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RESEARCH ARTICLE

Consistent loss of genetic diversity in isolated cutthroat trout populations independent of habitat size and quality

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Abstract Fragmentation and isolation of wildlife populations has reduced genetic diversity worldwide, leaving many populations vulnerable to inbreeding depression and local extinction. Nonetheless, isolation is protecting many native aquatic species from interactions with invasive species, often making reconnection an unrealistic conservation strategy. Isolation management is widely used to protect extant cutthroat trout (*Oncorhynchus clarkii*) populations from invasive species. Despite this, few studies have empirically examined how predictor variables including habitat length, population size, time since isolation and habitat quality, relate to levels of genetic diversity in isolated trout populations. We compared allelic richness of cutthroat trout across 14 microsatellite loci in two connected and 12 anthropogenically isolated populations of the Flathead River basin, Montana. Isolated populations in habitat fragments <8 km stream length had reduced genetic diversity, but diversity was not significantly related to any of our predictor variables. To broaden our scope, we analyzed seven geologically isolated populations from the same river basin occupying habitat fragments up to 18 km in length. These populations showed reduced diversity, regardless of fragment size. Furthermore, geologically isolated populations had significantly lower average allelic richness compared to streams recently isolated by anthropogenic activities. These results demonstrate a consistent loss of genetic diversity through time in isolated populations, emphasizing the need to explore strategies to minimize risks of inbreeding depression. Testing conservation theory and subsequent assumptions broadly across taxa is necessary to ensure efficacy of conservation efforts.

Keywords Cutthroat trout · Isolation management · 50/500 rule · Genetic drift · Habitat fragmentation

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Introduction

Habitat and population fragmentation is a primary factor leading to loss of genetic diversity across wildlife species (Dixon et al. 2007; Clark et al. 2010; Alexander et al. 2011; Vranckx et al. 2012). Due to the dendritic nature of stream networks, stream-dwelling organisms (e.g., salmonid fish) are particularly susceptible to fragmentation (Fagan 2002). Various human activities, such as dam construction, road building, water diversions, and agricultural practices have degraded habitat and caused population isolation, loss of migratory life histories, and reduced genetic diversity in salmonid populations world-wide (Dunham et al. 1997; Aarts et al. 2004; Wofford et al. 2005; Morita et al. 2009; Sato and Gwo 2011). Despite the demographic and genetic risks inherent to small isolated populations, intentional isolation is also a widely used conservation strategy to

address the immediate threats posed by nonnative invasive fishes (Fausch et al. 2009; Rahel 2013).

Due to increasing rates of nonnative species introductions, managers are often faced with the dilemma of choosing when to intentionally isolate native fish populations to prevent harmful interactions with invasive species versus restoring connectivity to promote gene flow and metapopulation dynamics (Fausch et al. 2006, 2009). Cutthroat trout (*Oncorhynchus clarkii,* hereafter "cutthroat") is one species for which these tradeoffs are of increasing concern. In connected riverscapes of western North America, many subspecies of cutthroat are threatened by hybridization and competition with expanding populations of introduced rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*). As a result, many of the remaining cutthroat populations across the Rocky Mountains are found in isolated habitat fragments (Dunham et al. 1997; Shepard et al. 1997; Kruse et al. 2000; Shepard et al. 2005), and managers often choose to maintain or construct barriers to fish passage in order to protect the remaining purebred populations of cutthroat from invasive species. Intentional and unintentional isolation of cutthroat populations is increasingly common on a landscape scale (Dunham et al. 1997; Kruse et al. 2001; Young and Harig 2001; Shepard et al. 2005; Young et al. 2005) and understanding the necessary requirements of maintaining the genetic diversity of these populations is critical for effective long-term conservation of the species.

Despite the strong influence of habitat connectivity on subpopulation persistence, many salmonid populations, including those of the cutthroat subspecies, have persisted above natural barriers such as waterfalls since the last glacial period (Taylor et al. 2003; Shepard et al. 2005; Wofford et al. 2005). This suggests isolated populations may be buffered against extirpation risk if habitat fragments are relatively large and contain suitable environmental conditions. Over the last decade substantial research on habitat characteristics and census population sizes for isolated trout populations has generally found that persistence is positively related to larger, higher quality habitats (Dunham et al. 2002; Harig and Fausch 2002; Morita and Yokota 2002; Novinger and Rahel 2003; Peterson et al. 2008; Fausch et al. 2009; Muhlfeld et al. 2012; Peterson et al. 2013). These same habitat characteristics have been associated with maintenance of genetic diversity. For example, Whiteley et al. (2013) found a positive relationship between patch size and levels of genetic diversity in populations of isolated brook trout in their native range. Research also suggests that life history characteristics may influence levels of genetic diversity. Neville et al. (2006) found higher levels of genetic diversity in Lahontan cutthroat trout (*O. c. henshawi*) populations with migratory life histories and in populations residing in relatively higher quality habitat characterized by cooler temperatures and more consistent (perennial) stream flows.

Genetic theory predicts that isolated populations will lose heterozygosity through drift at a rate of $1/(2N_e)$ per generation, indicating that smaller populations will lose genetic diversity more quickly than larger populations (Wright 1969). A common rule of thumb for preservation of genetic diversity is the "50/500" rule (Franklin 1980). This rule estimates that an N_e of 50 is desirable to reduce the short-term likelihood of extinction due to the harmful effects of inbreeding depression on population demography. An N_e of 500 is required for mutation to add genetic diversity back into a population at a similar rate to loss caused by genetic drift, thereby maintaining a population's long-term evolutionary potential. Estimates for wild Pacific salmon stocks equate an N_e of 500 to a census size of roughly 2500 (Allendorf et al. 1997). With these guidelines, studies estimated that for streams with relatively high trout abundances (0.3 fish/m >75 mm in total length), a minimum of 8 km of high-quality stream habitat would be necessary to sustain an inland trout population at this census size, and that larger habitat fragments would be necessary in poorer quality areas because habitat quality and volume are generally positively associated with fish density, population size, and genetic diversity (Hilderbrand and Kershner 2000; Young et al. 2005). Few studies have empirically examined habitat size, population size, and time since isolation with loss of genetic diversity and no studies have empirically evaluated the potential role of habitat quality (flow, temperature, riparian and watershed impacts) on loss of genetic diversity in small isolated populations. Research filling these gaps in knowledge will lead to a better understanding of how habitat length and habitat quality within the isolated refuge may influence potential viability and resilience of a population.

To address these gaps in knowledge, we studied populations of genetically pure westslope cutthroat trout (*O. c. lewisi*) in western Montana to address three main research questions:

(1) How do time since isolation, length of occupied habitat, population density, and abundance relate to levels of genetic diversity in anthropogenically isolated populations?
(2) In the small, anthropogenically isolated systems in this study, is there an influence of habitat characteristics (including fragment size, habitat quality, temperature, and stream flow) on genetic diversity?
(3) How do amounts of genetic diversity compare between streams that have been isolated on short time scales (anthropogenic isolation, 10s of generations) and those that have been isolated on very long time scales (geologic isolation, 100 to 1000s of generations)?

Materials and methods

Study area

The Flathead River watershed drains over 22,780 km^2 of land encompassing the headwaters of the Columbia River Basin. Stream flows in the basin are dominated by snowmelt runoff patterns, with the majority of annual discharge occurring during spring and early summer. Stream flows typically decline to base levels by late July to early August. Salmonids of the watershed include native mountain whitefish (*Prosopium williamsoni*) and westslope cutthroat and bull trout (*Salvelinus confluentus*), as well as introduced brown trout (*Salmo trutta*), brook trout, and rainbow trout. Hybridization between native cutthroat and invasive rainbow trout readily occurs in connected tributaries where populations are sympatric, and is pervasive throughout the larger river network (Boyer et al. 2008; Corsi 2011). Many of the remaining pure cutthroat populations in this watershed are found above anthropogenic barriers, such as perched culverts and irrigation canals installed over the last century or in first or second order streams above natural barriers, such as waterfalls formed by either glacial cutting, about 10,000 years ago, or base-level fall, at least 1000 years ago (A. Wilcox, Associate Professor of Fluvial Geomorphology, University of Montana, personal communication).

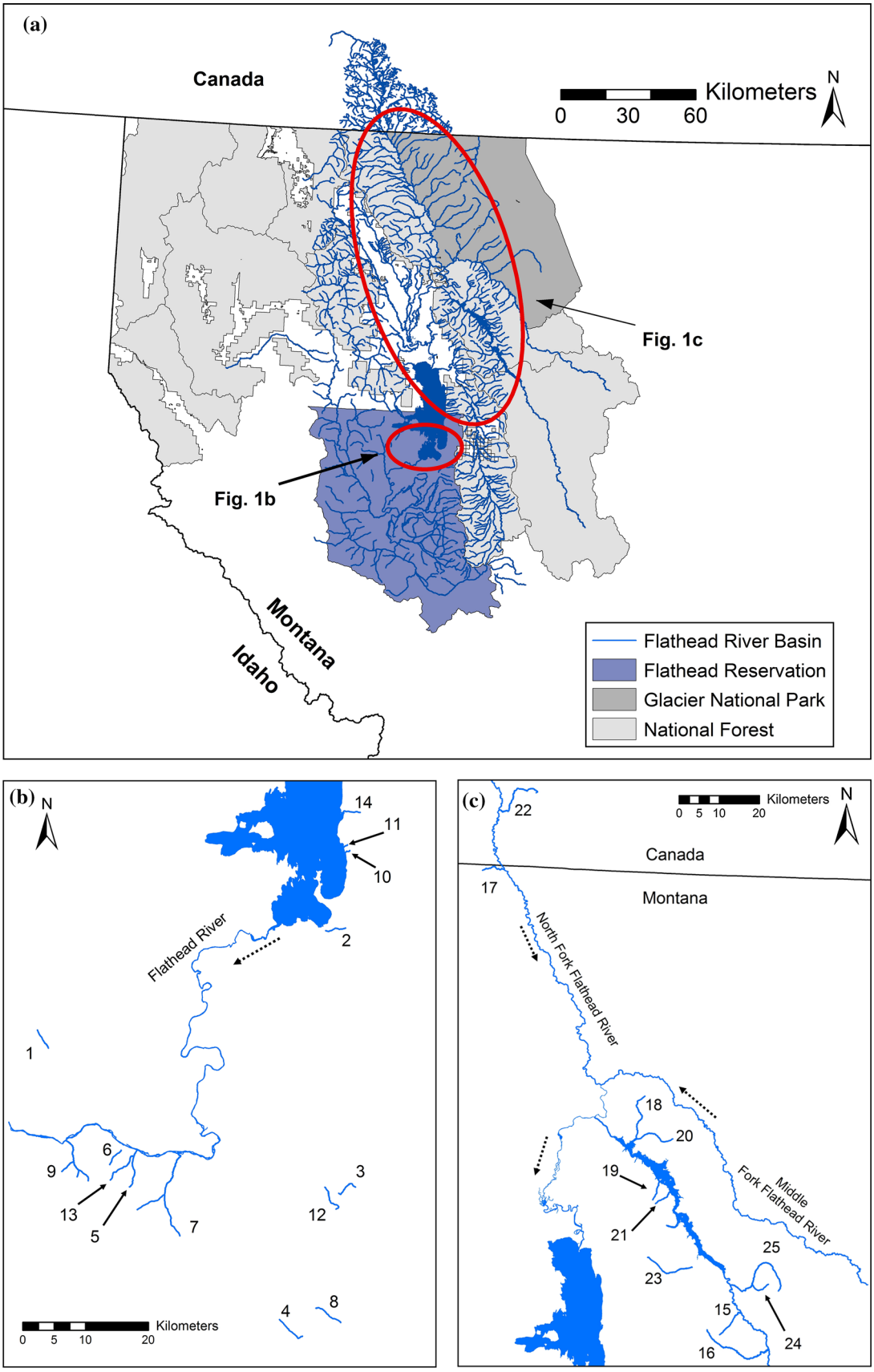
Populations included in this study reside in streams located in a range of habitat types, from high gradient mountain environments to low gradient grassland environments. The majority of the Lower Flathead River Basin (tributaries to Flathead Lake and waters downstream) drains through lands on the Flathead Indian Reservation. Human impacts on streams are common and associated with agricultural and ranching practices, including stream dewatering and cattle grazing (Fig. 1). Conversely, the Upper Flathead River Basin (upstream of Flathead Lake; where our geologically isolated sites are located) is relatively unimpacted by human activities, with a majority of the watershed draining through several national wilderness areas, Glacial National Park, and areas of the Flathead National Forest with little history of resource extraction.

Sample collection and habitat assessment in anthropogenically isolated populations

To compare genetic diversity between connected and anthropogenically isolated populations of cutthroat, we studied 2 connected and 12 isolated genetically pure populations located in headwater streams of the Lower Flathead River basin. We confirmed the purity of these cutthroat populations using eight diagnostic markers (see Genetic Analysis section below). Connected streams in this dataset drain into the Flathead River where migratory cutthroat are regularly observed. For these isolated streams, we used records from the Montana Department of Transportation and the Bureau of Indian Affairs Flathead Indian Irrigation Project to date all isolating barriers. If a perched culvert was the isolating barrier, we collected information on culvert dimensions, material and construction (e.g., corrugations), and we surveyed longitudinal profiles that extended through the culvert. We analyzed these data in FishXing program to ensure that culverts were impassible by *Oncorhynchus* species (FishXing 3; http://www.stream.fs.fed.us/fishxing/). Introduced trout existed downstream of all cutthroat populations isolated by perched culverts and a lack of invasion by rainbow trout and hybrids in these isolated populations further confirmed that upstream fish passage into these streams was not possible. Two populations were isolated by irrigation canals with no immediate barrier, such as fish screens, preventing individuals from moving in and out of the canal. However, fish were not observed in the canal, and there were no nearby streams supporting *Oncorhynchus* species that drained into the canal that could provide a source for gene flow.

Length of occupied habitat for cutthroat in streams of the Lower Flathead River was determined by electrofishing upstream from the confluence (connected streams), or the isolating barrier (isolated streams) until fish were no longer captured. At this location, GPS coordinates were recorded and we used Arc GIS (ESRI ArcMap 9.3) and stream data layers created by the Confederated Salish and Kootenai Tribes (unpublished data) to measure the length of occupied habitat in each stream between the isolating barrier and upper extent of the cutthroat distribution.

All density estimates were conducted between late June and early September of 2010–2013. We estimated fish densities in each stream using standard mark-recapture or depletion methods at sites that ranged from 120–155 m in length. For depletion estimates, we repeated collection passes until we captured less than 20 % of the number of fish caught in the first depletion pass. Recapture runs at mark-recapture estimate sites were conducted between 6 and 9 days after the marking run. Typically, two sites were sampled in each stream with one site located in the upper and lower half of the occupied habitat. In three streams (Teepee, Talking Water and Yellow Bay Creeks) density estimates were only performed at one site due to short total occupied habitat lengths ($\leq$1.4 km). Density estimates were repeated once in the years following the initial estimate at six sites across six streams (upper Camas, lower Centipede, lower Magpie Spring, upper West Magpie Creeks, and the single estimate sites in Teepee and Talking Water Creek). To obtain an average density estimate for an entire stream, we first averaged density estimates for any


(a)
Canada
Kilometers
0 30 60
N
Fig. 1c
Fig. 1b
Montana
Idaho
Flathead River Basin
Flathead Reservation
Glacier National Park
National Forest
(b)
N
14
11
10
Flathead River
2
1
6
9
13
5
7
3
12
4
8
Kilometers
0 5 10 20
(c)
22
Kilometers
0 5 10 20
N
Canada
Montana
17
North Fork Flathead River
18
20
19
21
Middle Fork Flathead River
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23
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16
24

◀**Fig. 1** Map of study locations in the Flathead River basin (**a**). **b** highlights the location of study streams in the Lower Flathead River basin, and **c** highlights study streams in the Upper Flathead River basin. Numbers correspond to "Map ID" in Table 1 and *dashed arrows* indicate direction of river flow

site with more than one estimate, and then took the average across all density estimate sites in a given stream.

To obtain a representative sample of each population's genetic diversity, we collected tissue samples at all density estimate sites, and at one to three additional locations interspersed throughout the length of occupied habitat in every stream. The average distance between sampling locations in a given stream was 0.74 km, with a maximum of 2.11 km in Revais Creek, the longest stream in the study.

To assess habitat quality, we asked two fisheries biologists and a hydrologist that have worked in these streams for 10–20 years, to independently complete an expert opinion survey on habitat quality as outlined in Peterson et al. (2013). Briefly, the survey asked experts to rate the portion of the stream above the isolating barrier as having high (1), moderate (2), or low (3) quality habitat based on anthropogenic and natural disturbances including road densities, logging and grazing activities, wildfire, floods and debris flow. Given the agreement in rankings among experts, we averaged the results from our three experts to obtain a single value of habitat quality for each stream.

We also measured stream temperature and summer base flows for all streams. Temperature was recorded at one location per stream targeting the middle of the cutthroat distribution. We used temperature loggers (HOBO and Tidbit V2 models, Onset Computer Corporation, Pocasset, Massachusetts, ±0.2 °C of accuracy) to record temperature at one-hour intervals at from 1 July through 8 September 2013 (70 days). We compared growing seasons by calculating growing degree days (GDD) above 0 °C for this 70-day period. Base flows were recorded (cubic meters per second; CMS) from August 6–8th, 2013 in the lower half of the cutthroat distribution in each stream (Table S1) using handheld Acoustic Doppler Velocimeter (FlowTracker).

Sample collection in geologically isolated populations

To quantify genetic diversity in geologically isolated populations of cutthroat, we analyzed samples from four connected and eight isolated populations located above waterfalls at least two meters in height in the Upper Flathead River. Connected streams in this dataset drain into the North Fork of the Flathead River and Hungry Horse Reservoir and contain resident and migratory cutthroat life history forms. For these streams, extent of occupied habitat was determined from Montana Fish Wildlife and Parks fish monitoring records and expert opinion of local fisheries biologists (Montana Fish Wildlife and Parks, unpublished data). We measured occupied habitat from either the confluence or isolating waterfall to the upper extent of fish bearing habitat. We obtained genetic samples previously collected by methods outlined in Boyer et al. (2008). Fish were captured by electrofishing in stream reaches separated by 250–1000 m to minimize sampling of related individuals, and to obtain a representative sample of the genetic diversity in the entire stream. All samples were collected between late July and early September in 2003 and 2004 (Middlepass and Colts Creeks), as well as 2009 and 2010 (all other creeks).

Total length (TL; mm) was recorded for all fish sampled in both the Lower and Upper Flathead basins. Because catchability of small fish is low, only individuals >75 mm TL were included in genetic analysis and population density calculations. A small portion of fin tissue was excised from individuals sampled for genetic analysis and stored individually in 95 % ethanol prior to analysis. Based on TL and the time of year at which they were collected (i.e., post-spawn), all fish sampled in connected streams were either resident life history forms or juvenile progeny, and thus native to their stream of capture.

Based on the length-frequency distributions of fish in these populations and information on size at age from isolated populations we were able to determine the age structure of each population (Carim 2013). We then compared the size structure with information on size at maturity in westslope cutthroat populations in western Montana (Downs et al. 1997) and estimated the generation time (i.e., the average age of reproductively mature individuals) to be 4 years. To obtain the number of generations each population was isolated, we determined the number of years between the date of isolation and the first year of sampling, and divided this number by four. For geologically isolated populations, we assumed 2500 generations of isolation, based on the estimated time of the last glacial retreat. Because population-level changes in genetic structure are detected on a time scale of generations, all sampling efforts were conducted within the span of a single generation.

Genetic analysis

Genetic analysis, including DNA extraction, PCR amplification, and ABI fragment analysis, was conducted following methods outlined in Muhlfeld et al. (2009). We analyzed 16 microsatellite loci, including Ogo8 (Olsen et al. 1998), Omm1037-1, Omm1037-2, Omm1019, Omm1050, Omm1060 (Rexroad et al. 2002) and Omy0004 (Holm and

Brusgaar 1999). Multiplex 2 consisted of Omy1001 (Spies et al. 2005), Ogo4, Ssa456, and Sfo8 (Small et al. 1998). Multiplex 3 consisted of Ogo3 (Olsen et al. 1998), Oki10 (Smith et al. 1998), Ots107 (Nelson and Beacham 1999), Ssa408, and Ssa407 (Cairney et al. 2000). Eight of these markers (Ogo8, Omm1019, Omm1050, Omm1060, Omy004, Ogo4, Sfo8, and Ssa408) are diagnostic for cutthroat and rainbow trout across watersheds tested in Montana (Sally Painter, Montana Conservation Genetics Laboratory, personal communication) and were used to ensure that each population contained only pure cutthroat. Omy004 and Ssa408 are monomorphic in pure cutthroat trout and were not included in genetic diversity analyses. All genetic analyses were conducted at the University of Montana Conservation Genetics Lab, Missoula, USA.

Statistical analyses

We used Arlequin v3.5 (Excoffier and Lischer 2010) to calculate expected heterozygosity and test for linkage disequilibrium and deviations from Hardy–Weinberg expectations. To quantify genetic diversity, we used FSTAT (Goudet 1995) to calculate the allelic richness (R_s) for each population. FSTAT uses rarefaction (see Leberg 2002) to estimate the number of alleles per population scaled to the population with the smallest sample size (Petit et al. 1998), which in this study was 25. To determine how levels of genetic diversity in all populations compared to recommendations for maintaining genetic diversity outlined by genetic theory, we estimated effective population size using LDNe (Waples and Do 2008).

Genetic diversity in anthropogenically isolated populations

Because connected populations had potential for gene flow, we assumed that levels of genetic diversity in connected populations were not appreciably altered by genetic drift, and thus represented a reference point for the highest level of genetic diversity that could be maintained in an isolated population in the same watershed (as in Whiteley et al. 2010). We used a Welch's two-sample *t* test to compare average allelic richness between open and isolated systems within Lower Flathead River. To explore the relationship between number of generations since isolation, length of occupied habitat, population density, and abundance on maintenance of genetic diversity of isolated populations, we used linear regressions to independently compare these variables to allelic richness.

To further explore how population and habitat characteristics collectively affect genetic diversity in anthropogenically isolated populations in small fragments (<8 km of habitat), we performed a multiple linear regression of allelic richness on length of occupied habitat, generations isolated, habitat quality, growing degree days, base flow, density, and abundance. We used 8 km of habitat as a threshold because this size of habitat has been estimated as the minimum stream length that could maintain a viable population large enough to avoid loss of genetic diversity through genetic drift (Hilderbrand and Kershner 2000; Young et al. 2005). Because length of occupied habitat and population density were used to calculate abundance, we created two global model sets, (separately examining model sets with abundance or density and length), and analyzed all subsets of these global models. We identified highly correlated independent variables using Pearson's correlation tests, and removed any variables from the multiple regression analysis with r^2 values stronger than ± 0.5 that were statistically significant ($\alpha = 0.05$). Additionally, to verify that multicollinearity between variables was not influencing our results, we also calculated a variance inflation factor of the variables in each global model and excluded any variables with a variance inflation factors >5.

For each global model set, the model with the lowest Akaike information criteria corrected for small sample size (AICc; Burnham and Anderson 2002) whose parameter estimates were all significantly different from zero was considered the best supported model. Because these analyses were meant to elucidate potential predictors of reduced genetic diversity in isolated populations, we did not include the open populations in these analyses.

Comparison between anthropogenically and geologically isolated populations

We used a Welch's two-sample *t*-test to compare average allelic richness between (1) geologically isolated populations and connected populations in the Upper Flathead River Basin; and (2) populations isolated on anthropogenic (short) versus geologic (long) time scales. All statistical analysis and graphical figures were conducted and created in R Statistical Software (R Developement Core Team 2012), and p-values were assessed at the level of $\alpha = 0.05$. All maps were created in Arc Map (ESRI Arc GIS v9.3).

Results

We analyzed tissue samples from 994 fish across 25 streams throughout the Upper and Lower Flathead watersheds. The number of individuals genotyped varied by population (Table 1). Excluding monomorphic loci within a given population, we performed a total of 210 tests for departure from Hardy–Weinberg equilibrium across all populations. After Bonferroni correction, no tests were

significant for departure from Hardy–Weinberg equilibrium (corrected $p = 0.00024$). Excluding monomorphic loci, we performed 2275 independent tests for linkage disequilibrium. After Bonferroni correction, only four tests for linkage disequilibrium spread across three populations were significant (corrected $p = 0.00002$; two tests in Seepay Creek, one test in Thorne Creek, and one test in West Magpie Creek). Heterozygosity and allelic richness were highly correlated across all populations with Pearson's correlation coefficient of 0.95 ($p < 0.001$). All isolated populations in this study had estimated effective population sizes less than 100 (Table 1) with the exception of Revais Creek, which had an estimated effective population size of 166. In some instances, low levels of genetic diversity resulted in negative estimates of effective population size (Goldie and Kneiff Creeks) and confidence intervals bounded by infinity (Teepee, Addition, Bunker, Goldie, Horse, Knieff, and Quintonkin Creeks).

Table 1 Map identification, stream name, number of individuals sampled (N), type of isolating barrier, number of significant tests for linkage disequilibrium after Bonferroni correction (LD), average heterozygosity (H_e) and average allelic richness (R_s) across all loci, length of occupied habitat (Length, km), estimated number of generations isolated for each population, and estimated effective population size (Ne), and average and range of density estimates (fish/m) at each site

Map ID	Stream	N	Barrier type	LD	H_e	R_s	Ne (95 % CI)	Length (km)	Generations isolated	Average density (range)
Lower Flathead River basin (includes anthropogenically isolated populations)										
1	Camas	53	Rerouted/dispersed into agricultural field	0	0.120	1.69	23 (8–95)	3.5	27.5	0.44 (0.13–0.80)
2	Centipede	55	Irrigation diversion/canal	0	0.193	1.74	25 (8–156)	2.7	22.75	0.5 (0.08–1.47)
3	Cold	62	Irrigation diversion/canal	0	0.419	3.75	44 (32–64)	4.9	21.25	0.71 (0.26–1.15)
4	Frog	44	Perched culvert	0	0.420	3.74	63 (38–139)	3.7	11.5	0.37 (0.25– 0.49)
5	Magpie[a]	55	NA	0	0.526	5.23	126 (79–268)	5.3	NA	0.57 (0.54–0.61)
6	Magpie spring	54	Perched culvert	0	0.435	3.29	28 (20–41)	2.9	18	0.18 (0.15– 0.29)
7	Revais	42	Irrigation diversion/canal	0	0.489	5.25	166 (84–1134)	14.0	23.75	0.31 (0.28–0.35)
8	Schley	46	Perched culvert	0	0.418	3.47	22 (16–31)	1.7	11.5	0.73 (0.69–0.77)
9	Seepay[a]	68	NA	2	0.501	5.18	93 (68–138)	10.4	NA	1.11 (0.36–1.84)
10	Talking water	40	Perched culvert	0	0.187	1.95	28 (11–165)	0.6	15.5	0.28 (0.27–0.30)
11	Teepee	36	Perched culvert	0	0.176	1.50	14 (2–Inf)	0.4	15.5	0.2 (0.17–0.23)
12	Thorne	51	Irrigation diversion/canal	1	0.416	3.48	17 (13–23)	3.5	24.5	0.35 (0.15–0.56)
13	West Magpie	44	Irrigation diversion/canal	1	0.233	2.30	8 (4–12)	4.6	12	0.21 (0.09– 0.33)
14	Yellow Bay	57	Perched culvert	0	0.189	1.93	17 (7–40)	1.4	15.5	0.61 (N/A)
Upper Flathead River Basin (includes geologically isolated populations)										
15	Addition	26	Waterfall	0	0.149	1.84	20 (4–Inf)	6.7	≥250	
16	Bunker	28	Waterfall	0	0.132	1.54	82 (5–Inf)	15.6	≥250	
17	Colts[a]	25	NA	0	0.431	4.19	42 (23–123)	5.4	NA	
18	Emery[a]	27	NA	0	0.439	4.33	38 (23–82)	8.0	NA	
19	Goldie	25	Waterfall	0	0.090	1.43	–21 (9–Inf)	2.1	≥250	
20	Hungry horse[a]	24	NA	0	0.439	4.14	83 (33–Inf)	9.0	NA	
21	Kneiff	25	Waterfall	0	0.060	1.14	−12 (0–Inf)	4.4	≥250	
22	Middlepass[a]	25	NA	0	0.420	4.11	25 (16–47)	10.9	NA	
23	Quintonkin	27	Waterfall	0	0.245	2.19	58 (14–Inf)	10.0	≥250	
24	South	27	Waterfall	0	0.285	2.13	20 (7–169)	2.8	≥250	
25	Upper twin	28	Waterfall	0	0.281	2.26	25 (9–425)	18.6	≥250	

Average density of fish was only estimated for populations in the Lower Flathead River Basin. Density was estimated at only one location in one year in Yellow Bay Creek, and so there was no range of density estimate values to report

[a] connected populations

Habitat characteristics, density and abundance estimates for anthropogenically isolated populations

For anthropogenically isolated populations, median length of occupied habitat above the isolating barrier was 3.2 km with a range of 0.4–14 km, compared to 5.3 and 10.4 km for the two connected systems in the same watershed (Table 1). Average density estimates of fish over 75 mm TL in isolated streams ranged from 0.18 to 0.73 fish/m (median = 0.36 fish/m) compared to 0.57 and 1.11 fish/m for the connected populations. Abundance estimates, which are a product of average density and length of occupied habitat, ranged from 89 to 4347 (median = 1239) fish in isolated streams, compared to 3044 and 11,567 in the two connected streams (Table 1).

Scores for habitat quality across all 16 streams in our data set ranged from 1 (high quality) to 3 (low quality) with a median of 1.9. Growing degree days and base flow ranged from 630 to 1134 GDD (median = 828) and 0.04–2.52 CMS (median = 0.29) respectively (Table S1).

Genetic diversity in anthropogenically isolated populations

Isolated populations had an average allelic richness (R_s) of 2.84, which was significantly lower than that of connected populations in the same region (R_s in connected populations = 5.21, $p < 0.001$; Table 1). Revais Creek, the only anthropogenically isolated stream over 8 km in our dataset, maintained R_s of 5.25, similar to that of the connected populations in the same watershed (Fig. 2a; Table 1). All other anthropogenically isolated populations examined were found in less than 8 km of occupied habitat and had lower allelic richness (average $R_s = 2.62 \pm 0.33$ S.E.). Across all anthropogenically isolated populations, length of occupied habitat and abundance were significant predictors of allelic richness ($p < 0.01$, $R^2 = 0.576$ and $p < 0.006$, $R^2 = 0.547$ respectively), but time since isolation and average density were not ($p = 0.791$, $R^2 = 0.007$ and $p = 0.760$, $R^2 = 0.01$ respectively). However, when analyzing populations isolated in less than 8 km of occupied habitat (i.e., excluding Revais Creek), the effect of habitat length and abundance on allelic richness was substantially reduced ($p = 0.103$, $R^2 = 0.268$ and $p = 0.112$, $R^2 = 0.256$) and time since isolation and average density still had no effect ($p = 0.625$, $R^2 = 0.028$ and $p = 0.414$, $R^2 = 0.075$).

Preliminary analyses revealed a significant negative correlation between growing degree days and base flow (Pearson's correlation = −0.72, $p < 0.02$) for all stream fragments with less than 8 km of occupied habitat (excluding Centipede Creek because the temperature logger failed in this stream). Given this correlation, we retained base flow (versus a temperature metric) in our global regression models for two reasons: First base flow is related to habitat volume and other potentially limiting habitat factors such as pools (Harig and Fausch 2002). Second, the summertime temperature range measured did not exceed the optimal growth range for cutthroat (9.5–16.4 °C, Bear and McMahon 2007).

The two global models tested to examine the relationship between variance in genetic diversity and habitat characteristics in our isolated populations were as follows:

$$\text{MODEL A}: Iso + CMS + Quality + Density + Length,$$

$$\text{MODEL B}: Iso + CMS + Quality + Abundance,$$

where Length represents length of occupied habitat in isolate habitat, and Iso represents generations of isolation in anthropogenically isolated populations, CMS represents base flow measured as cubic meters per second, Quality represents habitat quality as estimated by local experts, Density is average number of fish per meter, and Abundance is estimated population size. The variance inflation factor of each variable in these models was less than 5.

None of the multiple regression models relating habitat characteristics, length of occupied habitat, and time since isolation to genetic diversity for populations isolated in under 8 km of occupied habitat met our model selection criteria. Specifically, the multiple regression analyses did not produce any models in which all parameter estimates were significantly different from zero. The best supported model for A contained only length of occupied habitat ($R^2 = 0.268$, $p = 0.103$). The best supported model for B contained only population abundance ($R^2 = 0.256$, $p = 0.112$). For both model structures, there were no competing models within two AICc points of the best supported model.

Comparison between anthropogenically and geologically isolated populations

Geologically isolated streams had a median of 6.7 km (range 2.1–18.6 km) of occupied habitat, versus a median of 15.1 km (range 13.2–16 km) for the connected populations in the same watershed. Geologically isolated populations had an average R_s of 1.79, which was significantly lower than that of the connected populations in the same basin ($p < 0.001$, Fig. 2b). While several of the geologically isolated streams had occupied habitat of 10 km or more (Table 1), none of the geologically isolated systems demonstrated maintenance of genetic diversity compared to connected populations in the same basin. Although geologically isolated populations were found in substantially larger habitat fragments compared to anthropogenically isolated populations ($p < 0.05$), geologically isolated streams had significantly lower genetic diversity ($p = 0.01$).

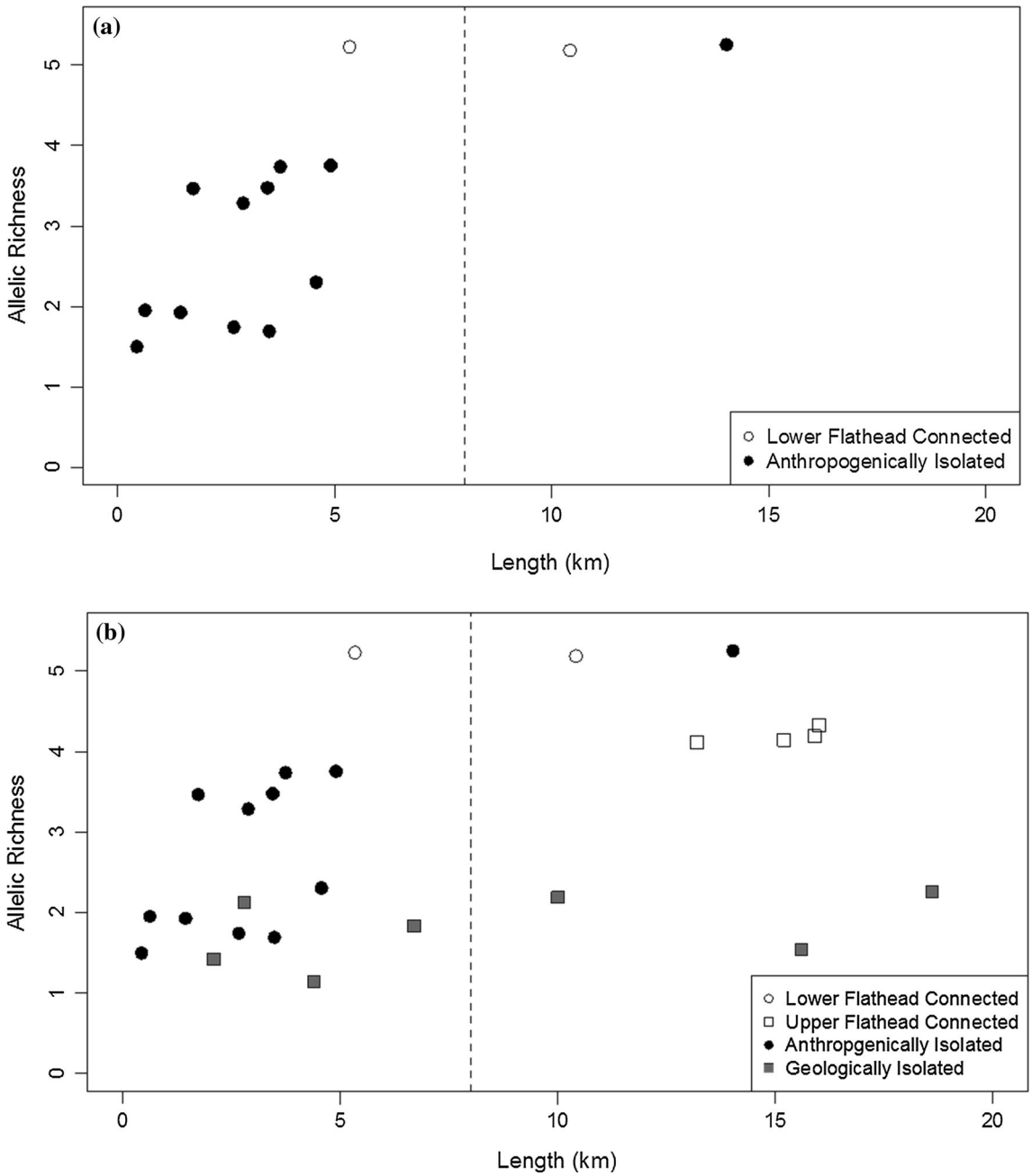

Fig. 2 Allelic richness versus length of occupied habitat in streams of the Lower Flathead River basin (**a**) and both the Lower and Upper Flathead River basins (**b**). The *dashed line* marks 8 km of occupied stream habitat. For the purposes of this study, a stream fragment with less than 8 km of occupied habitat is considered "small", while one with more than 8 km is considered "large"

Discussion

Loss of genetic diversity over geologic time appears to be a general phenomenon in isolated populations of cutthroat, even for populations in relatively large (>8 km) high-quality habitat. Compared to connected populations in this study, genetic diversity was not maintained in geologically isolated populations residing in fragments of large habitat unimpacted by human activities. These results suggest that large habitat fragments may not provide an effective long-term buffer against loss of genetic diversity. Rather, over longer time frames the genetic characteristics of isolated cutthroat populations may be largely shaped by stochastic events that cause population bottlenecks and genetic drift.

Estimated levels of heterozygosity for connected populations in this dataset are similar to other connected populations of cutthroat trout in western Montana. For example, expected heterozygosity of genetically pure populations in Wasson, Poorman, Nevada Spring, and Grantier Spring Creeks of the Upper Blackfoot River basin ranged from 0.416 to 0.45 when analyzed across 11 of the same polymorphic loci included in this study (Carim 2013). This highlights that the connected populations in this study are an appropriate baseline for comparison to the isolated populations in the Flathead River basin. Additionally, these results corroborate other studies documenting loss of genetic diversity in geologically isolated populations of cutthroat trout (Pritchard et al. 2007; Whiteley et al. 2010).

Unlike previous studies, our data also demonstrate that loss of genetic diversity in small, anthropogenically isolated fragments can occur in relatively few generations. For example, in two populations (Teepee and Talking Water Creeks) that had been isolated for less than a dozen generations at the time of samples, levels of allelic richness were three fold lower compared to connected populations. A similar rapid loss of genetic diversity has been observed in populations of brook trout isolated by anthropogenic barriers for less than 50 years (Whiteley et al. 2013). These results highlight that maintenance of genetic diversity in populations residing in small habitat patches likely rely on metapopulation dynamics (sensu Kritzer and Sale 2004), including regular dispersal between subpopulations, rather than maintenance of a large local population size. Thus, the potential for isolation lasting longer than several generations to be an effective management tool may be limited, particularly in the context of more pervasive conservation challenges such as increased environmental stochasticity associated with climate change and invasive species that are not limited by the same types of barriers.

The loss of genetic diversity we observed in all geologically isolated populations (including those in large habitat fragments) may be attributed to two potential explanations. First, rules of thumb for maintaining genetic diversity in isolated population are based on genetic theory, which assumes an ideal Fisher–Wright population– something that is rarely, if ever observed in the wild. Furthermore, the suggestion that an $N_e = 500$ is appropriate for maintenance of diversity in cutthroat populations assumes: (1) the estimated rate of functional mutations per generation equal to 0.001 for fruit flies is similar for inland trout (see Franklin 1980); and (2) the N_e:N ratio is similar between inland trout and Pacific salmon (see Allendorf et al. 1997). Nabholz et al. (2008) found that mutation rates in mammals varied greatly across mammalian taxa. Specifically, the authors found that mutation rates in the order Rodentia were $\sim$50 times faster than Carnivora, with a significant positive correlation between functional and neutral mutation rates. Although there is currently no estimate of how functional mutation rates may vary across salmonid species, Steinberg et al. (2002) estimated neutral mutation rates in pink salmon ranging from 0 to 0.0085 per generation. In addition to variable mutation rates, N_e:N ratios will also vary between species, and even among populations within a species, due to factors including, but not limited to, population size, historical population bottlenecks, variation in life history strategy, and whether variance or inbreeding N_e was estimated (Hedrick et al. 2000; Palstra and Ruzzante 2008; Hare et al. 2011; Gomez-Uchida et al. 2013). Given that mutation rates vary across species and considering that isolated populations face different life history tradeoffs than connected populations, the minimum effective population size for maintenance of genetic diversity is likely to vary at both the species and population level.

The second potential explanation for the loss of genetic diversity in geologically isolated populations relative to connected populations is that localized environmental stochasticity is inherently difficult to incorporate into broad rules of thumb for maintaining diversity in isolated trout populations. On a landscape scale, trout populations often persist as a group of subpopulations that interact through dispersal and periodically experience natural disturbance such as floods, drought, fire, and debris and ice flows (Resh et al. 1988; Dunham and Rieman 1999; Lake 2000; Rieman and Dunham 2000; Miller et al. 2003). When these events are severe, they can cause population bottlenecks and subsequent loss of genetic diversity (Hakala and Hartman 2004; Pujolar et al. 2011), particularly in isolated populations that lack potential for gene flow. Populations in connected habitat are expected to rebound from disturbances through dispersal (Roghair et al. 2002; Neville et al. 2006; Pierce et al. 2013), which may restore both population size and genetic diversity in a given habitat patch. Populations isolated in larger habitat fragments are expected to be less susceptible to bottlenecks and loss of genetic diversity because larger habitat will support larger populations and provide more refugia under adverse conditions (Dunham and Rieman 1999; Neville et al. 2009). However, this does not mean that populations in larger fragments are immune to bottlenecks or extirpation. For example, salmonid species observed above geological barriers commonly show lower levels of genetic diversity compared to connected populations in the same region (Costello et al. 2003; Neville et al. 2006; Guy et al. 2008; Whiteley et al. 2010). Additionally, viable habitat for cutthroat trout above geologic barriers is more likely to be unoccupied than connected habitat below (Hastings 2005). Over the course of several thousand generations, it is likely that all populations in this study have experienced stochastic environmental events capable of producing

strong demographic and genetic effects. Although we cannot reconstruct where and how historically occurring populations were extirpated, the observations in our study and others suggest that stochasticity is one of the most influential factors in the fate of isolated populations where immigration is not possible.

The low levels of genetic diversity we observed in geologically isolated populations should not be interpreted to mean that genetic diversity is not important for population persistence. While persistence of cutthroat above geological barriers is not uncommon, studies have shown that occurrence of cutthroat is more patchy in streams and stream networks above these natural barriers, even when ample high-quality habitat is present (Dunham et al. 1997; Hastings 2005). Research examining the association between inbreeding load and demographic vital rates is necessary to understand how and when inbreeding depression may be affecting these populations. Furthermore, because researchers have not closely observed and documented the extirpation of cutthroat under these circumstances, it is difficult to discern whether unoccupied habitats are the result of demographic factors, genetic factors, or some combination of the two.

No population in this dataset, including connected populations, had an estimated effective population size larger than 166. Two isolated geologically isolated populations had negative estimates of effective population size, which occurs when the correction for sampling from a finite population is larger than the observed linkage disequilibrium, indicating that the sample size for estimating effective population size is too small (Macbeth et al. 2013). Of the 12 anthropogenically isolated and five geologically isolated populations with positive estimates of effective population size, only four populations had estimated effective sizes $\geq$50 (Frog, Revais, Bunker and Quinitonkin; Table 1). However, overlapping generations in our dataset likely created a downward bias of Ne estimates. Waples et al. (2014) estimated that including individuals from all cohorts of a population to estimate Ne using linkage disequilibrium methods can result in a downward bias as high as 50 % in vertebrates. Yet, accounting for the most extreme downward bias by doubling the estimates of Ne in our dataset would still result in effective sizes $\leq$100 for most populations. If these populations met the underlying assumptions of the 50/500 rule, all populations in this dataset would be considered at high risk for inbreeding depression.

The consistent pattern of reduced genetic diversity in anthropogenically isolated salmonid populations both here and in other studies (Yamamoto et al. 2004; Morita et al. 2009; Horreo et al. 2011; Sato and Gwo 2011; Kitanishi et al. 2012; Whiteley et al. 2013) indicates that intentional isolation of populations to prevent invasion by nonnative species may need to be coupled with assisted geneflow to promote gene flow and avoid the risk of inbreeding depression (Whiteley et al. 2015). However, managers should closely analyze demographic parameters to determine if genetic rescue through assisted geneflow would actually benefit the population. Across salmonid species, including cutthroat, mounting evidence suggests that adaptation to local habitat characteristics is common, and can occur rapidly in as few as a few generations (Fraser et al. 2011; Drinan et al. 2012; Narum et al. 2013). It is commonly believed that loss of genetic diversity in small isolated populations is primarily driven by genetic drift. However, if selection pressure is sufficiently high, local adaptation could equally influence small isolated populations with natural selection quickly driving advantageous alleles to high frequencies (Allendorf and Luikart 2008). Under these circumstances, isolation would limit gene flow that could reduce the frequency of the most advantageous alleles. Thus, before proceeding with genetic rescue efforts in small isolated populations of trout, a better understanding is needed of how the introduction of fish that are ill-adapted to the local environment could result in outbreeding depression, and potential population decline. This is particularly relevant to populations in our study where time since isolation for all populations exceeded ten generations, but populations have persisted despite low diversity.

In our study, there was no significant relationship between habitat or demographic characteristics and the observed levels of genetic diversity in cutthroat populations isolated in less than 8 km of habitat. This result could be driven by lack of power in a small data set with a limited range in the predictor variables. With 14 km of occupied habitat, Revais Creek was the only stream in this dataset with a population residing in over 8 km of habitat. This population had the largest estimated abundance ($14\ \text{km} \times 0.31\ \text{fish/m} = 4416\ \text{fish}$), and was the only population that maintained levels of genetic diversity similar to populations in connected systems. While this is just a single example, it does suggest that genetic diversity may be maintained on shorter timescales by maximizing the size of high-quality habitat under isolation. This strategy may be useful for managers protecting native trout while performing eradication efforts to remove invasive species, and subsequently extend the amount of habitat available to the native population.

Many remaining populations of westslope cutthroat trout are isolated (Shepard et al. 2005), leaving them at increased risk of extirpation from genetic, demographic, and environmental stochasticity. Yet, for many of these cutthroat populations, isolation may still be the only reliable strategy for protection against invasive fish species. Genetic monitoring of isolated populations will be

important for identifying populations at risk of inbreeding depression. Furthermore, future research should focus on rigorous evaluation of how and where assisted geneflow could be used to rescue populations suffering from inbreeding depression and maintain populations and species-level genetic diversity.

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