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The Genetic and Demographic Outcomes of Mixed-Source Reintroductions of Westslope Cutthroat Trout in Montana

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By

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ABSTRACT

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Chairperson: Andrew Whiteley

Conservation reintroductions are now a critical tool in conservation biology to restore native species populations. Many remaining candidate source populations are small, isolated, and have limited genetic variation. As a result, conservation managers commonly reintroduce multiple source populations together into a single habitat, referred to as mixed-source reintroductions. Theoretically, mixing populations could increase and preserve remaining genetic variation, reduce negative genetic and demographic effects from decades of inbreeding and small population sizes, and increase the range-wide distribution and abundant of threatened species. However, when source populations have experienced extended periods of geographic isolation and developed local adaptations to specific habitats, mixing source populations may have negative consequences. In the face of anthropogenic effects pushing many populations towards extinction, understanding mixed-source reintroduction outcomes is imperative, especially as they are continually used as a species conservation tool.

Using several westslope cutthroat trout (*Oncorhynchus clarkii lewisii*) reintroductions in southwest Montana, we first described how genetic variation and mixing divergent source populations influenced population- and individual success. Secondly, we evaluated how different reintroduction methods and mixing divergent source populations influenced reintroduction success and population expansion. In several cases, we found that source populations with higher genetic variation were more successful relative to source populations with lower genetic variation. Individual fitness was also positively related to higher individual genetic variation. Generally, hybrids were more abundant than expected when two source populations were translocated together into the same reintroduction location. These results were consistent across sites and years within reintroduction streams. Population expansion was variable, but likely driven by the number of translocated individuals, where populations re-founded with more translocated individuals re-filled available habitat more quickly. Overall, our results described the interacting effects of various genetic and demographic processes involved in multiple mixed-source reintroductions, and highlighted factors important for reintroduction success.

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CHAPTER 1
INTRODUCTION

Anthropogenic effects continually fragment and isolate populations, which has contributed to the unprecedented worldwide decline in biodiversity (IPBES 2019; Johnson et al. 2017). Many species are now relegated to small, isolated habitats and commonly face stressors of changing environmental conditions, competition with nonnative species, and limited genetic variation (Allendorf et al. 2022; Cucherousset, Olden 2011; Frankham et al. 2017; Johnson et al. 2017). This is particularly true for many freshwater organisms, which are considered among the most imperiled species on the planet (Brauer, Beheregaray 2020; Su et al. 2021; Tickner et al. 2020). To mitigate these effects and conserve remaining populations, many conservation biologists must often eliminate nonnative species and reintroduce native species into protected, historically occupied habitats (e.g., Arnold et al. 2017; Clancey et al. 2019). Consequently, biologists are often faced with determining how many and which populations should be used for sourcing reintroductions (Jamieson, Lacy 2012), a decision requiring careful ecological and genetic consideration. However, reintroduction outcomes associated with such decisions are rarely monitored and generally not well understood, leaving biologists with little empirical information for decision-making.

When implementing reintroductions conservation managers often aim to release (i.e., translocate) individuals that will survive and reproduce, and for translocated individuals or populations (i.e., sources) to possess the necessary evolutionary potential for long-term population persistence (Biebach et al. 2019; Jamieson, Lacy 2012). Thus, when selecting single or multiple source populations, managers must often consider: (1) the genetic integrity of candidate source populations (i.e., differences in genetic diversity), (2) the environmental conditions inhabited by each prospective source population and in the planned site for reintroduction (i.e.,

accounting for potential local adaptations), and (3) the genetic divergence and extent of isolation between source populations (Biebach et al. 2019). These considerations and subsequent reintroduction decisions may, in theory, influence reintroduction outcomes.

In instances where remaining source populations are small and fragmented, with limited genetic variation (common in conservation populations at risk of extinction), theoretical evidence suggests that mixing populations in reintroductions could increase genetic variation in the subsequent population (Allendorf et al. 2022; Frankham et al. 2017). Specifically, mixing populations with low genetic variation may improve population and individual fitness by alleviating inbreeding effects and increasing genetic variation (Whiteley et al. 2015), which could in-turn provide populations with the necessary genetic material to adapt to changing environmental conditions (Frankham et al. 2017; Ralls et al. 2018; Razgour et al. 2019; Reed, Frankham 2003). Conversely, population genetic theory also alludes to potential negative genetic consequences arising when divergent populations are mixed (i.e., loss of genetic variation and reduced fitness) (Allendorf et al. 2022; Edmands 2007; Tallmon, Luikart & Waples 2004). For example, mixing populations might be problematic when the source populations are highly genetically divergent and have developed local adaptations due to extended periods of isolation (Allendorf et al. 2022; Frankham et al. 2017; Stockwell, Hendry & Kinnison 2003).

Conservation biologists are further challenged with deciding how many individuals should be sourced for reintroductions. Given remnant isolated populations are often small, with limited genetic variation, managers are commonly restricted by the number of individuals that can be removed from a source population due to risks of further genetically or demographically damaging source populations (Biebach et al. 2019; Groombridge et al. 2012). Subsequently, translocating too few source individuals into reintroduction sites could negatively influence

newly refounded reintroduction populations by increasing the likelihood of inbreeding and reducing population growth and reproduction (Deredec, Courchamp 2007). Mixed-source reintroductions can help managers mitigate these issues by reintroducing more individuals, sourced across multiple populations, while also lessening the likelihood of negatively impacting source populations.

Mixed-source reintroductions are an increasingly common practice for species conservation. Given the various potential theoretical costs and benefits of mixed-source reintroductions, it is paramount that we empirically describe and understand reintroduction outcomes. The recent proliferation of westslope cutthroat trout (*Oncorhynchus clarkii lewisii*) reintroductions in Montana serve as an ideal candidate to examine the genetic and demographic outcomes of mixed-source reintroductions. Westslope cutthroat trout (WCT, hereafter), a salmonid native to the western North America, are of significant conservation concern throughout their historical range (Shepard, May & Urie 2005). Habitat degradation, introduction of nonnative fishes, and hybridization with nonnative fish are all responsible for the decline of WCT (Bell et al. 2021; Shepard, May & Urie 2005). In the Missouri River basin in Montana, USA (i.e., the most eastern portion of WCT range), most WCT populations have been relegated to small, isolated headwater streams, most consisting of less than 10km of stream habitat (Shepard, May & Urie 2005). Their isolation is concerning considering the potential negative genetic and demographic effects that occur in small populations (Allendorf et al. 2022; Frankham et al. 2017). Moreover, many remnant populations have extremely low genetic variation, suggesting they have low adaptive potential and may be suffering from inbreeding depression (Kovach et al. 2021).

To prevent further decline in the abundance and distribution of WCT in Montana and preserve remaining population genetic variation across the landscape, biologists have used various mixed-source reintroduction methods. For instance, recent efforts have involved translocating between 60 and 330 individuals from 2 or 3 source populations into reintroduction streams. Commonly, source populations are translocated across years and sites, but this varies among reintroductions. Though WCT populations have not experienced extended periods of isolation (i.e., <200 years), translocated source populations are often extremely genetically divergent and have widely different levels of genetic variation, which often raises concerns about mixing populations. Thus, we need to better understand the genetic and demographic response following the application of different reintroduction methods. More broadly, previous WCT reintroduction efforts can be used to better understand the risk of harmful genetic effects potentially associated with various reintroduction methods and describe how to best preserve genetic variation and adaptive potential.

The goals of Chapter 2 were to describe source population mixing and the genetic representation of source individuals following WCT reintroductions and quantify fitness differences among source individuals with various genetic ancestry. In Chapter 2 we addressed these study goals using genetic data from four reintroductions in southwest Montana including the North and South Forks of Greenhorn (NFG and SFG), Ruby, and Peet Creeks. The goal of Chapter 3 was to describe relationships between different WCT reintroduction methods, source population mixing across sites and years, changes in genetic variation, and reintroduction population abundance expansion. For Chapter 3, we used genetic and demographic data collected from six reintroductions in southwest Montana including NFG, SFG, Meadow Fork of Greenhorn (MFG), Peet, Ruby, and Schultz Creeks.

In Chapter 2 we investigated how genetic variation and mixing divergent populations influenced population- and individual success among four WCT reintroductions (i.e., NFG, SFG, Peet, and Ruby Creeks). Specifically, we evaluated these processes at population- and individual-scales. In NFG, we found that offspring from the most genetically diverse population were three times more abundant than expected, while offspring from the least genetically diverse population were five times less abundant than expected. Using parentage assignments, we then found that family size was higher for populations with higher genetic variation, and individual probability of survival and reproduction and reproductive success was elevated for individuals with higher genetic variation. These analyses also described increased success in one of three potential hybrid crosses. In SFG, offspring from the least genetically variable population were underrepresented, while offspring from hybrids were more abundant than expected. Results were similar in Peet and Ruby Creeks, where we found that hybrids were nearly two times more abundant than expected in both reintroductions. However, it was difficult to interpret the role of genetic variation in Peet and Ruby Creek due to reintroduction methods and low sample sizes.

In Chapter 3 we evaluated how different reintroduction methods and mixing divergent source populations influenced reintroduction success and population expansion among six WCT reintroductions (i.e., NFG, SFG, MFG, Ruby, Peet, and Schultz Creek). We found that hybrids were more abundant than expected among reintroduction streams when two source populations were translocated together into the same reintroduction location. Generally, hybrids had increased success and genetic variation. On several occasions, higher source population genetic variation was positively related to reintroduction success, however, source populations with relatively low genetic variation still contributed to the first-generation in all reintroduction sites. Changes in reintroduction population genetic variation following one generation of reproduction

was variable relative to source population genetic variation and among reintroductions. Though hybrid offspring commonly had higher genetic variation compared to source populations. Population expansion following translocation was variable, likely due to the number of translocated individuals, where reintroduction populations involving more translocated individuals reached larger population sizes more quickly. Results also suggested that translocating source populations together into the same location may have promoted hybridization, which might have positively influenced population expansion.

The results presented in this thesis demonstrate that mixed-source reintroductions can serve as a useful conservation method for preserving among population genetic diversity, while also increasing genetic variation through source population hybridization. Particularly, these results show the efficacy of mixed-source reintroductions by direct translocation of source individuals into reintroduction streams, and that such practices can serve as a method for preserving among population genetic diversity, while also increasing genetic variation through source population hybridization. These results also emphasize that source population genetic variation and hybridization may be important to reintroduction success. Ultimately, this thesis describes the interacting effects of the various genetic and demographic processes involved in mixed-source reintroductions, and highlights factors important to reintroduction success.

CHAPTER 2
GENETIC VARIATION AND HYBRIDIZATION DETERMINE THE OUTCOMES OF CONSERVATION REINTRODUCTIONS

Abstract

The preservation of genetic variation is fundamental in biodiversity conservation, but empirical evidence directly linking genetic variation to individual and population success is extremely rare. One conservation strategy to improve genetic variation is to reintroduce individuals from multiple small, genetically depauperate populations with widely varying genetic variation into a single vacant habitat (i.e., mixed-source reintroduction). Population genetic theory predicts that individuals with higher genetic variation and hybrids among populations should have increased success. We tested these hypotheses by analyzing individual and population-scale data for translocated westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) and their offspring in four reintroductions. We found clear evidence that heterozygosity predicted individual reproductive- and population success. Among reintroductions, we also observed elevated abundances of hybrid offspring. Our results suggest a strong, positive relationship between genetic variation, hybridization, and reintroduction success.

Introduction

Amidst unprecedented declines in biodiversity, many imperiled species remain in small, isolated populations, fragmented across a once connected landscape (1, 2). Consequently, many populations are vulnerable to reduced fitness and future adaptability due to the loss of genetic variation through genetic drift and inbreeding depression (3, 4). Although genetic variation is theoretically fundamental to population persistence (5-7), its role in conservation is highly debated (8, 9), in part due to a lack of empirical data connecting genetic variation to conservation success. Thus, understanding how genetic variation influences conservation outcomes is crucial, especially as more species are pushed towards extinction.

Theoretically, individual and population fitness should be linked to genetic variation, where higher genetic variation equates to increased fitness, particularly when species are relegated to small, isolated populations (8). There is also growing evidence that hybridization between genetically depauperate populations can improve population and individual fitness due to the alleviation of inbreeding depression (heterosis) (10). Conversely, admixture between divergent populations may result in a reduction in fitness of hybrid (i.e., outcrossed) offspring compared to parental types, known as outbreeding depression (11, 12). These conflicting theories have left conservation biologists with an imperative question: How does genetic variation and mixing divergent populations influence conservation outcomes, and more importantly, success? The proliferation of mixed-source reintroductions, where individuals with widely varying genetic variation are moved into a single habitat, provides an excellent opportunity to address this key question in conservation biology by examining whether genetic variation and hybridization predict individual- and population success (i.e., fitness).

Freshwater organisms are among the most imperiled on the planet and are frequently threatened by population fragmentation and isolation (13-15). The westslope cutthroat trout (*Oncorhynchus clarkii lewisi*), a freshwater salmonid native to western North America, has been largely extirpated from its historical range due to habitat degradation and the introduction of nonnative species (16, 17). As a result, most remaining westslope cutthroat trout (WCT) populations are small and isolated with extremely low genetic variation (18). To reduce the negative genetic effects of isolation, preserve remaining genetic variation, and increase range-wide abundance and distribution, several conservation efforts have, eliminated nonnative species from historically occupied WCT streams and reintroduced multiple source populations into newly vacated habitat (19, 20). However, the genetic outcomes from such efforts are rarely monitored and generally not well understood. Here, we used genetic data from three mixed-source WCT reintroductions in the upper Missouri River basin Montana, USA to describe the genetic outcomes of mixed-source reintroductions at individual and population scales (Fig. 2.1, A).

Materials and Methods

We focus principally on Greenhorn Creek, where WCT individuals were translocated from three and two source populations into the North and South Forks of Greenhorn Creek (NFG and SFG), respectively (Fig. 2.1 and Table. S2. 1) (21). We quantified reproductive success and population contribution in NFG and SFG by sampling first-generation (F_1) offspring (22). We then used genetic parentage analysis (22) to assign NFG (n = 640) and SFG (n = 245) offspring to candidate parents and one of nine population crosses (crosstypes): parental (within source population matings; BN, BC, PR, MD, CW) and F_1 hybrids (among source population matings; BNxPR, BNxBC, PRxBC, CWxMD) (Table. S2. 3) (21). Simulation results revealed high

accuracy (>95%) in assigning offspring to NFG parents, however, SFG offspring could only be accurately assigned to crosstypes (Table. S2. 2) (21). Therefore, we evaluated individual success in NFG and population success in NFG and SFG.

Results

We compared observed to expected crosstype proportions in NFG and SFG to test the prediction that offspring would be more abundant from hybrid crosstypes or parental crosstypes with higher genetic variation (21). We then estimated expected heterozygosity (H_e) for all source populations (Fig. 2.1, B) to qualitatively describe population success as a function of genetic variation (21). Overall, observed crosstype proportions were considerably different from random expectation in NFG ($\chi^2 = 358.42$, df = 4, $p < 0.0001$) and SFG ($\chi^2 = 25.26$, df = 1, $p < 0.0001$) (Table. S2. 4). Specifically, offspring from BN, the most genetically diverse population (H_e = 0.368), were three times more abundant than expected, while offspring from BC (H_e = 0.062), the least genetically diverse population, were five times less abundant than expected (Fig. 2. 1, C and Table. S2. 4). We also found that offspring from hybrid crosstypes with BC ancestry were underrepresented, while offspring from PRxBN hybrids were more abundant than expected (Fig. 2.1, C and Table. S2. 4). Results were similar in SFG, where offspring from the least genetically variable parental crosstype (CW, H_e = 0.013) were less abundant than expected, and offspring from the hybrid crosstype (CWxMD) were more abundant than expected (Fig. 2.1, D and Table S2. 4).

To confirm population-level results and examine drivers of individual reintroduction success, we used parentage assignments in NFG to describe fitness differences among crosstypes and source individuals using both family size and reproductive success. We first found that family size was elevated for parental crosstypes with higher genetic variation BN (P = 0.0014)

and PR (H_e = 0.194; P = 0.0072) compared to the parental crosstype with the lowest genetic variation (BC) (Fig. 2.2, A and Table. S2. 5). We then observed larger family size for hybrid crosstypes involving the genetically variable source populations (BNxPR) and reduced family size when a hybrid crosstype included a parent with ancestry from the least genetically variable population (BNxBC and PRxBC) (Fig. 2.2, A). We also found that individuals with higher genetic variation among populations were more likely to survive and reproduce (P = 0.064) (Fig. 2.2, B and Table. S2. 6). Further, among populations and reproducing individuals, reproductive success was greater for individuals with higher genetic variation (P = 0.087) (Fig. 2.2, C and Table. S2. 6). We accounted for other biological factors potentially influencing reintroduction success by examining family size and reproductive success in NFG and found parent body length influenced family size (Dam, P = 0.025; Sire, P = 0.068), the probability of survival and reproduction (P = 0.004), and reproductive success (P = 0.0003) (Fig. S2. 1 and Tables. S2. 5 and S2. 6). The relationship between body length and fitness is well documented in salmonid species, as maternal and paternal length can often influence fecundity and reproductive success, respectively (23, 24). Though parent length positively influenced family size and reproductive success in NFG, individual- and population genetic variation still strongly predicted reintroduction success when length was held constant as shown in Figure 2.2 B and C.

We examined two additional WCT mixed-source reintroductions (Peet and Ruby Creeks) to test the generality of our findings that parental genetic variation and hybridization predict reintroduction success. Peet and Ruby were both refounded using two source populations (Fig. 2.1, A and Table. S2. 1). We sampled offspring following reintroductions and used population assignment tests (25) to assign Ruby (n = 102) and Peet (n = 93) offspring to one of six crosstypes: parental (BE, BR, MC, LC) and hybrids (BExBR and MCxLC) (Table. S2. 3) (21).

Comparisons of observed to expected crosstype proportions revealed that hybrids (BExBR and MCxLC) were nearly two times more abundant than expected in both reintroductions (Fig. 2.1, E and F, and Table. S2. 4) (21). These findings substantiate hybridization benefits described in SFG.

Across the four study sites, we described relationships between genetic variation, hybridization, and reintroduction success by comparing observed crosstype heterozygosity and crosstype type (parental or hybrid) to crosstype success (the difference between observed and expected offspring) (Table. S2. 4) (21). Crosstype success was positively related to hybridization and crosstype heterozygosity (Fig. 2.3), further demonstrating the effects of hybridization and genetic variation on reintroduction success. Generally, populations and crosstypes with the highest observed genetic variation had the highest relative success (Fig. 2.3). Overall, our results highlight that: (i) populations with lower relative genetic variation were less successful, (ii) individuals with lower genetic variation had reduced mating success and fewer offspring, and (iii) hybrid crosstypes were most successful when only two populations were used for reintroductions.

Discussion

Although we demonstrated that genetic variation and hybridization influenced reintroduction success, other biotic and abiotic aspects of source populations should also be considered when implementing mixed-source reintroductions. For example, we attempted to account for differences in population success due to environmental effects (or habitat mismatching) by comparing daily stream temperatures and timing of peak stream flows between NFG source populations and the reintroduction stream. We found no difference in timing of peak flows among streams, however, daily stream temperatures in the least successful source

population, BC, were most dissimilar to NFG (Figs. S2. 2 and S2. 3). These results highlight the complex abiotic and biotic dynamics involved in mixed-source reintroductions and further demonstrate the need to better describe how source population habitat mismatching could influence reintroduction efforts.

Additional research is needed to understand the fitness effects of hybridization between genetically divergent source populations beyond the F_1, when outbreeding depression may occur. Nevertheless, outbreeding depression in our system is unlikely to cancel our observed apparent heterosis and population-specific fitness effects because all source populations were translocated into new habitat, reducing potential extrinsic local adaptation disruptions, and we do not suspect any extreme differences in structural genomic variation since source populations have not been isolated long (<200 years) (4). Importantly, we also evaluated individual fitness within populations in NFG and found individual heterozygosity predicted survival and reproduction within source populations BN and BC, but not in PR (Fig. S2. 4, A). Within source populations, we also observed weak, but positive relationships between individual reproductive success and individual heterozygosity (Fig. S2. 4, B). These results suggest further research is also needed to understand individual fitness differences within source populations that could influence reintroductions success.

Though theoretical predictions have proposed fitness benefits of genetic variation and hybridization between genetically depauperate populations (3-7), we present consistent and replicated empirical tests demonstrating the benefits of genetic variation and hybridization among several conservation reintroductions. In the face of climate change and continued anthropogenic effects, reintroductions are likely to be increasingly common, and our results accentuate that genetic variation and hybridization are central components to reintroduction

success. More broadly, this study empirically describes and emphasizes the role of genetic variation in conservation biology, clearly demonstrating that genetic variation is a critical component to individual and population fitness.

References

1. C. N. Johnson, A. Balmford, B. W. Brook, J. C. Buettel, M. Galetti, L. Guangchun & J. M. Wilmshurst, Biodiversity losses and conservation responses in the Anthropocene. *Science*, **356**, 270-275 (2017).

2. IPBES, "Global assessment report on biodiversity and ecosystem services of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services" (IPBES secretariat, 2019).

3. F. Allendorf, C. Funk, S. Aitken, M. Byrne & G. Luikart, Eds., *Conservation and the genomics of populations* (Oxford University Press, Oxford, United Kingdom, ed. 3, 2022).

4. R. Frankham, J. D. Ballou, K. Ralls, M. D. B. Eldridge, M. R. Dudash, C. B. Fenster, R. C. Lacy, P. Sunnucks & K. McInnes, Eds., *Genetic management of fragmented animal and plant populations* (Oxford University Press, Oxford, United Kingdom, ed. First edition, 2017).

5. D. H. Reed & R. Frankham, Correlation between Fitness and Genetic Diversity. *Conservation Biology*, **17**, 230-237 (2003).

6. O. Razgour, B. Forester, J. B. Taggart, M. Bekaert, J. Juste, C. Ibáñez, S. J. Puechmaille, R. Novella-Fernandez, A. Alberdi & S. Manel, Considering adaptive genetic variation in climate change vulnerability assessment reduces species range loss projections. *Proceedings of the National Academy of Sciences*, **116**, 10418-10423 (2019).

7. K. Ralls, J. D. Ballou, M. R. Dudash, M. D. B. Eldridge, C. B. Fenster, R. C. Lacy, P. Sunnucks & R. Frankham, Call for a Paradigm Shift in the Genetic Management of Fragmented Populations. *Conservation Letters*, **11** (2018).

8. M. Kardos, E. E. Armstrong, S. W. Fitzpatrick, S. Hauser, P. W. Hedrick, J. M. Miller, D. A. Tallmon & W. C. Funk, The crucial role of genome-wide genetic variation in conservation. *Proceedings of the National Academy of Sciences*, **118**, 1 (2021).

9. J. C. Teixeira & C. D. Huber, The inflated significance of neutral genetic diversity in conservation genetics. *Proceedings of the National Academy of Sciences*, **118**, 1 (2021).

10. A. R. Whiteley, S. W. Fitzpatrick, W. C. Funk & D. A. Tallmon, Genetic rescue to the rescue. *Trends in ecology & evolution*, **30**, 42-49 (2015).

11. S. Edmands, Between a rock and a hard place: evaluating the relative risks of inbreeding and outbreeding for conservation and management. *Molecular Ecology*, **16**, 463-475 (2007).

12. D. A. Tallmon, G. Luikart & R. S. Waples, The alluring simplicity and complex reality of genetic rescue. *Trends in ecology & evolution*, **19**, 489-496 (2004).

13. C. J. Brauer & L. B. Beheregaray, Recent and rapid anthropogenic habitat fragmentation increases extinction risk for freshwater biodiversity. *Evolutionary Applications,* **13**, 2857-2869 (2020).

14. D. Tickner, J. J. Opperman, R. Abell, M. Acreman, A. H. Arthington, S. E. Bunn, S. J. Cooke, J. Dalton, W. Darwall, G. Edwards, I. Harrison, K. Hughes, T. Jones, D. Leclère, A. J. Lynch, P. Leonard, M. E. McClain, D. Muruven, J. D. Olden, S. J. Ormerod, J. Robinson, R. E. Tharme, M. Thieme, K. Tockner, M. Wright & L. Young, Bending the Curve of Global Freshwater Biodiversity Loss: An Emergency Recovery Plan. *Bioscience,* **70**, 330-342 (2020).

15. G. Su, M. Logez, J. Xu, S. Tao, S. Villéger & S. Brosse, Human impacts on global freshwater fish biodiversity. *Science,* **371**, 835-838 (2021).

16. B. B. Shepard, B. E. May & W. Urie, Status and Conservation of Westslope Cutthroat Trout within the Western United States. *North American Journal of Fisheries Management,* **25**, 1426-1440 (2005).

17. D. A. Bell, R. P. Kovach, C. C. Muhlfeld, R. Al-Chokhachy, T. J. Cline, D. C. Whited, D. A. Schmetterling, P. M. Lukacs & A. R. Whiteley, Climate change and expanding invasive species drive widespread declines of native trout in the northern Rocky Mountains, USA. *Science Advances,* **7**, eabj5471 (2021).

18. R. P. Kovach, R. F. Leary, D. Bell, S. Painter, A. Lodmell & A. R. Whiteley, Genetic variation in westslope cutthroat trout reveals that widespread genetic rescue is warranted. *Canadian Journal of Fisheries and Aquatic Sciences,* (2021).

19. P. T. Clancey, B. B. Shepard, C. G. Kruse, S. A. Barndt, L. Nelson, B. C. Roberts & B. R. Turner, Collaboration, commitment, and adaptive learning enable eradication of nonnative trout and establishment of native westslope cutthroat trout into one-hundred kilometers of Cherry Creek, a tributary of the Madison River, Montana. *American Fisheries Society Symposium,* **91**, 589-647 (2019).

20. J. L. Arnold, C. R. Detjens, B. D. Ertel, M. E. Ruhl & T. M. Koel, Westslope cutthroat trout and fluvial arctic grayling restoration. *Yellowstone Science,* **25**, 18-25 (2017).

21. Materials and Methods are available as supplementary materials.

22. A. Cockburn, J. V. Peñalba, D. Jaccoud, A. Kilian, L. Brouwer, M. C. Double, N. Margraf, H. L. Osmond, L. E. B. Kruuk & M. van de Pol, HIPHOP: improved paternity assignment among close relatives using a simple exclusion method for biallelic markers. *Molecular Ecology Resources,* **21**, 1850-1865 (2021).

23. C. C. Downs, R. G. White & B. B. Shepard, Age at Sexual Maturity, Sex Ratio, Fecundity, and Longevity of Isolated Headwater Populations of Westslope Cutthroat Trout. *North American Journal of Fisheries Management,* **17**, 85-92 (1997).

24. I. J. Koch & S. R. Narum, An evaluation of the potential factors affecting lifetime reproductive success in salmonids. *Evolutionary Applications*, **14**, 1929-1957 (2021).

25. M. J. Hubisz, D. Falush, M. Stephens & J. K. Pritchard, Inferring weak population structure with the assistance of sample group information. *Molecular Ecology Resources*, **9**, 1322-1332 (2009).

Figures

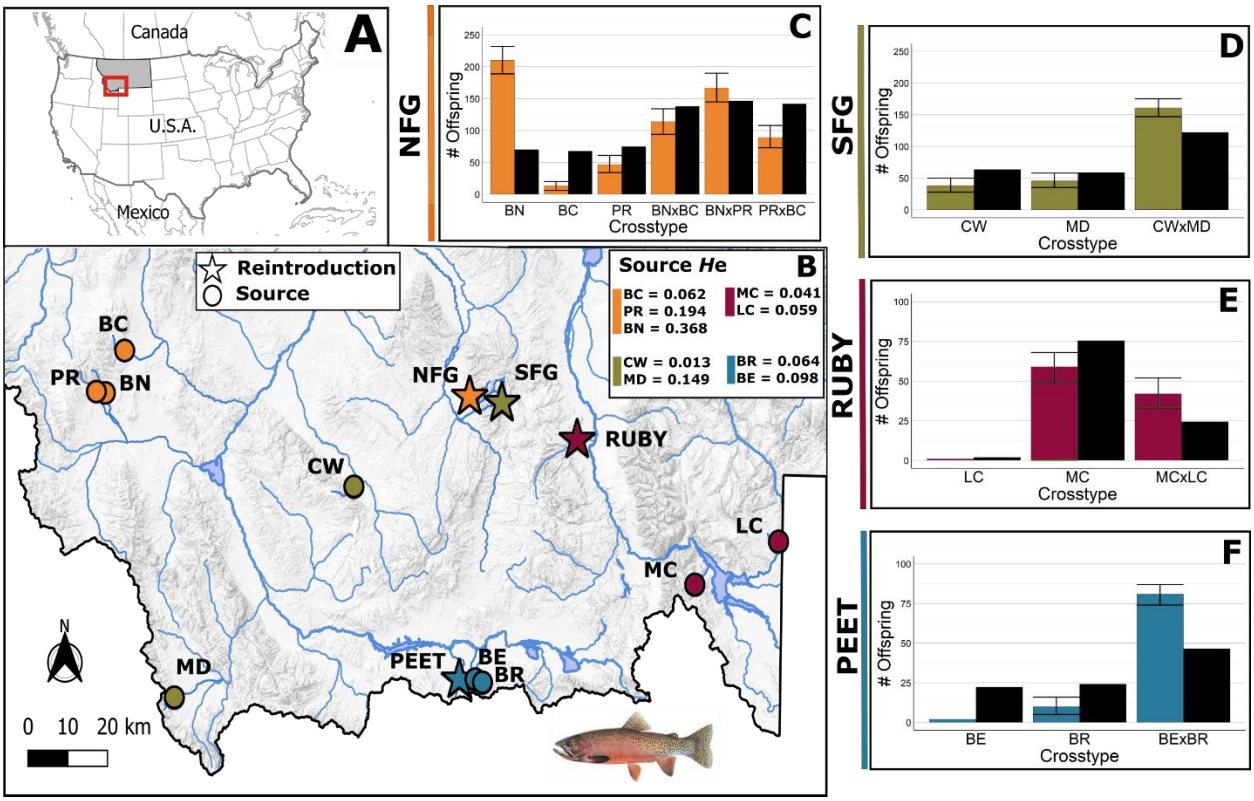

Figure 2. 1: Tests of crosstype success reveal greater offspring production of genetically variable populations and hybrid crosstypes. Map of Montana, USA (A) showing source populations and their corresponding, color-coded reintroduction locations**.** The expected heterozygosity of each source population (B) and number of observed (colored bars) vs expected (black bars) offspring assigned to crosstype following reintroduction efforts into NFG (C), SFG (D), Ruby (E), and Peet (F) Creeks. 95% confidence intervals are shown for observed offspring.

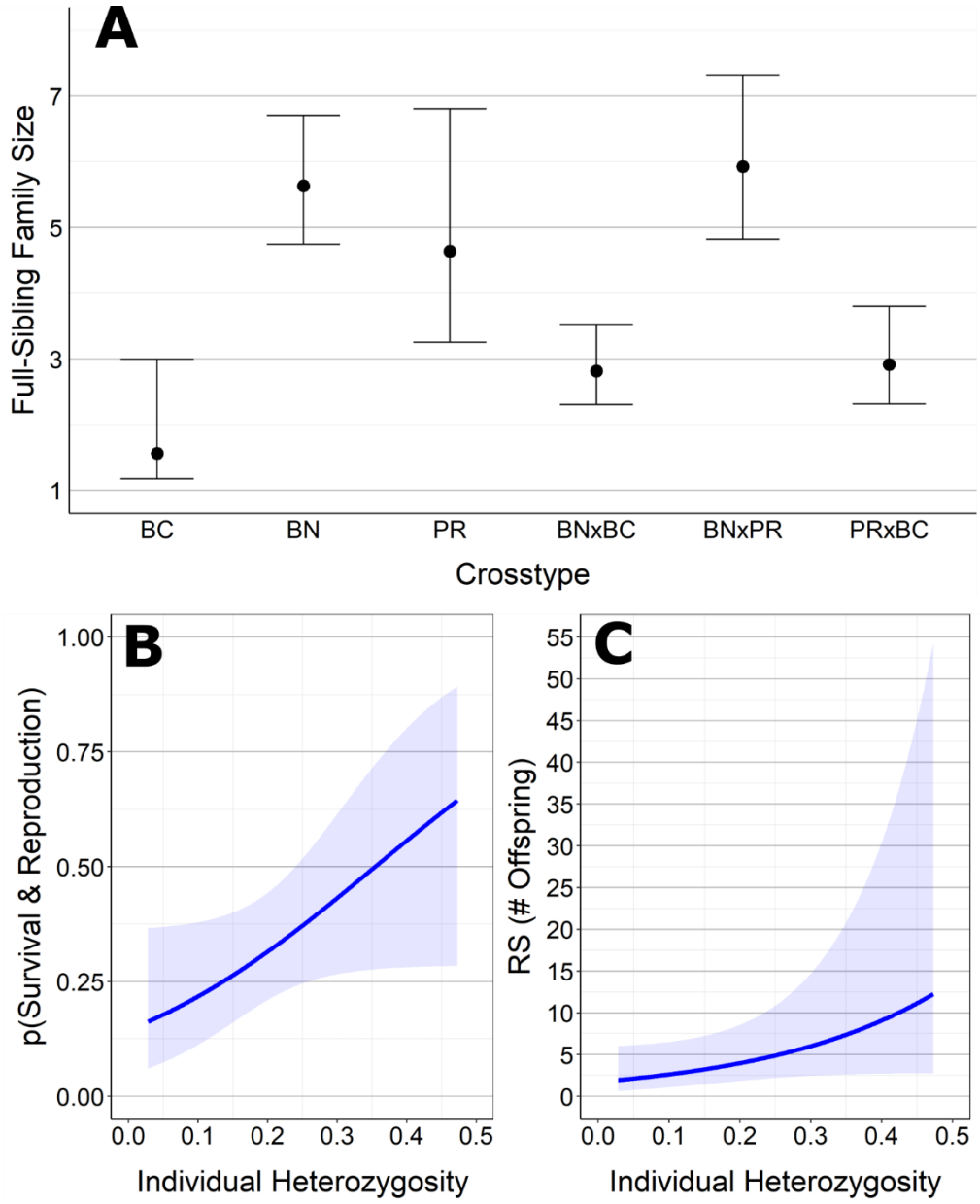

Figure 2. 2: Crosstype predicts family size, while individual heterozygosity predicts the probability of survival and reproduction, and reproductive success. Full-sibling family size (A) was predicted for each crosstype, while the probability of survival and reproduction (B), and reproductive success (C) were predicted as a function of individual heterozygosity. 95% confidence intervals were computed for each prediction.

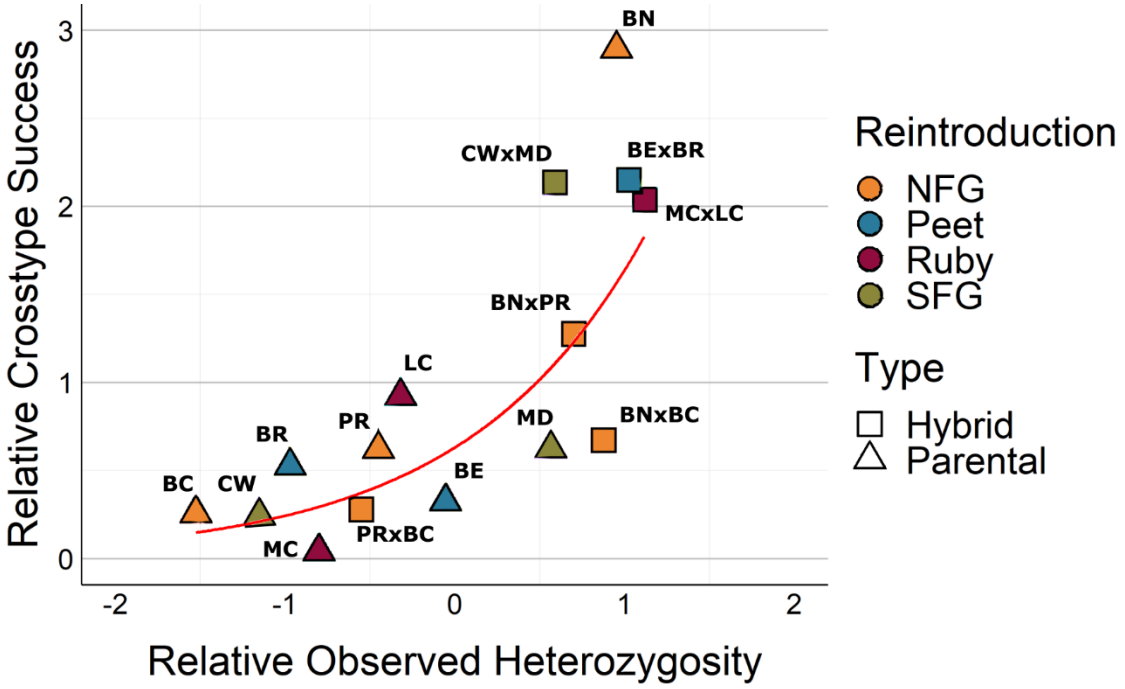

Figure 2. 3: Crosstypes with greater relative heterozygosity and hybrids had increased success. Colors describe reintroduction stream and shapes show crosstype type: parental or hybrid. Relative observed heterozygosity values are described by low (negative) and high (positive) heterozygosity values. Values along both axes were standardized by reintroduction stream to account for competitive dynamics. The red line describes the exponential relationship between crosstype success and heterozygosity.

Supplemental

Appendix 2.1 – Materials and Methods

Study Design and Sample Collection

Reintroductions

To restore westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) to historically occupied habitat, all nonnative species above artificial or natural stream barriers were removed from the North and South Forks of Greenhorn (NFG and SFG), Peet, and Ruby Creeks. Following nonnative removal efforts, source individuals were captured from nine geographically separated populations using a backpack electrofisher with power output settings adjusted to minimize negative effects on fish. Individuals were collected throughout each source stream to avoid capturing and translocating family groups. All fish were anesthetized, measured to the nearest millimeter, and translocated to recipient reintroduction streams (Fig. 2.1, A and Table. S2. 1). Translocated individuals were released near or in the same stream sections each year. Source individuals used to refound NFG and SFG had a small portion of their anal fin removed for genetic analyses. Yearly translocations of source individuals from the five populations used to refound NFG and SFG were mostly equal, whereas source translocations into Peet and Ruby were variable by population and year (Table. S2. 1).

Offspring Sampling

Reproduction between source individuals began in 2016 in Ruby and 2017 in NFG, SFG, and Peet. Given westslope cutthroat trout (WCT) typically do not reach sexual maturity until age three (1), we sampled all reintroduction streams in 2019 and 2020 to capture first-generation (F_1) offspring from source individual reproduction. To capture offspring for population-level analyses, we sampled 200-1,000 meters at release sites, then systematically sampled additional

200-1,000-meter reaches, spaced semi-evenly throughout SFG, Peet, and Ruby reintroduction streams. In NFG, we followed a similar sampling design, but doubled our sampling efforts at each site (1,000-1,500) to increase sample size and distance for NFG parentage analyses. Overall, these methods allowed us to cover different stream habitat throughout the entirety of each stream, minimize capturing offspring family groups, and maximize sampling size and distance. All offspring were captured using a backpack electrofisher with power output settings adjusted to minimize negative effects on fish. Upon capturing offspring, we collected genetic samples tissue samples from anal and caudal fins and lengths to the nearest millimeter.

Environmental Sampling

Since populations experiencing geographical isolation and limited gene flow can become genetically divergent and develop local adaptations to specific environments (2, 3), we measured environmental metrics in NFG and SFG source populations to describe the influence of environmental differences on population or reproductive success. Specifically, we installed HOBO MX water level data loggers (MX2001-04) within each source population stream and at the confluence of the NFG and SFG to continually measure stream temperature and water level from spring of 2020 to fall of 2021. Temperature and water level data were then used to describe source population success by making post-hoc comparisons between source and reintroduction stream environmental conditions.

<u>DNA Extraction, Library Preparation, & Sequencing</u>

DNA Extraction

We used tissue samples from every translocated individual (n = 686) and offspring (n = 1,257) to perform population and parentage assignment in NFG and SFG. For Peet and Ruby, we

used tissue samples from source populations BE (*n* = 24), BR (*n* = 25), LC (*n* = 29), and MC (*n* = 26), and offspring from Ruby (*n* = 102) and Peet (*n* = 93) to perform population assignment. In total, we prepared 2,242 samples for polymerase chain reaction (PCR) amplification using a modified DNA extraction procedure (4). For each extraction, a Liquidator 96 Manual 96-well Pipettor (Rainin) was used to add Lysis buffer (80 µL, 0.1M Tris-HCl, 0.1mM EDTA, and 1% SDS) to each well of a 96-well place. Fin clips measuring 2-25 mm^2 were placed into each well containing Lysis buffer. Then, a digestion master mix (40 µL, 4mls Liftons, 125 µL 20 mg/ml Proteinase K, and 330 µL 1M DTT) was added to each well. After mixing, the plate was sealed and incubated at 37°C for 12-24 hours or until tissue was completely dissolved. To a new plate, we added a mixture of Hybridization Buffer (8.5 mL, 1 g DTT, 29 g NaCl, and 50 g PEG 8000) and home-made Serapure beads (5), pipetting 100 µL of the mixture into each well. 80 µL of the crude lysate was then added to each well in the Hybridization Buffer/Serapure beads plate. After vortexing and incubating at room temperature for 15 minutes, the plate was placed on a magnet for 5 minutes. While remaining on the magnet, the supernatant was aspirated and discarded. Then, 170 µL of freshly prepared 80% ethanol was used to resuspend the Serapure beads. An additional 80% ethanol wash was performed, and the beads were allowed to air-dry on the magnet for 5 minutes. Lastly, beads were resuspended in 70 µL of low TE (10 nM Tris-HCl, 0.1 nM EDTA), incubated for 10 minutes at room temperature, and placed on magnet for 5 minutes. 60 µL of the eluted DNA was transferred to a fresh plate for subsequent library preparation steps. Above extraction steps generated 24 plates containing individuals from source and reintroduction streams. Within each plate, we used four wells as controls: two H_2O, one individual duplicated among plates, and one individual duplicated within each plate. We also filled half of the remaining empty wells in the final plate with within plate duplicates. We used well F2 for all

within plate duplicates, which allowed us to semi-randomly select within plate duplicates while having at least one duplicated individual from each WCT donor population.

Library Preparation

Extracted DNA was quantified using PicoGreen™ (Molecular Probs, Eugene, OR) to identify samples with low-quality DNA (< 10 ng/µL) and standardize DNA concentrations prior to library preparation. Low-quality DNA samples (n = 68) were moved to a new plate, evaporated, and reconcentrated. All samples were standardized between 20 and 60 ng/µL in concentration. Following protocols from Campbell et al. 2015, we prepared a PCR cocktail (371 µL Qiagen Plus multiplex master mix and 159 µL of pooled WCT GT-seq primers at ≈30 nM per primer) for multiplex PCR amplification of target loci. We added 5 µL of the PCR mixture and 2 µL of DNA extract to each well in a new plate. For the first PCR, thermal cycling was conducted in 96-well PCR plates with the following conditions: 95℃ - 15 min; 5 cycles [95℃ - 30 s, 5% ramp down to 57℃ - 30 s, 72℃ - 2 min]; 10 cycles [95℃ - 30 s, 65℃ - 30 s, 72℃ - 30 s]; 4℃ hold. Afterwards, we diluted the PCR plate by adding 133 µL of nuclease free H_2O to each well and transferred 3 µL of diluted PCR product to a new plate. On top of the diluted PCR product, we added 6 µL of i7 barcode cocktail (106 µL of 11 µM i7 index and 530 µL of Hot Start Master Mix) and 2 µL of i5 barcode in preparation for the second PCR. Thermal cycling conditions for the second PCR were 95℃ - 15 min; 10 cycles [98℃ - 10 s; 65℃ - 30 s; 72℃ - 5 min; 4℃ hold. Following the second PCR, each plate was normalized using Charm Biotech Normalization Kits (Charm Biotech, San Diego, CA) using the manufacturer's instructions. Then, 10 µL of each sample per 96-well plate was pooled into 24 1.5-mL Eppendorf tubes.

Lastly, we performed a purification step on each of the 24 pooled aliquots by mixing 250 µL of Agencourt® AMPure® XP magnetic beads with 500 µL of pooled library in a fresh 1.5-mL

Lo-Bind Eppendorf tube. Each tube was incubated at room temperature and placed on a magnet for 5 minutes. The supernatant was then transferred to a new 1.5-mL Lo-Bind Eppendorf tube with 350 µL of beads. After another round of incubating at room temperature and sitting on a magnet for 5 minutes, the supernatant was discarded, and the leftover beads were washed twice with 80% ethanol. Remaining beads were air dried for 5 minutes while on the magnet and eluted with 17 µL of low TE. The 17 µL of supernatant was then transferred to fresh 1.5-mL Lo-Bind Eppendorf tubes. Following purification, all 24 libraries were quantified using a Qubit 2.0 fluorometer (Life Technologies Inc.) with a Qubit™ dsDNA Assay Kit, normalized to a concentration of 4 nM, and equal volumes were pooled to create the final GT-seq library for sequencing.

SNP Sequencing, Genotyping & Filtering

Genetic samples from founder individuals and subsequent progeny were genotyped using genotyping-in-thousands by sequencing (GT-seq) (6). We used an existing GT-seq panel of 373 WCT single-nucleotide-polymorphisms (SNP's) (one of which was a sex ID marker) developed by the Idaho Fish & Game Eagle Fish Genetics Lab. All 24 prepared libraries were sequenced on a single lane of an Illumina NextSeq V2 and demultiplexed by the University of Colorado Next-Generation Sequencing Facility. We used a high output 75-cycle kit, single-read sequencing, and 2% PhiX for all sequencing runs.

Sequencing data were concatenated and genotyped using an updated python script described in Campbell et al. 2015. Briefly, genotypes were obtained with a python script that reads in locus information from a text file and uses each forward primer sequence and allele-specific internal probe sequences to identify and count the occurrence of each allele for every SNP. Reads containing the correct primer sequence and allele-specific internal probe sequence

were referred to as ‘on-target’ reads and were the only reads we used to make genotype calls. Once on-target allele counts were completed, the ratio of allele 1 to allele 2 counts was used to generate a genotype for each locus in a script in Program R (7). For each SNP, allele ratios >10.0 were called as homozygous for the 1 allele, ratios <0.1 were called homozygous for the 2 allele, and ratios between 0.33 and 3.0 were called heterozygous. Similar ratios were shown to generate accurate genotypes when genotyping-by-sequencing (6). Loci with <8 reads were not genotyped, as a minimum of 8 reads is expected to yield accurate genotypes via genotyping-by-sequencing (8). After making genotype calls, we used within and among plate controls to estimate genotyping error for all sequencing runs combined. Using among plate controls (n = 24) we detected a genotyping error of 0.036%, while within plate controls (n = 104) yielded a genotyping error of 0.014%.

Locus filtering was conducted with Program R (7) using Greenhorn Creek donor populations. To remove low quality data, we removed individuals with genotype missingness >25% and loci with genotype missingness >25%. By removing individuals with low genotyping success, we lost three source individuals from NFG and SFG analyses (1 BC, and 2 CW). For below filtering, we performed Hardy-Weinberg (HW) and linkage disequilibrium tests (LD) separately for each group of source populations used for reintroductions (i.e., tests were performed with NFG and SFG, Peet, and Ruby source populations separately). Using approaches from Waples (9) we calculated F_{IS} values with R package HIERFSTAT v0.5.9 (10) and removed loci fixed heterozygous. Then we tested conformance with HW proportions and LD at each locus with exact tests (11) using R packages PEGAS v1.1.0 (12) (ran using 10,000 dememorizations) and GENEPOP v1.1.7 (13) (ran using 10,000 dememorizations, 100 batches, and 5,000 iterations), respectively. For both tests, we adjusted significance values for multiple testing using

sequential Bonferroni procedures (14). After removing loci not conforming with HE proportions and showing patterns of linkage disequilibrium, we proceeded with parentage and population assignment testing using 251, 81, and 76 variable loci for NFG and SFG, Peet, and Ruby datasets, correspondingly.

Population and Parentage Assignments

To assess the accuracy of parentage assignments, we randomly simulated matings among all genotyped source parents (n = 538), producing approximately 9,500 offspring with variable genotype missingness (i.e., missingness 0-25%). We then used R package HIPHOP v0.0.1 (15), an exclusion-based parentage assignment program, to assign simulated offspring to source parents. Offspring were assigned to a parent using a homozygous opposite test (*hot* score) (16) and precluding heterozygous offspring where there were homozygous identical parents (*hiphop* score) (15). In some instances, one parent in a parent pair remained unclear (i.e., parentage was undecided between two sires or dams), in which case we selected the parent-pair where genotype missingness was lowest among parents and the offspring (*loci.tryad* score). This selection method yielded lower parentage assignment errors compared to other approaches when assigning offspring to parents from BN, PR, BC, and MD (Table. S2. 2). Consequently, we proceeded to only evaluate parentage with offspring from source populations translocated into the North Fork (i.e., BN, BN, and PR). Parentage assignment results were then used to assign and categorize offspring to one of nine population crosses (crosstypes): parental (BN, BC, PR, MD, CW) and F_1 hybrids (BNxPR, BNxBC, PRxBC, CWxMD). Though parentage assignment errors were high when assigning to parents with Cottonwood ancestry (due to a very small number of variable SNPs; n = 9), simulation results indicated population assignment was highly accurate (i.e., 100% for all populations), allowing us to evaluate population contribution for all donor populations.

After verifying our ability to accurately assign simulated offspring to NFG and SFG source populations and NFG parents, we used R package HIPHOP (15) to assign sampled offspring to source populations (i.e., crosstypes) and NFG parents. Once assigned, we removed offspring with *hothiphop.parents* scores >0.008 (i.e., the sum of the *hiphop* and *hot* mismatch scores for each parent), removing parentage assignments with higher uncertainty (n = 13). Then, we identified source and offspring individuals recaptured in 2019 and 2021 as individuals having zero or one mismatch using program CERVUS v3.0.7 (17). Using this approach, we found two PR source individuals with matching genotypes. One of these two individuals was removed.

To assign offspring to Ruby and Peet source populations we used a Bayesian population assignment program (STRUCTURE; (18)). For each reintroduction, we used an ADMIXTURE model in STRUCTURE with parameters set to: USEPOPINFO = 1, MAXPOPS = 2, ALPHA = 1, POPFLAG = 1, and POPDATA = 1. We ran STRUCTURE using 10,000 burn-ins and 100,000 MCMC repetitions. To corroborate STRUCTURE was correctly assigning offspring to source populations with low genetic variation, we ran SFG offspring and source populations with the same settings and compared STRUCTURE results to HIPHOP results. In doing so, we found 100% conformity between the two programs. Using STRUCTURE results for Peet and Ruby, we assigned offspring to one of six crosstypes: parental (BE, BR, MC, LC) and hybrids (BExBR and MCxLC). The total number of offspring used for all population assignment tests is described in Table S3.

Population and Parentage Analyses

Offspring from source populations with higher genetic variation or hybrid crosstypes were predicted to be more abundant than expected. To test these predictions, we quantified and compared observed to expected crosstype proportions in each reintroduction stream. Expected

crosstype proportions were estimated using the initial reintroduction proportions of each source population assuming random mating and equal survival and reproduction from 2016-2020 (19). Observed crosstypes were offspring sampled throughout each reintroduction stream from 2019-2020. To statistically evaluate the difference between expected and observed crosstype proportions, we performed a chi-square test between observed and expected proportions and adjusted significance values for multiple testing using sequential Bonferroni procedures (14). Statistical assessments of crosstype proportions were computed with the STATS package in R (7). Then, we used R package HIERFSTAT v0.5.9 (10) to estimate and compare mean observed (H_O) and expected (H_e) among source populations (Fig. 2.1, B and Table. S2. 1).

To describe potential fitness differences among crosstypes and source individuals, we evaluated reproductive success in NFG using two metrics: full-sibling family size (family size) and the number of offspring produced per parent (reproductive success), correspondingly. Starting with family size, we predicted family size would be elevated for hybrid crosstypes and crosstypes with parents from populations with higher genetic variation. Since family size is potentially influenced by parental body size due to size-fecundity relationships and sexual competition, we used each parent's length as covariates (1, 20). However, we only knew initial reintroduction lengths for each source individual, not size at maturity. In response, we used donor recapture data (n = 25) to fit a simple linear regression, modeling growth as a function of length with the STATS package in R (7). Briefly, there was a strong negative relationship between growth and length (R^2 = 0.64, P < 0.0001). This model was then used to predict the lengths of source individuals at year of reproduction, which we refer to as estimated length at reproduction (ELR). Furthermore, family size is potentially influenced yearly by density dependent interactions (21). To account for this bias, we used cohort year as a covariate and

removed all but age one offspring for each cohort year. Age one offspring (n = 436) were identified using offspring lengths within each family (i.e., progeny of the same family and cohort should be similar in length) and length frequency histograms by year and site. We then evaluated the influence of crosstype on family size using a zero-truncated Poisson generalized linear model (GLM) with a log link function coded in R (7) (Table. S2. 5). A zero-truncation was used because observed family size was always greater than one. We used a Poisson error structure after determining that family size data was only mildly over dispersed (i.e., dispersion < 1) and residuals were mostly randomly distributed (22). R packages VGAM v1.1.5 (23) and GGEFFECTS v1.1.1 (24) were used for model implementation and predictions, respectively. Our model predicted full-sibling family size as a function of fixed effects: crosstype, sire ELR, dam ELR, and cohort year.

Secondly, we investigated how individual genetic variation affects an individual's reproductive success. We predicted that parents with higher genetic variation would produce more offspring. Like family size, reproductive success is potentially influence by parental body size due to size-fecundity relationships and sexual competition (1, 20, 21). Therefore, we used parent length and sex as covariates. The growth model described above was used to predict the 2017 length of 2016 source individuals, making parent length comparable among all translocated parents. We referred to this covariate as comparable parent length (CPL). Since reproductive success is also likely affected by translocation year (i.e., 2016 translocated individuals had an extra year of reproduction), we used translocation year as a covariate. Lastly, source population (BN, PR, BC) was included as a covariate to account for remaining population-level differences possibly contributing to reproductive success including variation in source population heterozygosity. We then examined the influence of individual heterozygosity on reproductive

success by modeling two separate processes: (i) whether an individual survived and reproduced as a function of individual heterozygosity, sex, CPL, translocation year, and population of origin, and (ii) if an individual reproduced, then reproductive success as a function of the same covariates. We described the first process as "Survival and Reproduction" by assuming that an individual did not survive or reproduce between 2016-2019 if we did not capture subsequent offspring in 2019 or 2020. However, this is not truly "survival" given we did not account for capture probabilities and unsampled stream reaches. A Bernoulli GLM with a logit link function was used to model the first process (logistic regression model), while a zero-truncated Negative Binomial GLM with a log link function was applied for the second process (count model) (Table. S2. 6). This model, commonly known as a hurdle model, has been employed for similar analyses (25, 26). A zero-truncation was used for the count because observed offspring counts were always greater than one. We used a Negative Binomial error structure rather than a Poisson due to the severe overdispersion of offspring count data (i.e., dispersion > 1) and distribution of residuals (22). Because our covariate "population of origin" was mostly accounting for among population variation within this model, we also used this same modeling framework to evaluate survival and reproduction, and reproductive success as a function of individual heterozygosity, sex, CPL, and translocation year, separately for each source population (i.e., account for within population variation) (Fig. S2. 4 A and B). R packages glmmTMB v1.1.2.3 (27) and GGEFFECTS v1.1.1 (24) were used for model implementation and predictions, correspondingly.

Lastly, we summarized relationships between genetic variation, hybridization, and crosstype success among study sites. We estimated observed crosstype heterozygosity (n =15) with R package HIERFSTAT v0.5.9 (10) and standardized heterozygosity estimates (i.e., derived Z-scores) by reintroduction stream. Then, we derived an estimate of crosstype success by taking

the difference between observed and expected offspring counts for each crosstype. Positive and negative values of crosstype success were then standardized (i.e., obtained Z-scores) by reintroduction stream and plotted against observed crosstype heterozygosity. We used the STATS package in R (7) to describe the relationship between observed crosstype heterozygosity and crosstype success by fitting an exponential regression model.

Appendix 2.2 – References

1. C. C. Downs, R. G. White & B. B. Shepard, Age at Sexual Maturity, Sex Ratio, Fecundity, and Longevity of Isolated Headwater Populations of Westslope Cutthroat Trout. *North American Journal of Fisheries Management*, **17**, 85-92 (1997).

2. F. Allendorf, C. Funk, S. Aitken, M. Byrne & G. Luikart, Eds., *Conservation and the genomics of populations* (Oxford University Press, Oxford, United Kingdom, ed. 3, 2022).

3. R. Frankham, J. D. Ballou, K. Ralls, M. D. B. Eldridge, M. R. Dudash, C. B. Fenster, R. C. Lacy, P. Sunnucks & K. McInnes, Eds., *Genetic management of fragmented animal and plant populations* (Oxford University Press, Oxford, United Kingdom, ed. First edition, 2017).

4. O. A. Ali, S. M. O'Rourke, S. J. Amish, M. H. Meek, G. Luikart, C. Jeffres & M. R. Miller, RAD capture (Rapture): flexible and efficient sequence-based genotyping. *Genetics (Austin)*, **202**, 389-400 (2016).

5. N. Rohland & D. Reich, Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. *Genome research*, **22**, 939-946 (2012).

6. N. R. Campbell, S. A. Harmon & S. R. Narum, Genotyping-in-Thousands by sequencing (GT-seq): A cost effective SNP genotyping method based on custom amplicon sequencing. *Molecular ecology resources*, **15**, 855-867 (2015).

7. R Core Team. , R: a language and environment for statistical computing (R Foundation for Statistical Computing, 2020).

8. D. Hall, W. Zhao, U. Wennström, B. Andersson Gull & X. Wang, Parentage and relatedness reconstruction in Pinus sylvestris using genotyping-by-sequencing. *Heredity*, **124**, 633-646 (2020).

9. R. S. Waples, Testing for Hardy–Weinberg proportions: have we lost the plot? *The Journal of heredity*, **106**, 1-19 (2015).

10. J. Goudet & T. Jombart, hierfstat: estimation and tests of hierarchical F-statistics. (2021).

11. S. W. Guo & E. A. Thompson, Performing the Exact Test of Hardy-Weinberg Proportion for Multiple Alleles. *Biometrics*, **48**, 361-372 (1992).

12. E. Paradis, pegas: an R package for population genetics with an integrated–modular approach. *Bioinformatics*, **26**, 419-420 (2010).

13. F. Rousset, genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular ecology resources*, **8**, 103-106 (2008).

14. W. R. Rice, Analyzing tables of statistical tests. *Evolution*, **43**, 223 (1989).

15. A. Cockburn, J. V. Peñalba, D. Jaccoud, A. Kilian, L. Brouwer, M. C. Double, N. Margraf, H. L. Osmond, L. E. B. Kruuk & M. van de Pol, HIPHOP: improved paternity assignment among close relatives using a simple exclusion method for biallelic markers. *Molecular Ecology Resources,* **21**, 1850-1865 (2021).

16. J. Huisman, Pedigree reconstruction from SNP data: parentage assignment, sibship clustering and beyond. *Molecular ecology resources,* **17**, 1009-1024 (2017).

17. S. T. Kalinowski, M. L. Taper & T. C. Marshall, Revising how the computer program cervus accommodates genotyping error increases success in paternity assignment. *Molecular ecology,* **16**, 1099-1106 (2007).

18. M. J. Hubisz, D. Falush, M. Stephens & J. K. Pritchard, Inferring weak population structure with the assistance of sample group information. *Molecular Ecology Resources,* **9**, 1322-1332 (2009).

19. D. D. Huff, L. M. Miller & B. Vondracek, Patterns of ancestry and genetic diversity in reintroduced populations of the slimy sculpin: implications for conservation. *Conservation genetics,* **11**, 2379-2391 (2010).

20. I. J. Koch & S. R. Narum, An evaluation of the potential factors affecting lifetime reproductive success in salmonids. *Evolutionary Applications,* **14**, 1929-1957 (2021).

21. G. G. Nicola, A. Almodóvar, B. Jonsson & B. Elvira, Recruitment variability of resident brown trout in peripheral populations from southern Europe. *Freshwater biology,* **53**, 2364-2374 (2008).

22. A.F. Zuur & E. N. Ieno, Eds., *Beginner's guide to zero-inflated models with R* (Highland Statistics Ltd, Newburgh, United Kingdom, 2016).

23. T.W. Yee, Eds., *Vector Generalized Linear and Additive Models: With an Implementation in R* (Springer New York, New York, NY, ed. 1st ed. 2015, 2015).

24. D. Lüdecke, ggeffects: Tidy data frames of marginal effects from regression models. *Journal of open source software,* **3**, 772 (2018).

25. A. E. Samuelson, R. J. Gill, M. J. F. Brown & E. Leadbeater, Lower bumblebee colony reproductive success in agricultural compared with urban environments. *Proceedings of the Royal Society. B, Biological sciences,* **285**, 20180807 (2018).

26. J. Huisman, L. E. B. Kruuk, P. A. Ellis, T. Clutton-Brock & J. M. Pemberton, Inbreeding depression across the lifespan in a wild mammal population. *Proceedings of the National Academy of Sciences - PNAS,* **113**, 3585-3590 (2016).

27. M. Brooks E, K. Kristensen, Benthem, Koen,J.,van, A. Magnusson, C. Berg W, A. Nielsen, H. Skaug J, M. Mächler & B. Bolker M, glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *The R journal*, **9**, 378 (2017).

Appendix 2.3 – Figures and Tables

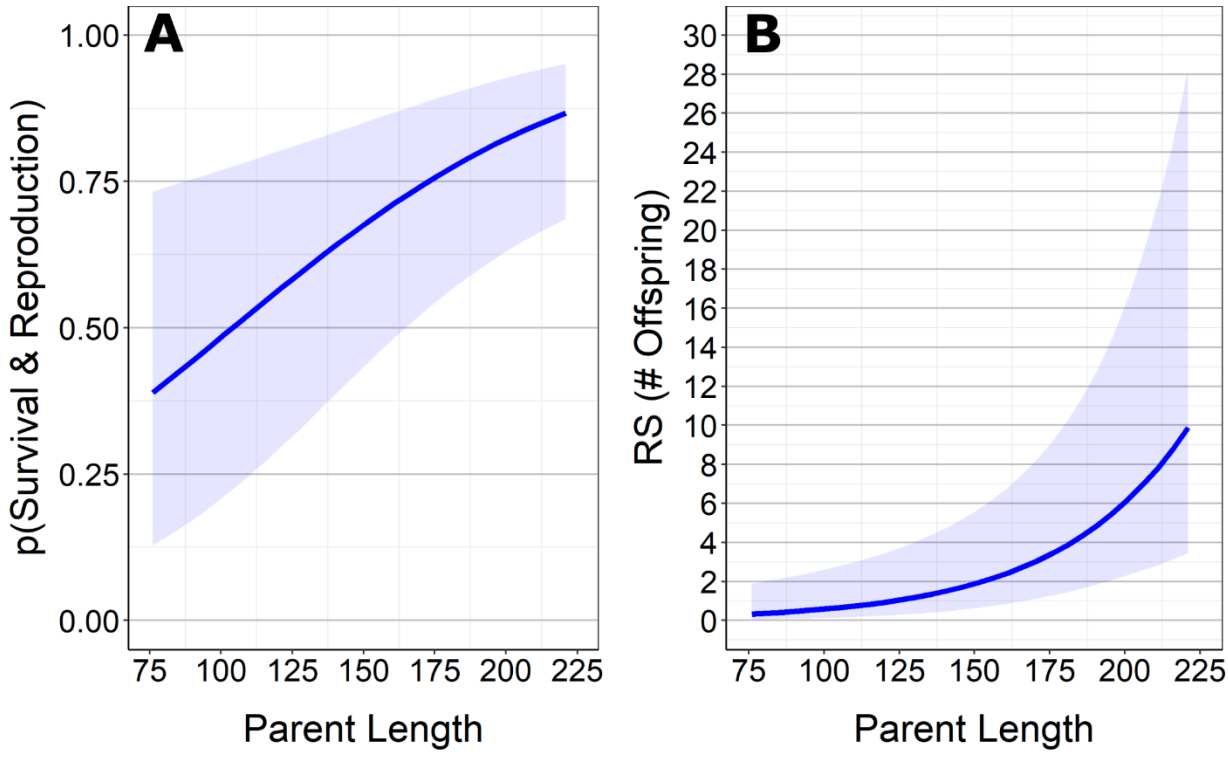

Figure S2. 1: Predicted metrics of reintroduction success plotted against parent length. The probability of survival and reproduction (A), and the number of offspring per parent (reproductive success) (B) predicted as a function of individual parent length. 95% confidence intervals were computed for each prediction.

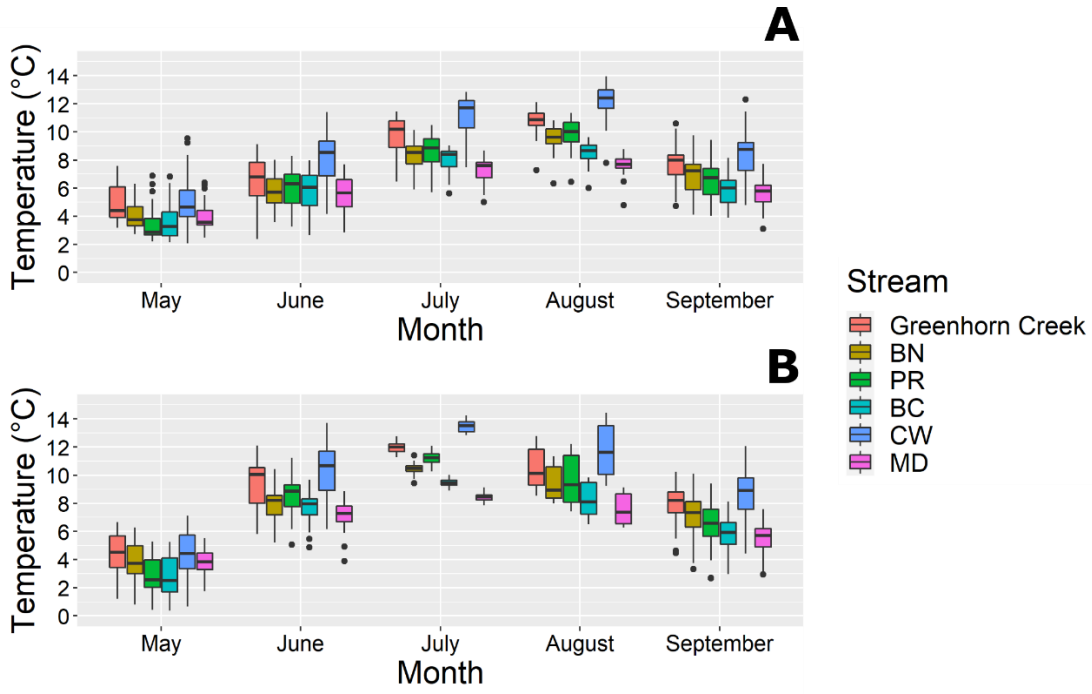

Figure S2. 2: Monthly stream temperatures for NFG and corresponding source populations. Temperatures were measured in 2020 (A) and 2021 (B).

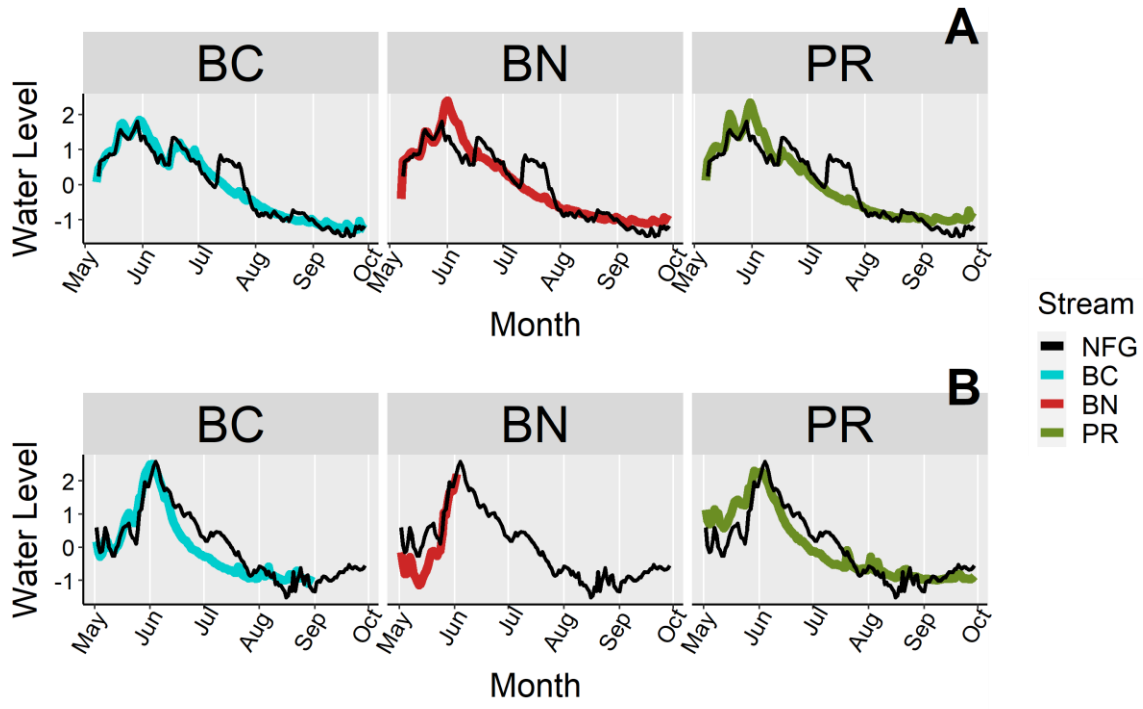

Figure S2. 3: Daily stream flows for NFG and corresponding source populations. Flows were measured in 2020 (A) and 2021 (B).

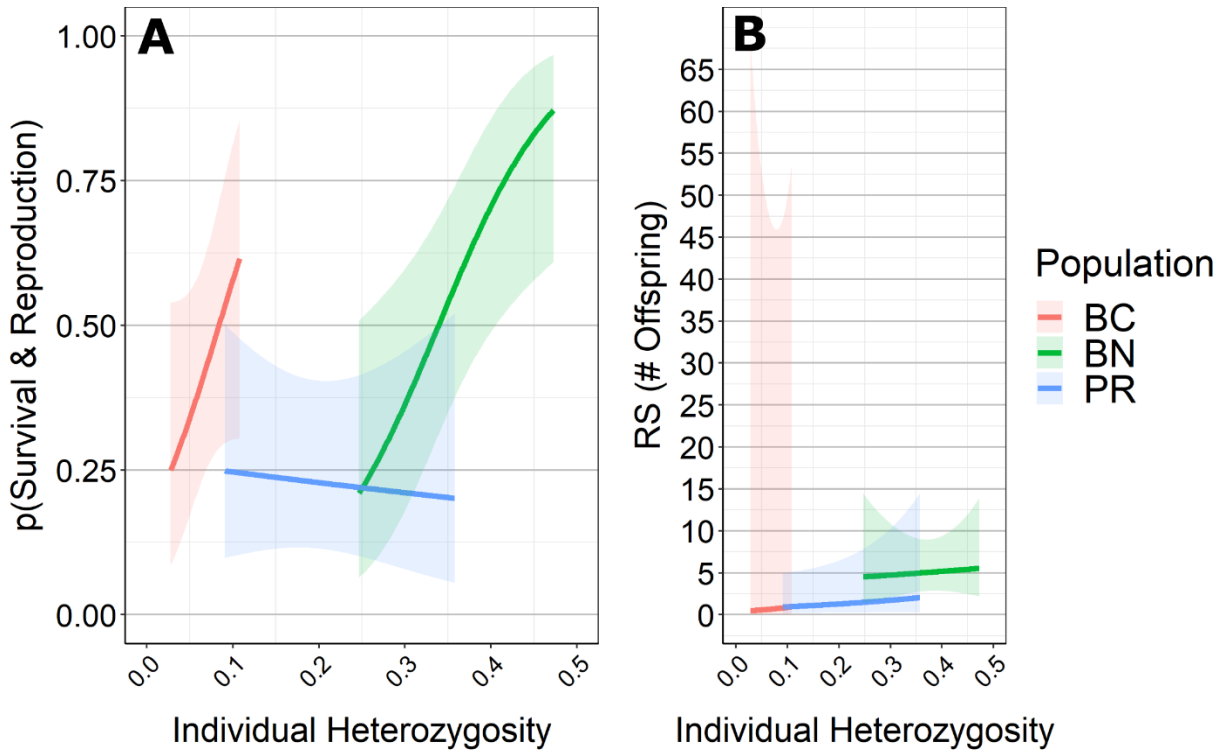

Figure S2. 4: Predicted fitness effects of individual heterozygosity for individuals within each source populations. The probability of survival and reproduction (A) and reproductive success (B) were predicted as a function of individual heterozygosity separately for each source population (color). In other words, this figure depicts the output from three different hurdle models, one for each source population.

Table S2. 1: Total number of individuals translocated between 2015-2018 from one of nine source streams to one of four reintroduction streams. Total translocated is the total number of source individuals translocated across years. Expected (H_e) and observed (H_o) heterozygosity were calculated for each source stream.

Source Population	Reintroduction Stream	2015	2016	2017	2018	Total Translocated	*He*	*Ho*
BC	NFG	0	50	55	0	105	0.062	0.06
BN	NFG	0	55	52	0	107	0.368	0.365
PR	NFG	0	50	60	0	110	0.194	0.182
CW	SFG	0	61	50	0	111	0.013	0.013
MD	SFG	0	55	50	0	105	0.149	0.144
BE	Peet	0	25	0	23	48	0.098	0.094
BR	Peet	0	0	26	25	51	0.064	0.061
LC	Ruby	0	10	0	0	10	0.059	0.059
MC	Ruby	20	14	15	12	61	0.041	0.038

Table S2. 2: Parentage simulation results describing estimated parentage assignment error for each possible crosstypes within and among the North and South Forks of Greenhorn Creek.

Crosstype	Offspring Assigned	Assignment Error
BN	406	0%
BNxPR	810	0%
MDxBN	705	0%
MD	365	0%
MDxPR	685	0%
PR	403	0%
BNxBC	798	0.25%
MDxBC	751	0.27%
PRxBC	734	0.68%
BC	323	5.57%
CWxBC	869	46.14%
MDxCW	772	50.39%
CWxBN	644	56.68%
CWxPR	828	57.25%
CW	415	92.05%

Table S2. 3: Total number of offspring sampled from each reintroduction stream in 2019 and 2020. Sampled is the total number of offspring sampled in each stream. Analyzed is the total number of offspring used for population and/or parentage analyses after individual and locus filtering steps.

Reintroduction Stream	2019	2020	Sampled	Analyzed
NFG	500	414	914	640
SFG	145	133	278	245
Peet	25	68	93	93
Ruby	0	120	120	102

Table S2. 4: NFG, SFG, Peet, and Ruby crosstype observed vs. expected analyses. Exp is the number of expected offspring from each crosstype assuming random mating, and Obs is the number of offspring from each crosstype sampled in each reintroduction stream from 2019-2020. Deviations between observed and expected are presented by Obs-Exp. Chi-square values are represented by $\chi 2$. The *P value* is reported for observed vs expected tests in each reintroduction stream.

Crosstype	Reintroduction Stream	Exp	Obs	(Obs-Exp)	$\chi 2$	*P value*
BN	NFG	70.4	210	139.6	276.82	
BC	NFG	67.8	13	-54.8	44.29	
PR	NFG	74.9	47	-27.9	10.39	
BNxBC	NFG	138.2	114	-24.2	4.24	
BNxPR	NFG	146.6	167	20.4	2.84	
PRxBC	NFG	142.1	89	-53.1	19.84	
Total	NFG	640	640	0	358.42	2.67E-76
CW	SFG	63.7	38	-25.7	10.37	
CWxMD	SFG	122.5	161	38.5	12.1	
MD	SFG	58.8	46	-12.8	2.79	
Total	SFG	245	245	0	25.26	5.01E-07
BE	Peet	22.3	2	-20.3	18.5	
BR	Peet	24.2	10	-14.2	8.3	
BExBR	Peet	46.5	81	34.5	25.6	
Total	Peet	93	93	0	52.4	4.53E-13
MC	Ruby	75.5	59	-16.5	3.6	
MCxLC	Ruby	24.5	42	17.5	12.5	
LC	Ruby	2	1	-1	0.5	
Total	Ruby	102	102	0	16.6	4.62E-05

Table S2. 5: Full-sibling family size model output describing family size as a function of given coefficients.

Coefficients	Estimate	Std. Error	z value	*p*
(Intercept)	-0.9498	1.1729	-0.8098	0.4181
BNxBC	0.9881	0.5610	1.7614	0.0782
BNxBN	1.7553	0.5510	3.1859	0.0014
BNxPR	1.8071	0.5545	3.2590	0.0011
PRxBC	1.0317	0.5665	1.8211	0.0686
PRxPR	1.5551	0.5792	2.6848	0.0073
Cohort Year (2020)	-0.7881	0.1439	-5.4750	0.0000
Dam ELR	0.0097	0.0043	2.2465	0.0247
Sire ELR	-0.0055	0.0030	-1.8261	0.0678

Table S2. 6: Hurdle model output describing probability of survival and reproduction (LogR) and reproductive success (Count) as a function of translocation year, source population (BN, PR, BC), Sex, length (CPL), and individual heterozygosity (Ind H_e).

Coefficients	Model	Estimate	Std. Error	z value	*p*
(Intercept)	Count	-3.7641	1.3760	-2.7356	0.0062
Translocation Year (2017)	Count	0.2381	0.3418	0.6966	0.4861
BN	Count	0.0158	0.7757	0.0204	0.9838
PR	Count	0.3646	0.4752	0.7674	0.4428
Sex Male	Count	0.3249	0.2654	1.2243	0.2209
CPL	Count	0.0234	0.0065	3.5752	0.0003
Ind He	Count	4.1124	2.4053	1.7097	0.0873
(Intercept)	LogR	2.7453	1.0582	2.5944	0.0095
Translocation Year (2017)	LogR	0.5089	0.3152	1.6147	0.1064
BN	LogR	1.4043	0.8613	1.6305	0.1030
PR	LogR	1.0743	0.4815	2.2312	0.0257
Sex Male	LogR	-0.1953	0.2403	-0.8127	0.4164
CPL	LogR	-0.0160	0.0056	-2.8450	0.0044
Ind He	LogR	-5.0247	2.7110	-1.8535	0.0638

CHAPTER 3
THE GENETIC AND DEMOGRAPHIC OUTCOMES OF MULTIPLE WESTSLOPE CUTTHROAT TROUT REINTRODUCTIONS VIA DIRECT TRANSLOCATIONS

Abstract

Native freshwater species at risk of extinction are often threatened by adverse effects of small population sizes, low genetic diversity, and limited habitat availability. Mixed-source reintroductions, where individuals from multiple source populations are reintroduced together into a single, large vacant habitat, have become an increasingly common method to conserve species threatened by these stressors. Mixing populations with low genetic diversity could theoretically increase and preserve genetic variation, reduce negative genetic effects from decades of inbreeding and small population sizes, and increase species' range-wide abundance and distribution. However, reintroduction outcomes are rarely described in nature, leaving management biologists with limited empirical information for decision-making. We used genetic and demographic data from six mixed-source reintroductions of westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) to evaluate reintroduction outcomes. Our results suggest hybridization, genetic variation, and reintroduction methods may influence reintroduction success. In general, we found that mixed-source reintroductions can serve as a method for preserving among population genetic diversity and increasing genetic variation through source population hybridization.

Introduction

The introduction of nonnative species has severely contributed to the biodiversity crisis, often directly leading to the extirpation of many native species (Cucherousset, Olden 2011). In response, conservation biologists must often eliminate nonnative species and reintroduce native species into historically occupied habitat (e.g., Arnold et al. 2017; Clancey et al. 2019). Consequently, conservation biologists are often faced with determining if more than one source population (if available) should be used for sourcing reintroductions, a decision typically influenced by the extent of isolation and genetic divergence between source populations, remaining source population genetic diversity, and environmental differences between source populations and the reintroduction site (Biebach et al. 2019; Jamieson, Lacy 2012). In many instances however, remnant populations have low genetic diversity, which is not indicative of long-term population persistence (Allendorf et al. 2022; Frankham et al. 2017). Thus, reintroductions involving multiple source populations (i.e., mixed-source reintroductions) have become an increasingly common conservation method to reduce negative genetic and demographic effects of isolation, increase and preserve genetic variation, and ultimately, increase a species' range-wide abundance and distribution (Allendorf et al. 2022; Biebach et al. 2019; Frankham et al. 2017; Jamieson, Lacy 2012). Though mixing and reintroducing multiple populations into viable, vacant habitat (mixed-source reintroductions) could serve as a crucial conservation tool to achieve those objectives, conservation managers are tasked with balancing theoretical benefits and risks without empirical evidence that can inform decision-making.

When remnant populations are small and have little genetic variation, mixed-source reintroductions could be a valuable conservation tool for increasing genetic variation, which is fundamental to population persistence (Ralls et al. 2018; Razgour et al. 2019; Reed, Frankham 2003).

There is growing evidence that higher genetic variation and hybridization between genetically depauperate populations can improve population and individual fitness (Feuerstein et al. 2022; Bell et al. 2022), likely due to the alleviation of inbreeding depression and increase of genetic variation (i.e., heterosis) (Whiteley et al. 2015). This reintroduction strategy could be critical for the long-term persistence and maintenance of genetic variation, providing populations with the necessary genetic material to adapt to changing environmental conditions (Frankham et al. 2017).

However, when populations are mixed, strong genetic divergence and local adaptations among populations could result in a reduction in fitness of hybrid offspring, known as outbreeding depression (OD) (Allendorf et al. 2022; Frankham et al. 2017). Particularly, incompatibilities among populations can occur when populations experience extended periods of geographic isolation and accumulate neutral or advantageous mutations to specific habitats (Allendorf et al. 2022; Edmands 2007). In this case, negative fitness effects from OD may arise in when parental gene combinations (i.e., coadapted gene complexes) are disrupted (Edmands 2007; Turelli, Orr 2000). This process is termed intrinsic OD and will often not arise until the second-generation of matings, or even later. Furthermore, OD can occur in the first-generation when local adaptations are directly disrupted due to a negative interaction between phenotype and environment (i.e., extrinsic OD) (Allendorf et al. 2022; Edmands 2007). Thus, OD could directly influence the success of source populations in mixed-source reintroductions. This exemplifies the need to better describe the genetic outcomes of mixed-source reintroductions.

Conservation biologists are further challenged with determining the number of individuals to be used in a reintroduction. Commonly, this decision is restricted by risks of removing too many individuals from small, genetically depauperate source populations (Biebach et al. 2019; Groombridge et al. 2012). However, translocating too few individuals into

reintroduction sites could influence the initial demographic response in newly re-founded populations through genetic drift, inbreeding depression, and limited reproduction (Biebach et al. 2019; Groombridge et al. 2012). Translocating too few individuals could then affect population growth, genetic variation, and overall reintroduction success (Deredec, Courchamp 2007). As a result, biologists are tasked with maximizing population growth by translocating enough individuals to quickly reach a large population size, while minimizing risks of negatively influencing the genetic and demographic viability of source populations (Jamieson, Lacy 2012). Mixed-source reintroductions can increase the number of translocated individuals by sourcing more individuals spread across multiple populations, and therefore, decrease the likelihood of negative genetic and demographic effects of small population size and slow expansion.

Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) provide an excellent opportunity to empirically describe the genetic and demographic outcomes of mixed-source reintroductions via direct translocation. Westslope cutthroat trout (WCT), a freshwater salmonid native to western North American, have been largely extirpated from their historical range due to habitat degradation and the introduction of nonnative species (Bell et al. 2021; Shepard, May & Urie 2005). Previous WCT studies have described high genetic differentiation (Kovach et al. 2021), variable life-history characteristics (Downs, White & Shepard 1997), and evidence for local adaptation (Drinan et al. 2012) among populations, all of which increase the likelihood of outbreeding depression in reintroduction populations. However, many remnant populations are small, isolated, and have extremely low genetic variation (Kovach et al. 2021) suggesting they have low adaptive potential and may be suffering from inbreeding depression. Competition and hybridization with nonnative species further threaten the persistence of remnant WCT populations (Bell et al. 2021). As a result, biologists have begun removing nonnative species and

reintroducing multiple genetically divergent WCT populations in large, vacant habitat (Arnold et al. 2017; Clancey et al. 2019). In Montana, this conservation strategy is commonly implemented, in part, to achieve the primary management goal of ensuring the long-term self-sustaining persistence of WCT in its historical range (Montana Department of Fish, Wildlife & Parks 2007). The genetic and demographic outcomes of mixed-source reintroductions via direct translocation are unknown, leaving managers with the potential theoretical costs or benefits to make conservation decisions. Broadly, previous WCT reintroduction efforts can be used to better explain how to best preserve genetic variation and adaptive potential, while minimizing the risk of harmful genetic effects (i.e., OD) potentially associated with various restoration and translocation methods.

We present six case studies describing the genetic and demographic outcomes of mixed-source WCT reintroductions to evaluate how different reintroduction methods and mixing divergent source populations influenced reintroduction outcomes. The implementation of each mixed-source reintroduction and resulting response was variable among reintroductions, differing by how individuals were translocated among years and sites, source population genetic variation, and the number of translocated individuals and source populations. To describe how reintroduction methods and population mixing influenced reintroduction success, we quantified and compared the following post-reintroduction demographic and genetic outcomes: (1) source population contribution and mixing, (2) distribution of source population contribution and mixing across sites and years, (3) change in genetic diversity and polymorphic loci, and (4) population abundance and expansion.

Materials and Methods

Study systems. – To restore WCT to historically occupied habitat, all nonnative species above artificial or natural stream barriers were removed from the North, South, and Meadow Forks of Greenhorn (NFG, SFG, and MFG), Peet, Ruby, and Schultz Creeks. Study systems were spread among three subbasins in Montana, however, NFG, SFG, and MFG reintroductions were all within the same watershed (Greenhorn Creek). We analyzed reintroductions in Greenhorn Creek separately since source populations reintroduced in NFG, SFG, and MFG mostly stayed in their respective reintroduction streams due to barriers or lack of among tributary movement. We describe the Greenhorn Creek study system information (i.e., study area history and nonnative removal efforts) in case study 1: NFG.

Sample collection and laboratory procedures. – Non-native species removal was performed by Montana Fish, Wildlife & Parks in partnership with governmental and non-governmental agencies including the U.S. Forest Service, U.S. Bureau of Land Management, and Turner Enterprises Inc. Following nonnative removals, source individuals were captured from thirteen geographically separate populations using a backpack electrofisher. Individuals were collected throughout each source stream to avoid capturing and translocating family groups. All fish were anesthetized, measured to the nearest millimeter, and translocated to recipient reintroduction streams (Figure 3.1; Table. S3. 1). Source individuals used to refound NFG, SFG, and MFG had a small portion of their anal fin removed (tissue sample) to perform population and parentage assignments within corresponding reintroduction streams. Tissue samples were not collected from Peet, Ruby, and Schultz Creek source individuals upon translocation. Thus, we relied on population tissue samples collected in years prior to translocations from Peet, Ruby, and Schultz Creeks to perform population assignments within reintroduction streams.

Reproduction between source individuals began in 2016 in Ruby Creek, 2017 in NFG, SFG, MFG, and Peet, and 2018 in Schultz Creek. We sampled all reintroduction streams in 2019 and/or 2020 to capture first-generation (F_1) offspring from translocated individual reproduction. For all reintroductions, we sampled offspring 200-1,000 meters at translocation release sites, then systematically sampled additional 200-1,000-meter reaches, spaced semi-evenly throughout each system or near areas where reproduction mostly occurred. In NFG, SFG, MFG, and Peet Creeks we performed three-pass 100-meter depletions to estimate abundance. In Ruby Creek, we derived abundance estimates from one-pass electrofishing efforts. Overall, these methods allowed us to sample different stream habitat throughout the entirety of each stream, minimize capturing offspring family groups, and maximize sample size and distance. All offspring were captured using a backpack electrofisher. We collected lengths to the nearest millimeter and genetic tissue samples from all offspring captured.

Briefly, we extracted DNA from all sampled offspring and source individuals (or source populations), then genotyped individuals using genotyping-in-thousands by sequencing (GT-seq) (Campbell, Harmon & Narum 2015). We used an existing GT-seq panel of 373 WCT single-nucleotide-polymorphisms (SNP's) (one of which was a sex ID marker) developed by the Idaho Fish & Game (IDFG) Eagle Fish Genetics Lab (Matt Campbell, IDFG, personal communication). Specific DNA extraction, library preparation, and SNP genotyping and filtering steps are described in Feuerstein et al. (2022).

Since genetically isolated populations can become genetically divergent and develop local adaptations to specific environments (Allendorf et al. 2022; Frankham et al. 2017), we measured environmental metrics in NFG and SFG as an attempt to account for the influence of environmental differences on source population reintroduction success. We installed HOBO MX

water level data loggers (MX2001-04) within each source population stream and at the confluence of NFG and SFG to continually measure stream temperature and water level from spring of 2020 to fall 2021. We used water level data to describe timing of maximum stream flows. Temperature and water level data were compared between source and reintroduction stream to describe environmental differences. Specifically, we estimated the mean daily stream temperature and water level from May to October for NFG and SFG source populations.

Population assignments. – We used programs STRUCTURE (Hubisz et al. 2009) and R package HIPHOP (Cockburn et al. 2021; R Core Team 2020) to assign sampled offspring to source populations and crosstypes (Feuerstein et al 2022). Crosstype assignment results were then used to assign offspring into specific categories, either parental (offspring from within source population matings) or F_1 hybrids (offspring from among source population matings). Here, hybrid refers to within-species matings of individuals from genetically divergent WCT populations. We used STRUCTURE parameter settings and offspring filtering steps described in Feuerstein et al. (2022) and supplemental material.

Population and demographic analyses. – To describe differences in the proportions of parental and hybrid types in NFG, SFG, Ruby, and Peet Creeks, we quantified and compared observed to expected crosstype proportions in each reintroduction stream. We omitted offspring sampled from second and third pass depletion sites from observed offspring counts to avoid overrepresenting certain crosstypes. Expected crosstype proportions were estimated using the initial reintroduction proportions of each source population assuming random mating and equal survival and reproduction from 2016-2020 (Huff, Miller & Vondracek 2010). We performed a chi-square test between observed and expected crosstype proportions to statistically evaluate the difference between expected and observed crosstype proportions. Significance values were

adjusted for multiple testing using sequential Bonferroni procedures (Rice 1989). Statistical assessments of crosstype proportions were computed with the STATS package in R (R Core Team 2020). We also estimated and compared mean source population expected heterozygosity (H_e) and genetic differentiation (F_{ST}) (Weir, Cockerham 1984) among source populations using R package STRATAG (Archer, Adams & Schneiders 2017) and HIERFSTAT (Goudet, Jombart 2021), respectively. Furthermore, we used parentage results from NFG described in Feuerstein et al. (2022) to evaluate the number of translocated NFG individuals that reproduced. We could not make observed vs expected comparisons in MFG and Schultz Creek due to how source populations were translocated, but we were able to broadly evaluate how source populations mixed relative to translocation method. We also evaluated crosstype proportions throughout MFG and Schultz Creek to evaluate how crosstypes were distributed across each system.

We used expected heterozygosity (H_e) and the number of polymorphic loci (P) as measures of source and reintroduction stream population genetic variation to evaluate changes in genetic variation following reintroductions and population mixing. Kovach et al. (2021) described the utility of using both H_e and P as measures of genetic variation due to their opposing strengths and weaknesses. Briefly, H_e provides an accurate measure of population genetic variation based on allele frequencies at loci but, as a result, does not effectively describe low frequency alleles. P treats the presence and absence of all alleles equally and provides a measure for describing low frequency alleles within a population. Thus, we used both statistics to best summarize genetic variation. We also estimated observed heterozygosity (H_o) for crosstypes in each reintroduction stream to describe how population mixing influenced genetic variation relative to source populations. R package STRATAG (Archer, Adams & Schneiders 2017) was used to estimate H_e and P for source populations and reintroduction populations.

To describe how quickly reintroduced WCT populations re-saturated (i.e., filled) habitat in NFG, SFG, MFG, Peet, and Ruby Creeks, we compared abundances of nonnative salmonids prior to WCT reintroduction efforts (i.e., pre-reintroduction) to WCT abundances from 2019-2021 (i.e., post-reintroduction). Juvenile (<100mm) and adult (>100mm) abundances were estimated separately due to widely differing capture probabilities between small and large fish. For post-reintroduction abundances in Peet Creek and all abundances in NFG and SFG, we estimated abundance, capture probabilities, and 95% confidence intervals using the Carle-Strub method (Carle, Strub 1978) implemented within R package FSA (Ogle, Wheeler & Dinno 2021). In MFG and Schultz Creek, we did not have pre-reintroduction abundance data, and therefore, could not make pre- and post-reintroduction abundance comparisons. For pre- and post-reintroduction abundances in Ruby Creek and pre-reintroduction abundances in Peet Creek, abundance was measured using one-pass surveys. Capture probabilities are often similar among headwater WCT streams. Thus, we used NFG and SFG capture probabilities to generate 95% confidence intervals and derive medians for abundance estimates in Ruby and Peet Creeks.

Results

Case 1: NFG, Greenhorn Creek, Montana. – Greenhorn Creek is a large tributary of the Upper Ruby River, located in the Gravely mountains of southwest Montana, USA. Historically, WCT inhabited Greenhorn Creek and its tributaries (i.e., NFG, SFG, and MFG) (Figure. 3.1), but they were mostly replaced by nonnative brook trout (*Salvelinus fontinalis*; EB) and rainbow trout (*O. mykiss*; RBT) over the last several decades. Prior to WCT restoration efforts, NFG and SFG were sampled periodically between 2002 and 2013 to monitor population abundances and hybridization between WCT and RBT. These monitoring efforts described low densities of non-

hybridized WCT and high densities of WCT by RBT hybrids and EB, affirming the need for restoration efforts.

In 2013, an artificial stream barrier was constructed several hundred meters below the confluence of NFG and SFG, isolating a total of approximately 42km of stream habitat and preventing future nonnative species invasions (Figure. 3.2). Following barrier placement, Greenhorn Creek and its tributaries were treated with piscicides in 2013 and 2014 to remove all nonnative salmonids above the artificial barrier. In 2015, extensive eDNA sampling and backpack electrofishing was conducted throughout the stream to verify nonnative removal efforts were successful (Carim et al. 2020). In 2016 and 2017, 323 individual WCT were translocated into NFG from three non-hybridized source populations residing in the Beaverhead River subbasin (Figure. 3.1). Specifically, individuals from BC (32%), BN (33%), and PR (34%) were reintroduced together to a lower (NF05) and upper (NF07) site in 2017 and 2016, respectively (Figure. 3.2; Table. S3. 1). Additionally, a relatively small, remnant population of non-hybridized WCT remained in Dark Hollow Creek (DH; a tributary of NFG). To salvage this remnant non-hybridized population, individuals were removed from the stream during treatment and released back into DH shortly after. With DH included, a total of four genetically divergent WCT populations were initially present within the NFG study area at the beginning of this study.

We sampled 500, 414, and 357 offspring in 2019, 2020, and 2021, correspondingly in NFG (Table. S3. 2). Sampling efforts in 2019 and 2020 were used for genetic and demographic analyses, while sampling in 2021 was only used for demographic analyses. We sampled four locations among sampling years, distributed semi-evenly throughout NFG and spread across locations where source populations were initially introduced (Figure. 3.2). At each sampling site, we performed a 100-meter three-pass depletion. In 2019 and 2020, we sampled approximately

500 meters above and below the three highest depletion sites (i.e., NF05, NF06, NF07) and approximately 200 meters above and below the lowest site (i.e., NF04). Less stream habitat was sampled in the lowest site due to low offspring densities (i.e., colonization of stream habitat initially occurred within upper reaches). Overall, we sampled roughly 4km of stream habitat among sample sites within NFG in 2019 and 2020.

Case 1 Results & Discussion

To describe differences in crosstype proportions and evaluate how crosstypes were distributed throughout NFG, we used HIPHOP (Cockburn et al. 2021) to assign 640 F_1 offspring to parents and parental or hybrid crosstypes (Supplemental; Table. S3. 2). Parental types included BN, BC, and PR crosstypes, and hybrid types consisted of BNxPR, BNxBC, and PRxBC crosstypes. Observed crosstypes were considerably different from random expectation in NFG ($\chi^2 = 358.42$, df = 4, $p < 0.0001$) (Figure. 3.3 A; Table. S3. 3). Specifically, offspring from the most genetically variable parental crosstype (BN; $H_e = 0.368$) were five times more abundant than expected, while offspring from BC, the least genetically diverse parental crosstype ($H_e = 0.062$) were three times less abundant than expected. We also found that offspring from a hybrid cross between the two most genetically diverse populations (BNxPR) were more abundant than expected, while offspring from hybrid crosstypes with BC ancestry were underrepresented.

Parentage results demonstrated that individuals with higher genetic variation had increased reproductive success (i.e., number of offspring and family size) and had a higher probability of survival and reproduction (Feuerstein et al. 2022). Parentage results also described increased fitness of the hybrid crosstype between the two most genetically variable populations (BNxPR) and decreased fitness of hybrid crosstypes with BC ancestry (BNxBC and PRxBC) (Feuerstein et al. 2022). Although BN parents had the most offspring, parentage results revealed

that 40-60% of translocated individuals from each source population typically reproduced each year (Figure. 3.3 B), which highlights a high contribution of all source populations. This range only represents the source individual reproduction that we captured, and thus, may be higher than what we captured. Generally, these results showed: (1) higher parental type success possibly due to higher population- and reproductive success for individuals and source populations with higher genetic variation, (2) variable success for hybrid crosstypes likely due to negative effects associated with having BC ancestry, and (3) a high percentage of reproducing translocated individuals among source populations and years.

NFG population genetic variation increased by 77% and 34% relative to BC and PR source populations, correspondingly, but decreased by 14% compared to BN (Table. S3. 4). The large decrease in genetic variation relative to BN was likely driven by the contribution (mostly by hybridization) of BC paired with their lower genetic variation. The number of polymorphic loci in NFG increased relative to all source populations (Table. S3. 4). The increase of polymorphic loci relative to BN was described by a couple of variable alleles from BC that were not variable or present in BN. The increases in polymorphic loci relative to BC and PR were similarly explained by the variable alleles from BN that were not variable or present in BC and PR.

Crosstype proportions among sample sites and years were consistent with observed vs. expected results, especially in upper reaches (NF05-NF07) where most reproduction occurred. (Figure. 3.3 C). BN offspring abundances were consistently elevated across space and years, while BC offspring were rare. BNxPR offspring were common among sites and years, however, BNxBC and PRxBC offspring abundances varied. Parental crosstype movement from SFG and MFG into NFG was limited, as we observed few CW (n = 4), MD (n = 6), and JK (n = 2)

offspring throughout NFG (Figure. 3.3 C). We did not sample any DH or MF offspring in NFG, suggesting a lack of downstream movement from DH and MFG. Conversely, hybrid crosstype movement from SFG to NFG (CWxMD, *n* = 21) accounted for 85% of offspring sampled in 2019 at site NF04 (Figure. 3.3 C). SFG abundance at site NF04 in 2019 was extremely low (*n* = 7 per 100m) and decreased substantially by 2020 as NFG crosstypes expanded. We suspect that site NF04 initially consisted of primarily SFG crosstypes due to the locations where NFG individuals were initially translocated and reproduced paired with a lack of downstream movement. These results suggest SFG, MFG, and DH source individuals are mostly remaining within their respective translocation or remnant tributaries, and NFG crosstypes initially re-saturated habitat near translocation sites or in upper NFG locations. Additionally, we observed three offspring in NFG from matings between NFG and SFG source populations. All three offspring were from the same MDxBN family. Matings between MD and BN indicate that mixing between SFG, MFG, and NFG source populations will likely occur in the next several generations.

We compared nonnative pre-reintroduction abundances to WCT post-reintroduction abundances and found that WCT had either met or surpassed pre-reintroduction abundances at most sites (NF05-NF07) by 2020 (four years after the start of reintroductions) (Figure. 3.3 D). At the uppermost site (NF07), WCT abundances were nearly two times higher than any recorded nonnative abundance. Site NF04 (the lowest site in NFG) was re-saturated by 2021. Overall, WCT were fully established in NFG within four to five years following translocations of 323 individuals from three source populations. Altogether, WCT were fully established in NFG within seven to eight years following artificial barrier placement and nonnative removal efforts.

Case 2: SFG, Greenhorn Creek, Montana. – In 2016 and 2017, 216 individual WCT were translocated into SFG from two non-hybridized source populations residing in two different subbasins (Figure. 3.1). Individuals from CW (51%) and MD (49%) were reintroduced together to a lower site (SF01) in 2017 and an upper site (SF03) site in 2016. (Figure. 3.2; Table. S3. 1). Following reintroductions, we sampled 145, 133, and 63 offspring in 2019, 2020, and 2021, respectively (Table. S3. 2). Sampling efforts in 2019 and 2020 were used for genetic and demographic analyses, while sampling in 2021 was only used for demographic analyses. We sampled three locations among sampling years, distributed semi-evenly throughout SFG and spread across locations where source populations were initially introduced (Figure. 3.2). At each sample site, we performed a 100-meter three-pass depletion. In 2019 and 2020, we sampled approximately 200 meters above and below all three depletion sites (i.e., SF01, SF02, SF03). Overall, we sampled roughly 1.5km of stream habitat among sample sites within SFG in 2019 and 2020.

Case 2 Results & Discussion

To describe differences in crosstype proportions and evaluate how crosstypes were distributed throughout SFG, we used HIPHOP (Cockburn et al. 2021) to assign 245 F_1 offspring to parental or hybrid types (Supplemental Methods; Table. S3. 2). Parental types included CW and MD crosstypes, and hybrid types consisted of CWxMD crosstypes. Overall, observed crosstypes were substantially different from random expectation in SFG ($\chi^2 = 25.26$, df = 1, $p < 0.0001$) (Figure. 3.4 A; Table. S3. 3). Offspring from CW, the least genetically diverse population (H_e = 0.013) were less abundant than expected, while offspring from the hybrid crosstype (CWxMD) were more abundant than expected. Given results described in NFG by Feuerstein et al. (2022), genetic variation may have influenced CW source population success. Furthermore, more hybrid

offspring than expected could suggest hybridization between source populations increased individual fitness, thereby elevating crosstype success.

SFG population genetic variation increased by 85% relative to CW but decreased by 18% compared to MD (Table. S3. 4). The large decrease in genetic variation relative to MD was likely driven by the contribution of CW (mostly by hybridization) combined with their lower genetic variation. The number of polymorphic loci in SFG increased relative to all source populations (Table. S3. 4). However, this large increase (94% and 28%) relative to source populations CW and MD was mostly driven by variation from two BN source individuals that moved into SFG from NFG.

Crosstype proportions among sites in SFG were fairly consistent among sampling sites, further emphasizing the excess of hybrids throughout SFG (Figure. 3.4 B). Translocating source populations together, paired with a lack of source population movement, might have helped promote hybridization. However, translocation methods do not explain higher hybrid abundances than expected. Moreover, CW offspring were abundant at site SF02 in 2019, but they were practically nonexistent at the same site in 2020. This may suggest CW initially colonized mid-section reaches and were then outcompeted by MD and hybrid crosstypes. Lastly, movement from NFG source populations or offspring into SFG was mostly nonexistent. In 2020 we captured a BN parent at the lowest SFG site (SF01) that was initially translocated to NF05 in 2017. We also captured one offspring from a MDxBN crosstype at site SF01 from a different BN parent. Given the success of BN individuals in NFG, we suspect BN ancestry will gradually continue to increase in SFG.

We compared nonnative pre-reintroduction abundances to WCT post-reintroduction abundances in SFG and found that WCT had met or surpassed pre-reintroduction abundances at

the uppermost sites (SF02 and SF03) by 2019 (three years after the start of reintroductions) (Figure. 3.4 C). At the uppermost site (SF03), WCT abundances were consistently two to three times higher than any recorded nonnative abundance. Site SF01 (the lowest site in SFG) was re-saturated last, meeting pre-reintroduction abundances by 2020. Although we did not capture juveniles among all SFG sites in 2021, WCT were still likely reestablished throughout SFG by 2020 (four years after the start of reintroductions). The absence of nonnative juveniles at site SF02 in 2013, suggest that large density fluctuations might be common within SFG and could be related to environmental variation influencing spawning success and adult or juvenile survival. It is also possible that most source individuals died by 2020 (given most translocated individuals were adults) and first-generation offspring are not yet reproductively mature. Nevertheless, WCT were completely established in SFG within three to four years following translocations of 216 individuals from two source populations. In total, WCT were fully established in NFG within seven to eight years following artificial barrier placement and nonnative removal efforts.

Case 3: MFG, Greenhorn Creek, Montana. – In MFG, a relatively small, remnant population of non-hybridized WCT (MF) was present prior to reintroduction efforts (Figure. 3.1). To salvage MF genetic ancestry, individuals (n = ~25) were removed from the stream during treatment and released back into MFG shortly after. MFG was then sampled using electrofishing and eDNA methods (prior to JK reintroductions) to evaluate the presence of nonnative and MF individuals. In 2015, these efforts confirmed at least one MF individual survived (Carim et al. 2020). Between 2016 and 2018, 148 individual WCT were translocated into MFG and the North Fork (NF) of NFG from a single non-hybridized source population (JK) residing in the Ruby River subbasin (i.e., the same subbasin as Greenhorn Creek) (Figure. 3.1; Table S3. 1). These JK translocations were split between the NF of NFG and MFG, where 49 and 99 individuals were translocated into

NF of NFG and MFG, respectively (Figure. 3.2). In 2019 we sampled 65 individuals at site MF09 within MFG, where JK individuals were initially translocated. At site MF09 we sampled 100 meters, then sampled 200 meters below (Below) and above (Above) the 100-meter reach. These sampling efforts were only used for genetic analyses since nonnative abundance data was not collected for demographic comparisons. Overall, we sampled approximately 500 meters of stream habitat within MFG in 2019.

Case 3 Results & Discussion

We used genetic data to evaluate the presence and reproduction of remnant MF individuals in MFG. We used program STRUCTURE (Hubisz et al. 2009) to assign 65 individuals to three crosstypes: parental (MF and JK) and hybrid (JKxMF) (Supplemental Methods). Crosstype assignments revealed that MF individuals successfully reproduced (Figure. 3.5). Specifically, 22% of individuals sampled within MFG had MF ancestry. We also observed several hybrids (n = 12) and MF parental crosstypes (n = 2) at two of the three sample locations (Figure. 3.5). Though sample size and effort were low within MFG, our results suggest that a few remnant MF source individuals were able to contribute to the first-generation. Given extremely few MF individuals were detected after release in 2015 and only 40-60% of those remaining individuals likely reproduced (as described in NFG), it is quite possible that MF ancestry, and hybrids specifically, were overrepresented in this case study (i.e., relative to the number of MFG individuals that survived). However, we could not empirically test this hypothesis.

MFG population genetic variation increased by 8% relative to JK but decreased by 11% compared to MF (Table. S3. 4). The large decrease in genetic variation relative to MF was likely due to the high contribution of JK paired with their lower genetic variation. The number of polymorphic loci in MFG increased relative to all source populations (Table. S3. 4). The increase

of polymorphic loci relative to JK was described by several variable alleles in MF that were not variable or present in JK and vice versa.

Case 4: Peet Creek, Montana. – Peet Creek is a tributary of the Upper Red Rock River, located in the Centennial mountains of southwest Montana, USA (Figure. 3.1). Historically, WCT inhabited Peet Creek and its tributaries. However, Peet Creek WCT were found to be hybridized with nonnative RBT in 2012 (data not shown). RBT and WCT hybridization occurred within approximately 11.4km of stream habitat, isolated by an existing barrier and impoundment (pond). Prior to WCT restoration efforts, three locations were sampled throughout Peet Creek in 2012 to describe population abundances and the extent of RBT and WCT hybridization. These monitoring efforts displayed high densities of WCT by RBT hybrids, affirming the need for restoration efforts.

In 2013 and 2014, Peet Creek and its tributaries were treated with piscicides to remove all salmonids above the existing barrier. In 2015, extensive eDNA sampling and backpack electrofishing was conducted throughout the stream to verify removal efforts were successful. Between 2016 and 2018, 99 individual WCT were then translocated into Peet Creek from two non-hybridized source populations residing in the Red Rock subbasin (i.e., same subbasin as Peet Creek) (Figure. 3.1; Table S3. 1). Individuals from BE (n = 25) were translocated directly into the pond/barrier in 2016, and BR individuals (n = 26) were translocated slightly above the barrier in 2017. In 2018, both BE (n = 23) and BR (n = 25) individuals were reintroduced together to a higher site, between sites LP02 and LP03 (Figure. 3.6; Table. S3. 1). Following reintroductions, we sampled 25 and 68 individuals in 2019 and 2020, respectively (Table. S3. 2). We sampled five locations among sampling years, distributed throughout Peet Creek and spread across locations where source populations were initially introduced or reproducing (Figure. 3.6). We

performed a single pass 100-meter abundance estimate at two sites in 2019 (LP02 and MF) and a three-pass 100-meter depletion at four sites (LP02, LP03, MF, and WF) in 2020. We also sampled approximately 200 meters below site MF (UP) in 2020 to increase sample size near reaches where reproduction occurred. All sampling efforts in 2019 and 2020 were used for genetic and demographic analyses. Overall, we sampled roughly 800m of stream habitat among sampling sites and years within Peet Creek.

Case 4 Results & Discussion

To describe differences in crosstype proportions and evaluate how crosstypes were distributed throughout Peet Creek, we used program STRUCTURE (Hubisz et al. 2009) to assign 93 individuals to parental or hybrid types (Supplemental Methods; Table. S3. 2). Parental types included BE and BR crosstypes, and hybrid types consisted of BExBR crosstypes. Overall, observed crosstypes were substantially different from random expectation in SFG ($\chi^2 = 52.4$, df = 1, $p < 0.0001$) (Figure. 3.7 A; Table. S3. 3). Crosstype observed vs expected comparisons revealed that hybrids (BExBR) were nearly two times more abundant than expected, while both parental types were more than two times less abundant than expected. The lack of offspring from both parental crosstypes in Peet Creek may be partially explained by initial translocation methods. For example, BE source individuals were released directly into the above barrier pond in 2016, which has recently been recognized as potentially poor habitat (Lucas Bateman, MTFWP, Personal Communication). BE source individuals may have also been influenced by beaver dams directly above the pond impeding upstream movement. Since BR source individuals were translocated directly above the pond the following year, they might have had higher chances of survival and reproduction simply due to habitat quality differences. Nevertheless, if translocation location issues were severely influencing crosstype success, we should have also

observed fewer hybrid crosstypes. Instead, hybridization between source populations might have increased individual fitness, and thus elevated hybrid crosstype success and abundance.

Peet Creek population genetic variation increased by 1% and 35% relative to BE and BR source populations (Table. S3. 4). The increase in genetic variation relative to BE and BR was likely explained by the excess of among population matings (i.e., hybridization; BExBR) and lack of within population matings (BE and BR). The number of polymorphic loci in Peet Creek increased relative to all source populations (Table. S3. 4). The increase of polymorphic loci relative to BR was described by a several variable alleles in BE that were not variable or present in BR, and vice versa.

Crosstype proportions among sample sites in Peet Creek were mostly consistent with observed vs. expected analyses, further showing the excess of hybrids throughout Peet Creek (Figure. 3.7 B). Particularly, hybrid crosstypes accounted for 80-100% of individuals sampled at the three uppermost sites (UP, MF, and WF). Parental crosstype BR mostly occupied lower reaches (LP02 and LP03) near where source individuals were initially translocated in 2016 and 2017, while parental crosstype BE was only observed at one sampling site near upper stream reaches. These results further suggest translocation methods in 2018 whereby source populations were translocated together in a higher stream reach, may have had positive effects on upstream abundance by promoting hybridization. However, translocation methods do not explain higher hybrid abundances than expected.

We compared nonnative pre-reintroduction abundances to WCT post-reintroduction abundances in Peet Creek and found that WCT had almost re-saturated habitat in the uppermost sites (MF and WF) by 2020 (Figure. 3.7 C), which consisted of both juvenile and adult WCT. Conversely, WCT did not re-saturate habitat in lower reaches (LP02 and LP03) by 2020, and

only adults were observed. These results indicate reproduction mostly occurred within the uppermost reaches of Peet Creek. Though WCT abundances increased from 2019 to 2020, overall WCT abundances did not meet pre-reintroduction abundances by 2020 (five years following the first reintroduction).

Case 5: Ruby Creek, Montana. – Ruby Creek is a large tributary of the Upper Madison River, located in the Gravely mountains of southwest Montana, USA (Figure. 3.1). Historically, 12km of Ruby Creek was unoccupied by salmonids due to a natural stream barrier (waterfall) at the lower end of the stream. However, sampling efforts in 1997 confirmed RBT were previously stocked above the waterfall, warranting removal efforts prior to WCT reestablishment. In 1997, an abundance estimate occurred at an upper and lower location.

In 2012 and 2013, Ruby Creek and its tributaries were treated with piscicides to remove all salmonids above the waterfall barrier. In 2014, extensive eDNA sampling and backpack electrofishing was conducted throughout the stream to verify removal efforts were successful. Between 2015 and 2018, 71 individual WCT were translocated into Ruby Creek from two non-hybridized source populations residing in the Madison River subbasin (i.e., same subbasin as Ruby Creek) (Figure. 3.1; Table S3. 1). Individuals from MC were translocated separately to an upper site in 2015 (n = 20) and lower site in 2017 (n = 15) and 2018 (n = 12). In 2016, both MC (n = 14) and LC (n = 10) individuals were reintroduced together and between the two previous reintroduction sites (Figure. 8; Table. S1). Following reintroductions, we sampled 102 WCT in 2020 (Table. S3. 2). We sampled twelve locations distributed semi-evenly throughout Ruby Creek and spread across locations where source populations were initially introduced (Figure. 3.8). We performed a single pass at each site, sampling 150-200-meter reaches. All sampling

efforts in 2020 were used for genetic and demographic analyses. Overall, we sampled roughly 2,200m of stream habitat among sampling sites within Ruby Creek.

Case 5 Results & Discussion

To describe differences in crosstype proportions and evaluate how crosstypes were distributed throughout Ruby Creek, we used program STRUCTURE (Hubisz et al. 2009) to assign 102 individuals to parental or hybrid types (Supplemental Methods; Table. S3. 2). Parental types included MC and LC crosstypes, and hybrid types consisted of MCxLC crosstypes. Overall, observed crosstypes were substantially different from random expectation in Ruby Creek (χ^2 = 16.6, df = 1, $p < 0.0001$) (Figure. 3.9 A; Table. S3. 3). Crosstype comparisons revealed that hybrids (MCxLC) were nearly two times more abundant than expected, while both observed parental types were slightly less abundant than expected. An excess of hybrids suggests hybridization may have had positive fitness effects and ultimately elevated hybrid crosstype success and abundance. Although LC source individuals only accounted for 14% of the total source individuals translocated into Ruby Creek, 61% of individuals sampled in 2020 had LC ancestry (either hybrid or parental). The elevated abundance of LC ancestry throughout Ruby Creek indicates few source individuals can successfully contribute to population reestablishment when managers cannot source many individuals from small, isolated populations. Also, observed offspring abundances from the parental crosstype with higher genetic variation (LC; H_e = 0.059) were closer to expected abundances compared to the parental crosstype with the lowest genetic variation (MC; H_e = 0.041). However, it was difficult to make any inferences regarding a relationship between crosstype success and genetic variation due to small sample size.

Ruby Creek population genetic variation increased by 45% and 8% relative to MC and LC source populations (Table. S3. 4). The increase in genetic variation relative to MC and LC

was likely explained by the excess of among population matings (i.e., hybridization; MCxLC) and lack of within population matings (MC and LC). The number of polymorphic loci in Ruby Creek increased relative to all source populations (Table. S3. 4). The increase of polymorphic loci relative to MC was described by a several variable alleles in LC that were not variable or present in MC, and vice versa.

Crosstype proportions among sampling sites in Ruby Creek were mostly consistent with observed vs. expected analyses, further emphasizing the excess of hybrids throughout Ruby Creek (Figure. 3.9 B). Specifically, hybrid crosstypes were present within every site throughout Ruby Creek, while parental crosstypes varied between upper and lower sites. Hybrid crosstypes made-up at least 50% of individuals sampled at five sampling locations. Parental crosstype LC only occupied a single lower reach (i.e., $n = 2$), and parental crosstype MC was only observed throughout upper reaches. Crosstype proportions by site paired with abundance data indicate reproduction mostly occurred within the upper reaches of Ruby Creek, as juveniles were only found just upstream of the 2016 reintroduction site. Interestingly, the 2016 reintroduction site was the only site where both LC and MC source individuals were translocated, suggesting these translocation methods may have positively influenced abundance by promoting hybridization. However, translocation methods do not explain higher hybrid abundances than expected.

We compared nonnative pre-reintroduction abundances to WCT post-reintroduction abundances in Ruby Creek and found that WCT were below nonnative abundances at one lower and upper site (Figure. 3.9 C). Additionally, at ten of the twelve sampling sites we only observed adults. Juveniles were only observed within upper reaches 8 and 9. It was difficult to make inferences regarding WCT re-saturation rates due to a lack of pre-reintroduction sampling data.

Nonetheless, the low abundances of juveniles suggest WCT were not reestablished in Ruby by 2020 (five years following the first reintroduction).

Case 6: Schultz Creek, Montana. – Schultz Creek is a small tributary of the Big Hole River, located in the Anaconda mountains of southwest Montana, USA (Figure. 3.1). Historically, 6.5km of Schultz Creek was thought to be unoccupied by salmonids due to a natural stream barrier (cascades) at the lower end of the stream. However, sampling efforts in 2014 confirmed nonnative Yellowstone cutthroat trout (*Oncorhynchus clarkii bourvieri*; YCT) existed above the cascade barrier, warranting removal efforts prior to WCT reestablishment.

In 2015 and 2016, Schultz Creek and its tributaries were treated with piscicides to remove all salmonids above the barrier. Shortly after treatment in 2016, eDNA sampling and backpack electrofishing was conducted throughout the stream to verify removal efforts were successful. In 2017, 60 individual WCT were then translocated into Schultz from two non-hybridized source populations residing in the Big Hole subbasin (i.e., same subbasin as Schultz Creek) (Figure. 3.1; Table S3. 1). Individuals from PM (n = 30) and HR (n = 30) were translocated separately to an upper and lower site, respectively (Figure. 3.10; Table. S3. 1). Following reintroductions, we sampled 176 individual WCT in 2020 (Table. S3. 2). We sampled seven locations distributed between locations where source populations were initially introduced (Figure. 3.10). We performed a single pass at each site, sampling 150-200-meter reaches. These sampling efforts were only used for genetic analyses since nonnative abundance data were not collected for demographic comparisons. Overall, we sampled roughly 1,100m of stream habitat among sampling sites within Schultz.

Case 6 Results & Discussion

To describe crosstype proportions among sampling sites we used program STRUCTURE (Hubisz et al. 2009) to assign 157 offspring to parental or hybrid crosstypes. Initial genetic analyses clearly identified the presence of YCT ancestry (i.e., YCT hybrids were not successfully removed from Schultz Creek). Therefore, we assigned offspring to one of six potential crosstypes: parental (PM, HR, or YCT) or hybrid (PMxHR, PMxYCT, or HRxYCT). Here, PMxYCT and HRxYCT are nonnative hybrids. Crosstype proportions among sampling sites confirmed nonnative YCT removal efforts were not successful and that YCT hybrids readily hybridized with translocated WCT following reintroductions. Specifically, YCT hybridization occurred within two sampling locations located between WCT reintroduction sites (Figure. 3.11). Furthermore, crosstype proportions indicate WCT source populations mostly remained close to their reintroduction locations and did not mix during three years of occupancy, despite translocation locations being only about 1.5km apart. Though YCT could have influenced mixing between source populations, these results suggest initial source population movement from reintroduction sites can be rare.

Schultz Creek population genetic variation increased by 44% relative to HR and decreased by 21% compared to PM (Table. S3. 4). The large decrease in genetic variation relative to PM was likely due to hybridization events between PM and nonnative YCT hybrids, where genetic variation from PM was essentially lost upon hybridizing with YCT hybrids. The number of polymorphic loci in Schultz Creek increased relative to all source populations (Table. S3. 4). The increase of polymorphic loci relative to HR was described by a several variable alleles in PM that were not variable or present in HR, and vice versa.

Discussion

We aimed to empirically describe the outcomes of several mixed-source reintroductions to better understand how translocation methods and mixing divergent populations influence reintroduction success. Since genetic variation is fundamental to population persistence (Ralls et al. 2018; Razgour et al. 2019; Reed, Frankham 2003), and in-turn may influence the success of translocated source populations, we described relationships between reintroduction success and source population genetic variation. Genetic results from three of our four study systems indicate that genetic variation may have influenced reintroduction success. Specifically, parentage results in NFG demonstrated that genetic variation predicted reintroduction success, where individuals with higher genetic variation had increased fitness. Similarly, population results in SFG and Ruby Creek suggested parental crosstypes with higher genetic variation were more successful relative to parental crosstypes with lower genetic variation. Although we were unable to link genetic variation to fitness in SFG and Ruby Creek, combined reintroduction outcomes in NFG, SFG, and Ruby Creek suggest individual- and population genetic variation may influence reintroduction success. These results do not imply that population genetic variation should be the only consideration when selecting source populations. Alternatively, genetic variation should be considered as an essential conservation management tool when implementing mixed-source reintroductions. For instance, translocating different populations with similar levels of genetic variation might be beneficial for balancing success and promoting reproduction. On the other hand, when managers are left with few candidate source populations with widely differing genetic variation, our genetic results among six reintroductions revealed that all source populations contributed to the next generation, regardless of limited genetic variation.

Mixing small, genetically depauperate populations may serve as a method for improving population and individual fitness (Whiteley et al. 2015), and therefore, we described relationships between reintroduction success and source population hybridization. Our genetic results from five study systems suggest that hybridization positively influenced reintroduction outcomes. Particularly, we observed an excess of hybrids in SFG, Ruby, and Peet Creeks. Though we could not empirically describe hybrid abundances in MFG, we suspect hybrids were also more abundant than expected. Additionally, we found elevated hybrid abundances in one of three hybrid crosstypes in NFG, however, hybrid crosstype success was likely negatively influenced by ancestry from the least genetically diverse source population (BC). In general, hybrids were abundant throughout each study system, which suggest hybrid crosstype success was not primarily driven by stream location (i.e., specific habitats). Overall, these results cumulatively suggest hybridization among source populations increased crosstype success, which may have also positively influenced population expansion. These results do not imply that hybridization between genetically divergent populations will always be beneficial. Specifically, hybridization between genetically divergent populations might be disadvantageous when populations have been isolated for extended periods of time, have developed local adaptations to specific environmental conditions, or are highly genetically divergent (Allendorf et al. 2022; Biebach et al. 2019; Frankham et al. 2017; Jamieson, Lacy 2012). Under such circumstances, hybridization may lead to outbreeding depression, which typically does not arise until later generations (i.e., beyond the first-generation) (Edmands 2007). Even though genetic differentiation between source populations was high (Table. S3. 5) and our results only include first-generation offspring, outbreeding depression in these systems is unlikely to cancel our results because: (1) all source populations were translocated into new habitat, reducing potential

extrinsic local adaptation disruptions, and (2) we do not suspect any extreme differences in structural genomic variation since source populations have not been isolated long (< 200 years) (Frankham et al. 2017).

Although we demonstrated that genetic variation and hybridization influenced reintroduction success, other biotic and abiotic aspects of source populations should also be considered when implementing mixed-source reintroductions. For example, Feuerstein et al. (2020) showed that parent body length influenced fitness (i.e., reintroduction success). This relationship is well documented in salmonid species, as maternal and paternal length can often influence fecundity and reproductive success, respectively (Downs, White & Shepard 1997; Koch, Narum 2021). However, the relationship between parental body size and fitness highlights that parental body size should be considered when translocating individuals, as larger source individuals could initially have increased success relative to smaller individuals. Furthermore, environmental differences between source and reintroduction streams may influence source population reintroduction success.

We attempted to account for differences in population success due to environmental effects (or habitat mismatching) by comparing daily stream temperatures and timing of peak stream flows between NFG and SFG source populations and Greenhorn Creek. We found no difference in timing of peak flows among streams (Figure. S3. 1), however, stream temperatures in the least successful NFG source population (BC) were most dissimilar to Greenhorn Creek (Figure. 3.12). We also found that stream temperatures in SFG source population CW were consistently warmer relative to Greenhorn Creek, while source population MD was consistently cooler. Such differences might have influenced CW and MD parental crosstype success. These results suggest environmental differences between source and reintroduction streams exist and

may play a role in source population success, ultimately demonstrating the need to better describe how source population habitat mismatching could influence reintroduction efforts.

The number of individuals used in reintroductions, along with how translocated individuals are reintroduced, may affect reintroduction outcomes (Deredec, Courchamp 2007; Groombridge et al. 2012). Specifically, translocation methods might influence how quickly, or slowly reintroduced populations re-fill habitat. Abundance in Peet and Ruby Creeks had not met historical nonnative abundances four to five years following initial reintroductions. Conversely, NFG and SFG demographic data suggested that reintroduced populations had either met or surpassed pre-reintroduction abundances four to five years following initial reintroductions. Nearly twice as many individuals were reintroduced into NFG and SFG compared to Peet and Ruby Creeks, suggesting translocating more individuals positively influenced population expansion, as expected. However, population expansion in Peet Creek was likely negatively influenced by initial translocation methods, where individuals were possibly released in poor habitat. Peet and Ruby Creek reintroductions were also restricted by source population size, and therefore, fewer individuals were translocated to avoid damaging source populations. In such instances, reintroductions that involved translocating individuals from two or more source populations into the same location may have promoted hybridization between source populations, which might have positively influenced population expansion. Though we could not empirically test this observation, we observed increased population abundance and hybrids throughout multiple study systems where source populations were translocated together, suggesting introducing source populations together might be beneficial for increasing population expansion.

Lastly, we evaluated changes in genetic variation between source and reintroduction populations and found variable results among reintroductions (Table. S3. 4). In NFG, SFG, MFG, and Schultz Creek we found that genetic variation in the reintroduction population was always less than the most genetically diverse source population. In Schultz we suspect that hybridization between WCT and nonnative YCT hybrids negatively influenced population genetic variation. However, lower genetic variation in NFG, SFG, and MFG was likely driven by the success of source populations with low genetic variation through hybridization. Alternatively, Peet and Ruby Creek genetic variation increased relative to source population genetic variation. We suspect increased genetic variation in Peet and Ruby Creeks was driven by the excess of hybrids and lack of parental crosstypes, particularly the parental crosstypes with higher genetic variation. These results highlight that mixed-source reintroductions will not always increase genetic variation, and instead, changes in genetic variation will likely vary among reintroductions depending on source individual survival, reproduction, contribution, and hybridization. Furthermore, we observed an increase in the number of polymorphic loci among all reintroductions relative to source populations, suggesting mixed-source reintroductions generally increase the number of polymorphic sites across SNP's. We also described changes in crosstype genetic variation to evaluate how hybridization influenced genetic variation and found that hybrids typically had higher genetic variation compared to source populations (Figure. 3.13). Oddly, the little genetic variation left in CW was also present in MD, and thus, CWxMD genetic variation only slightly increased. Source population BN is one of the most genetically diverse WCT populations east of the continental divide, and harbors much of the same genetic variation left in source populations BC and PR. Therefore, hybridization among source populations BC, BN, PR did not increase genetic variation. Overall, these results suggest hybridization generally

increases the genetic variation of hybrid crosstypes, however, increased genetic variation through hybridization will ultimately depend on source population genetic variation and contribution.

Conclusions

In general, our results demonstrate the utility and efficacy of mixed-source reintroductions when remnant populations are small, isolated, and genetically depauperate. We directly show that mixed-source reintroductions can serve as a method for preserving among population genetic diversity, while also increasing genetic variation through source population hybridization, which can increase reintroduction success. We also demonstrate how mixed-source reintroductions can be used to quickly restore new populations into protected habitats across historically occupied watersheds. These results are relevant to many freshwater fish species worldwide, especially as populations are continually pushed towards extinction by anthropogenic effects (Jelks et al. 2008; Su et al. 2021). Although mixed-source reintroductions are likely a viable tool for freshwater fish conservation, it is imperative that these efforts are continually monitored to further understand the genetic and demographic consequences associated with such practices.

References

Allendorf, F., C. Funk, S. Aitken, M. Byrne, and G. Luikart. 2022. Conservation and the genomics of populations, 3rd edition. Oxford University Press, Oxford, United Kingdom.

Archer, F. I., P. E. Adams, and B. B. Schneiders. 2017. Stratag: An r package for manipulating, summarizing and analysing population genetic data. Molecular Ecology Resources 17(1):5-11.

Arnold, J. L., C. R. Detjens, B. D. Ertel, M. E. Ruhl, and T. M. Koel. 2017. Westslope cutthroat trout and fluvial arctic grayling restoration. Yellowstone Science 25(1):18-25.

Bell, D. A., R. P. Kovach, C. C. Muhlfeld, R. Al-Chokhachy, T. J. Cline, D. C. Whited, D. A. Schmetterling, P. M. Lukacs, and A. R. Whiteley. 2021. Climate change and expanding invasive species drive widespread declines of native trout in the northern rocky mountains, USA. Science Advances 7(52):eabj5471.

Biebach, I., D. M. Leigh, K. Sluzek, and L. F. Keller. 2019. Genetic issues in reintroduction. Pages 149-184 *in* Genetic issues in reintroduction. Reintroduction of fish and wildlife populations. University of California Press, Berkeley.

Campbell, N. R., S. A. Harmon, and S. R. Narum. 2015. Genotyping-in-thousands by sequencing (GT-seq): A cost effective SNP genotyping method based on custom amplicon sequencing. Molecular Ecology Resources 15(4):855-867.

Carim, K. J., N. J. Bean, J. M. Connor, W. P. Baker, M. Jaeger, M. P. Ruggles, K. S. McKelvey, T. W. Franklin, M. K. Young, and M. K. Schwartz. 2020. Environmental DNA sampling informs fish eradication efforts: Case studies and lessons learned. North American Journal of Fisheries Management 40(2):488-508.

Carle, F. L., and M. R. Strub. 1978. A new method for estimating population size from removal data. Biometrics 34(4):621-630.

Clancey, P. T., B. B. Shepard, C. G. Kruse, S. A. Barndt, L. Nelson, B. C. Roberts, and B. R. Turner. 2019. Collaboration, commitment, and adaptive learning enable eradication of nonnative trout and establishment of native westslope cutthroat trout into one-hundred kilometers of cherry creek, a tributary of the madison river, montana. American Fisheries Society Symposium 91:589-647.

Cockburn, A., J. V. Peñalba, D. Jaccoud, A. Kilian, L. Brouwer, M. C. Double, N. Margraf, H. L. Osmond, L. E. B. Kruuk, and M. van de Pol. 2021. HIPHOP: Improved paternity assignment among close relatives using a simple exclusion method for biallelic markers. Molecular Ecology Resources 21(6):1850-1865.

Cucherousset, J., and J. D. Olden. 2011. Ecological impacts of nonnative freshwater fishes. Fisheries (Bethesda) 36(5):215-230.

Deredec, A., and F. Courchamp. 2007. Importance of the allee effect for reintroductions. Écoscience (Sainte-Foy) 14(4):440-451.

Downs, C. C., R. G. White, and B. B. Shepard. 1997. Age at sexual maturity, sex ratio, fecundity, and longevity of isolated headwater populations of westslope cutthroat trout. North American Journal of Fisheries Management 17(1):85-92.

Drinan, D. P., A. V. Zale, M. A. H. Webb, M. L. Taper, B. B. Shepard, and S. T. Kalinowski. 2012. Evidence of local adaptation in westslope cutthroat trout. Transactions of the American Fisheries Society (1900) 141(4):872-880.

Edmands, S. 2007. Between a rock and a hard place: Evaluating the relative risks of inbreeding and outbreeding for conservation and management. Molecular Ecology 16(3):463-475.

Frankham, R., J. D. Ballou, K. Ralls, M. D. B. Eldridge, M. R. Dudash, C. B. Fenster, R. C. Lacy, P. Sunnucks, and K. McInnes. 2017. Genetic management of fragmented animal and plant populations, First edition. Oxford University Press, Oxford, United Kingdom.

Goudet, J., and T. Jombart. 2021. Hierfstat: Estimation and tests of hierarchical F-statistics.

Groombridge, J. J., C. Raisin, R. Bristol, and D. S. Richardson. 2012. Genetic consequences of reintroductions and insights from population history. Pages 395-440 *in* Genetic consequences of reintroductions and insights from population history. Reintroduction biology. John Wiley & Sons, Ltd, Chichester, UK.

Hubisz, M. J., D. Falush, M. Stephens, and J. K. Pritchard. 2009. Inferring weak population structure with the assistance of sample group information. Molecular Ecology Resources 9(5):1322-1332.

Huff, D. D., L. M. Miller, and B. Vondracek. 2010. Patterns of ancestry and genetic diversity in reintroduced populations of the slimy sculpin: Implications for conservation. Conservation Genetics 11(6):2379-2391.

Jamieson, I. G., and R. C. Lacy. 2012. Managing genetic issues in reintroduction biology. Pages 441-475 *in* Managing genetic issues in reintroduction biology. Reintroduction biology. John Wiley & Sons, Ltd, Chichester, UK.

Jelks, H. L., S. J. Walsh, N. M. Burkhead, S. Contreras-Balderas, E. Diaz-Pardo, D. A. Hendrickson, J. Lyons, N. E. Mandrak, F. McCormick, J. S. Nelson, S. P. Platania, B. A. Porter, C. B. Renaud, J. J. Schmitter-Soto, E. B. Taylor, and M. L. Warren. 2008. Conservation status of imperiled north american freshwater and diadromous fishes. Fisheries (Bethesda) 33(8):372-407.

Koch, I. J., and S. R. Narum. 2021. An evaluation of the potential factors affecting lifetime reproductive success in salmonids. Evolutionary Applications 14(8):1929-1957.

Kovach, R. P., R. F. Leary, D. Bell, S. Painter, A. Lodmell, and A. R. Whiteley. 2021. Genetic variation in westslope cutthroat trout reveals that widespread genetic rescue is warranted. Canadian Journal of Fisheries and Aquatic Sciences.

Montana Department of Fish, Wildlife & Parks. 2007. Memorandum of understanding and conservation agreement for westslope cutthroat trout and yellowstone cutthroat trout in montana.

Ogle, D. H., P. Wheeler, and A. Dinno. 2021. FSA: Fisheries stock analysis, 0.8.32. https://github.com/droglenc/FSA.

R Core Team. 2020. R: A language and environment for statistical computing, R Foundation for Statistical Computing.

Ralls, K., J. D. Ballou, M. R. Dudash, M. D. B. Eldridge, C. B. Fenster, R. C. Lacy, P. Sunnucks, and R. Frankham. 2018. Call for a paradigm shift in the genetic management of fragmented populations. Conservation Letters 11(2).

Razgour, O., B. Forester, J. B. Taggart, M. Bekaert, J. Juste, C. Ibáñez, S. J. Puechmaille, R. Novella-Fernandez, A. Alberdi, and S. Manel. 2019. Considering adaptive genetic variation in climate change vulnerability assessment reduces species range loss projections. Proceedings of the National Academy of Sciences 116(21):10418-10423.

Reed, D. H., and R. Frankham. 2003. Correlation between fitness and genetic diversity. Conservation Biology 17(1):230-237.

Rice, W. R. 1989. Analyzing tables of statistical tests. Evolution 43(1):223.

Shepard, B. B., B. E. May, and W. Urie. 2005. Status and conservation of westslope cutthroat trout within the western united states. North American Journal of Fisheries Management 25(4):1426-1440.

Su, G., M. Logez, J. Xu, S. Tao, S. Villéger, and S. Brosse. 2021. Human impacts on global freshwater fish biodiversity. Science 371(6531):835-838.

Turelli, M., and H. A. Orr. 2000. Dominance, epistasis and the genetics of postzygotic isolation. Genetics (Austin) 154(4):1663-1679.

Weir, B. S., and C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution 38(6):1358-1370.

Whiteley, A. R., S. W. Fitzpatrick, W. C. Funk, and D. A. Tallmon. 2015. Genetic rescue to the rescue. Trends in Ecology & Evolution 30(1):42-49.

Figures

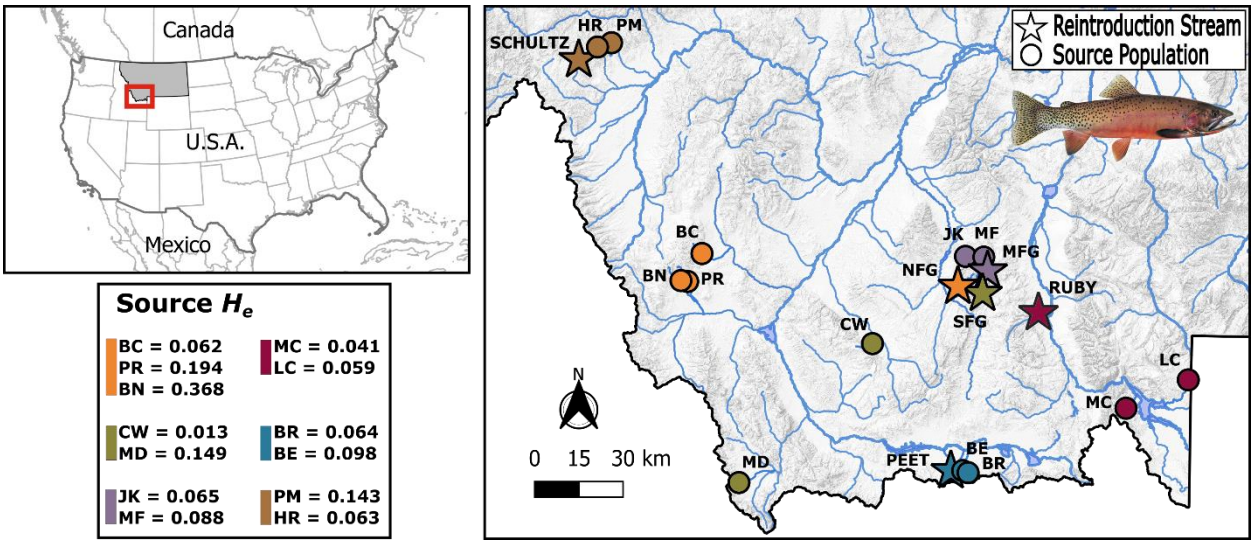

Figure 3. 4: Study area in Southwest Montana showing all reintroduction sites (stars), source populations (circles), and source population expected heterozygosity (Source H_e). Colors correspond to each case study.

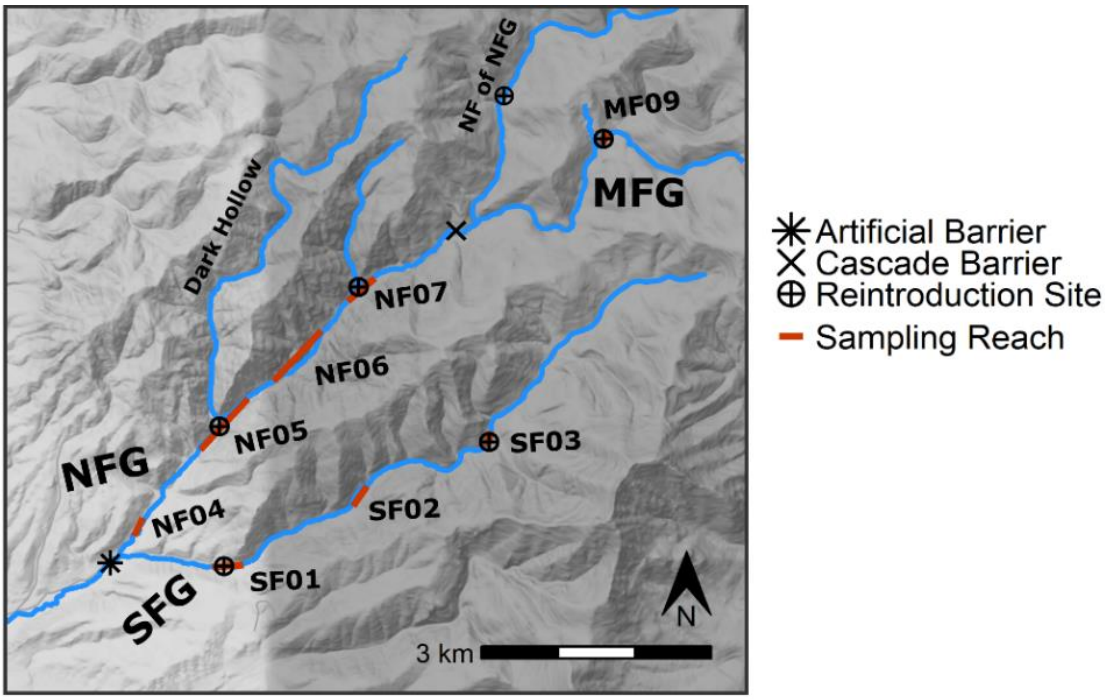

Figure 3. 2: Greenhorn Creek Montana, USA study system including NFG, SFG, and MFG reintroduction, barrier, and sampling locations. Sampling reaches (red bars) depict all habitat sampled from 2019 to 2021.

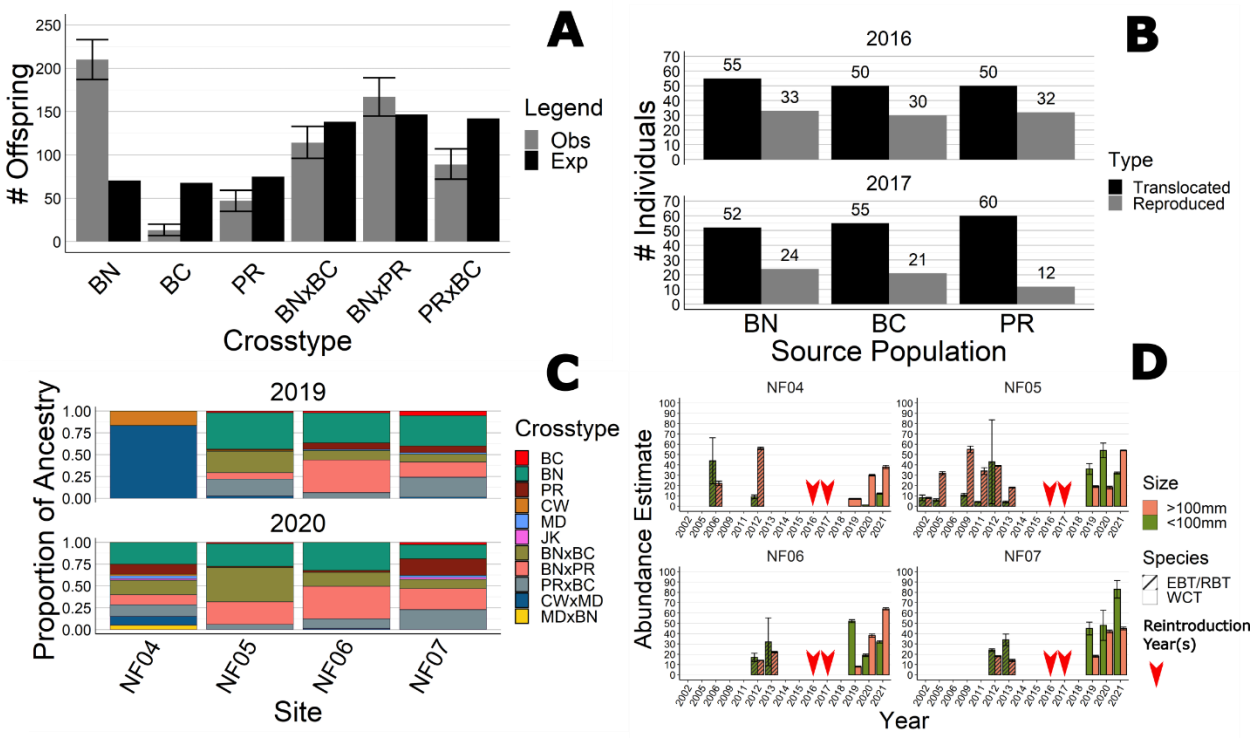

Figure 3. 3: NFG genetic and demographic results. (A) The number of observed (grey bars) and expected (black bars) first-generation offspring assigned to crosstypes (parental or hybrid). (B) The number of individuals translocated (black bars) and that reproduced (grey bars, based on parentage analysis) from each source population and translocation year. (C) Proportion of offspring crosstypes shown among sites and years. (D) Pre-reintroduction nonnative abundances of brook trout (EB) and rainbow trout (RBT) compared to post-reintroduction WCT abundances. Sample sites are ordered from the lower (NF04) to upper (NF07) stream sites from left to right.

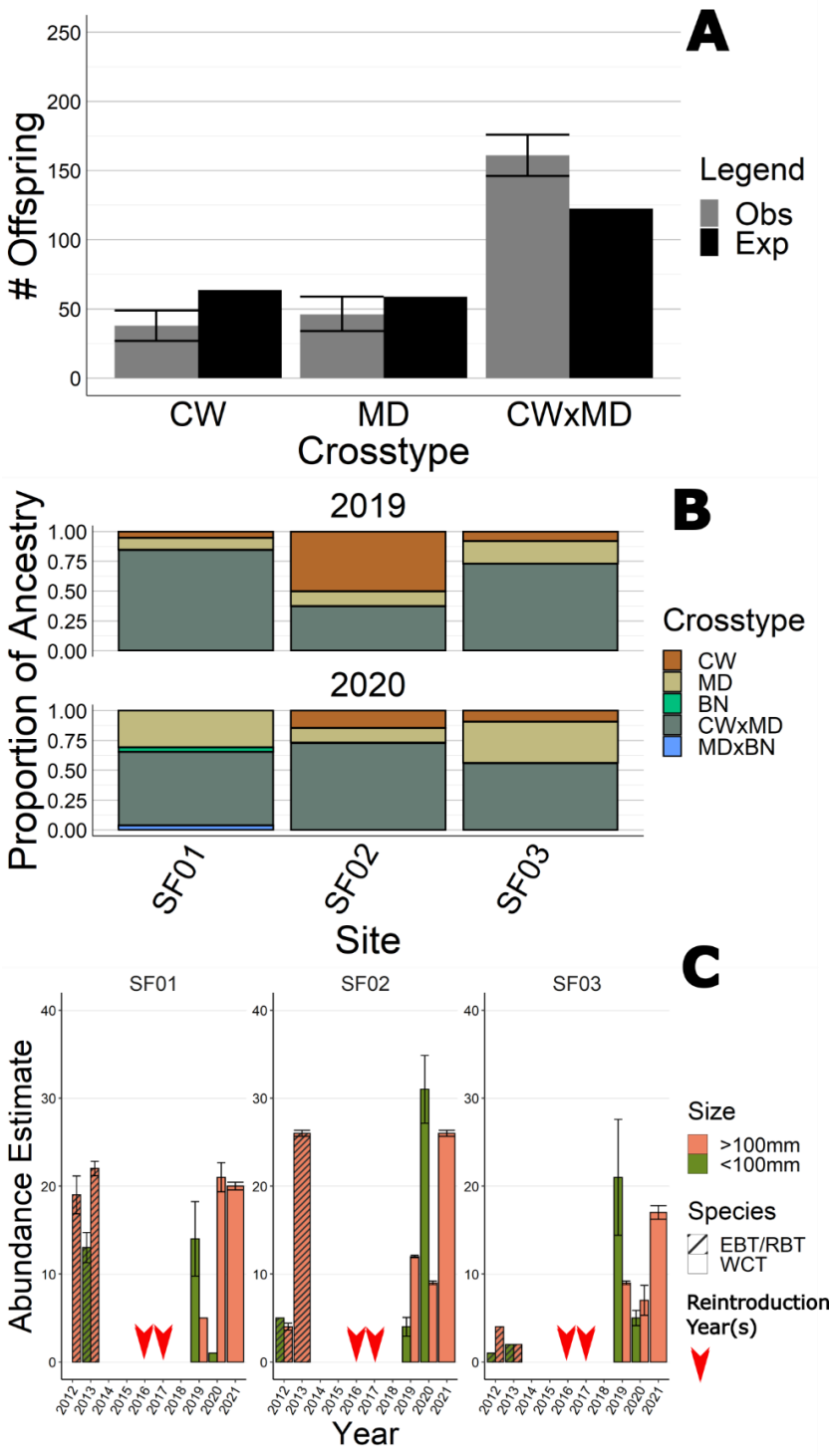

Figure 3. 4: SFG genetic and demographic results. (A) the number of observed (grey) and expected (black) first-generation offspring assigned to crosstypes (parental or hybrid). (B) Proportion of offspring crosstypes shown among sites and years. (C) Pre-reintroduction nonnative abundances of brook trout (EB) and rainbow trout (RBT) compared to post-reintroduction WCT abundances. Sampling sites are ordered from the lower (SF01) to upper (SF03) stream sites from left to right.

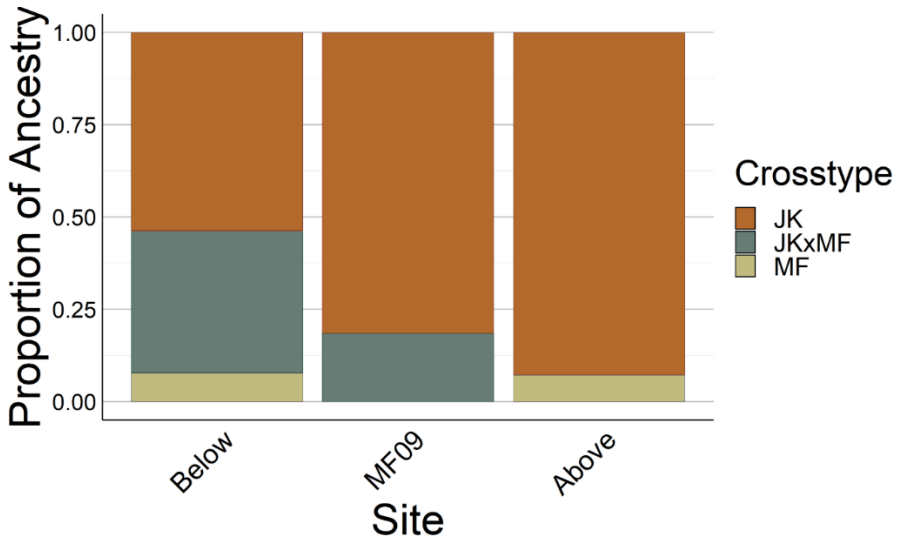

Figure 3. 5: MFG genetic results showing crosstype proportions by site. Sampling sites are ordered from the lowest (Below) to the uppermost (Above) stream sites from left to right.

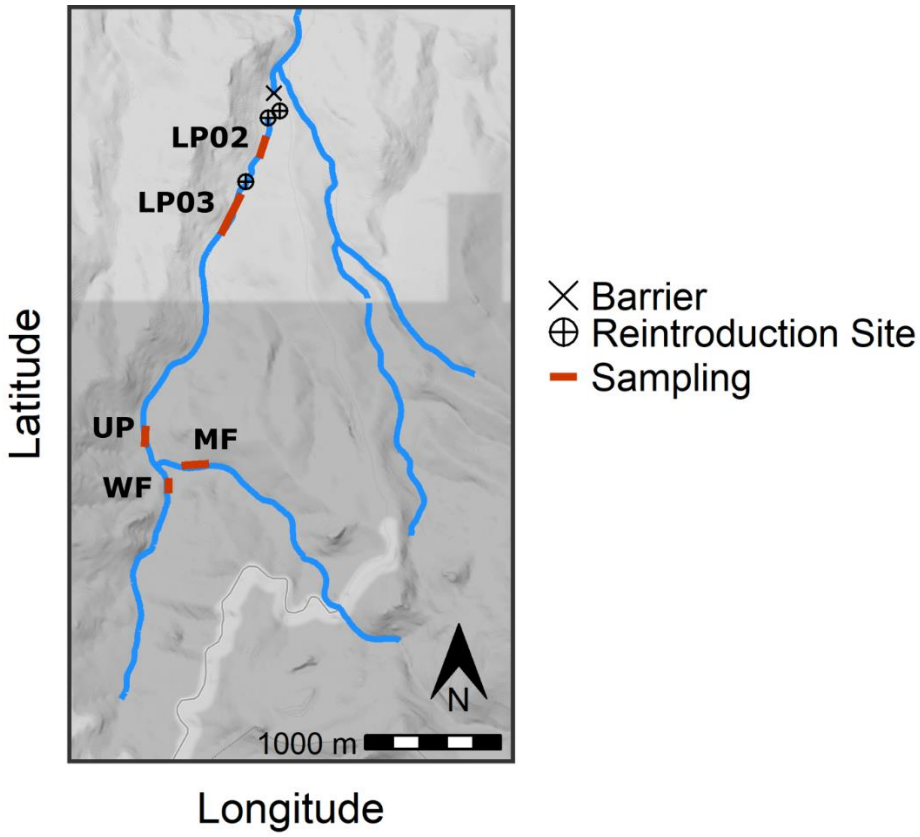

Figure 3. 6: Peet Creek Montana, USA study system including reintroduction, barrier, and sampling locations. Sampling reaches depict all habitat sampled in 2019 and 2020.

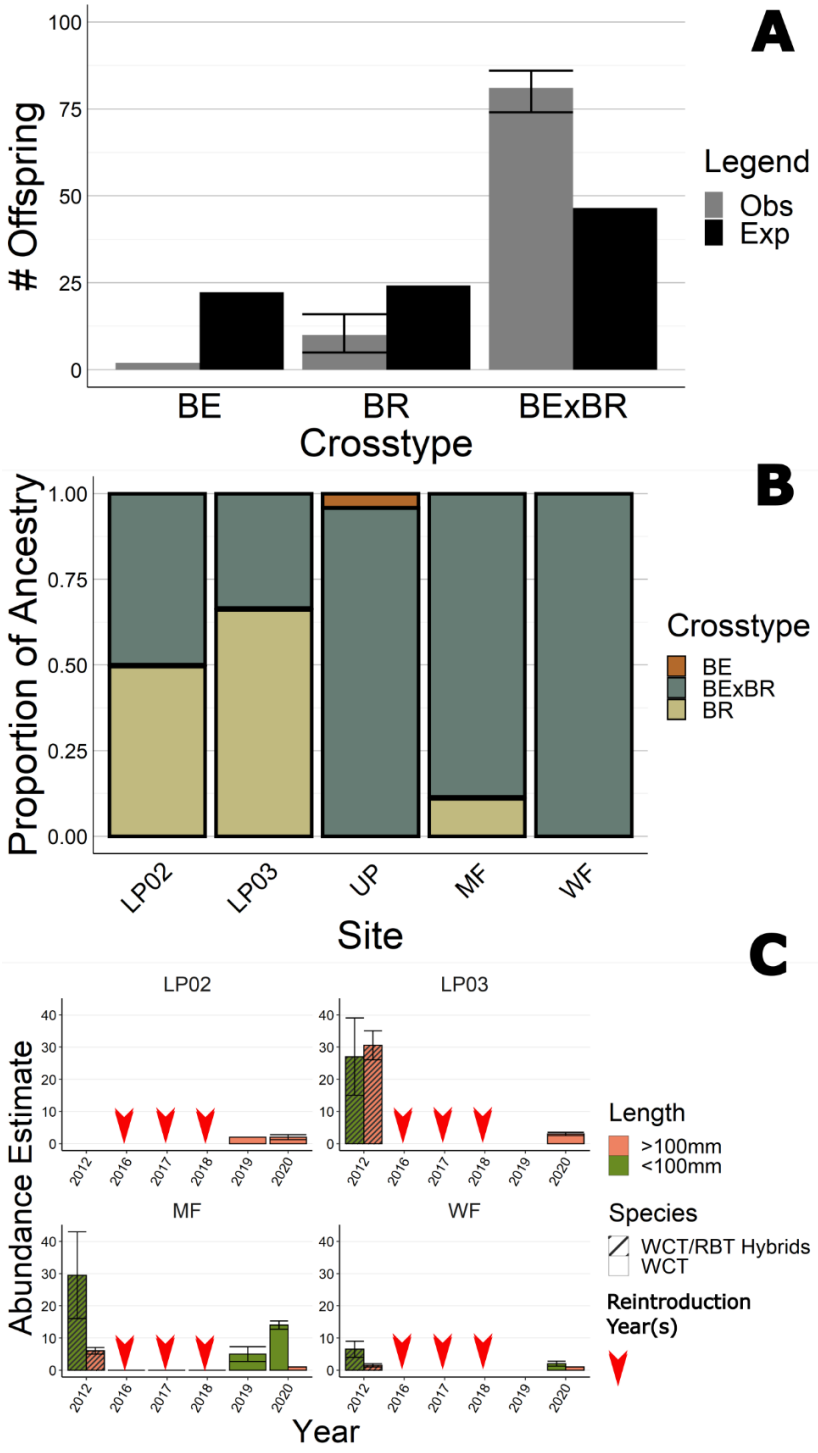

Figure 3. 7: Peet Creek genetic and demographic results. (A) the number of observed (grey) and expected (black) first-generation offspring assigned to crosstypes (parental or hybrid). (B) Proportion of offspring crosstypes shown among sites. (C) Pre-reintroduction nonnative abundances of WCT by rainbow trout (RBT) hybrids compared to post-reintroduction WCT abundances. Sampling sites are ordered from the lower (LP02) to upper (MF and WF) stream sites from left to right.

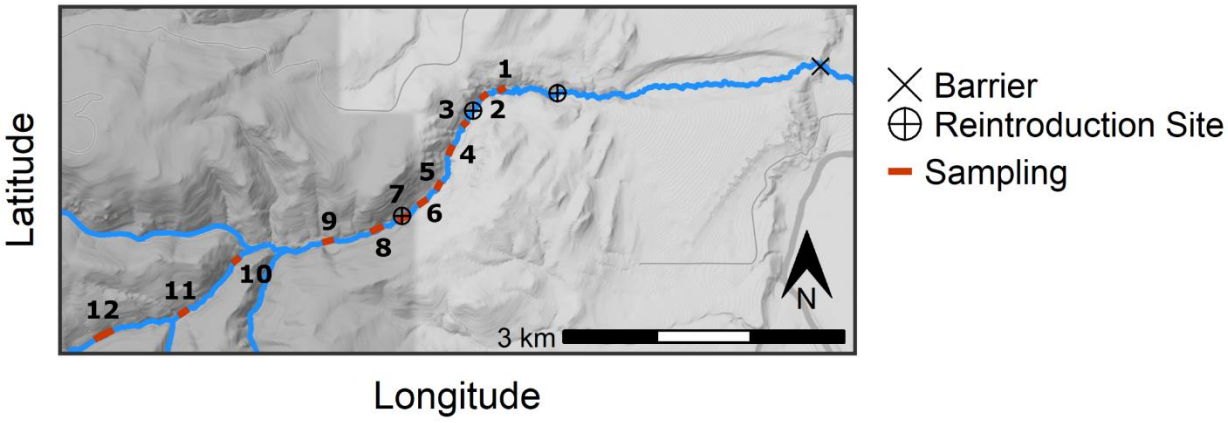

Figure 3. 8: Ruby Creek Montana, USA study system including reintroduction, barrier, and sampling locations. Sampling reaches depict all habitat sampled in 2020.

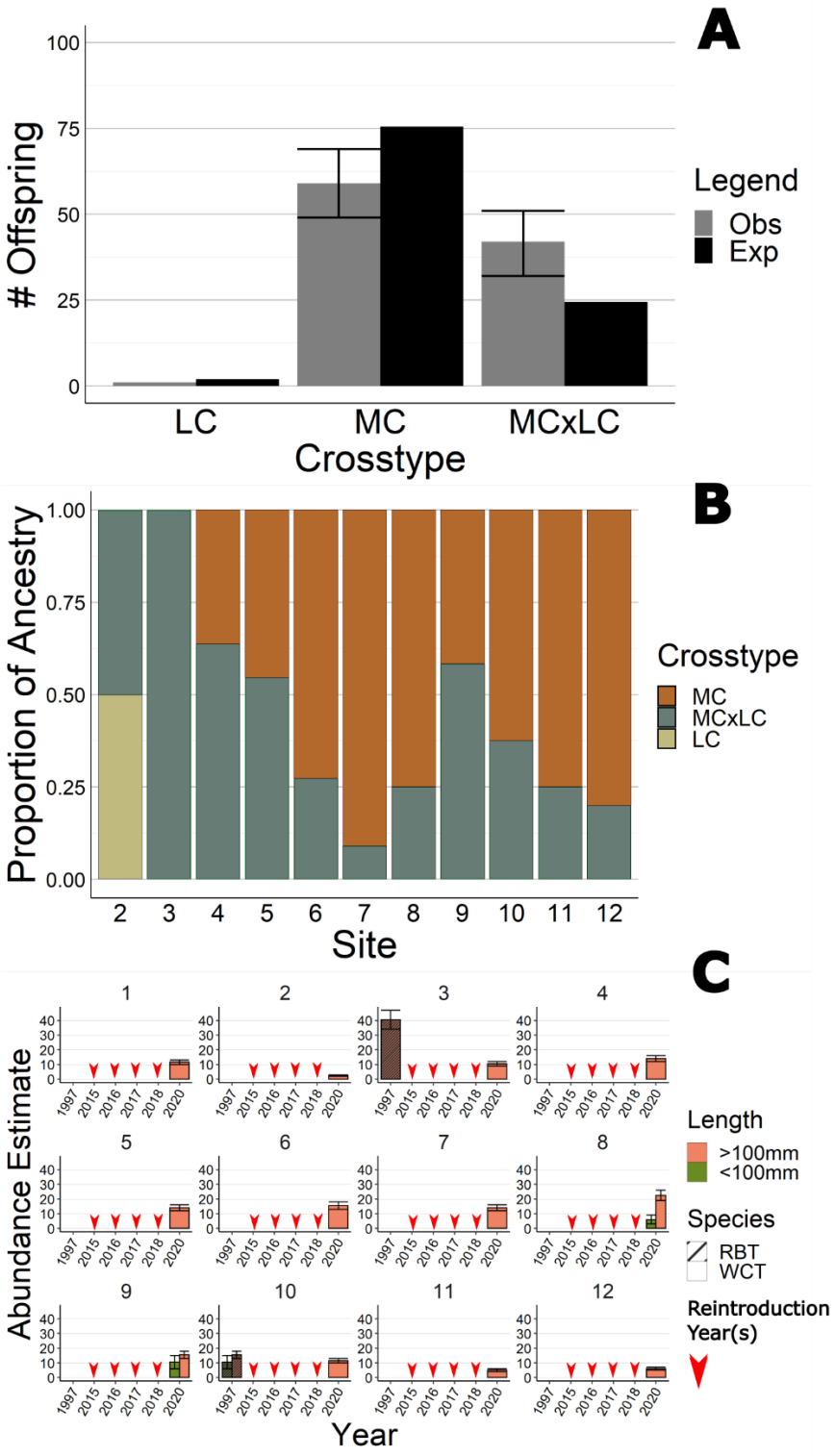

Figure 3. 9: Ruby genetic and demographic results. (A) the number of observed (grey) and expected (black) first-generation offspring assigned to crosstypes (parental or hybrid). (B) Proportion of offspring crosstypes shown among sites. (C) Pre-reintroduction nonnative abundances of rainbow trout (RBT) compared to post-reintroduction WCT abundances. Sampling sites are ordered from the lower (1) to upper (12) stream sites from left to right.

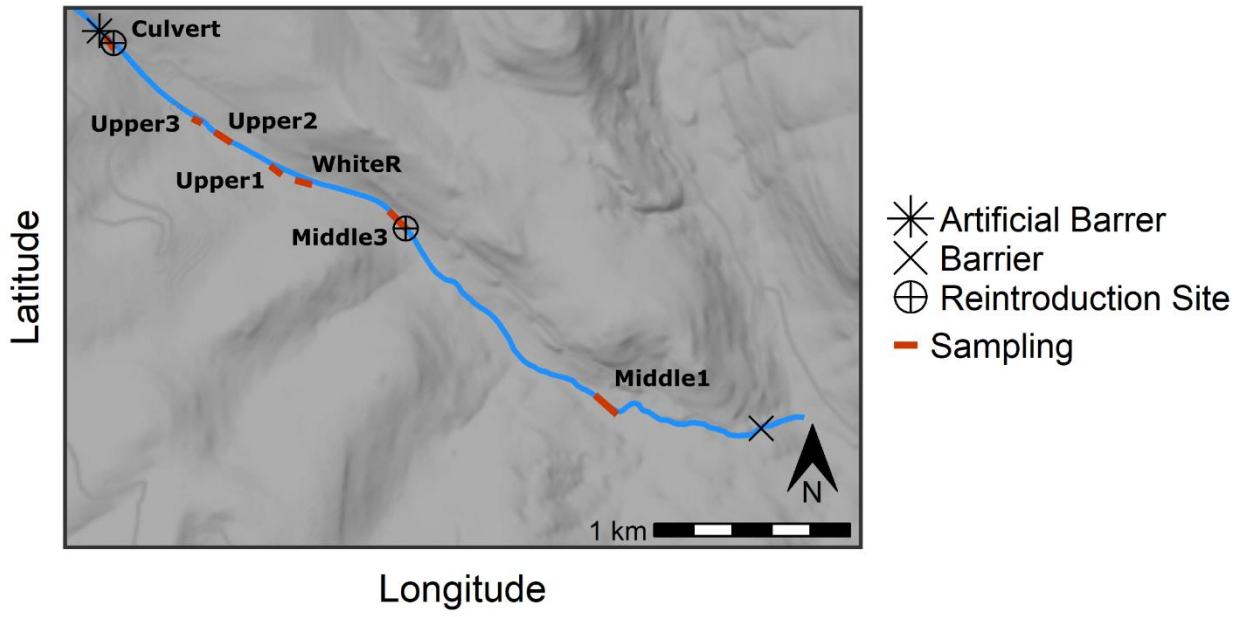

Figure 3. 10: Schultz Creek Montana, USA study system including reintroduction, barrier, and sampling locations. Sampling reaches depict all habitat sampled in 2020.

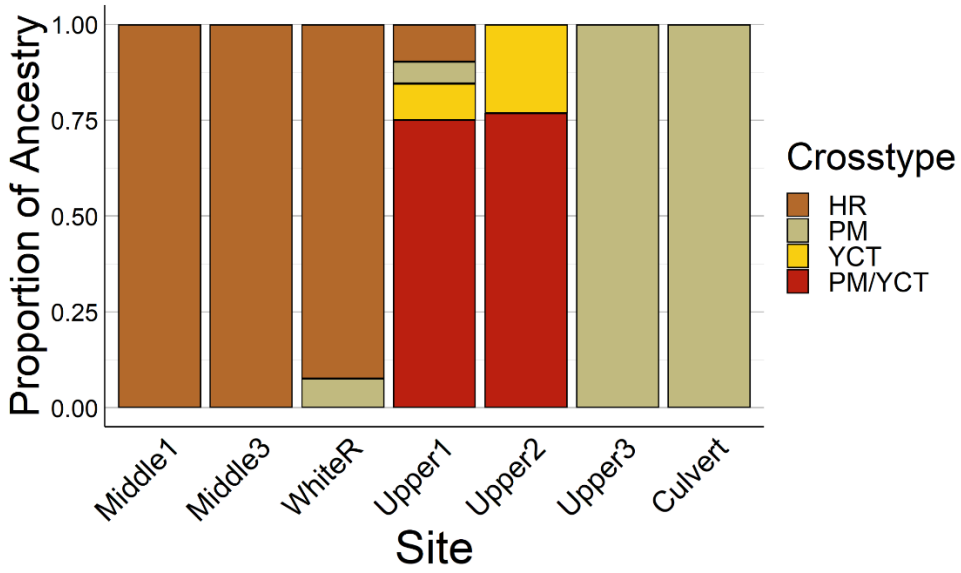

Figure 3. 11: Schultz genetic results showing crosstype proportions by site. Sampling sites are ordered from the lowest (Middle1) to the uppermost (Culvert) stream sites from left to right. Any crosstype including YCT ancestry is a nonnative hybrid crosstype.

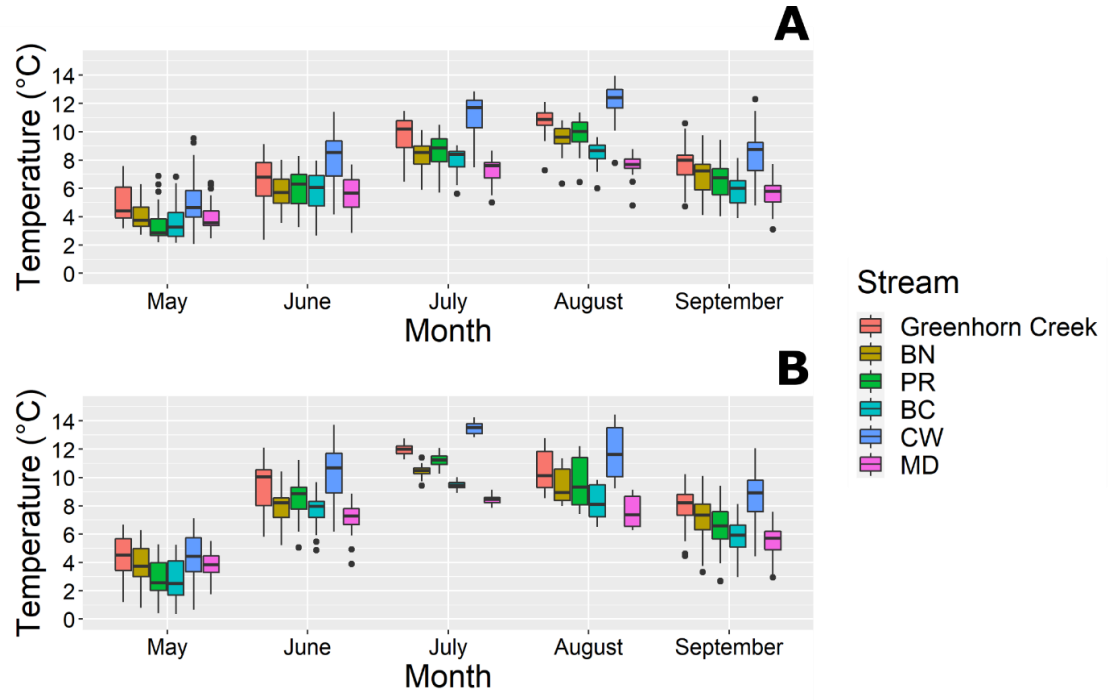

Figure 3. 15: Monthly stream temperatures of Greenhorn Creek (watershed containing NFG and SFG reintroduction streams) and NFG and SFG source population streams. Temperatures were measured in (A) 2020 and (B) 2021.

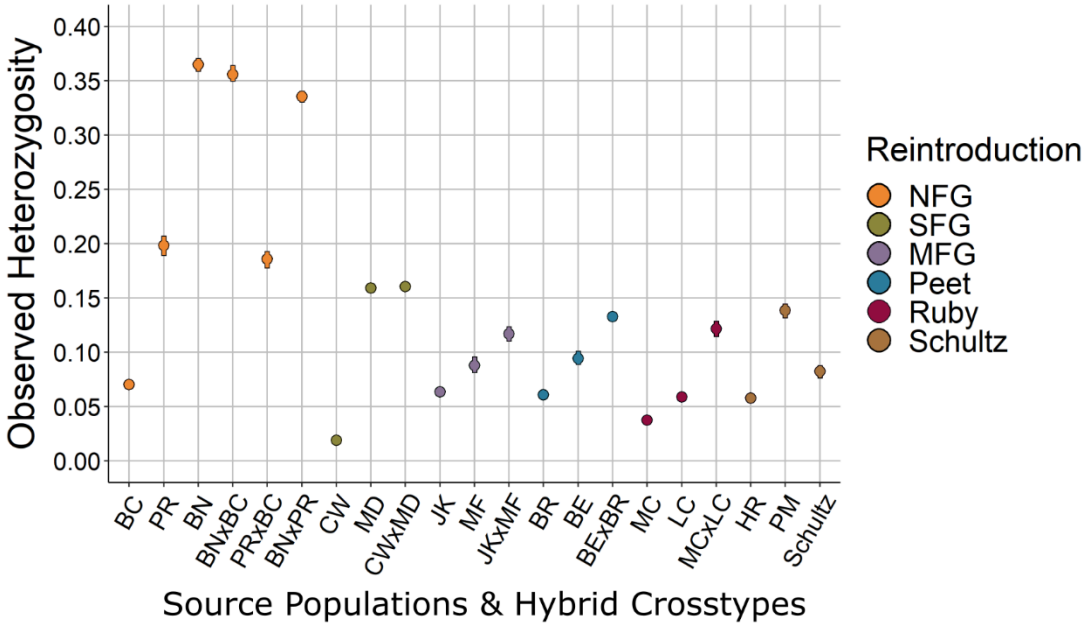

Figure 3. 16: Observed heterozygosity of source populations and hybrid crosstypes for each reintroduction. Colors correspond to reintroduction stream. WCT hybrids were not detected in Schultz, and therefore, the Schultz crosstype includes only parental crosstypes. 95% confidence intervals are provided for each estimate of observed heterozygosity.

Supplemental

Appendix 3.1 – Genetic Methods by Study Stream

Case 1-3: NFG, SFG, & MFG, Greenhorn Creek, Montana. – We performed all locus and individual filtering steps jointly for NFG, SFG, and MFG study streams since mixing could occur among all translocated source populations. We removed low quality offspring and source population data by removing individuals with genotype missingness >25% (n = 56) and loci with genotype missingness >25% (n = 38). By removing individuals with low genotyping success, we lost six source individuals from NFG, SFG, and MFG analyses (1 BC, 2 CW, and 2 JK). Loci invariable in all source populations (n = 55) were also removed. Then we used NFG, SFG, and MFG source populations to evaluate loci for Hardy-Weinberg (HW) and linkage disequilibrium (LD) issues. Specifically, we used approaches from Waples (2015) to calculate F_{IS} values with R package HIERFSTAT (Goudet, Jombart 2021) and remove loci fixed heterozygous (F_{IS} = -1; n = 7). Then we tested conformance with HW proportions and LD at each locus with exact tests (Guo, Thompson 1992) using R packages PEGAS (Paradis 2010) (ran using 10,000 dememorizations) and GENEPOP (Rousset 2008) (ran using 10,000 dememorizations, 100 batches, and 5,000 iterations), respectively. For both tests, we adjusted significance values for multiple testing using sequential Bonferroni procedures (Rice 1989). After removing loci not conforming with HE proportions (n = 4) and showing patterns of LD (n = 17), we proceeded with parentage and population assignment testing with 251 variable loci.

We used R package HIPHOP (Cockburn et al. 2021) to assign sampled offspring to source populations (i.e., crosstypes) and NFG parents. Parent and population assignment accuracies were verified by Feuerstein et al. (2022). Once assigned, we removed offspring (n = 13) with

high assignment uncertainty (Feuerstein et al. 2022). We then used program CERVUS (Kalinowski, Taper & Marshall 2007) to identify and remove source (n = 29) and offspring (n = 46) individuals recaptured in 2019 and 2021 as having zero or one genotype mismatch. Using this approach, we found two PR source individuals with matching genotypes. One of these two individuals was removed.

Case 4: Peet Creek, Montana. – We evaluated genotype missingness for Peet Creek offspring and source population (BE and BR) individuals. Subsequently, we found and removed 2 source population individuals with genotype missingness >25% and 41 loci with genotype missingness >25%. We also removed 241 invariable loci that were fixed for the same alleles. Then, we used HW and LD testing approaches (described for NFG) to evaluate loci for HW or LD issues among Peet Creek source populations. We removed loci that were fixed heterozygous (F_{IS} = -1; n = 6), did not conform with HE proportions (n = 0), and showed patterns of LD (n = 3). We proceeded with population assignment testing with 81 variable loci.

To assign offspring to Peet Creek source populations we used a Bayesian population assignment program (STRUCTURE; Hubisz et al. 2009). We used an ADMIXTURE model in STRUCTURE with parameters set to: USEPOPINFO = 1, MAXPOPS = 2, ALPHA = 1, POPFLAG = 1, and POPDATA = 1. We ran STRUCTURE using 10,000 burn-ins and 100,000 MCMC repetitions. Peet Creek offspring were assigned to crosstypes using q-values. Specifically, offspring with q-values >= 0.9 were assigned to parental sources (BE or BR), and offspring with q-values <0.9 and >0.1 were assigned as a hybrid crosstype (BExBR). To corroborate STRUCTURE was correctly assigning offspring to source populations with low genetic variation, we ran SFG offspring and source populations with the same settings and

compared STRUCTURE results to HIPHOP results. In doing so, we found 100% conformity between the two programs.

Case 5: Ruby Creek, Montana. – We evaluated genotype missingness for Ruby Creek offspring and source population (MC and LC) individuals. Subsequently, we found and removed 1 source population individual with genotype missingness >25% and 30 loci with genotype missingness >25%. We also removed 259 invariable loci that were fixed for the same alleles. Then, we used HW and LD testing approaches (described for NFG) to evaluate loci for HW or LD issues among Ruby Creek source populations. We removed loci that were fixed heterozygous (F_{IS} = -1; n = 7), did not conform with HE proportions (n = 0), and showed patterns of LD (n = 0). We proceeded with population assignment testing with 76 variable loci.

To assign offspring to Ruby Creek source populations we used STRUCTURE with parameter settings set as described for Peet Creek. Ruby Creek offspring were assigned to crosstypes using q-values. Specifically, offspring with q-values >= 0.9 were assigned to parental sources (LC or MC), and offspring with q-values <0.9 and >0.1 were assigned as a hybrid crosstype (MCxLC).

Case 6: Schultz Creek, Montana. – We evaluated genotype missingness for Schultz Creek offspring and source population (PM and HR) individuals. Subsequently, we found and removed 9 offspring with genotype missingness >25% and 48 loci with genotype missingness >25%. We also removed 203 invariable loci that were fixed for the same alleles. Then, we used HW and LD testing approaches (described for NFG) to evaluate loci for HW or LD issues among Schultz Creek source populations. We removed loci that were fixed heterozygous (F_{IS} = -1; n = 6), did

not conform with HE proportions (n = 0), and showed patterns of LD (n = 0). We proceeded with population assignment testing with 119 variable loci.

To assign offspring to Schultz Creek source populations we used STRUCTURE with parameter settings set as described for Peet Creek. However, we allowed for three populations (k=3) rather than two to assign offspring to source populations because previous genetic data (not described here) showed Yellowstone cutthroat trout (*Oncorhynchus clarkii bourvieri*) ancestry in Schultz Creek. Schultz Creek offspring were assigned to crosstypes using q-values. Specifically, offspring with q-values >= 0.9 were assigned to parental sources (PM, HR, or YCT), and offspring with q-values <0.9 and >0.1 were assigned as a hybrid crosstype (PMxHR, PMxYCT, or HRxYCT).

Appendix 3.2 – Figures and Tables

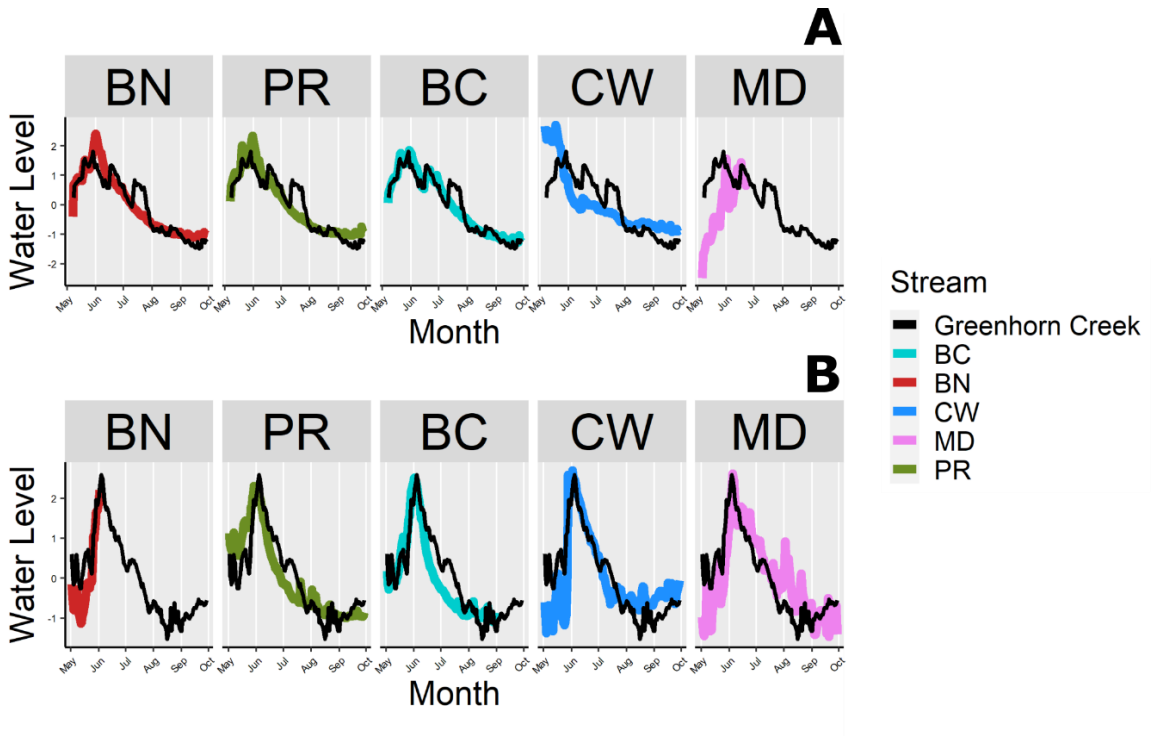

Figure S3. 7: Relative water level (peak flows) of Greenhorn Creek (watershed containing NFG and SFG reintroduction streams) and NFG and SFG source population streams. Water levels were measured in (A) 2020 and (B) 2021.

Table S3. 1: Total number of individuals translocated between 2015-2018 from one of 14 source populations to one of six reintroduction streams. Total translocated is the total number of source individuals translocated across years. Expected (H_e) and observed (H_o) heterozygosity were calculated for each source stream. Source populations with n/a were remnant WCT already in the reintroduction stream, and therefore we did not know the starting number of individuals.

Source Population	Reintroduction Stream	2015	2016	2017	2018	Total Translocated	*He*	*Ho*
BC	NFG	0	50	55	0	105	0.062	0.06
BN	NFG	0	55	52	0	107	0.368	0.365
PR	NFG	0	50	60	0	110	0.194	0.182
DH	NFG	n/a	n/a	n/a	n/a	n/a	0.08	0.079
CW	SFG	0	61	50	0	111	0.013	0.013
MD	SFG	0	55	50	0	105	0.149	0.144
JK	MFG	0	50	49	49	148	0.053	0.05
MF	MFG	n/a	n/a	n/a	n/a	n/a	0.071	0.067
BE	Peet	0	25	0	23	48	0.098	0.094
BR	Peet	0	0	26	25	51	0.064	0.061
LC	Ruby	0	10	0	0	10	0.059	0.059
MC	Ruby	20	14	15	12	61	0.041	0.038
HR	Schultz	0	0	30	0	30	0.063	0.058
PM	Schultz	0	0	30	0	30	0.143	0.138

Table S3. 2: Total number of offspring sampled from each reintroduction stream in 2019, 2020, and 2021. Sampled is the total number of offspring sampled in each reintroduction stream. Analyzed is the total number of offspring used for genetic analyses.

Reintroduction Stream	**2019**	**2020**	**2021**	**Sampled**	**Analyzed**
NFG	500	414	357	1271	640
SFG	145	133	63	341	245
MFG	65	0	0	65	65
Peet	25	68	0	93	93
Ruby	0	120	0	120	102
Schultz	0	176	0	176	157

Table S3. 3: NFG, SFG, Peet, and Ruby population analyses. Exp is the number of expected offspring from each crosstype assuming random mating. Obs is the number of offspring from each crosstype sampled in each reintroduction stream from 2019-2021. Chi-square values are represented by $\chi 2$. The P value is reported for observed vs. expected tests in each reintroduction stream.

Crosstype	**Reintroduction Stream**	**Exp**	**Obs**	**$\chi 2$**	***p***
BN	NFG	70.4	210	276.82	
BC	NFG	67.8	13	44.29	
PR	NFG	74.9	47	10.39	
BNxBC	NFG	138.2	114	4.24	
BNxPR	NFG	146.6	167	2.84	
PRxBC	NFG	142.1	89	19.84	
Total	NFG	640	640	358.42	2.67E-76
CW	SFG	63.7	38	10.37	
CWxMD	SFG	122.5	161	12.1	
MD	SFG	58.8	46	2.79	
Total	SFG	245	245	25.26	5.01E-07
BE	Peet	22.3	2	18.5	
BR	Peet	24.2	10	8.3	
BExBR	Peet	46.5	81	25.6	
Total	Peet	93	93	52.4	4.53E-13
MC	Ruby	75.5	59	3.6	
MCxLC	Ruby	24.5	42	12.5	
LC	Ruby	2	1	0.5	
Total	Ruby	102	102	16.6	4.62E-05

Table S3. 4: Expected heterozygosity and number of polymorphic loci for all reintroduction (H_e(R); P(R)) and corresponding source streams (H_e(S); P(S)). Percent change in expected heterozygosity between reintroduction and source stream is shown by ΔH_e. Percent change in the number of polymorphic loci between reintroduction and source stream is shown by ΔP.

Source Stream	**Reintroduction Stream**	H_e**(S)**	P**(S)**	H_e**(R)**	P**(R)**	ΔH_e	ΔP
BC	NFG	0.073	77	0.319	266	77%	71%
PR	NFG	0.21	241	0.319	266	34%	9%
BN	NFG	0.369	264	0.319	266	-14%	1%
CW	SFG	0.02	14	0.136	227	85%	94%
MD	SFG	0.165	164	0.136	227	-18%	28%
JK	MFG	0.065	55	0.071	66	8%	17%
MF	MFG	0.08	61	0.071	66	-11%	8%
BE	Peet	0.098	74	0.099	76	1%	3%
BR	Peet	0.064	47	0.099	76	35%	38%
MC	Ruby	0.041	33	0.066	60	38%	45%
LC	Ruby	0.059	55	0.066	60	11%	8%
HR	Schultz	0.063	51	0.113	124	44%	59%
PM	Schultz	0.143	107	0.113	124	-21%	14%

Table S3. 5: Pairwise estimates of genetic differentiation (F_{ST}) between source populations used for six reintroductions.

Reintroduction Stream	**Source Stream1**	**Source Stream2**	F_{ST}
NFG	PR	BC	0.243
NFG	BN	BC	0.354
NFG	PR	BN	0.166
SFG	MD	CW	0.43
MFG	MD	JK	0.547
Peet	BR	BE	0.395
Ruby	LC	MC	0.648
Schultz	PM	HR	0.395