis of local adaptation and ultimately, reproductive isolation.

We utilized a native fish reintroduction program that provided a unique opportunity to investigate outbreeding effects in a natural setting. The timing, number, and origin of reintroduced individuals were known; the source populations had distinct genetic and ecologically relevant physical traits; and there were nine discrete reintroduced populations in close geographic proximity that provided an unusual degree of replication for a field study of this type. We determined the potential for adaptive differences between source populations through laboratory growth trials, and from evidence that one of the source strains was more persistent in the reintroduced populations. We accordingly hypothesized that two fitness indicators, body size and growth rate (Petty and Grossman 2004), would differ in pure-strain individuals by ancestral origin in the reintroduced populations. We further examined the result of outbreeding on fitness indicators in first and second generation hybrid crosses and backcrosses. Finally, we compared relative body condition, a trait that is generally considered a good indicator of fitness in fish (Danzmann et al. 1988, Rakitin et al. 1999, Thelen and Allendorf 2001), among all of the pure strain and hybrid crosstypes to corroborate potential differences in growth rate and body size.

Methods

Study organism and reintroduction project

The slimy sculpin (*Cottus cognatus* Richardson) is a small (< 130 mm), cryptic, freshwater fish that occupies benthic habitats in lakes, rivers, and small streams from Virginia, USA to Labrador in eastern Canada and northwest across Canada to eastern Siberia (Scott and Crossman 1979). *Cottus* spp. are often locally abundant and are frequently a prominent constituent of ecosystems suited to trout and other cold-water fish (Petrosky and Waters 1975, Goyke and Hershey 1992). Slimy sculpins in the study region spawn once per year during the early spring at age II, or rarely at age I, and live up to 6 years (Petrosky and Waters 1975).

The study area is located in the Driftless Region of southeast Minnesota, USA (Fig. 1). Prior to major settlement by European immigrants beginning in 1850, nearly all of the spring-fed streams in the region held brook trout (*Salvelinus fontinalis*) and presumably slimy sculpins. In subsequent years, slimy sculpin and other cold-water fish abundance declined because of severe habitat degradation and overexploitation (Waters 1977, Leopold and Sewell 2001). Since the 1940s, the Minnesota Department of Natural Resources (MNDNR) and other organizations have completed hundreds of in-stream improvement projects (Waters 1977, Thorn et al. 1997, MNDNR 2003). In locations where the habitat has been restored sufficiently, slimy sculpins were recently reintroduced by MNDNR personnel. The goal was to reestablish viable, self-sustaining populations where native populations were likely present historically, but had been

extirpated. Nine recipient streams were stocked from 2003-2005 in mid-autumn with a mixture of sculpins from three source streams. The source streams are all are small tributaries within separate sub-drainages that enter the Mississippi River within approximately 40 river kilometers of each other. We focus our analysis on only two of these source ancestries: Beaver Creek (Beaver) and Garvin Brook (Garvin). Although three source populations were used in reintroductions, for this evaluation we removed descendents from one source, Cold Spring Brook, because initial analyses indicated very low overall ancestry in most reintroduced populations. There were a total of 1230 Beaver and Garvin sculpins stocked in equal proportions across all nine reintroduction sites.

Sampling

Sampling was conducted in fall 2007 at all source and recipient sites except Little Pickwick Creek and Pickwick Creek. Little Pickwick Creek was additionally sampled in fall 2008 and fall 2009, and Pickwick Creek was sampled in spring and autumn 2008 and autumn 2009. Fish were collected using a Wisconsin™ Abp-3 pulsed DC backpack electrofisher with power output settings adjusted to minimize effects on the reintroduced fish (Cowx and Lamarque 1990). Each fish was anesthetized using tricaine methanosulfate (MS-222) (Summerfelt and Smith 1990), weighed, and measured for standard length. A small amount of tissue was clipped from the left pelvic fin of each fish and preserved in 95% ethanol for genetic analysis. After processing, all fish were returned to the streams except those captured in 2008 and 2009 at Pickwick and Little

Pickwick sites, which were euthanized with a lethal dose of anesthetic (1 $g*L^{-1}$ of MS-222) and retained for otolith analysis. None of the visibly marked, originally stocked fish were sampled.

DNA extraction and amplification

For all samples we initially used eight microsatellite loci developed for other *Cottus* species that resolved genetic variation in *C. cognatus*: Cgo18, Cgo42, Cgo310, and Cgo1033 (Englbrecht et al. 1999); Cott290, Cott686, and CottES1 (Nolte et al. 2005b); and Cba14 (Fiumera et al. 2002). We extracted DNA for polymerase chain reaction (PCR) amplification using a chelating resin as described in Fujishin et al. (2009). Microsatellite amplification was performed in 15 µL reactions containing 1x polymerase buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton® X-100), 1.5 mM MgCl2, 0.2 mM each dNTP, 0.5 µM of the forward and reverse primers, with the forward primer labeled with a fluorescent dye 6FAM, VIC, NED or PET, and 0.5 units Taq DNA polymerase (Promega, Madison, WI). Amplification was carried out in a thermocycler (Hybaid Omn-E, Thermo-Hybaid U.S., Franklin, MA) with 35 cycles at the following temperature profile: 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min; followed by a 20 min extension at 72°C. We submitted PCR products to the Biomedical Genomics Center (University of Minnesota, St. Paul) for electrophoresis on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). We scored alleles using the software program GENOTYPER 2.5 (Applied Biosystems 2001). For samples that were determined to be advanced generation crosses, a second round of amplification was carried out with a set of six newly-

developed microsatellite loci for *C. cognatus* (Fujishin et al. 2009): Cco01, Cco09, Cco10, Cco14, Cco15, and Cco17.

Crosstype Assignment

We used multilocus genotype data to assign individual fish to parental (Beaver, Garvin), or first (F_1) or second (F_2, F_1xB, and F_1xG) generation hybrid crosstypes. First, data from three source populations were evaluated in MICROCHECKER v2.2.3 to detect evidence of null alleles or scoring errors due to large allele drop-off (Van Oosterhout et al. 2004). Conformance with Hardy-Weinberg expectations and linkage equilibrium was tested using GENEPOP v4.0.4 (Raymond and Rousset 1995). Significance values for both tests were adjusted using sequential Bonferroni procedures (Rice 1989).

The proportion of each individual's ancestry derived from the three source populations was estimated using the Bayesian clustering algorithm implemented in the program STRUCTURE (V. 2.2.3; (Pritchard et al. 2000); also refer to http://pritch.bsd.uchicago.edu). The number of populations (K) was set to 3, the known number of genetically distinct source populations, with an admixture model and correlated allele frequencies. The program was run with a 50,000 burn-in period followed by 100,000 Monte Carlo simulations. Baseline individuals were included in the runs without population identification to assist resolution of genetically differentiated clusters and determine the ability of STRUCTURE to correctly determine the ancestry of known fish. Individuals with probable Cold Spring ancestry ($q > 0.125$) were removed

from the dataset and subsequent analyses were conducted with only Beaver Creek and Garvin Brook descendents.

The software NewHybrids (Anderson and Thompson 2002) was used to classify individual fish as pure (parental) strain or hybrid categories assuming that only first-generation, (F_1) or second-generation (F_2), backcrosses to Beaver (F_1xB), or backcrosses to Garvin (F_1xG) descendents of founders were present. This assumption is reasonable as reintroduced populations were sampled within three years of initial spawning and sculpins typically mature at age 2 (Petrosky and Waters 1975). Individuals from the two source populations were included as a baseline in the analyses. Each run had a 50,000 burn-in period followed by 150,000 simulations, using Jeffrey's priors for allele frequencies and mixing proportions. Runs were repeated using different seeds to verify that consistent solutions were found. Individuals were classified into a pure strain or hybrid category if their probability of membership was greater than or equal to 0.70; otherwise the classification of the individual was considered uncertain. Second-generation hybrids were difficult to distinguish, so we genotyped the previously mentioned additional six loci for all individuals whose combined probability of membership across all three second-generation cross-types exceeded 0.70.

Laboratory growth trial

Seven similar sized (mean weight; Garvin=0.84g ± 0.003 SE, Beaver=0.86g ± 0.001 SE) young-of-the-year sculpins were collected from each of the source sites in mid December 2006 and acclimated to laboratory conditions at University of Minnesota

(Saint Paul, MN, U.S.A) facilities for two weeks prior to growth trials. Each replicate consisted of one fish from each strain in a 10-L aquarium, for a total of 7 aquaria. Flow rate to each aquarium was 1000 mL/min. Each fish was marked with a strain-specific colored visible elastomer implant tag (Northwest Marine Technology, Inc., Seattle WA) for identification. Water temperatures, intended to mimic natural conditions, ranged from 6-7° C during the winter months and were raised to 9.5-10.5° C beginning in April through the end of the trials in late July 2007. Light-dark cycles were maintained at natural day length using an outdoor light sensor. Fish were fed frozen brine shrimp (*Artemia salina*), *ad libitum*. The weight and length of each fish was measured every 2-3 weeks for the duration of the 188 day experiment. We calculated mean daily growth for each fish and compared means for Beaver and Garvin using a two tailed t-test (Zar 1999).

Statistical and analytical methods

Expected quantities of each strain within the reintroduced populations were estimated for the Fall 2007 sampling season using a two-generation multinomial expansion of crosstypes based on the quantity of individuals from each strain that were stocked and assuming null conditions: equal survival, reproduction, and random mating among lineages (see Epifanio and Philipp 2000). The first and second generations contributed to the total population in a 1:2 ratio, and originally stocked fish were subtracted from the totals for each corresponding pure-strain category. We considered a 1:2 ratio a conservative approximation of overall population growth based on population estimates

(Chapter 3) that indicate abundances from 2 to 10 times greater than were originally stocked in the reintroduced populations. This ratio would also tend to overestimate expected pure-strain and F_1 individuals because more of these are produced in the first generation of admixture than the second (Epifanio and Philipp 2000). Likewise, expected quantities of F_2, F_1xB, and F_1xG would be underestimated by our chosen ratio. Our estimate included only first generation cross-types (Beaver, Garvin, and F_1) for the two Pickwick sites based on the timing of the sampling relative to stocking. Statistical assessment of the divergence between expected and observed values for each category were made using the Median test (Zar 1999), a version of the Kruskal-Wallis ANOVA that frames the computation in terms of a contingency table.

We used a mixed effects model (Weisberg 1993, Weisberg et al. 2010) to analyze differences among crosstypes in incremental growth rates using otoliths collected from 418 sculpins from Pickwick and Little Pickwick reintroduction sites. Because extracting otoliths from fish is lethal, we sacrificed fish from only two of the populations. These two populations were stocked once in autumn 2005, so there were only Beaver, Garvin, and F_1 crosstypes present in 2007. Otoliths were collected from these populations in autumn of 2008 and 2009 to ensure that there would be enough F_2, F_1xB, and F_1xG crosstypes to develop a growth model. We modeled the growth increments for each fish as a function of: Age (levels=0, 1, and 2), Stream (levels=Pickwick and Little Pickwick), Crosstype (levels=Garvin, Beaver, F_1, F_2, F_1xB, and F_1xG), Sex (levels=Male, Female, and Unknown) as fixed effects. Following specifications from Weisberg et al. (2010), we also

included Year, Year-Age interaction and Unique ID as random effects because of year-to-year variation likely to occur between the sequential years of sampling and natural variation likely to occur among individual fish. The Year-Age interaction was included in the model to allow for separate year effects during each year of a fish's life. Models were fit using the function *lmer* (Bates and Maechler 2009) in R (v 2.10.0) using maximum likelihood procedures. Starting with the full model containing all fixed parameters and 2 level interactions, we used backward model selection to select the most parsimonious model as determined by the lowest Akaike Information Criterion (AIC) corrected for sample size (Burnham and Anderson 1998). We do not report results of the marginal F-tests, only the parameter estimates of the final model. The *lmer* function does not produce p-values for model parameters so these were calculated using the *pvals.fnc* function in R (Baayen 2009), which computes p-values and Markov Chain Monte Carlo (1000 iterations) confidence intervals for mixed models. Post-hoc multiple comparisons of means (Tukey's) among crosstypes were calculated using the *glht* function in R (Hothorn et al. 2008).

Because there are possible age differences between first (Beaver, Garvin, and F_1) and second (F_2, F_1xB, and F_1xG) generation crosstypes which could be due to differential survival or recent stocking, we categorized all fish into two age categories that included young-of-the-year and "older" fish to corroborate differences among crosstypes found in an all-ages dataset. All young-of-the-year fish were designated by length (<41mm, n=91), based on age-length relationships from the Pickwick otolith data and

supplementary otolith data from Beaver (n=38) and Garvin (n=39) source sites (see Appendix A).

We estimated body condition by calculating relative condition factor (K_n), which has previously been employed as a fitness related trait in *Cottus* species (Knaepkens et al. 2002), for each fish. Relative condition factor is defined as $K_n=W/W_{pred}$, where W is the observed weight, and W_{pred} is the predicted weight from a third-order polynomial based on a weight-length relationship (LeCren 1951, Wootton 1998b) developed for each reintroduced population.

Results

Persistence and growth rates

We observed faster mean daily growth in weight for Garvin than Beaver over the 188 d period of the growth trial (t-test; P=0.05, t=2.31, 7df) under identical laboratory conditions (Fig. 2). Mean growth rate was 0.017 and 0.010 $g*day^{-1}$ for Garvin and Beaver, respectively.

The microsatellite data indicated strong differentiation between the source populations (F_{ST}=0.32, p<0.05). Within each population, all loci were in Hardy-Weinberg and linkage equilibrium. We detected no evidence for null alleles or large allele dropout. Simulated genotypes for the eight initial loci in NewHybrids estimated error rates of 2-6% for Beaver, Garvin and F_1 crosstypes; these errors caused assignment to F_2, F_1xB, and F_1xG, whereas 8-12% of F_2, F_1xB, and F_1xG crosstypes were erroneously assigned to Beaver, Garvin and F_1 crosstypes. Simulations using all 13 loci estimated error rates of 0-0.3% for Beaver, Garvin and F_1 crosstypes and 2-5% for F_2, F_1xB, and F_1xG. For F_2, F_1xB, and F_1xG crosstypes, 1-3% of backcrosses erroneously assigned to F_2, while 12% of F_2 erroneously assigned to backcrosses.

Assignment of crosstypes for a total of 1230 slimy sculpins revealed that there were more sculpins of Beaver ancestry (531; 43% of total) at the reintroduction sites than any other crosstype. F_1 was the next most numerous crosstype (315; 26% of total), followed by Garvin (196; 16% of total). F_2, F_1xB, and F_1xG crosstypes made up the remaining 16% (188 individuals) of the samples. Beaver individuals occurred in a

significantly greater frequency than expected (Fig. 3) based on null conditions (Median test; median=31.0, Chi-Square=5.6, p=0.02). All other crosstypes occurred at lower frequencies than expected, but only F_2 (Median test; median=14.4, Chi-Square=7.1, p=0.01) and F_1xG (Median test; median=5.0, Chi-Square=7.1, p=0.01) were significantly lower.

Comparison of growth rate, weight and body condition

The most parsimonious otolith incremental growth model that included fish of all ages (Fig.4, top-left panel) had the following fixed effects ($p<0.001$ for all variables): age ($F=23.7$, 3 df), stream ($F=148.5$, 1 df), sex ($F=32.3$, 2 df), and crosstype ($F=7.2$, 5 df). Otolith growth rates in Pickwick and Little Pickwick populations concurred with laboratory growth for Beaver and Garvin. In the reintroduced populations, Garvin grew faster than Beaver (Tukey's; $p<0.05$), whereas F_1 had an intermediate mean growth rate and was not significantly different from either Beaver or Garvin. Otolith growth in F_2, F_1xB, and F_1xG crosstypes were generally lower than in Beaver, Garvin, or F_1 (Tukey's; $p<0.05$, except Beaver vs. F_2, $p=0.07$), and F_1xG was not significantly different from Beaver or F_1.

Mean weights from 1230 samples (Fig.4, top-right panel) differed significantly (ANOVA, $p<0.001$ for all variables) among stream ($F=32.9$, 8 df), season ($F=74.5$, 1 df), crosstype ($F=11.9$, 5 df), and age category ($F=92.4$, 1 df). This pattern in crosstype mean weight mirrored the pattern in otolith growth among crosstypes. Garvin were heavier than Beaver (Tukey's, $p<0.05$), F_1 fish were intermediate to Garvin and Beaver, and

mean weights in F_2, F_1xB, and F_1xG crosstypes were lower than Beaver, Garvin, and F_1 crosstypes.

Otolith growth for young-of-the-year fish only (Fig. 4, bottom-left panel) differed significantly (ANOVA, $p<0.05$ for all variables) among age at capture ($F=3.9$, 1 df), stream ($F=40.1$, 2 df), crosstype ($F=11.5$, 5 df), and sex ($F=7.5$, 2 df). Otolith growth in F_2, F_1xB, and F_1xG crosstypes were lower overall than in the first generation (Tukey's; $p<0.05$). In contrast with the dataset for all ages, young-of-the-year growth rates were fastest for Beaver, although not significantly greater than Garvin or F_1.

Young-of-the-year weights (Fig.4, bottom-right panel) differed significantly (ANOVA, $p<0.001$ for all variables) among stream ($F=8.0$, 5 df) and crosstype ($F=4.1$, 5 df). This pattern in crosstype mean weight mirrored the results seen for otolith growth in the young-of-the-year category: Beaver was heavier than Garvin and F_1 (Tukey's, $p<0.07$) and mean weights in F_2, F_1xB, and F_1xG crosstypes were lower than in Beaver, Garvin, and F1.

Mean body condition (Fig. 5) varied significantly (ANOVA, $p<0.05$ for both variables) by stream ($F=61.4$, 8 df) and crosstype ($F=2.27$, 5 df). Mean F_2 body condition was significantly (Tukey's, $p<0.05$) depressed relative to Beaver, Garvin, and F_1 crosstypes, whereas there were no significant pairwise differences among Beaver, Garvin, F_1, F_1xB, or F_1xG. Mean body condition for back-crosses was highly variable, especially for F_1xG.

Discussion

The generality of small, subdivided populations with restricted gene flow and the extent to which different selective pressures, combined with genetic drift, will lead to local adaptation (Hanski and Gaggiotti 2004), and ultimately speciation is a fundamental question in evolutionary biology. The reduced success of hybrid offspring observed in this study could result in barriers to gene flow caused by variation in traits, such as young-of-the-year growth rate. These traits may be mapped in the genome to allow strain or species boundaries to be genetically quantified, whereas field transplant studies can establish the importance and magnitude of natural barriers to gene flow (Noor and Feder 2006). Genetic divergence in our source populations and elsewhere has been detected on very small spatial scales (Edmands 1999, Waser et al. 2000). Species such as the slimy sculpin, with restricted dispersal abilities (Schmetterling and Adams 2004), strong philopatry, patchy breeding distributions, and small effective population sizes (Fiumera et al. 2002) tend to show the strongest genetic differentiation among populations. These qualities, combined with a broad distribution, make the slimy sculpin a good model organism for studies of local adaptation, the consequences of outbreeding, and early stages of speciation (Sweigart 2009).

Crosstype proportions within reintroduced populations

The source populations in our study are geographically close (< 40 km apart), with similar habitats; however, based on high F_{ST} values there has been little recent gene flow between them (Holsinger and Weir 2009). Growth rates in the laboratory were

faster for the Garvin than the Beaver population under common conditions, suggesting that these populations are ecologically and evolutionarily divergent. However, measured fitness components do not always translate into population growth (Leberg 1993), which is often more relevant to both conservation and evolutionary questions. Although they evolved in very similar surroundings, each strain may have developed distinct genetic mechanisms and ecological strategies for survival (Crandall et al. 2000, Rader et al. 2005). The reintroduction sites are geographically close and have similar habitats (Chapter 3). Consequently, our null hypothesis was that reintroduced Beaver and Garvin strains would occur in equal proportions, given equal fitness and random mating among source lineages.

We observed that Beaver was the only crosstype that exceeded expectations for persistence; it was more numerous than all other crosstypes at eight of nine sites and made up 43% of the individuals in the study. All other crosstypes were either significantly less (F_2, F_1xG), or tended to be less abundant than expected. This deviation from null expectations suggests either fitness differences or non-random mating (or both) among crosstypes. Differential initial fitness at the reintroduction sites between Beaver and Garvin could plausibly explain low proportions of hybrid crosstypes (F_1, F_2, F_1xB, and F_1xG). For example, if Beaver sculpins had higher survival the first winter after stocking before spawning in the spring, fewer Garvin and therefore F_1, would be present in the first generation of offspring. A similar outcome is possible with differential initial reproductive success. Therefore, although depressed hybrid fitness may have

contributed to the observed patterns in crosstype persistence, lower than expected quantities of hybrids does not, by itself, provide conclusive evidence of reduced hybrid fitness. However, these results are consistent with the hypothesis that Garvin has a lower relative fitness than Beaver among reintroduced populations.

Beaver young-of-the-year versus Garvin adult body size and growth

We sought to quantify fitness related traits to test the hypothesis that Beaver had greater relative fitness compared to Garvin in the reintroduced populations. We used otolith increments to extract a growth history for reintroduced sculpins both as adults and young-of-the-year. The adult growth model is more comparable to the laboratory growth trial because sculpins were collected for the laboratory study after their first summer. These two analyses agree, in that both demonstrated that Garvin grew faster than Beaver. The dataset used to calculate body size, that included all populations and ages, was dominated by adults and confirmed that Garvin were larger than Beaver individuals. These results seem inconsistent with the pattern of greater Beaver persistence in the reintroduced populations, since growth rate and body size are generally accepted as fitness related traits in fish (Petty and Grossman 2004, McClelland and Naish 2007). In contrast, the young-of-the-year datasets corroborated higher persistence of Beaver in the reintroduced populations. The estimated growth rate for Beaver young-of-the-year fish, although not statistically significant, appears higher than for Garvin young-of-the-year fish. This trend may be more difficult to detect because these data consisted of only two sites and a single year of growth. There was however, a

significant and pronounced weight advantage in Beaver young-of-the-year that corroborated an apparent growth advantage in Beaver young-of-the-year. The early survival of fishes are often strongly affected by growth (Miller et al. 1988, Houde 1989), and Beaver fish overwhelmingly outnumber Garvin fish in the reintroduced populations. This is consistent with a growth advantage in Beaver young-of-the-year as an explanation of fitness differences between these two strains among the reintroduced populations.

Fitness of F_2, F_1xB, and F_1xG relative to Beaver, Garvin, and F_1

Our nine reintroduction sites represent multiple common garden experiments in which there is a significant genetically-based fitness depression in F_2, F_1xB, and F_1xG crosstypes. This pattern of hybrid fitness is consistent with disruption of co-adapted gene complexes in the second generation of hybrid crosstypes. In every comparison of growth, weight or body condition, the F_2 crosstype was significantly lower than either parental type or F_1. There was a greater degree of variability within the F_1xB and F_1xG crosstypes, but these crosstypes exhibited a similar pattern in fitness traits to F_2. The additional variability could be due in part to the potential for backcrosses to regress toward the parental condition through restored epistatic interactions (Ellison and Burton 2008). This regression was conceivable for F_1xB, in which we observed a slight to moderate increase in growth, weight, and body condition relative to F_2. Lower numbers of F_1xG fish may, at least partially, explain additional variability in body condition, but regression toward the parental condition is also plausible.

Our work indicates that outbreeding studies should include as much of the life cycle as possible because the ontogenetic timing of outbreeding effects may differ greatly (McGinnity et al. 2003, Edmands 2007). A faster young-of-the-year growth rate appears to confer a selective advantage at most reintroduction sites. F_1 young-of-the-year had slower growth and lower weight than Beaver young-of-the-year. Greater weight and body condition for Garvin and F_1 when older fish are included in the analysis could result from reduced survival of slower growing young-of-the-year individuals. Despite the potential for reduced early survival, a high overall body condition in surviving Garvin and F_1 crosstypes may facilitate subsequent generations of crosses back to the more persistent Beaver. If selection against hybrid genotypes is not strong enough, then introgression will continue until a hybrid swarm develops and the pure strain genotype is lost (Epifanio and Philipp 2000, Allendorf et al. 2001).

Errors in crosstype classification

Classification errors were unlikely to alter our major interpretations and conclusions. The strong differentiation among source populations provided sufficient power to distinguish crosstypes. Simulations indicated potential bias toward increased estimates of Beaver, Garvin, and F_1 crosstypes, but this is contrary to our observations of fewer than expected Garvin and F_1 crosstypes. Furthermore, classification error would have obscured differences in growth rate and condition factor among crosstypes resulting in underestimation of true differences.

Future research and conservation implications

Introductions of sculpins and other fishes have provided excellent opportunities to study the ecological basis for reproductive isolation and the progression of evolution (Hendry et al. 2000, Nolte et al. 2006). Future research should investigate the underlying genetic mechanisms of reproductive isolation in sculpins (Nolte et al. 2009), explore behavioral and maternal components of hybrid survival, and monitor the long-term fate of hybrids in natural settings. Investigation of the role of additive genetic effects versus epistatic interactions may also yield interesting insights, because local adaptation based on epistasis may accelerate speciation (Carroll et al. 2003).

A worldwide decline of some animals has led conservationists to advocate increasing genetic diversity in declining, isolated populations through translocations (Wolf et al. 1996, Fischer and Lindenmayer 2000). A perception that hybrid vigor is a common phenomenon (Rhymer and Simberloff 1996) and a pervasive "small population" paradigm (Caughley 1994), in which inbreeding depression is the major concern, may lead conservation managers to conclude that intentionally mixing source populations should be the default option in the absence of information that would indicate an alternative course of action. However, outbreeding depression may occur as frequently as hybrid vigor and there is very little quantitative data to support assumptions otherwise (Edmands 2007). Therefore, a precautionary approach should include careful consideration of the risks associated with crossing genetically divergent sources. Laboratory research with fruit flies suggests that outbreeding depression may

be a temporary phenomenon and will rapidly disappear if the population can survive the initial reduced fitness (Annest and Templeton 1978, Templeton 1986). However, for beleaguered populations that are already small and reproduce slowly, a short-term fitness reduction could have catastrophic consequences.

Figure Captions

Fig. 1 - Source (□) and recipient (□) sites in southeast Minnesota. The Driftless Region where study sites are located is shown in the inset, indicated by the cross-hatched area covering portions of Minnesota, Wisconsin, Iowa, and Illinois.

Fig. 2 - Mean weight of sculpins from Garvin Brook (solid line) and Beaver Creek (dashed line) under laboratory conditions over 188-d. Mean daily growth rates are significantly higher in fish from Garvin Brook (weight; 2 sided t-test, $p= 0.05$, $t=2.31$, 7df).

Fig. 3 - Median (closed square), 1^{st} and 3^{rd} quartile (rectangle), and range (whisker) for observed versus expected numbers of sculpins in the reintroduced populations for each crosstype. Significant differences between observed and expected quantities ($p>0.05$, median test) are indicated with an asterisk. The dashed line in each panel represents the overall median.

Fig. 4 - Least-squares mean otolith growth (left panels) and mean weight (right panels) by crosstype for all ages (top panels) and young-of-the-year (bottom panels). The same letter above each crosstype category indicates a lack of a significant pairwise difference (Tukey's HSD; $p<0.05$) among crosstypes. Otolith growth (left panels) was modeled with a mixed-effects least-squares ANOVA using data collected at two reintroduction sites: Pickwick and Little Pickwick Creek (n=418). Mean weight (right panels) was calculated across all nine reintroduction sites (n=1230).

Fig. 5 - Means (closed square) and 95% confidence intervals (whiskers) for body condition by crosstype across all reintroduction sites. The F_2 mean value is significantly

different (Tukey HSD; $p < 0.05$) from Garvin, Beaver, and F_1; no other pairwise comparisons are significantly different. The number of samples for each crosstype is shown in parentheses.

Figure 1

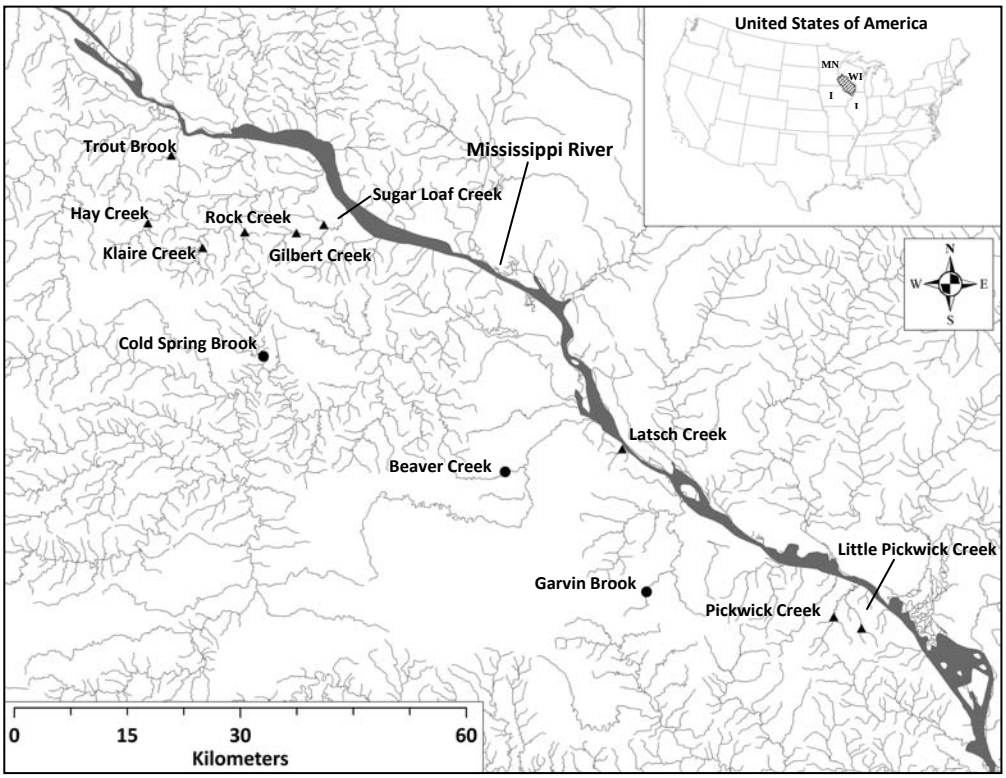

United States of America
MN
WI
I
I
Trout Brook
Mississippi River
Sugar Loaf Creek
Hay Creek
Rock Creek
Klaire Creek
Gilbert Creek
N
W
E
S
Cold Spring Brook
Latsch Creek
Beaver Creek
Little Pickwick Creek
Garvin Brook
Pickwick Creek
0
15
30
60
Kilometers

Figure 2

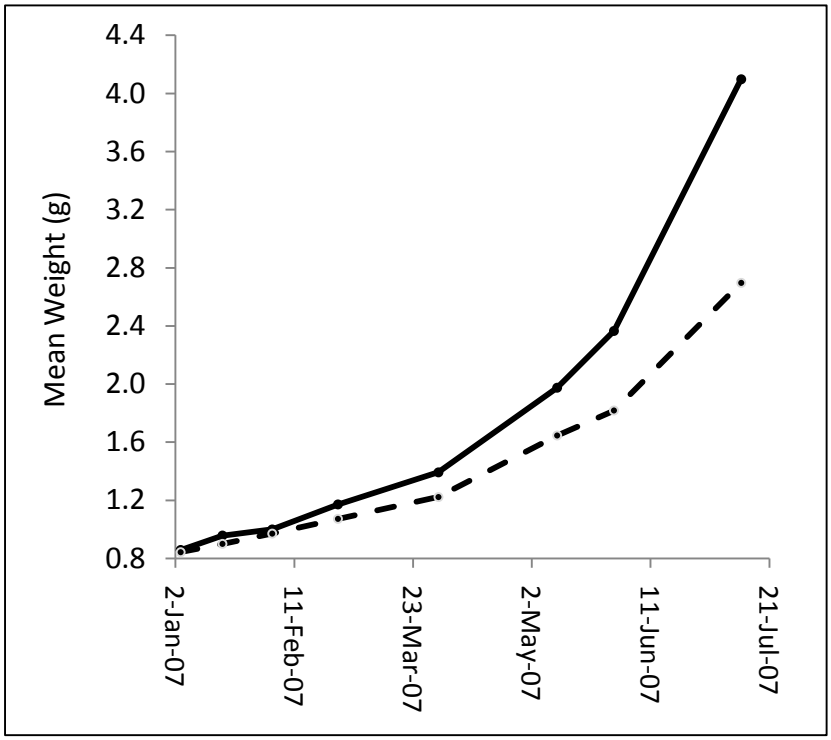
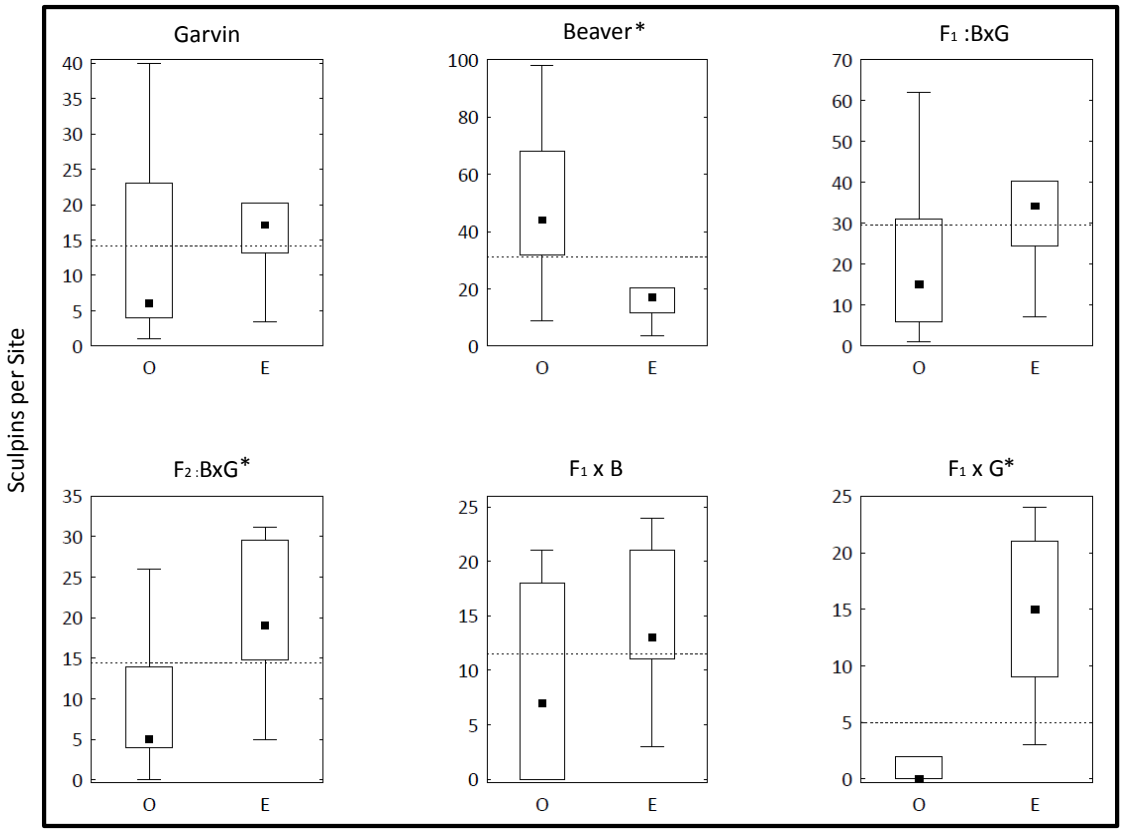

Mean Weight (g)
4.4
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
2-Jan-07
11-Feb-07
23-Mar-07
2-May-07
11-Jun-07
21-Jul-07

Figure 3



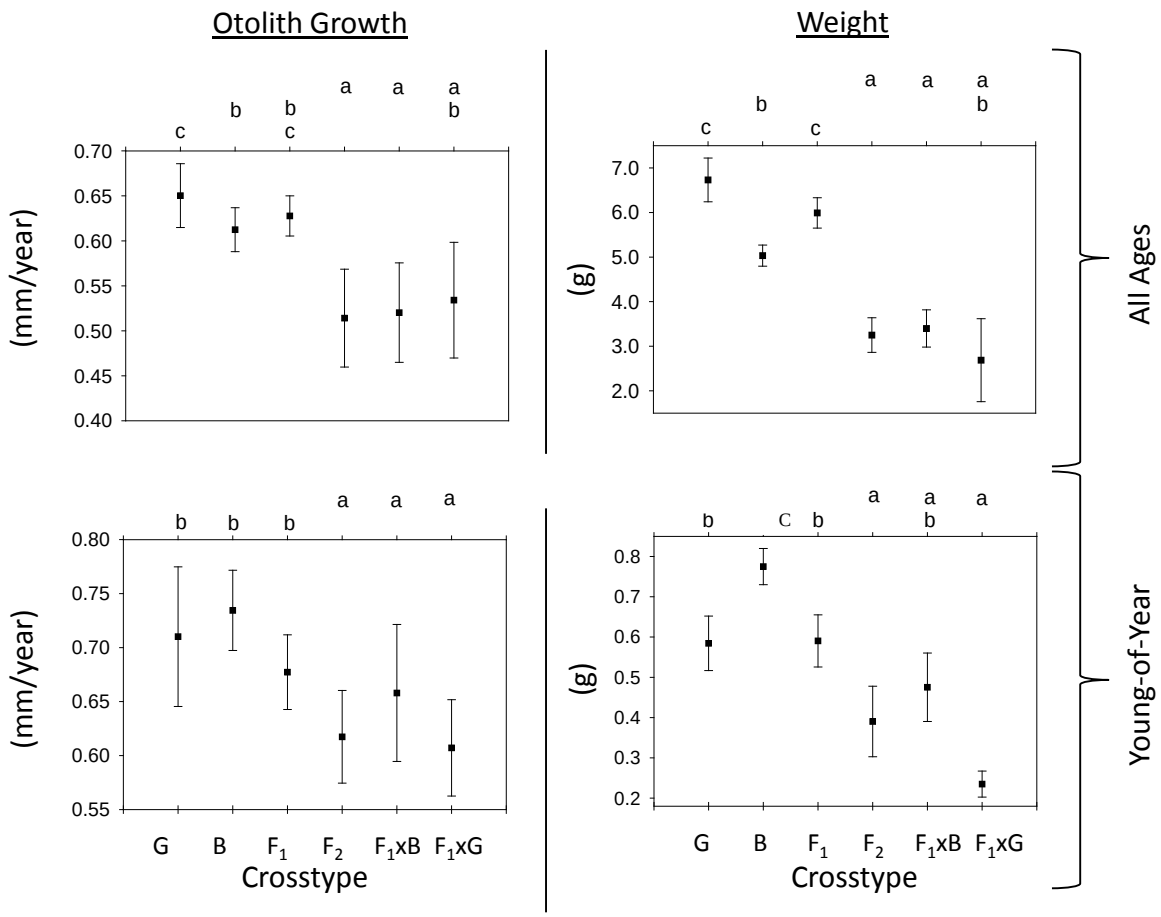
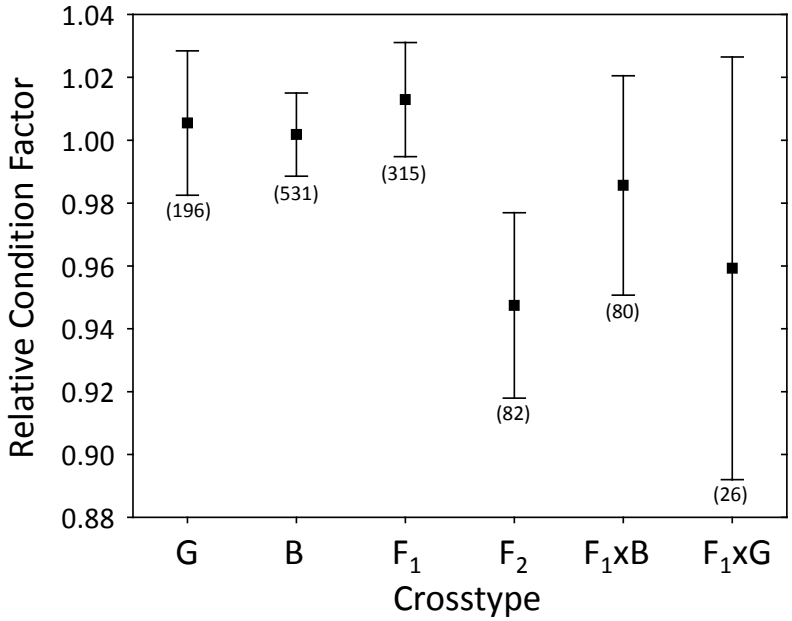

Otolith Growth
Weight
(mm/year)
(g)
All Ages
Young-of-Year
Crosstype
G
B
F1
F2
F1xB
F1xG

Figure 5


1.04
1.02
1.00
0.98
0.96
0.94
0.92
0.90
0.88
Relative Condition Factor
(196)
(531)
(315)
(82)
(80)
(26)
G
B
F1
F2
F1xB
F1xG
Crosstype

Chapter 3

A simulated reciprocal transplant experiment: local adaptation in reintroduced populations of a native North American fish

Introduction

Many fish species have formed distinct strains (or "stocks") over evolutionary history because the particular local conditions were divergent enough that locally adapted populations arose. These populations have been characterized as having a spawning location or timing that is distinct from other populations, which produces reproductive isolation (Ricker 1972). By this definition, a given region may have many strains. Distinct populations are critical for evolutionary studies because they are often precursors of new species (Magurran et al. 1999), provide models for studies of local adaptation (Foster et al. 2003), and increase understanding of the consequences of outbreeding on early stages of speciation (Sweigart 2009). Intraspecific genetic variation provides adaptive potential for evolutionary processes (Ehrlich 1988, Foster et al. 2003) and may be important to a species' viability (Soulé 1987). Reintroductions, which attempt to re-establish a species within its former range (IUCN 1998), are a common practice (Fischer and Lindenmayer 2000, Seddon et al. 2007) and are integral to a high proportion of recovery plans for imperiled species in North America (Williams et al. 1988). Successful reintroduction programs should avoid disrupting important adaptations (Crandall et al. 2000, Rader et al. 2005), reintroducing poorly adapted organisms (Greig 1979) or contaminating historically based population divergence (Moritz 1999).

The designation of an evolutionarily distinct population may be determined through molecular techniques that identify historically divergent lineages (Moritz 1994, Waples and Gaggiotti 2006) or through phenotypic divergence of certain characters

between geographically disjunct groups (Hickman and Behnke 1979, Behnke 1992). However, some components of the phenotype may not be adaptive (Gould and Lewontin 1979) and different genotypes may produce similar phenotypes or viabilities (Mayr 1970). Long-term persistence of a population that is relatively isolated within a particular habitat may, by itself, suggest local adaptation (Cohan 1984). However, functional divergence among populations may develop without a long history of isolation, even with high levels of gene flow (Dieckmann and Doebeli 1999). Therefore, local adaptations may be difficult to detect without experimental manipulation.

The study of genetically based variation between populations may be confounded by the exceptional capacity, possessed by many species, for non-genetic modification of the phenotype, or phenotypic plasticity (e.g. Imre et al. 2002, Abdoli et al. 2007). Therefore, one of the difficulties in determining whether a population is locally adapted is distinguishing among traits that have a genetic basis, versus those that are due to phenotypic plasticity. Common garden and reciprocal transplant experiments are often used to evaluate whether traits have a genetic basis or represent phenotypic plasticity. In common garden experiments, individuals from different populations are grown under the same conditions. If traits differ, a genetic basis may be inferred. The primary disadvantage of a common garden experiment is that no strain is grown in its native environment. Therefore, differences detected in measured responses may not be fitness-related or ecologically relevant where the strain is indigenous. Reciprocal transplant experiments require transferring individuals between the original divergent

environments. If traits for a given strain remain the same in both habitats, then a genetic basis for the traits may also be inferred. Thus, reciprocal transplant experiments allow local adaptations to be detected because the relative fitness of each strain may be compared in both habitats. Generally, locally adapted populations should be better suited to their native environments (but see Crespi 2000). Therefore, trait differences between strains that are ecologically relevant may be expressed in terms of greater fitness in their respective native habitats. Lack of a difference in fitness implies that a population is not locally adapted within the scale of the experiment. A major drawback of a reciprocal transplant is that it is very seldom feasible to release mobile non-native individuals at a site without some type of enclosure that may substantially alter natural conditions. Furthermore, there is a significant risk of causing harm by introducing exotic genotypes into a population should the experimental organisms escape (Fraser et al. 2008). Natural fish habitats are complex and local adaptations are, by definition, dependent on a particular environment, thus laboratory experiments have limited usefulness for determining whether local adaptation exists and which traits might be most relevant to adaptation in the wild.

Mixed-source reintroductions may provide opportunities to study local adaptation at the population level (Armstrong and Seddon 2008). Reintroductions are usually intended to restore a species to a previously occupied habitat that has been deemed suitable, and if potentially divergent strains are grown under identical conditions, they represent a common garden experiment. In this study, we utilized a

native stream fish reintroduction program in southeast Minnesota, U. S. A. to detect local adaptation in wild fish populations. Three genetically distinct source populations were used for the reintroductions (Chapter 1), but because initial analyses indicated very few individuals from one source in most reintroduced populations, we focused our analysis on two of the source strains. Because the reintroductions were carried out at nine isolated sites in close geographic proximity, our goal was to model the response of fitness related traits in the reintroduced populations to the variation in habitat across the sites. In an earlier study it was demonstrated that fitness indicators in these populations, persistence, body size, and growth rate (Chapter 2), differed by ancestry in the reintroduced populations. We therefore developed strain-response habitat models, and then input habitat variables from the source locations to make response variable predictions in the source habitats. Consequently, with a combination of multiple linear regression and ordination methods, we simulated a reciprocal transplant experiment by utilizing multiple common garden configurations.

We measured variables that we expected to influence the natural selection of sculpin morphological and physiological traits for use in the models. These included biotic variables such as macroinvertebrate density and community composition, physical habitat, and water temperature. We hypothesized that if a local adaptation was present in either strain, the predicted response variables at the source sites would indicate greater fitness for each strain in its respective habitat. We then characterized the source habitats relative to the reintroduction site habitats in multivariate space using

ordination. Finally, we characterized the relationship of the response variables to habitat at the source and reintroduction sites.

Methods

Study organism and reintroduction project

The slimy sculpin (*Cottus cognatus* Richardson) is a small (< 130 mm), cryptic, freshwater fish that occupies benthic habitats in lakes, rivers, and small streams from Virginia to the northwest across Canada to eastern Siberia (Scott and Crossman 1979). Our study area is located in the Driftless Region of southeast Minnesota, USA (Fig. 1). Prior to major human settlement beginning in 1850, nearly all of the spring-fed streams in the region presumably held slimy sculpins, but their abundance declined in subsequent years because of habitat degradation related agricultural practices (Waters 1977). Since the 1940s, there have been hundreds of in-stream improvement projects in this region that have restored some stream habitats sufficiently for reintroduction of slimy sculpins (Waters 1977, Thorn et al. 1997).

The Minnesota Department of Natural Resources carried out the reintroductions; its goal was to reestablish viable, self-sustaining populations, where native populations were likely present historically, but were extirpated. Nine streams were stocked in autumn 2003-2005 with a mixture of sculpins from three donor streams that are small tributaries entering the Mississippi River within 40 river kilometers of each other (Fig. 1). We focus our analysis on only two of these source populations: Beaver Creek (Beaver) and Garvin Brook (Garvin). Although three source populations were used in reintroductions, for this evaluation we removed descendents from one source, Cold Spring Brook, because initial analyses indicated very few pure strain

individuals were present in most reintroduced populations (Chapter 1). A total of 1230 B and Garvin sculpins were stocked in equal proportions among all study sites.

Population estimates, genetic analysis, habitat and macroinvertebrate surveys

Genetic sampling and population estimates were conducted in autumn 2007 at all study sites, except genetic samples Little Pickwick Creek and Pickwick Creek, which were collected in autumn 2009. Sculpins were collected using a Wisconsin™ Abp-3 pulsed DC backpack electrofisher (Cowx and Lamarque 1990). Each fish was anesthetized using tricaine methanosulfate (MS-222) (Summerfelt and Smith 1990), weighed, measured for standard length, and a small amount of tissue was clipped from the left pelvic fin of each fish for genetic analysis. None of the visibly marked, originally stocked fish were sampled. Population sizes were estimated using multiple-pass depletion (Lockwood and Schneider 2000).

We used eight microsatellite loci, developed for other *Cottus* species, that resolved genetic variation in *C. cognatus*: Cgo18, Cgo42, Cgo310, and Cgo1033 (Englbrecht et al. 1999); Cott290, Cott686, and CottES1 (Nolte et al. 2005b); and Cba14 (Fiumera et al. 2002). DNA extraction and amplification were performed using standard methodology (Chapter 1). We used multilocus genotype data to assign individual fish to either Beaver or Garvin source ancestry (Chapter 1).

Macroinvertebrate samples were collected with a Waters-Knapp modification of a Hess sampler (0.11 m^2). Five samples were taken randomly in erosional habitat and combined into a single sample. Samples were sorted and subsampled to 500 individuals

and macroinvertebrates were identified to the lowest possible taxonomic level, usually genus (Klemm et al. 2003). Singleton and doubleton taxa among the reintroduction sites and any taxa that did not occur at both source sites were removed from the analysis. Temperatures were recorded at 30-minute intervals using Hobo® temperature loggers deployed at the study sites for one year (autumn 2008-2009). We calculated the seven day average of the daily maximum temperatures and chose a single metric date for autumn, winter and summer by identifying the date with maximum among-site temperature variability within each season. Physical habitat data were collected from 10 cross-sectional transects evenly spaced along a 150 meter stream reach (Simonson 1993) that included the reintroduction point. Systematic "pebble" counts provided substrate characterizations by calculating percentages of observations within five size classes (Kaufmann et al. 1999). Width, depth, velocity, and channel unit (riffle, glide, and pool) were also quantified along the transects at each site (Lazorchak et al. 1998).

Statistical analysis

We estimated body condition for each fish by calculating relative condition factor (K_n), which has previously been employed as a fitness related trait in *Cottus* species (Knaepkens et al. 2002). Relative condition factor is defined as $K_n=W/W_{pred}$, where W is the observed weight, and W_{pred} is the predicted weight from a third-order polynomial based on a weight-length relationship (LeCren 1951, Wootton 1998b) developed for each reintroduced population. The "persistence" of each strain was the number of pure Beaver and Garvin individuals identified within the sample at each site.

We developed four statistical models to test our hypothesis that predicted body condition and persistence for each strain, which were chosen to indicate fitness, would be higher in their respective habitats than the other strain based on multiple linear regression (MLR) models developed from reintroduction site data. That is, Beaver > Garvin in Beaver habitat and Garvin > Beaver in Garvin habitat. We carried out a multistage approach for selecting habitat variables and building predictive MLR models. First, we selected variables from the candidate list (Table 1) by assessing the multicollinearity of the variables and selecting those with Pearson correlations (with each response variable) that were significant ($p < 0.10$); this reduced the number of variables for inclusion in full, unreduced MLR models. Second, we reduced each full regression model with the candidate variables (no interactions) using backward selection implemented in R (v2.10.0; Ihaka and Gentleman 1996). We chose the most parsimonious model based on the lowest Akaike Information Criterion corrected for sample size (Burnham and Anderson 1998). For final models, we limited the number of independent variables to three and did not include interaction or quadratic terms in the regression equation to help avoid over-fitting. Finally, we used the multiple regression equations to estimate each of the corresponding response variables at both source sites. Standard errors for source site predictions were based on the errors associated with the parameter estimates.

We performed an indirect gradient analysis to describe the relationship among model variables, reintroduction sites, source sites, and response variable gradients. All

variables from the reduced models were used to construct a non-metric multidimensional scaling (NMS) ordination (Kruskal 1964, Mather 1976) based on average Bray-Curtis dissimilarity distances among the reintroduction sites (Bray and Curtis 1957) using PC-ORD software (McCune and Mefford 2006). The initial starting configuration for the NMS was random and we determined the appropriate number of dimensions for the final ordination by examining stress (a measure of distortion in ordination space from the original distance matrix) by dimension plots. The final solution was achieved when the standard deviation in stress over the preceding 10 iterations reached 0.00001. Variable ordination scores were calculated by weighted averaging of the site score values for a given variable. Persistence and body condition response gradients were represented as vectors and then overlaid as a joint plot originating from the center of the ordination, with relative length scaling determined by r-values between the response variable and the axes. We calculated *post-hoc* NMS site scores for the two source sites based on habitat variable values with an algorithm designed to calculate NMS scores for new sites based on a prior ordination without altering the positions of the original points (McCune et al. 1997). Finally, we plotted the predictor variable NMS scores versus each of the four response variables represented as a contour-gradient to investigate the relationship of the predictor variables to the reintroduction and source site gradients (Ihaka and Gentleman 1996, Oksanen 2004). Contours were constructed using non-parametrically smoothed surfaces that were fitted using general additive models with thin plate splines. The degree of smoothing was

determined using cross-validated r^2 to determine goodness-of-fit. The significance of each contour surface was tested with an ANOVA.

Results

The percentage of individuals identified as Beaver exceeded Garvin at most reintroduction sites and ranged from 5 to 54% (median = 23%) for Beaver and 1 to 25% (median = 3%) for Garvin. Population estimates varied greatly among the reintroduction sites (Table 2). Three of four of the higher sculpin density sites (Rock Creek, Little Pickwick Creek, and Hay Creek) had more equivalent proportions of Beaver and Garvin sculpins (Gilbert Creek was the exception) than the other sites with lower densities, which tended to overwhelmingly favor Beaver. Body condition ranged from 0.91-1.11 (median = 1.00) for Beaver and from 0.80-1.15 (median = 0.98) for Garvin.

We included 18 invertebrate taxa from both source and reintroduction sites of the 87 taxa present across all study sites (Table 1). All but four of the taxa were genera in the orders Diptera, Ephemeroptera, Trichoptera, Coleoptera, and Amphipoda. The remaining four taxa were families in the orders mentioned above except for the subclass Acari.

Regression analysis

Of the 32 candidate habitat variables quantified for potential inclusion in the MLR models and the NMS analysis (Table 1), only ten were used in the full, unreduced models. Models to predict Beaver and Garvin body condition and persistence were composed of biotic, physical habitat and temperature variables (Table 3). Beaver body condition was positively influenced by the abundance of *Dicranota* and *Hydropsyche* and warmer winter water temperatures. The Garvin body condition model was best

predicted by decreased abundance of *Neoplasta* and proportion of fine sediment. Beaver persistence was best predicted by a decreased abundance of *Micropsectra* and greater abundance of *Optioservus*, but not by physical habitat or temperature variables. Garvin persistence was not well predicted by biotic variables but by stream characteristics and increased with increasing proportion of cobble and faster stream velocity.

Predicted Beaver body condition and persistence indicated adaptation to local conditions in the source stream. Predicted Garvin persistence marginally indicated local adaptation, whereas Garvin body condition did not (Table 3). Three regression models were significant ($p < 0.05$) and predicted substantial differences in fitness between Beaver and Garvin that favored strains from their respective habitats, but only 95% confidence intervals (1.96 x S. E.) for Beaver body condition had no overlap. Beaver body condition and persistence were both predicted to be greater in Beaver habitat than Garvin habitat, and Garvin persistence was predicted to be greater in Garvin habitat than Beaver habitat, but there was no detectable difference between Garvin body conditions in Beaver or Garvin habitats.

Indirect gradient analysis

We described the habitat gradient across the reintroduction sites and its relationship to the habitat at the source sites using an NMS ordination with plotted sites that are based on variation in the final MLR predictor variables (Fig. 2). A two-dimensional solution was chosen after 250 Monte Carlo runs obtained a p-value = 0.04, indicating a low

probability that a similar final stress could have been obtained by chance. The final NMS solution with 42 iterations had a final stress of 1.8. The proportion of variance represented by both axes characterized by the r^2 between the original Bray-Curtis distances and the ordination distances was 0.96, with about two-thirds of the variance being represented on the first axis (axis 1, $r^2 = 0.62$; axis 2, $r^2 = 0.33$).

Predicted NMS scores for Beaver and Garvin source sites were fitted simultaneously to optimize the overall position of the fitted points in the ordination. A good fit was obtained for these sites (stress; mean = 7.4, SD = 1.9) and the range of scores in the original plot were comparable to fitted source site scores. Most of the habitat gradient between Garvin and Beaver sample plots was represented along axis 1. Garvin body condition and persistence had no relationship to axis 1 scores; the variance was represented on axis 2 ($r^2 = 0.35$ and 0.06 respectively). Beaver body condition was represented on both axes (axis 1, $r^2 = 0.50$; axis 2, $r^2 = 0.08$), as was Beaver persistence (axis 1, $r^2 = 0.57$; axis 2, $r^2 = 0.26$).

Because axis 1 essentially represented a linear habitat gradient between Garvin and Beaver source sites, the linear relationship between axis 1 scores and the predictor variables (Table 4) revealed several trends. First, winter temperature was most strongly related to axis 1 ($r^2 = 0.83$), with temperature decreasing from Garvin to Beaver source sites (left to right). *Micropsectra* and *Neoplasta* also decreased from Garvin to Beaver, whereas the three remaining biotic variables: *Optioservus*, *Hydropsyche*, and *Dicranota* increased from Garvin to Beaver. The remaining abiotic habitat variables (velocity,

cobble, and fine sediment) had no relationship to the Beaver to Garvin gradient. Interestingly, velocity, cobble, and fine sediment also were included in the two Garvin MLR models, along with *Neoplasta*, which had the weakest correlation of the biotic variables with axis 1. Sediment had an appreciable relationship with NMS axis 2 (r^2 = 0.31), as did winter temperature (r^2 = 0.35); both increased from the bottom to the top of the ordination. The linear gradient in velocity and cobble on either axis was negligible.

We used fitted contour surfaces on the same NMS ordination plotted with only the habitat predictor variables to visualize the gradient of each MLR response variable relative to the source and reintroduction site habitat gradient. Fitted contour surfaces (Fig. 3) were all significant at $p < 0.001$. The circumscribed region, which approximates the gradient represented by the reintroduction sites shown in Figure 2, runs diagonally from the top left corner to the bottom right. Thus, the habitat gradient across the reintroduction sites is correlated with Garvin to Beaver gradient, which is represented entirely along axis 1 (see above). The contour gradient for Beaver body condition (Fig. 3; upper left panel), which had the best MLR model (r^2 = 0.82) and indicated the strongest local adaptation, was roughly perpendicular to the Garvin to Beaver gradient. The gradient in Beaver persistence (Fig. 3; lower left panel) was perpendicular to the longest dimension of the habitat gradient represented by the reintroduction sites. The Garvin persistence gradient (Fig. 3; lower right panel) was perpendicular to the shortest dimension of the habitat gradient represented by the reintroduction sites. Finally, the

Garvin body condition gradient (Fig. 3; upper right panel) was non-linear, but approximated a parallel gradient along axis 1 and corresponded to the weakest MLR model (r^2 = 0.49) and provided no indication of local adaptation.

Discussion

Studies that identify local adaptations in wild populations are relevant to conservation efforts and are vital to our understanding of divergent evolution as the basis of local adaptation, reproductive isolation, and ultimately speciation. There is no universally "best" genotype because conditions that favor one genotype may vary to favor another within a short distance, as environments are variable in space and time (Mayr 1970). Although our source sites were close geographically, our regression models indicated divergent local adaptations in these strains through differential habitat responses at the reintroduction sites. We used multivariate gradient analysis to describe habitat patterns in reintroduction sites relative to the source sites. We recognize that the habitat gradient in reintroduction sites was less than optimal for modeling local adaptations in the sources, because source and reintroduction habitat gradients were oblique, rather than parallel to one another. This configuration resulted in a shorter gradient with which to develop models than we would have preferred, had we initially chosen the sites with the intention of testing our hypothesis. However, one of the major benefits of this study has been to illustrate the feasibility of capitalizing on an existing "field experiment," to develop satisfactory response models and identify potential mechanisms for local adaptations (Seddon et al. 2007).

Habitat and local adaptation

Our interpretation of the multivariate analysis is that winter water temperature is the major gradient differentiating Garvin and Beaver habitats which are represented along

NMS axis 1. Relatively warm winter water temperatures in this region indicate systems strongly influenced by groundwater, which also have colder summer temperatures. Of the macroinvertebrate variables that correlate with axis 1, *Micropsectra* is known to be associated with colder summer temperatures (pers. comm. L. C. Ferrington), whereas *Optioservus* and *Hydropsyche* are associated with warmer summer temperatures (Huff et al. 2006). These taxa are likely representative of macroinvertebrate communities that are dominated by different thermal regimes, and are therefore redundant signals of variation in water temperature. However, different prey preferences and life history strategies have potentially developed as each population continued to live in divergent selective environments.

Although slimy sculpins are known to inhabit uniformly cold, groundwater dominated systems in this region, even minor variations in temperature affect metabolic rate and influence a variety of other physiological functions in fish such as growth rate, reproduction, and swimming performance (Fry 1970, Wootton 1998a). As a result, temperature also affects species interactions (Baltz et al. 1982, Reeves et al. 1987, Taniguchi et al. 1998) in which fish distinguish and compete for habitats along thermal gradients to define their thermal niche (Magnuson et al. 1979, Huff et al. 2005). Divergent life history strategies have been documented for a closely related species, *Cottus gobio* in which growth rates of young versus older sculpins responded differently depending on the thermal regime of the local population (Abdoli et al. 2007). In a reciprocal transplant experiment in England, growth performance, maturation date, and

life span of *Cottus gobio* also varied significantly depending on temperature, but these differences were attributed to phenotypic plasticity and the study used relatively few fish that were held in enclosures (Mann et al. 1984).

The other major abiotic gradient across the reintroduction sites, primarily along NMS axis 2, was gradient that represented increasing fine sediment substrate. *Neoplasta* and *Micropsectra* were influential along axis 2 as well, in the same direction as increasing winter temperatures. These two genera are found near groundwater springs, which tend to have a shallower elevation gradient and slower velocity in this region, and consist of more depositional habitat that accumulates fine sediment. The cranefly, *Dicranota*, increased strongly with decreasing fine sediment. Together, these two gradients caused the diagonal orientation of the habitat variables relative to the gradient between Garvin and Beaver. As with temperature, substrate is important for aquatic communities. Many benthic macroinvertebrates have a high affinity for a particular substrate type and may respond negatively to high levels of fine sediment (Brusven and Prather 1974).

We predicted a higher body condition and persistence for each strain in its original habitat; however, Garvin results were more ambiguous. When the fitted contours in Figure 3 were more perpendicular to the Garvin to Beaver gradient, we could construct better and more sensitive regression models. The Beaver body condition model, for example, predicted a higher body condition for Beaver in the Beaver habitat than for Beaver in the Garvin habitat with very low error.

Conservation Implications

There may be considerable risk in assuming that observed interpopulation variation in life history traits result from phenotypic plasticity rather than local adaptation. Assumed phenotypic plasticity may be considered an advantage in adapting to warmer environmental conditions, but in reality, populations on the edge of their ranges that are vulnerable to thermal regime shifts may be genetically distinct and of considerable conservation value (Lesica and Allendorf 1995). As managers consider global warming adaptation scenarios, they should consider evaluating a population's potential for temperature adaptations, even in widespread species. Likewise, although sedimentation is a naturally occurring phenomenon, land-use changes have resulted in an increase in anthropogenically induced fine sediment deposition (Wood and Armitage 1997), especially in agricultural regions, such as southeast Minnesota. An understanding of population-specific adaptations may provide opportunities to better maintain the range of species despite anthropogenic influences on habitat. For example, if a particular strain thrives in a higher sediment or warmer environment than others, then it may be more appropriate to reintroduce it to locations where degraded aquatic habitat will never practicably achieve a full recovery. This idea has been explored for brook trout (*Salvelinus fontinalis*) in which a strain was sought that tolerated a naturally acidic environment to reintroduce populations lost to acid rain in Quebec (Lachance et al. 2000). The choice of the appropriate genetic source for the reintroductions depends on

the scale and magnitude of local adaptations and how novel a given environment is because of anthropogenic disturbance (Lesica and Allendorf 1999, Jones 2003).

Knowledge of local adaptations are relevant when considering the use of ecological and genetic exchangeability (Crandall et al. 2000, Rader et al. 2005) to distinguish populations with unique evolutionary trajectories and conservation importance (Waples and Gaggiotti 2006). Maintenance of evolutionary processes and historical population structure within distinct populations are principle strategies in species conservation (Moritz 1999). However, Crandall et al. (2000) contend that focus on historical population structure maintenance is overemphasized. They assert that molecular genetic data is increasingly used to the exclusion of ecological data and that the suggested reciprocal monophyly criterion (Moritz 1999) can be too stringent for populations with paraphyletic histories that have important adaptive differences. Furthermore, reciprocal monophyly may also be too discerning in some cases because of high resolution molecular techniques that may over-identify differences. Instead, Crandall et al. (2000) assert that distinct populations should include measures of adaptive diversity defined by survival-enhancing heritable traits, along with genetic isolation. The practical feasibility of employing this concept in conservation has been a major criticism (Zink 2007) and Rader et al. (2005) endeavored to facilitate its use by describing a test procedure for determining ecological exchangeability. Ecologically exchangeable populations can switch locations while still occupying the same niche (Rader et al. 2005). They are identified by parsing phenotypic plasticity from genetically

based local adaptation via common garden or reciprocal transplant experiments that measure differences in phenotypic traits (e.g., growth, body size, fecundity and survivorship) and molecular analyses of neutral markers that distinguish interpopulation genetic differences. Ecological exchangeability provides a conceptual framework for alleviating uncertainties surrounding reintroduction source choice and potential impacts of genetic introgression on conspecifics.

Exchangeability tests are most applicable to reintroductions when habitat is relatively undisturbed, representatives of ancestral lineages are available for release, and gene flow between populations of evolutionary unique lineages may be reestablished. Reintroducing source populations that are exchangeable with remnant populations may bolster their viability and prevent reestablished gene flow from disrupting local adaptations (Storfer 1999). If molecular and ecological analyses are feasible, ecological exchangeability may identify sources that take full advantage of genetic adaptive potential and maintain a distinct population's integrity (Jones 2003).

Because of the potential ubiquity of local adaptations in natural populations, it is likely impractical to attempt to preserve all populations that are locally adapted (Zink 2007). Likewise, many populations are not likely to be genetically or ecologically divergent. The difficulty in recognizing a particular local adaptation precludes having sufficient knowledge of it in every case. Therefore, the effort needed to demonstrate ecological exchangeability might need to be justified with additional information such as the importance of the species role in an ecosystem or the uniqueness of a particular

adaptation (e.g., the cave spider example in Crandall et al. 2000). Conversely, if there is evidence of genetic similarity (Moritz 1994), if populations have become recently isolated, or if they are influenced by significant gene flow, they are less likely to have distinct local adaptations.

Another potential application of our method for identifying local adaptations lies in invasion biology. Data from recent introductions of non-native stocks in a region could be employed to identify adaptations if there are enough individuals with un-hybridized genotypes at enough locations to adequately characterize a habitat gradient. Following this, fitness related traits may be modeled and invasion potential could be assessed with ordination methods to understand how well the calibration habitat gradient represented the new locations. Regression predictions combined with an examination of the predictor variables could be used to characterize the conditions under which an invasion would be successful.

Finally, some reintroduction and population supplementation practices provide ideal model systems to address questions about the importance of local adaptations and it is important to take advantage of these systems (Sarrazin and Barbault 1996, Armstrong and Seddon 2008). Many restoration and reintroduction projects may also become experiments in ecological genetics. Most of what is required lies in simple documentation of the source locations, quantities and locations of the organisms stocked, and a measure of performance (such as persistence, growth, or body condition). By addressing key questions in the emerging field of reintroduction biology,

we may improve species recoveries and produce research that will advance the field. Research such as this will lead to a better understanding of the biology of species and provide guidance for managers to improve reintroductions and other conservation practices for conserving imperiled species.

Figure Captions

Fig. 1 - Source (ô) and recipient (õ) sites in southeast Minnesota. The Driftless Region where study sites are located is shown in the inset, indicated by the cross-hatched area covering portions of Minnesota, Wisconsin, Iowa, and Illinois.

Fig. 2 - Two-dimensional NMS plot based on habitat variables at reintroduction sites (ó). Habitat variable values (+) are weighted averages of site scores for each variable. Joint plot (vectors) indicate the four response variable gradients across ordination space. Garvin Brook and Beaver Creek source sites (ó) were calculated *post-hoc* and plotted on the existing ordination using habitat variables with a predictive algorithm analogous to a multiple regression equation.

Fig. 3 - Non-metric multi-dimensional scaling (NMS) ordination (the same as in Fig. 2) with contour-gradients (regression surfaces; Wood 2000). Contours show smooth trends of the relationship between MLR response variables and habitat variable NMS scores. Labeled black dots indicate positions of predictor variables. The habitat gradient between Garvin and Beaver NMS sample plots is represented along axis 1. The circumscribed region approximates the gradient represented by the reintroduction sites shown in figure 2.

Figure 1

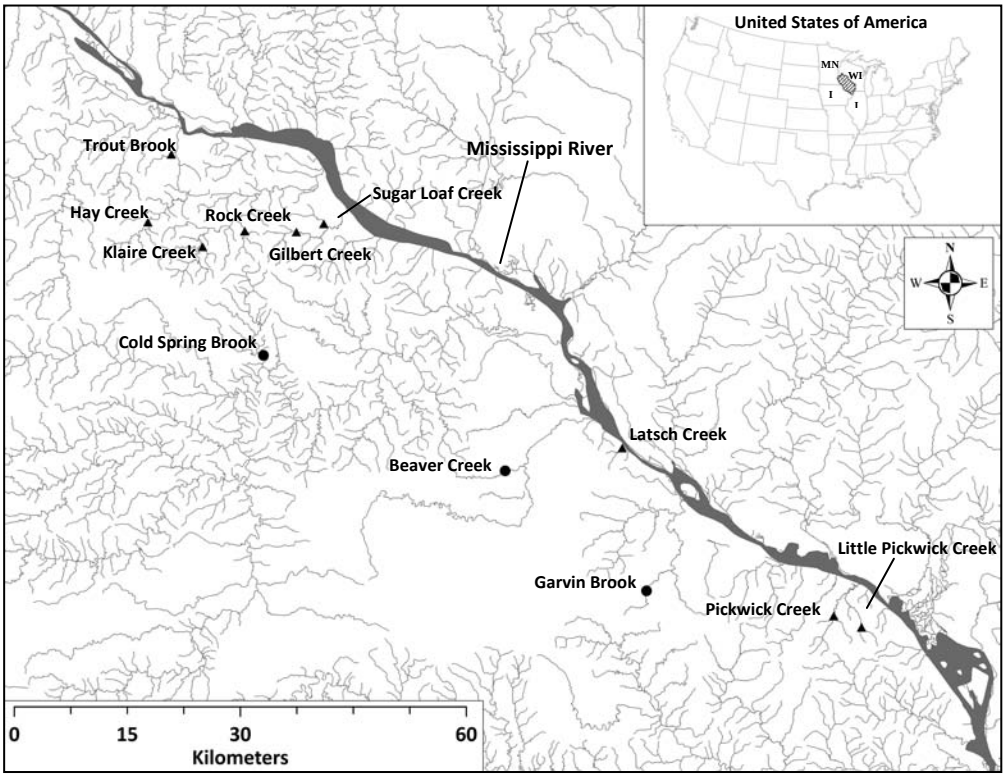

United States of America
MN
WI
Trout Brook
Mississippi River
Sugar Loaf Creek
Hay Creek
Rock Creek
Klaire Creek
Gilbert Creek
N
W
E
S
Cold Spring Brook
Latsch Creek
Beaver Creek
Little Pickwick Creek
Garvin Brook
Pickwick Creek
0
15
30
60
Kilometers

Figure 2

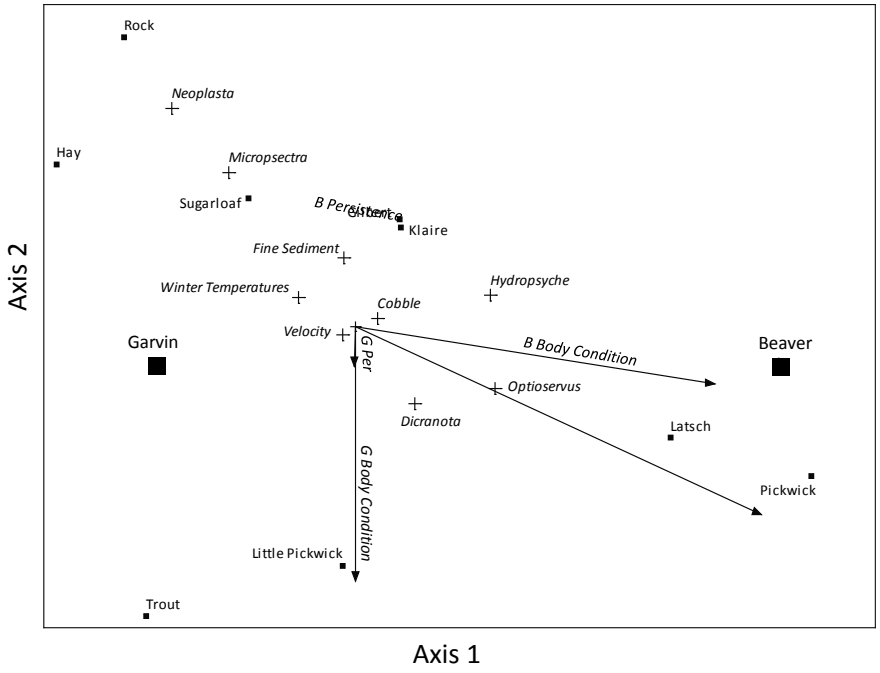

Rock
Neoplasta
Hay
Micropsectra
Sugarloaf
B Persistence
Klaire
Fine Sediment
Winter Temperatures
Hydropsyche
Cobble
Velocity
Garvin
G Per
B Body Condition
Beaver
Optioservus
Dicranota
Latsch
G Body Condition
Pickwick
Little Pickwick
Trout
Axis 2
Axis 1

Figure 3

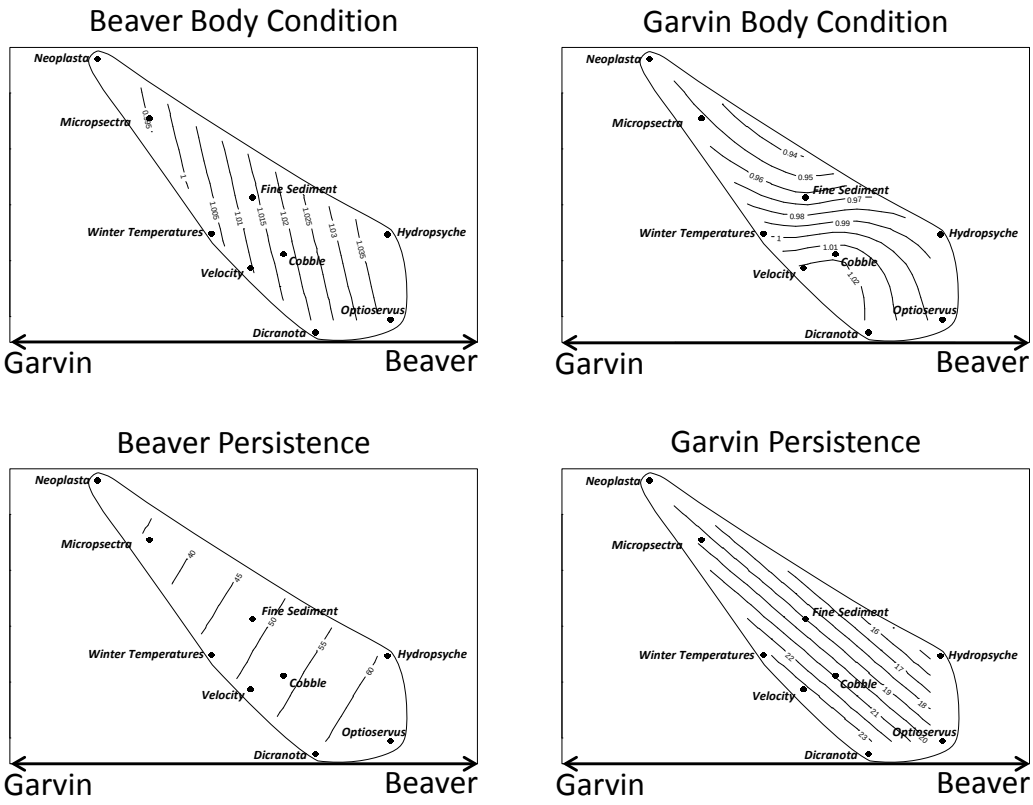

Beaver Body Condition
Neoplasta
Micropsectra
Fine Sediment
Winter Temperatures
Hydropsyche
Cobble
Velocity
Optioservus
Dicranota
Garvin
Beaver
Garvin Body Condition
Neoplasta
Micropsectra
Fine Sediment
Winter Temperatures
Hydropsyche
Cobble
Velocity
Optioservus
Dicranota
Garvin
Beaver
Beaver Persistence
Neoplasta
Micropsectra
Fine Sediment
Winter Temperatures
Hydropsyche
Cobble
Velocity
Optioservus
Dicranota
Garvin
Beaver
Garvin Persistence
Neoplasta
Micropsectra
Fine Sediment
Winter Temperatures
Hydropsyche
Cobble
Velocity
Optioservus
Dicranota
Garvin
Beaver

Table 1. Candidate habitat and macroinvertebrate variables quantified in this study. Models are indicated for variables if correlations with the response variable were strong enough ($p<0.10$) for inclusion as candidate variables for the multiple linear regression analysis.

Variable	Model
Biotic	
Acari	-
Baetidae	-
Brachycentrus	-
Ceratopsyche	-
Cheumatopsyche	-
Dicranota	B-BC, B-P
Elmidae	-
Ephemerellidae	-
Eukiefferiella	-
Gammarus	-
Glossosoma	-
Hydropsyche	B-BC
Micropsectra	B-P
Neoplasta	G-BC, B-P
Optioservus	B-P
Orthocladius	-
Simuliidae	-
Tvetenia	-
Macroinvertebrate Density	-
Sculpin Density	-
Physical Habitat	
Depth	-
Width	-
Velocity	G-P
Riffle to Glide Ratio	-
Fine Sediment	G-BC, G-P
Sand	-
Fine Gravel	-
Coarse Gravel	-
Cobble	G-P
Water Temperature	
Fall Temperature	B-BC
Winter Temperature	B-BC, B-P
Summer Temperature	-

B=Beaver, G=Garvin, BC=Body Condition, P=Persistence

Table 2. Percent of total (%) and number of individuals (No.) per source strain ancestry, mean body condition (BC) and population estimates for each of the reintroduced populations. Percentages do not total to 100% because sculpins with hybrid and Cold Spring ancestry were removed from the analysis. Population estimates (± 95% confidence) were made in autumn 2007 at all sites.

Population	Beaver			Garvin			Population
	%	No.	BC	%	No.	BC	Estimate
Latsch Creek	54	98	1.07	1	2	0.98	895 ± 178
Pickwick Creek	37	68	1.05	13	23	1.11	136 ± 19
Sugar Loaf Creek	35	59	0.98	2	4	1.05	211 ± 25
Klaire Creek	28	44	1.11	3	5	0.96	213 ± 14
Gilbert Creek	23	42	1.03	1	1	0.80	3147 ± 87
Little Pickwick Creek	21	80	1.00	20	76	1.04	1483 ± 111
Rock Creek	19	30	0.98	25	40	0.97	2984 ± 153
Trout Brook	17	32	1.00	3	6	1.15	324 ± 3
Hay Creek	5	9	0.91	5	9	0.99	1273 ± 82

Table 3. Regression model predictions for Beaver Creek and Garvin Brook with standard errors and adjusted correlation coefficient, p-value and variables included in each model. In a given source habitat, predicted persistence values are the estimated number of individuals; predicted body condition values are the estimated relative condition factors.

Response Model	Beaver Habitat	Garvin Habitat	r^2	p	Predictor Variables
Beaver Persistence	83±15	47±7	0.51	0.05	*Micropsectra*, *Optioservus*
Beaver Body Condition	1.06±0.02	0.94±0.02	0.82	0.01	*Dicranota*, *Hydropsyche*, Winter Temperature
Garvin Persistence	97±19	111±22	0.71	0.01	Cobble, Velocity
Garvin Body Condition	1.01±0.04	1.01±0.03	0.49	0.06	*Neoplasta*, Fine Sediment

Table 4. Pearson correlation values for each of the final regression model predictor variables versus NMS axis scores. Variables are sorted from highest to lowest r^2 for axis 1.

Predictor Variable	Axis 1		Axis 2	
	r	r^2	r	r^2
Winter Temperature	-0.91	0.83	0.59	0.35
Optioservus	0.78	0.61	-0.44	0.19
Hydropsyche	0.75	0.56	0.22	0.05
Micropsectra	-0.59	0.35	0.90	0.81
Dicranota	0.54	0.29	-0.88	0.77
Neoplasta	-0.50	0.25	0.75	0.56
Velocity	-0.15	0.02	-0.12	0.01
Cobble	0.10	0.01	0.04	0.00
Fine Sediment	-0.08	0.01	0.55	0.31

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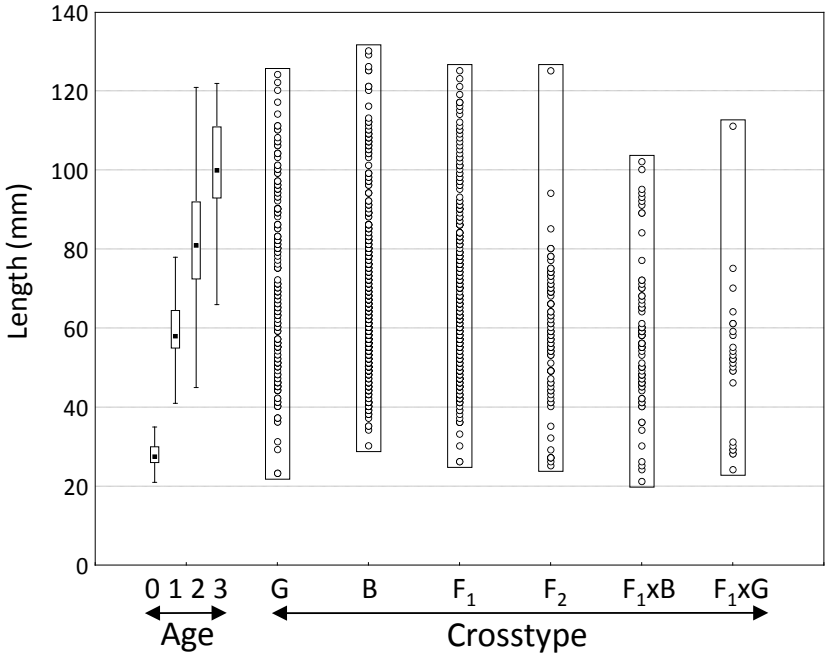
Appendix A



Appendix A - Age distribution by length for otolith data (n=495) and variance plots of raw length data at all nine reintroduction sites (n= 1230) grouped by crosstype. Box and whisker plots shown for fish ages 0(n=30), 1(n=196), 2(n=260) and 3(n=9) indicate the range (whiskers), quartile (boxes) and median (points) lengths at each age.