

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

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Reintroductions are commonly employed to preserve intraspecific biodiversity in fragmented landscapes. However, reintroduced populations are frequently smaller and more geographically isolated than native populations. Mixing genetically, divergent sources are often proposed to attenuate potentially low genetic diversity in reintroduced populations that may result from small effective population sizes. However, a possible negative tradeoff for mixing sources is outbreeding depression in hybrid offspring. We examined the consequences of mixed-source reintroductions on several fitness surrogates at nine slimy sculpin (*Cottus cognatus*) reintroduction sites in south-east Minnesota. We inferred the relative fitness of each crosstype in the reintroduced populations by comparing their growth rate, length, weight, body condition and persistence in reintroduced populations. Pure strain descendents from a single source population persisted in a greater proportion than expected in the reintroduced populations, whereas all other crosstypes occurred in a lesser proportion. Length, weight and growth rate were lower for second-generation intra-population hybrid descendents than for pure strain and first-generation hybrids. In the predominant pure strain, young-of-the-year size was significantly greater than any other crosstype. Our results suggested that differences in fitness surrogates among crosstypes were consistent with disrupted co-adapted gene complexes associated with beneficial adaptations in these reintroduced populations. Future reintroductions may be improved by evaluating the potential for local adaptation in source populations or by avoiding the use of mixed sources by default when information on local adaptations or other genetic characteristics is lacking.

Keywords: *Cottus*, fish conservation, fitness, hybridization, sculpin, translocation

Received 23 July 2010; revision received 21 July 2011; accepted 3 August 2011

Introduction

Reintroductions, which are intended to re-establish a species within its former range, are a common practice (IUCN 1998; Fischer & Lindenmayer 2000; Seddon *et al.* 2007) and are integral to a high proportion of recovery plans for imperiled fishes in North America (Williams *et al.* 1988; George *et al.* 2009). As habitats continue to

be degraded, there is concern that fragmented wild animal populations are vulnerable to inbreeding depression and reduced evolutionary potential (Keller & Waller 2002; Jenkins 2003). Yet, reintroduction practices may hasten a decline in genetic variability by reducing the effective population size of the source and reintroduced populations (Lande & Barrowclough 1987; 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), restore genetic diversity to historic levels and provide novel

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genetic combinations required for rapid evolutionary change (Lewontin & Birch 1966; Stockwell *et al.* 2003).

An increase in fitness known as hybrid vigour that may result from either the suppression of deleterious recessive alleles or the beneficial overdominance has been recognized as a useful application of outbreeding (or hybridization) for centuries (Darwin 1876; Lynch 1991). However, intentional mixing of distinct source populations poses risks (Lesica & Allendorf 1999; Jones 2003); among these is a decline in fitness among offspring that are the product of mating between genetically disparate individuals, known as outbreeding depression (Lynch 1991; Tallmon *et al.* 2004). Two mechanisms may lead to outbreeding depression. First, interpopulation hybridization may decrease fitness because introduced nonlocal alleles cause a population to become less suited to local environmental conditions by producing intermediate phenotypes (Hatfield & Schluter 1999). Reduced fitness by this mechanism would be apparent in the F_1 generation. Outbreeding depression may also occur by a second mechanism, the disruption of positive epistatic interactions (i.e., co-adapted gene complexes), which would only occur 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 that inbreeding depression may not be relevant to wild populations (Pusey & Wolf 1996), there is ample evidence that it occurs (Keller & Waller 2002). Conversely, recent literature has highlighted the potential importance of outbreeding depression for conservation, but there is a relative paucity of reports that document it (Edmands 2007; McClelland & 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 & Naish 2007). While laboratory studies focus on purely genetic components that require little environmental context, field studies can provide opportunities to measure the genetic and ecological constituents of hybrid fitness acting in concert. This 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.

Outbreeding depression has been demonstrated in a variety of organisms, including fish (Endler 1977; Edmands 2007; McClelland & Naish 2007). Nevertheless, a meta-analysis of the consequences of unrelated fish population crosses by McClelland & Naish (2007) demonstrated that overall the response to outbreeding

was positive and there was no relationship between the genetic distance among parental populations and its effect on life history, behavioural, morphological or physiological traits. Yet many of the comparisons they reported were inconclusive. Failure to detect positive or negative outcomes of outbreeding may have been associated with small sample sizes and a lack of power to detect statistical significance, especially in the F_2 generation. Ultimately, they concluded that outbreeding consequences may be difficult to predict and that it might be unreasonable to make broad generalizations because of the variety of processes by which an outbreeding effect may occur. They also identified several gaps in the understanding of outbreeding outcomes in fishes. Among these were documenting responses to outbreeding beyond the F_1 generation, responses of fitness related traits within a single species, the influence of environment on hybrid response and the relationship between outbreeding depression and genetic distance.

We utilized a native fish reintroduction programme 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 were genetically distinct and there were nine discrete reintroduced populations in close geographic proximity that provided an unusual degree of replication for a field study (See Huff *et al.* 2010 for additional details). We recognized the potential for adaptive differences between source populations because one of the source strains was far more persistent in the reintroduced populations. We accordingly hypothesized that the fitness surrogates body size, growth rate and body condition would differ among pure strain individuals in the reintroduced populations depending on ancestral origin. Furthermore, we investigated the consequences of outbreeding on fitness surrogates in first- and second-generation hybrid crosses and backcrosses among young-of-the-year (hatched to age I) and over-yearlings (older than age I). Our aim in including second-generation crosstypes in the analysis was to enable a greater understanding of the genetic mechanisms underlying outbreeding if we detected a difference among crosstypes in fitness surrogates.

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 & 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 & Waters 1975; Goyke & 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 & Waters 1975).

The study area is located in the Driftless Region of south-east Minnesota, USA (Fig. 1). Prior to major settlement by European immigrants beginning in 1850, nearly all of the spring-fed streams in the region presumably held slimy sculpins and brook trout (*Salvelinus fontinalis*). In subsequent years, slimy sculpin and other cold-water fish abundance declined because of severe habitat degradation and overexploitation (Waters 1977; Leopold & Sewell 2001). Since the 1940s, the Minnesota Department of Natural Resources (MNDNR) and other organizations completed hundreds of in-stream improvement projects (Waters 1977; Thorn *et al.* 1997; MNDNR 2003). In locations where they improved habitat sufficiently, MNDNR personnel recently reintroduced slimy sculpins. The goal was to re-establish viable, self-sustaining populations where native populations were likely present historically, but had been extirpated. Nine recipient streams were stocked from 2003 to 2005 in mid-autumn with a mixture of sculpins from three source streams. The source streams are all small tributaries in separate sub-drainages that enter the Mississippi River within approximately 40 river kilometers of each other (Fig. 1). We focus our analysis on only two of these source ancestries: Beaver Creek (Beaver) and Garvin Brook (Garvin). Although three source populations were originally reintroduced, for

this evaluation, we did not consider descendents from one source, Cold Spring Brook, because initial analyses indicated very low overall ancestry in most reintroduced populations and they were not stocked some years in certain streams. A total of 1230 Beaver and Garvin sculpins were stocked in equal proportions across all nine reintroduction sites. Huff *et al.* (2010) provide additional details for the reintroduction programme.

Sampling

We conducted sampling in autumn 2007 at all source and recipient sites except Pickwick Creek, which we sampled in spring and autumn 2008 and autumn 2009. We additionally sampled Little Pickwick Creek in autumn 2008 and autumn 2009. We collected fish using a Wisconsin™ Abp-3 pulsed DC backpack electrofisher with power output settings adjusted to minimize effects on the reintroduced fish (Cowx & Lamarque 1990). We anaesthetized fish with tricaine methanesulfate (MS-222) (Summerfelt & Smith 1990); then we weighed and measured standard length for each fish. We clipped a small amount of tissue from the left pelvic fin of each fish and preserved it in 95% ethanol for genetic analysis. After processing, we returned all fish to the streams, except those captured in autumn 2008 and 2009 at Pickwick and Little Pickwick sites. We euthanized these fish with a lethal dose of anaesthetic (1 g/L of MS-222) and retained them for otolith analysis. We did not capture any of the visibly marked, originally stocked fish during these sampling events.

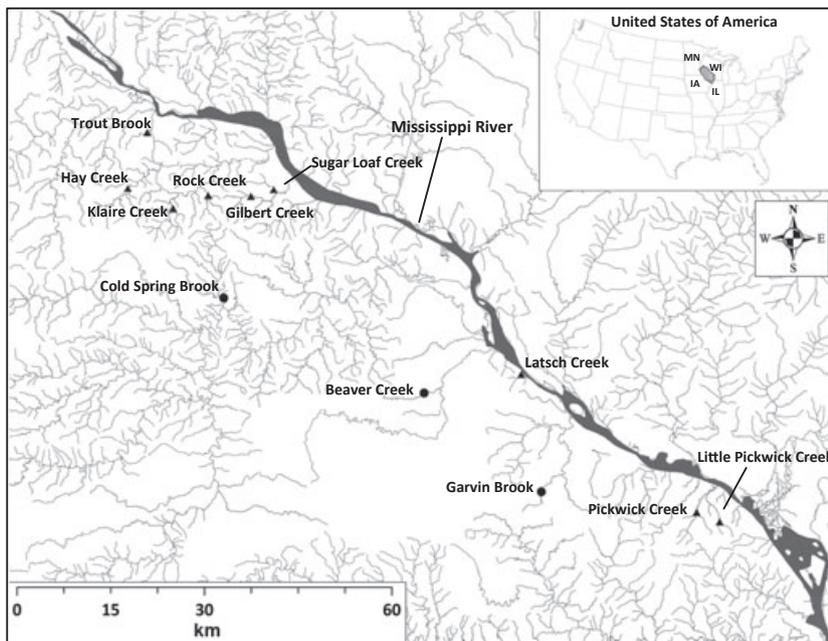


Fig. 1 Source (closed circle) and recipient (closed triangle) sites in south-east Minnesota. The Driftless Region where study sites are located is shown in the inset, indicated by the cross-hatched area covering portions of Minnesota, Wisconsin, Iowa and Illinois.

DNA extraction and amplification

For 2007 and 2008 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.* 2005); and Cba14 (Fiurera *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 μ L reactions containing 1 \times 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 μ M of the forward and reverse primers, with the forward primer labelled with a fluorescent dye 6FAM, VIC, NED or PET, and 0.5 units Taq DNA polymerase (Promega, Madison, WI, USA). Amplification was carried out in a thermocycler (Hybaid Omn-E; Thermo-Hybaid US, Franklin, MA, USA) 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, MN, USA) for electrophoresis on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). We scored alleles using the software program GENOTYPER 2.5 (Applied Biosystems 2001). For samples that we determined to be advanced generation crosses, we carried out a second round of amplification with six newly developed microsatellite loci for *C. cognatus* (Fujishin *et al.* 2009): Cco01, Cco09, Cco10, Cco14, Cco15 and Cco17. Autumn 2009 samples from Little Pickwick Creek and Pickwick Creek were genotyped at 12 loci, with the lower variation markers Cott290 and Cott686 removed from the original set.

Crosstype assignment

We used multilocus genotype data to assign individual fish to one of six crosstypes: parental (Beaver, Garvin), first-generation hybrids (F_1) or second-generation hybrids [F_2 , backcrosses to Beaver ($F_1 \times B$), or backcrosses to Garvin ($F_1 \times G$)]. First, we evaluated data from three source populations in MICROCHECKER v2.2.3 to detect evidence of null alleles or scoring errors because of large allele drop-out (Van Oosterhout *et al.* 2004). Conformance with Hardy–Weinberg expectations and linkage equilibrium was tested using GENEPOP v4.0.4 (Raymond & Rousset 1995). We adjusted significance values for both tests using sequential Bonferroni procedures (Rice 1989).

We next removed fish with Cold Spring ancestry from the dataset. 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 three, which was the known number of genetically distinct source populations, with an admixture model and correlated allele frequencies. We ran the program 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 determine the ancestry of known fish. Individuals with probable Cold Spring ancestry ($q > 0.125$) were removed from the dataset, and we conducted subsequent analyses with only Beaver Creek and Garvin Brook descendants.

The software NewHybrids (Anderson & Thompson 2002) was used to classify individual fish to crosstypes assuming no more than second-generation descendants of founders were present. This assumption is reasonable as reintroduced populations were sampled within 3 years of initial spawning and sculpins typically mature at age 2 (Petrosky & 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 the Jeffreys prior option for allele frequencies and mixing proportions. Runs were repeated using different seeds to verify that consistent solutions were found. We classified individuals into a pure strain or hybrid category if their probability of membership was ≥ 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 and repeated the NewHybrids analysis.

Statistical analysis

Expected quantities of each strain within the reintroduced populations were estimated for the autumn 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, equal reproduction and random mating among lineages (see Epifanio & Philipp 2000). The first and second generations comprised 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 (Huff

2010) 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 & Philipp 2000). Likewise, expected quantities of F_2 , $F_1 \times B$ and $F_1 \times G$ would be underestimated by our chosen ratio. Statistical assessment of the divergence between expected and observed values for each category was made using the median test (Zar 1999), a version of the Kruskal–Wallis ANOVA that frames the computation in terms of a contingency table. Pickwick and Little Pickwick populations were stocked once in autumn 2005, so there were only Beaver, Garvin and F_1 crosstypes present in 2007 and spring 2008. Our estimate, therefore, included only first-generation crosstypes (Beaver, Garvin, and F_1) for the two Pickwick sites based on the timing of the sampling relative to when these sites were stocked.

We used a fixed effects model (Weisberg 1993; Weisberg *et al.* 2010) to analyse 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 killed fish from only two of the populations. Otoliths were collected from these populations in autumn of 2008 and 2009 to ensure that there would be enough F_2 , $F_1 \times B$ and $F_1 \times G$ crosstypes to develop a growth model. We modelled the growth increments for each fish as a function of four fixed effects: age (levels = 0, 1, and 2), stream (levels = Pickwick and Little Pickwick), crosstype (levels = Garvin, Beaver, F_1 , F_2 , $F_1 \times B$ and $F_1 \times G$) and sex (levels = Male, Female and Unknown). 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 & 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 & Anderson 1998). 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 , $F_1 \times B$ and $F_1 \times G$) generation crosstypes that could be because of differential survival or recent stocking, we categorized all fish into two age categories that included young-of-the-year and over-yearlings. All young-of-the-year fish were designated by length (<41 mm, $n = 91$), 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 S1).

In addition to crosstype comparisons for weight and length, 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 & Allendorf 2001) to corroborate potential differences in growth rate and body size. 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 $K_n = W/W_{\text{pred}}$, 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 1998) developed for each reintroduced population.

Results

Genetic markers and error simulations

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 , $F_1 \times B$ and $F_1 \times G$, whereas 8–12% of F_2 , $F_1 \times B$ and $F_1 \times G$ crosstypes were erroneously assigned to Beaver, Garvin and F_1 crosstypes. Simulations using all 12 loci estimated error rates of 0–0.3% for Beaver, Garvin and F_1 crosstypes. For F_2 , $F_1 \times B$ and $F_1 \times G$ crosstypes, 2–5% erroneously assigned to Beaver, Garvin or F_1 , and 1–3% of backcrosses erroneously assigned to F_2 , while 12% of F_2 erroneously assigned to backcrosses.

Persistence

Assignment of crosstypes for 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 , $F_1 \times B$ and $F_1 \times G$ crosstypes made up the remaining 16% (188 individuals) of the samples. Beaver individuals occurred in a significantly greater frequency than expected (Fig. 2) based on null conditions (median test; median = 31.0, $\chi^2 = 5.6$, $P = 0.02$). All other crosstypes occurred at lower frequencies than expected, but only F_2 (median test; median = 14.4, $\chi^2 = 7.1$, $P = 0.01$) and $F_1 \times G$ (median test; median = 5.0, $\chi^2 = 7.1$, $P = 0.01$) were significantly lower.

Comparison of growth rate, weight, length and body condition

We divided the dataset into young-of-the-year and over-yearlings to investigate fitness surrogate patterns among crosstypes at different life stages. There were 531 young-of-the-year and 699 over-yearling sculpins in the total 1230 fish sample. Figure 3 graphically compares means and 95% confidence intervals and Table 1 summarizes P -values from pairwise comparisons among crosstypes (Tukey's honestly significant difference test) for all fitness surrogate models including growth rate, weight, length and body condition for both young-of-the-year and over-yearlings. With the exception of growth rate (described in the Methods), we performed a main effects analysis of variance that included crosstype and stream as factors. The pattern of differences in crosstypes among young-of-the-year fitness surrogates was generally similar. Beaver surrogates were greater than the other crosstypes, whereas second-generation crosstypes (F_2 , $F_1 \times B$ and $F_1 \times G$) were over-

all lower and tended to be highly variable. Over-yearling results were comparatively consistent; however, in contrast to young-of-the-year results, Garvin fitness surrogates were superior to all other crosstypes. We did not analyse each reintroduction site individually because of the sparse distribution of some crosstypes among various sites and the concern that an uneven lack of sensitivity among the sites could produce inconclusive and potentially misleading results. A supplemental table (Appendix S2) includes mean weights, lengths and body condition values for each crosstype by reintroduction site.

The most parsimonious otolith incremental growth model for over-yearlings (Fig. 3) had the following fixed effects (ANOVA, $P < 0.001$ for all variables): age ($F = 23.7$, 3 d.f.), stream ($F = 148.5$, 1 d.f.), sex ($F = 32.3$, 2 d.f.) and crosstype ($F = 7.2$, 5 d.f.). Over-yearling otolith growth in F_2 , $F_1 \times B$ and $F_1 \times G$ crosstypes was lower than in Beaver, Garvin or F_1 . Garvin over-yearlings grew significantly faster than Beaver, F_2 , $F_1 \times B$ and $F_1 \times G$ crosstypes, and Beaver growth was only significantly faster in a pairwise comparison with $F_1 \times B$, although Beaver also grew notably faster than F_2 (Table 1). F_1 over-yearlings grew significantly faster than F_2 and $F_1 \times B$ crosstypes. Young-of-the-year otolith growth (Fig. 3) differed significantly (ANOVA, $P < 0.05$ for all variables) among age at capture ($F = 3.9$, 1 d.f.), stream ($F = 40.1$, 2 d.f.), crosstype ($F = 11.5$, 5 d.f.) and sex ($F = 7.5$, 2 d.f.). Otolith growth in F_2 , $F_1 \times B$ and $F_1 \times G$ crosstypes was significantly lower than in Beaver, Garvin or F_1 (Table 1). However, in contrast to over-yearlings, young-of-the-year growth rates were fastest for Beaver rather than Garvin.

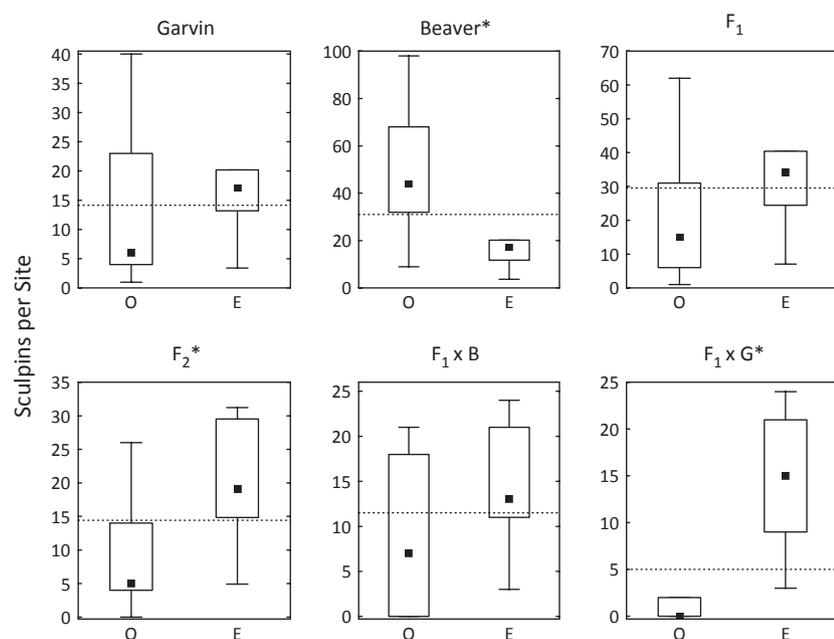


Fig. 2 Median (closed square), 1st and 3rd quartile (rectangle), and range (whisker) for observed versus expected numbers of sculpins in the reintroduced populations for each crosstype. Significant differences between observed and expected quantities ($P > 0.05$, median test) are indicated with an asterisk. The dashed line in each panel represents the overall median.

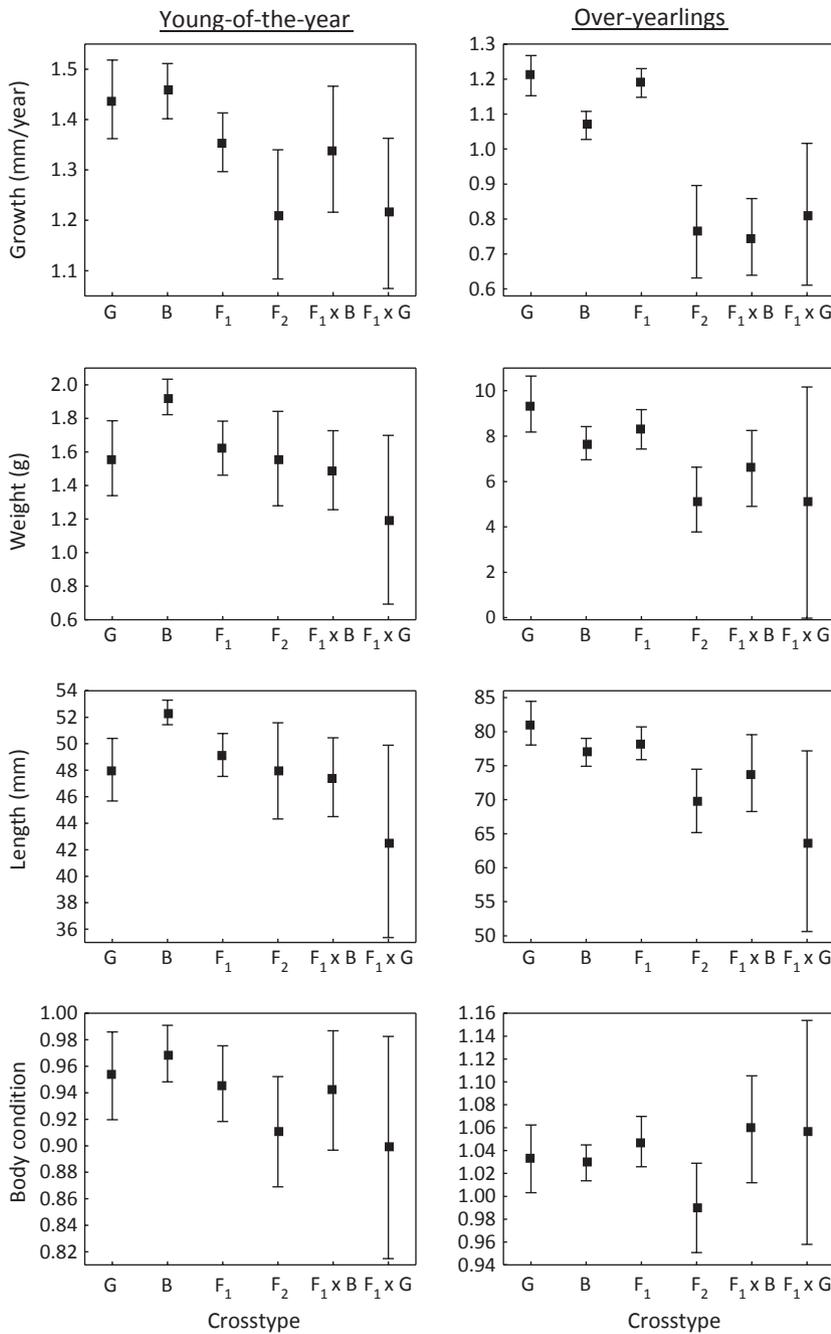


Fig. 3 Mean (closed square) and 95% confidence intervals (whisker) for otolith growth, weight, length and body condition by crosstype for young-of-the-year (left panels) and over-yearlings (right panels). Significant pairwise comparisons are given in Table 1. Otolith growth (top panels) was modelled with a fixed effects least-squares ANOVA using data collected at two reintroduction sites: Pickwick and Little Pickwick Creek ($n = 418$). All other values were calculated across all nine reintroduction sites ($n = 1230$).

For over-yearlings, mean weights (Fig. 3) differed by stream (ANOVA; $F = 25.57$, 8 d.f., $P \ll 0.001$) and crosstype (ANOVA; $F = 11.36$, 5 d.f., $P \ll 0.001$). Garvin was significantly heavier than Beaver and F₂ crosstypes. F₁ fish were intermediate to Garvin and Beaver, but were significantly heavier than F₂ (Table 1). Mean weights in F₂, F₁ × B and F₁ × G crosstypes were lower than Beaver, Garvin and F₁ crosstypes. Young-of-the-year mean weights (Fig. 3) differed significantly among streams (ANOVA; $F = 31.60$, 5 d.f., $P \ll 0.001$) and crosstype (ANOVA; $F = 3.86$, 5 d.f., $P = 0.002$). Beaver was

significantly heavier than all other crosstypes (Table 1).

For over-yearlings, mean lengths (Fig. 3) differed by stream (ANOVA; $F = 31.76$, 8 d.f., $P \ll 0.001$) and crosstype (ANOVA; $F = 13.86$, 5 d.f., $P \ll 0.001$). Garvin were longer than Beaver, F₂ and F₁ × G crosstypes. F₁ fish were intermediate in length to Garvin and Beaver, but were significantly longer than F₂ and F₁ × G. Mean lengths in F₂, F₁ × B and F₁ × G crosstypes were lower than Beaver, Garvin and F₁ crosstypes (Table 1). Young-of-the-year mean lengths (Fig. 3)

Table 1 *P*-Values from Tukey's honestly significant difference test among crosstypes for growth rate, weight, length and body condition. Young-of-the-year values are in the bottom diagonal, and over-yearling values are in the top diagonal. Bold *P*-values are ≤ 0.05 . ANOVA univariate tests of significance for each full model are given in the text

Growth rate	G	B	F ₁	F ₂	F ₁ × B	F ₁ × G
G	–	0.01	0.23	<0.001	<0.001	0.05
B	1.00	–	0.62	0.08	0.01	0.59
F ₁	1.00	1.00	–	0.02	<0.001	0.30
F ₂	<0.001	<0.001	<0.001	–	1.00	1.00
F ₁ × B	<0.001	<0.001	<0.001	0.89	–	0.99
F ₁ × G	<0.001	<0.001	<0.001	1.00	0.94	–
Weight	G	B	F ₁	F ₂	F ₁ × B	F ₁ × G
G	–	0.04	0.48	<0.001	0.12	0.17
B	<0.001	–	0.84	0.10	0.90	0.69
F ₁	0.99	<0.001	–	0.02	0.61	0.47
F ₂	1.00	0.02	1.00	–	0.92	1.00
F ₁ × B	0.99	<0.001	0.89	1.00	–	0.98
F ₁ × G	0.43	<0.001	0.22	0.49	0.70	–
Length	G	B	F ₁	F ₂	F ₁ × B	F ₁ × G
G	–	0.07	0.50	<0.001	0.15	0.01
B	<0.001	–	0.92	0.06	0.90	0.07
F ₁	0.92	<0.001	–	0.02	0.66	0.04
F ₂	1.00	<0.001	0.94	–	0.87	0.88
F ₁ × B	1.00	<0.001	0.75	1.00	–	0.45
F ₁ × G	0.08	<0.001	<0.001	0.12	0.18	–
Body condition	G	B	F ₁	F ₂	F ₁ × B	F ₁ × G
G	–	1.00	0.85	0.34	0.88	0.99
B	0.97	–	0.49	0.36	0.77	0.98
F ₁	1.00	0.79	–	0.05	1.00	1.00
F ₂	0.71	0.17	0.77	–	0.14	0.59
F ₁ × B	1.00	0.85	1.00	0.92	–	1.00
F ₁ × G	0.80	0.47	0.85	1.00	0.92	–

differed significantly among streams (ANOVA; $F = 34.79$, 5 d.f., $P \ll 0.001$) and crosstype (ANOVA; $F = 5.62$, 5 d.f., $P \ll 0.001$). Beaver young-of-the-year were significantly longer than all other crosstypes, and F₁ was longer than F₁ × G (Table 1).

Mean body condition generally showed similar trends among crosstypes to the other fitness surrogates, but few pairwise comparisons were significant. For over-yearlings, mean body condition (Fig. 3) differed by stream (ANOVA; $F = 58.26$, 8 d.f., $P \ll 0.001$) and crosstype (ANOVA; $F = 2.05$, 5 d.f., $P = 0.03$), but the only significant pairwise difference between crosstypes was that F₁ > F₂ (Table 1). Young-of-the-year mean body condition (Fig. 3) differed significantly among streams (ANOVA; $F = 5.84$, 5 d.f., $P \ll 0.001$), but not by crosstype (ANOVA; $F = 1.31$, 5 d.f., $P = 0.26$).

Discussion

A fundamental question in the fields of evolution and conservation biology is the extent to which small,

subdivided populations with restricted gene flow are influenced by selective pressures and genetic drift that lead to local adaptation, and ultimately speciation (Hanski & Gaggiotti 2004). Genetic divergence in our source populations occurs on a very small spatial scale, as has been detected elsewhere for other species (Edmands 1999; Waser *et al.* 2000). Species similar to the slimy sculpin, with restricted dispersal abilities (Schmetterling & 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. The slimy sculpin is an especially appropriate organism for studies of local adaptation, the consequences of outbreeding, and early stages of speciation because it has a very broad distribution and lives in a wide variety of habitats (Sweigart 2009). The reduced success of hybrid offspring observed in this study, potentially arising from variation in traits such as young-of-the-year body size or growth rate could result in barriers to gene flow among distinct populations.

The source populations in our study are geographically close (<30 km apart), with similar habitats. Consequently, our null hypothesis was that reintroduced Beaver and Garvin strains would occur in equal proportions, given equal fitness and random mating among lineages. However, high F_{ST} values indicate little gene flow between populations (Holsinger & Weir 2009), and this is likely a long-standing condition as the populations are in separate watersheds draining into the Mississippi River, which is unsuitable habitat for slimy sculpins. 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). We observed that Beaver was the only crosstype that exceeded expectations for persistence; it was more numerous than other crosstypes at eight of nine sites and made up 43% of the individuals in the study. All other crosstypes were less abundant than expected, significantly so for F_2 and $F_1 \times G$ crosstypes. These deviations from null expectations suggest both fitness differences, and possibly nonrandom mating, among crosstypes at the reintroduction sites (Huff *et al.* 2010). Differential initial fitness at the reintroduction sites between Beaver and Garvin could plausibly explain low proportions of hybrid crosstypes (F_1 , F_2 , $F_1 \times B$ and $F_1 \times G$). For example, if Beaver sculpins had higher survival over 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 given differential initial reproductive success. Therefore, although depressed hybrid fitness could 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, greater Beaver persistence is consistent with the hypothesis that it had higher fitness than Garvin in reintroduced populations.

Our results for over-yearlings, that Garvin are larger and grow faster, seem inconsistent with the pattern of greater Beaver persistence in the reintroduced populations, as growth rate and body size are commonly assumed to have a strong relationship to fitness in fish (Petty & Grossman 2004; McClelland & Naish 2007). Greater size or growth rate for Garvin and F_1 over-yearlings could result from reduced survival of smaller or slower growing young-of-the-year individuals that leave behind mostly faster growing over-yearlings. 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 was not strong enough, then introgression would continue until a hybrid swarm

developed and the pure strain genotype was lost (Epifanio & Philipp 2000; Allendorf *et al.* 2001). Our work indicated 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 greater young-of-the-year size, and perhaps a faster growth rate, in Beaver vs. Garvin and F_1 may confer a selective advantage on Beaver at most reintroduction sites. However, growth rate differences during the first year of life may not be the only explanation for larger body size in Beaver. For example, if Beaver \times Beaver mating occurred earlier or in better locations, then offspring may have more time or better conditions under which to grow. Additional studies regarding the timing and location of spawning and growth data could support different interpretations of our results.

Our analysis of fitness surrogates suggested potential mechanisms that may explain why Beaver was more persistent than Garvin in the reintroduced populations. The early survival of fishes is often strongly affected by size (Miller *et al.* 1988; Houde 1989). A size advantage in Beaver young-of-the-year is a consistent explanation for persistence differences between these two strains in the reintroduced populations. An ecological condition that may promote differential size and growth in young-of-the-year sculpins in our study sites is groundwater-mediated growing season length (Huff 2010). Temperature-dependent divergent life history strategies have been documented for a closely related species, *Cottus gobio* in which growth rates of young vs. older sculpins responded differently depending on the thermal regime of the local population (Abdoli *et al.* 2007). Sculpins in Garvin Brook may experience a longer growing season owing to warmer winter temperatures because streams such as Garvin Brook, with greater groundwater input (Huff 2010), will tend to remain relatively warmer in the winter and tend to cool more slowly in the autumn. Beaver Creek was more similar to most reintroduction streams in that there was comparatively less groundwater input than in Garvin; therefore, these sculpins experienced a shorter growth season, a colder winter and a warmer summer. Because of differences in temperature regimes between source streams, attainment of a larger size by the end of summer may have been more important for sculpin survival in Beaver Creek than in Garvin Brook. Size selective mortality in the first winter may enhance the fitness of faster growing sculpins that have shorter growing seasons. This has been observed among many species and has been shown to be a factor in year-class strength in fish (Shuter *et al.* 1980; Henderson *et al.* 1988; Hurst 2007), including a sculpin species (Rashleigh & Grossman 2005). Slower young-of-the-year growth rate for fish in

Garvin Brook would be consistent with a hypothesis in which slower growth rate phenotypes provide an advantage (Conover 1990) perhaps because of inferior swimming ability in larger fish (Billerbeck *et al.* 2000), increased susceptibility to predation (Lankford *et al.* 2001), or the costs of growth combined with the consumption of larger meals at an early life stage (Munch & Conover 2003). Variations in winter temperatures between the source streams may cause divergent selective pressures on metabolic rates and physiological functions, swimming performance and growth (Fry 1970; Wootton 1998). Subsequently, variations and relative stability in temperature and other environmental features among the reintroduction sites may have been, overall, more favourable for sculpins adapted to conditions in Beaver Creek vs. conditions in Garvin Brook. However, environmental variations at some sites may favour, to a greater or lesser degree, sculpins adapted to conditions that are more similar to those in Garvin Brook. This could account for the high degree of variation in fitness surrogate values among streams between Garvin and Beaver sculpins.

Fitness surrogate data are consistent with the hypothesis that outbreeding depression contributed to the deficit of hybrid crosstypes. Our nine reintroduction sites represent multiple common garden experiments in which there is a significant genetically based fitness surrogate depression in $F_1 \times B$, $F_1 \times G$ and especially F_2 crosstypes. This pattern among crosstypes is consistent with disruption of advantageous co-adapted gene complexes in the second generation of hybrid crosstypes. In comparisons of growth, size or body condition, the F_2 crosstype was significantly lower than parental types or F_1 . There was a greater degree of variability within the $F_1 \times B$ and $F_1 \times G$ crosstypes, but these crosstypes exhibited a similar pattern in fitness surrogates to F_2 . Additional variability could be due in part to the potential for backcrosses to regress toward the parental condition through restored epistatic interactions (Ellison & Burton 2008). This regression was conceivable for $F_1 \times B$, in which we observed a slight to moderate increase in growth, and body condition relative to F_2 . Fewer $F_1 \times G$ fish may explain, at least partially, their additional variability in body condition.

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. The F_2 crosstype may have been underestimated because it had the highest error rates, but most erroneously assigned to backcrosses, which also had

fewer observed than expected (significantly for $F_1 \times G$). Furthermore, classification error would have obscured differences in fitness surrogates among crosstypes resulting in underestimation of true differences.

Field transplant studies such as this provide advantages such as establishing the importance and magnitude of ecological barriers to gene flow (Noor & Feder 2006) and may be of greater relevance to specific conservation scenarios. However, our study has limitations in that we used previously reintroduced populations that were not originally intended for an outbreeding study. As such were not able to choose the reintroduction locations or proportions of individuals stocked. Therefore, we urge caution when interpreting the broader implications of our results, especially considering that potential outbreeding consequences may be difficult to generalize given the variety of mechanisms that may cause them. Laboratory research with fruit flies suggests that in the long term, outbreeding depression is a temporary phenomenon and may disappear if the population can survive the initial reduced fitness (Annett & Templeton 1978; Templeton 1986). Moreover, populations that have recently fragmented because of anthropogenic causes are less likely to experience outbreeding depression relative to populations with little historical gene flow. A worldwide decline of some animals has led conservationists to advocate increasing genetic diversity in declining, isolated populations through translocations of genetically disparate individuals (Wolf *et al.* 1996; Fischer & Lindenmayer 2000). A perception that hybrid vigour is a common phenomenon (Rhymer & 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 vigour, 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. For populations that are already small, especially if they reproduce slowly, a short-term fitness reduction may be an unnecessary risk and could produce negative consequences.

Acknowledgements

We would like to thank Ray Newman, Eric Merten, Karen Oberhauser, Andrew Simons and three anonymous reviewers for helpful comments on the manuscript. We also thank Jason Breeggemann for assistance with otolith analysis, and Lorissa Fujishin and Keith Barker for microsatellite marker develop-

ment. Thanks also to Vaughn Snook and Minnesota Department of Natural Resources personnel for providing technical support and resources for the project. Funding was provided by a fellowship from Austen Cargill II; a doctoral dissertation fellowship by the Graduate School of the University of Minnesota; and the Minnesota Department of Natural Resources, U.S. Geological Survey Cooperative Research Units Program. The Minnesota Cooperative Fish and Wildlife Research Unit is jointly sponsored by the U.S. Geological Survey, University of Minnesota, Minnesota Department of Natural Resources, and Wildlife Management Institute. Any use of trade, product or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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Data accessibility

Individual microsatellite, ancestry estimates and weight-length data used in this study are available from the Dryad data repository, DOI: 10.5061/dryad.pf318.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 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.

Appendix S2 Number (N) and mean weight (W), length (L), and body condition (BC) by crosstype for each reintroduction site by young-of-the-year (YoY) and over-yearlings (O-Y).

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