

Environmental and anthropogenic correlates of hybridization between westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) and introduced rainbow trout (*O. mykiss*)

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Abstract Hybridization with introduced taxa is one of the major threats to the persistence of native biodiversity. The westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) is found in southeastern British Columbia and southwestern Alberta, Canada, and adjacent areas of Montana, Idaho, and Washington State, USA. Through much of this area, native populations are threatened by hybridization with introduced rainbow trout (*O. mykiss*). We surveyed 159 samples comprising over 5,000 fish at 10 microsatellite DNA loci to assess the level of admixture between native westslope cutthroat trout (wsct) and introduced rainbow trout in southwestern Alberta. Admixture levels (q_{wsct} of 0 = pure rainbow trout, q_{wsct} of 1.0 = pure westslope cutthroat trout) ranged from <0.01 to 0.99 and averaged from 0.72 to 0.99 across seven drainage areas. Regression tree analyses indicated that water temperature, elevation, distance to the nearest stocking site, and distance to the nearest railway line were significant components of a model that explained 34 % of the variation across sites in q_{wsct} across 58 localities for which habitat variables were available. Partial dependence plots indicated that admixture with rainbow trout increased with increasing water temperature and distance to the nearest railway line, but decreased with increasing elevation and distance from

stocking site to sample site. Our results support the hypothesis that westslope cutthroat trout may be less susceptible to hybridization with rainbow trout in colder, higher elevation streams, and illustrate the interaction between abiotic and anthropogenic factors in influencing hybridization between native and introduced taxa.

Keywords Hybridization · Microsatellites · Admixture analyses · Regression trees · Westslope cutthroat trout · Threatened species · Salmonidae

Introduction

Hybridization between native and introduced species is an on-going conservation issue (Rhymer and Simberloff 1996; Allendorf et al. 2001; Levin 2002). In freshwater fishes, this problem has arisen in large part due to efforts to enhance recreational fisheries (Larson and Moore 1985; Allendorf and Leary 1988) or by habitat alteration (e.g., Hubbs 1955; Vonlanthen et al. 2012) or their interaction. Many situations include fishes that have evolved in isolation from one another such that reproductive isolation may be incomplete (Allendorf and Leary 1988), and following introduction of non-indigenous species widespread hybridization often results (Rhymer and Simberloff 1996). One of the possible outcomes of hybridization is genomic extinction (Epifanio and Philipp 2000; Allendorf et al. 2004; Muhlfeld et al. 2009a). In fact, introgressive hybridization is so pervasive in freshwater salmonids (trout, salmon and their relatives) it has been called the most important factor responsible for the loss of native trout species (Allendorf and Leary 1988).

The westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) is one of at least ten and perhaps as many as 14

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subspecies of cutthroat trout (*O. clarkii*), and is native to interior drainages of southeastern British Columbia (BC), southwestern Alberta, Canada, and adjacent watersheds in Idaho and Montana and scattered areas in western Washington and Oregon in the United States (US, Behnke 1992). Often considered as an indicator species for pristine environments, westslope cutthroat trout are slow-growing and have a relatively narrow range of suitable living and spawning conditions (DFO 2009; Rasmussen et al. 2010). Adults require cool, clear water with in-stream structural complexity and riparian cover. They are typically found in water temperatures between 9 and 12 °C. Different life stages use different habitat types; silt-free, well-oxygenated water with clean gravel is necessary for spawning, shallow pools with low water velocity for juveniles, and deep pools are needed for summer and overwintering (Cleator et al. 2009). These habitat requirements can span large geographic distances suggesting that connectivity is essential to the persistence of the species and, even in the absence of hybridization, may make them particularly susceptible to local extirpation. Westslope cutthroat trout are unusual in that much of their genetic variation results from alleles that are found in high frequency in relatively few localities and barriers and watershed divisions isolate populations from one another such that genetic divergence between populations of westslope cutthroat can be relatively high (Allendorf and Leary 1988; Taylor et al. 2003). Consequently, strategies to ensure high levels of genetic diversity within the subspecies call for the conservation of many different populations (Allendorf and Leary 1988). Further, hybridization with rainbow trout may result in increased fragmentation of populations as non-admixed populations of westslope cutthroat trout become isolated from one another by intervening admixed populations (Rhymer and Simberloff 1996; Cleator et al. 2009; DFO 2009).

The abundance of westslope cutthroat trout has declined across much of its range in BC, Alberta, and parts of the US (Schmetterling 2001; Weigel et al. 2003; Rubidge and Taylor 2005; Mayhood and Taylor 2011). In Alberta, westslope cutthroat trout inhabit less than 20 % of their historical range owing to overexploitation as well as habitat loss and degradation and are thought to number fewer than 5,000 adults (COSEWIC 2007; Mayhood and Taylor 2011). Populations of westslope cutthroat trout persist primarily in the headwater tributaries of the Oldman and Bow rivers' drainage systems which form part of the western headwaters of the South Saskatchewan River system (Mayhood and Taylor 2011). Early records of rainbow trout stocking date back to the mid-1920's, and some 60 million fish have been stocked since that time (Alberta Sustainable Resource Development, Airdrie, Alberta, unpublished data) and hybridization with rainbow trout is considered to be widespread across extant

populations (DFO 2009; Mayhood and Taylor 2011). Furthermore, Muhlfeld et al. (2009a) demonstrated that westslope cutthroat trout with admixture levels of 20 % rainbow trout experienced reductions in correlates of fitness of up to 50 % in nature. Concerns over declines in abundance and hybridization led to the conservation status assessments of Threatened and Special Concern for native westslope cutthroat trout in Alberta and BC, respectively, by Canada's Committee on the Status of Endangered Wildlife in Canada (COSEWIC). While clearly recognizing the threat of hybridization to westslope cutthroat trout, the US Fish and Wildlife Service has determined that a listing of Endangered or Threatened under the *Endangered Species Act* is not warranted at this time in the US (Kaeding 2003; see also Shepard et al. 2005).

Although hybridization in freshwater fishes has a long history of study (e.g., Hubbs 1955; Taylor 2004; Hansen and Mensberg 2009), the biological, environmental, and anthropogenic factors that influence the extent and spatial distribution of hybridization are not well known. Heath et al. (2010), presented evidence that a combination of anthropogenic habitat alteration (e.g., logging and urbanization) and stocking intensity were important drivers of spatial variation in hybridization between rainbow trout and coastal cutthroat trout (*O. c. clarkii*) in southwestern BC. At the intraspecific level, Marie et al. (2012) demonstrated an effect of stocking intensity, habitat size, dissolved oxygen levels and pH among wild lake-dwelling populations of brook char (*Salvelinus fontinalis*) in Québec, Canada. Previous work on rainbow trout and westslope cutthroat trout hybridization has indicated that some environmental features, especially water temperature and elevation, seem to co-vary with admixture levels (e.g., Rubidge and Taylor 2005; Muhlfeld et al. 2009b; Rasmussen et al. 2010), while in other cases spatial arrangement of populations also seems to be important (e.g., Hitt et al. 2003; Boyer et al. 2008).

In this study, we report the results of an extensive survey of admixture levels across more than 150 localities with westslope cutthroat trout in southwestern, Alberta, and adjacent portions of southeastern BC. We then use these data to test for an effect of key habitat and stocking variables that may influence spatial variation in levels of hybridization. By conducting this analysis we hoped to independently test the idea that certain environmental and anthropogenic factors (e.g., water temperature, human habitat disturbance) are important in influencing the degree of hybridization between rainbow trout and westslope cutthroat trout as has been suggested in other areas and species. The identification of natural variables influencing hybridization may also be relevant to understanding factors important in the evolution of reproductive isolation between these species (e.g., Culumber et al. 2012), while

the identification both of natural and anthropogenic-related variables could be useful when designing programs to limit the spread of hybridization or mitigate its effects.

Materials and methods

Sampling

Tissue samples consisted of either fins clips stored in 95 % ethanol or dried and stored in paper envelopes. All samples were obtained from populations of westslope cutthroat trout across localities in southwestern Alberta and a small adjacent region of British Columbia (BC) largely between 2006 and 2009, although a small number of samples dated to 1999 (Fig. 1, Supplementary Table 1). Most of our sampling localities spanned two major watersheds: the Bow, and Oldman rivers and 26 sub-basins within these watersheds and included samples from four national parks (Banff, Jasper, Kootenay and Yoho National Parks). The Bow and Oldman rivers are tributaries of the South Saskatchewan River (Hudson Bay drainage) which represents the northern limit of the natural range of westslope cutthroat trout east of the Continental Divide. We also examined samples from several tributaries of the upper Kootenay River (Columbia River drainage) in southeastern BC. Altogether, we assayed 6,072 fish across 220 localities, but we limited population-level analyses to localities with at least 15 samples ($N = 159$).

Genetic analyses

Genomic DNA was extracted from the fin samples using standard phenol–chloroform methods. Individuals were characterized for allelic variation using ten microsatellite based markers: *Ssa85* and *Ssa197* (O'Reilly et al. 1996), *Ssa456* (Slettan et al. 1995), *Ots3*, *Ots4* (Olsen et al. 1996), *Ots104*, and *Ots107* (Nelson and Beacham 1999), *Oki3a* (P. Bentzen, Dept. of Biology, Dalhousie University, Halifax, NS, unpublished data), *Omy77* (Morris et al. 1996), and *Occ16* (Ostberg and Rodriguez 2002). Only *Occ16* appears to be strictly diagnostic between the two species (Ostberg and Rodriguez 2002; Taylor unpubl. data), but all other loci (except *Ssa197*) showed major differences in allele size ranges and frequencies. For instance, in our final learning dataset (see below under Admixture analyses) $F_{ST}(\theta)$ (Weir and Cockerham 1984) between the species averaged 0.50 (SD = 0.08) and ranged from 0.17 (*Ssa197*) to 1.0 (*Occ16*). These loci were also scored in 150 “learning samples” of allopatric rainbow trout and 75 westslope cutthroat trout that previous analyses indicated had no detectable admixture and that represented a range of

populations from BC and Alberta (Taylor et al. 2003, 2007; Tamkee et al. 2010).

Polymerase chain reactions were performed in 20 μ l total volumes using the Qiagen Multiplex PCR Kit following the manufacturer's instructions. An average of 30 individuals were assayed per sampling location and PCR products were evaluated using fluorescently labeled primers and assayed on a Beckman-Coulter CEQ 8000 automated genotyper.

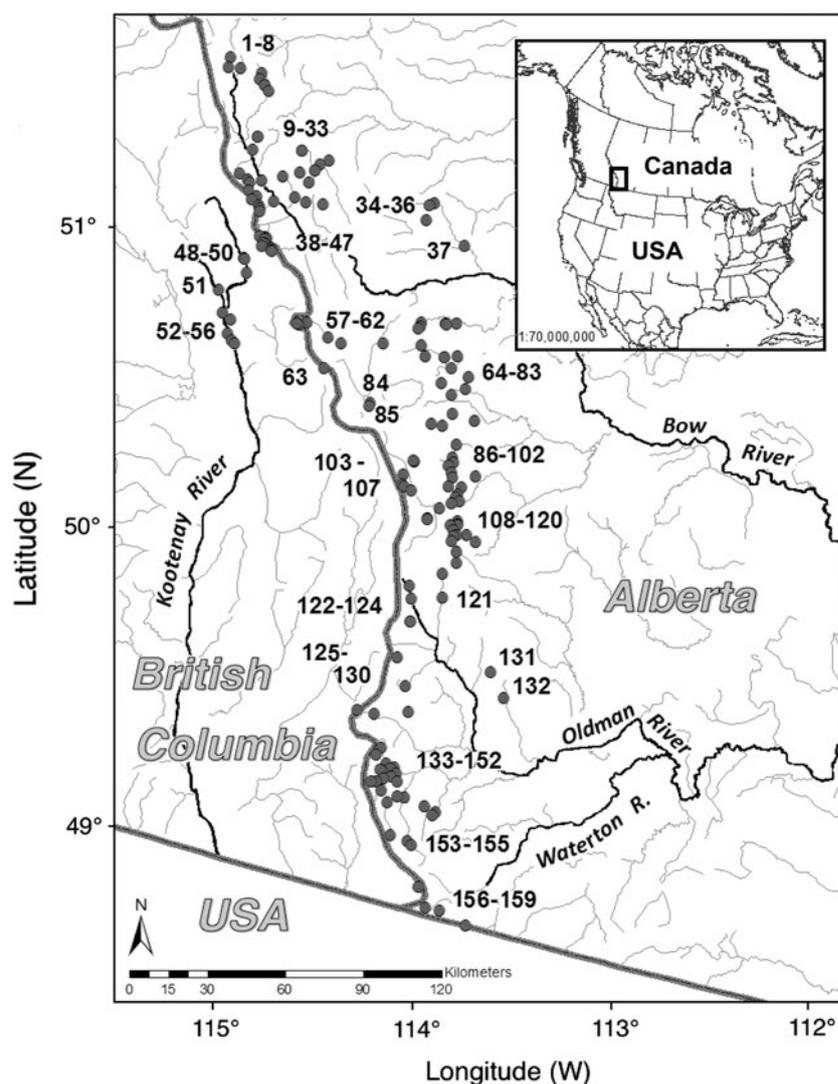
Population genetic analyses

We used MICRO-CHECKER (van Oosterhout et al. 2004) to check for the presence of null alleles or PCR artifacts that could compromise subsequent analyses. Thereafter, basic descriptive statistics of sample size (N), number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosity were compiled using FSTAT ver 2.9 (Goudet 2001). Tests for deviations from Hardy–Weinberg equilibrium were performed for each locus–population combination using an exact test in which probability values were estimated using a Markov chain method using GENEPOP ver. 4.0 (Rousset 2008). Tests for genotypic linkage disequilibrium for all combinations of locus pairs within a population were also made using a Markov chain method with GENEPOP default values.

Admixture analyses

Individual admixture values (q -values) and posterior probability intervals were estimated for each locality using STRUCTURE (Pritchard et al. 2000). We set K (number of genetic populations) to $K = 2$ to represent the two species which were clearly distinct from one another in preliminary analyses (e.g., ordination analyses, assignment tests). Models were run under the admixture model and assuming correlated allele frequencies with a burn-in of 100,000 steps and subsequent runs of 450,000 steps. We also calculated admixture values for a sample of 100 simulated hybrids between the two species. The simulated hybrid genotypes were generated by using the HYBRIDLAB (Nielsen et al. 2006) program by randomly selecting alleles for each locus from the allopatric, non-admixed populations of rainbow trout and westslope cutthroat trout. We performed admixture analyses in two steps. First, we ran simulations using the allopatric, non-admixed populations (“learning samples”) and all other population samples using five replicated analyses. Here, the non-admixed samples were used as priors in the model, i.e., the program was forced to consider these individuals as known rainbow trout and westslope cutthroat trout by invoking the USE-POPINFO model in STRUCTURE (Pritchard et al. 2000). The admixture value, q , was expressed as the proportion of

Fig. 1 Map of 159 localities in southwestern Alberta and southeastern British Columbia, Canada, which were sampled for westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) and subject to microsatellite DNA analyses



the genome estimated to stem from westslope cutthroat trout, q_{wsct} (0 = pure rainbow trout, 1.0 = pure westslope cutthroat trout). The mean q_{wsct} value across the five replicate analyses was then calculated for all of the non-learning samples. From this analysis, three populations of westslope cutthroat trout for which all individuals had q_{wsct} values of ≥ 0.99 were added to the learning sample group and the admixture analyses was rerun across five further replicates. We conducted the admixture analyses in two steps because: (i) the initial learning sample of westslope cutthroat trout was much smaller (75) than that of the rainbow trout, and (ii) the initial learning sample contained no non-admixed populations from Alberta and we wanted to account for possible genetic differentiation between BC and Alberta populations of westslope cutthroat which are separated by the Continental Divide. The final learning sample set consisted of 150 rainbow trout and 165 westslope cutthroat trout. The final values of q_{wsct} for Alberta populations of westslope cutthroat trout

represent the averages calculated across the five replicate analyses.

Stream characteristics and anthropogenic variables

Variables were organized into three broad categories: stream geomorphology/environments, stocking history, and variables representing aspects of anthropogenic-based habitat disturbance (Table 1). Environmental variables were chosen based on those considered to influence hybridization in western trout (Hitt et al. 2003; Weigel et al. 2003; Rubidge and Taylor 2005; Muhlfeld et al. 2009b; Heath et al. 2010). Also, the number of stocking events and the total number of rainbow trout introduced both represent aspects of stocking intensity that may influence the potential for admixture (e.g., Ruzzante et al. 2004; Hansen and Mensberg 2009; Marie et al. 2010). Finally, habitat disturbance from human development has long been considered an important variable influencing

Table 1 Definition of variables potentially explaining variation in admixture levels between native westslope cutthroat trout and introduced rainbow trout

Variable	Definition
Stream variables	
Depth1	Maximum water depth at sample site
Depth2	Mean water depth at sample site
Water temperature	Water temperature at time of sampling
Order	Strahler stream order of sample site (1–5)
Elevation	Elevation in meters at sample site
Anthropogenic disturbance variables	
Road (RoadD)	Euclidean distance from sample site to nearest road
Pipeline (PipeD)	Euclidean distance from sample site to nearest gas/oil pipeline
Railway (RailD)	Euclidean distance from sample site to nearest rail line
Powerline (PowerD)	Euclidean distance from sample site to nearest power line
Stocking variables	
Distance (StockD)	Fluvial distance to nearest stocking site
Stocking intensity (StockI)	Total number of stocking events at the stocking site
Number of fish stocked (StockN)	Total number of rainbow trout stocked at the stocking site
Year of last stocking (StockY)	Total years between 2010 and year of last stocking at stocking site

interspecific hybridization in fishes (Hubbs 1955). More recently, Heath et al. (2010) found evidence of a positive association between logging activity and urban development and hybridization between naturally sympatric rainbow trout and coastal cutthroat trout (*O. c. clarkii*) in southwestern BC.

Locality and stocking data were available for a total of 58 streams (Alberta Sustainable Resource Development, Airdrie, AB; Supplementary Table 2). These data were mapped using ArcGIS 10.0 (ESRI, Redlands, CA, USA). Elevation data was extracted using a 25-metre Digital Elevation Model and stream order, a measure of stream branching, was assessed in ArcGIS using the Strahler method (Horton 1945; Strahler 1952). Spot water temperature was recorded with hand-held thermometers during daytime fish sampling. To assess how well such instantaneous measures might reflect longer term, relative differences between localities, we obtained longer term measures of air temperature near each locality through WorldClim (www.worldclim.org). These data layers are generated by interpolating monthly climate data from the Global Historical Climatology Network (GHCN), the Food and Agriculture Organization of the United Nations, and the World Meteorological Organization, and several other sources of climate data for the period 1950–2000 (see

Hijmans et al. 2005 for details). Our locality water temperatures obtained during daytime fish sampling were positively correlated with mean annual air temperature ($r = 0.31$, $df = 57$, $P = 0.002$), maximum air temperature of the warmest month ($r = 0.26$, $P = 0.01$) as well average air temperature over the warmest three month period ($r = 0.40$, $P < 0.0001$). Further, elevation and water temperature are expected to be negatively correlated with one another in natural systems (e.g., Paul and Post 2001; Rasmussen et al. 2010) as was observed in our data ($r = -0.51$, $P < 0.001$). These associations with elevation and longer term air temperature trends suggest that locality water temperatures recorded at time of fish sampling represent a reasonable proxy for relative differences between localities. Human impact variables (e.g., distance of sample site to nearest road) were measured as Euclidean distances and distance from the sample site to the stocking location (stockD) was calculated as the fluvial distance in ArcGIS (Table 1). In some streams, stocking of rainbow trout took place in tributaries other than that in which genetic samples were obtained. In others, apparent upstream migration barriers (mapped in ArcGIS) separated stocking and genetic sample localities within tributaries. Preliminary analyses, however, indicated no difference in average q_{wsct} values between localities where stocking took place in the same or different tributaries or separated by potential upstream migration barriers (Yau and Taylor, unpubl. data) so we did not include these potential effects in our analysis.

Stocking intensity was represented by the number of stocking events at the stocking location nearest to the genetic sample locality. Other variables, included the total number of rainbow trout stocked at each locality, and the year of last stocking, which was assessed to investigate how time scale may influence admixture levels.

Statistical analysis

Differences in the mean values (calculated across samples with a sample size of ≥ 10) of q_{wsct} were tested for significance using variants of one-way ANOVA and subsequent pairwise t-tests suitable for samples with unequal variances using PAST (version 2.12), a general spreadsheet-based statistical package (Hammer et al. 2001).

To identify habitat and human impact variables that might influence admixture levels we used a nonlinear, regression tree approach implemented in the Random Forest algorithm described by Breiman (2001). Regression trees use decision tree analysis to help to resolve relationships between a response variable (in our case mean admixture level) and several potential predictor variables that may behave in a non-linear fashion and have multiple interactions amongst them. Regression trees and their use in ecological data analyses have been described by De'ath

and Fabricus (2000) and Moisen (2008) and have seen recent application in landscape genetics (e.g., Murphy et al. 2010; Hether and Hoffman 2012). Random Forest analysis uses bootstrapped, learning datasets to build an assemblage of regression trees each of which generates predictions of the dependent variable (q_{wsct}) based on the independent variables. These predictions are then averaged across all bootstrapped iterations to yield a final prediction. Examining many bootstrapped samples coupled with random sampling of subsets of predictor variables during tree construction helps to reduce the variance amongst different regression tree results. Random forests also have the desirable qualities of being insensitive to autocorrelation, are distribution free, do not require transformation of original variables, and they can assess complex interactions amongst many variables (De'ath and Fabricus 2000; Moisen 2008). We combined the approaches of Murphy et al. (2010) and Hether and Hoffman (2012) by first analyzing a full model incorporating all 13 potential predictor variables, evaluating sub-models with subsets of predictor variables, running a final “best” sub-model and assessing the relative importance of each predictor variable and overall model significance using the RANDOMFOREST package v 4.5-28 with R (Liaw and Wiener 2002; R Development Core Team 2012) under the regression mode with 10,000 trees.

We converted the measures of variable importance (I_p) that are provided in RANDOMFOREST to model improvement ratios (MIRs) by dividing each I_p by the maximum I_p observed (Hether and Hoffman 2012). We then used the MIR to iteratively select the best sub-models from the full model. First, we ran a sub-model that incorporated only those variables that had MIR of at least 0.3 (i.e., they improved the model to a degree of at least 30 % of the best variable). Next, we ran 20 independent RANDOMFOREST analyses for each of a series of sub-models that had one of the predictor variables removed, starting with the variable with the lowest MIR. We calculated the mean and 95 % confidence intervals for the resulting pseudo- R^2 (Liaw and Wiener 2002) values for each such sub-model. We then chose a final sub-model that had the fewest predictor variables, but whose mean pseudo- R^2 had a 95 % confidence interval overlapping that of the best sub-model (Hether and Hoffman 2012). We used partial dependence plots to assess the marginal effects of each retained predictor variable on admixture (i.e., the effect not explained by other predictor variables, Liaw and Wiener 2002; Cutler et al. 2007). Finally, we tested the significance of the derived sub-model following the randomization (of admixture values, $N = 1,000$) procedure and examining the randomized distribution of the simulated pseudo- R^2 values relative to our observed value as detailed in Hether and Hoffman (2012). We also explored alternative methods

of analysis of the data (e.g., standard multiple regression on the original data or on scores from a principal components analyses of correlation matrices), but the RANDOMFOREST procedure consistently produced lower mean square errors, higher R^2 , simpler models, identified the same major variables as predictors, and had the added advantage of being able to analyze the original variables without the need for transformations. Our analyses employed the mean q value for each stream which is uninformative concerning the variation amongst individuals within streams in admixture level. We subjected the data to the same analyses using the median q value (which as expected showed a correlation r of >0.9 with the mean q value) and very similar results were obtained (i.e., overall R^2 value of 0.32, same five variables chosen in same relative ranking of explanatory power—see below).

Where appropriate, adjustments for multiple simultaneous statistical tests incorporated the false discovery rate procedure of Narum (2006).

Results

Genetic analyses

Analysis of genetic data for all localities with at least 15 fish sampled indicated that evidence for null alleles was rare and scattered across individual loci and populations. Of 1,590 analyses (159 samples \times 10 loci), five suggested that null alleles might be present at *Ots107* in some samples. We found, however, that there were no individuals that were null-null homozygotes at *Ots107* suggesting that null alleles would not be a significant factor in subsequent analyses (cf. Taylor et al. 2003; Tamkee et al. 2010).

Across localities and loci, observed and expected heterozygosities, and the number of alleles averaged (SD) 0.32 (0.15), 0.34 (0.16), and 3.2 (1.4), respectively, and F_{IS} averaged 0.072 (0.14) and 29 of 159 permutation tests indicated F_{IS} values significantly greater than 0 (Supplementary Table 1).

Of the 159 samples examined, we found a broad range of estimated admixture levels ranging from $q_{\text{wsct}} = 0.010$ to 0.994. There was a strong right skew to the data with most localities having q_{wsct} values >0.90 . Pure rainbow trout (i.e., $q_{\text{wsct}} < 0.05$), however, were found at 12 localities, six localities had average q_{wsct} values of <0.10 and 34 of the localities exhibited extensive hybridization with q -values below 0.90 (Supplementary Table 1). Three localities were identified from admixture analysis and stocking records as Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*) or Yellowstone cutthroat trout \times westslope cutthroat hybrids and removed from further analyses. There was significant variation in mean

admixture levels across drainage groups (Welch’s F-test, $df = 38.7$, $P = 0.0002$) and this overall effect was driven by differences between the lower Bow River and the upper Bow River, Oldman River, and Kootenay River (max $P = 0.011$, Fig. 2). The Oldman River, exclusive of the Waterton River sub-basin, and the Kootenay River supported the greatest number of populations with q_{wsct} values greater than 0.95.

Of those localities with q_{wsct} values between 0.10 and 0.90 ($N = 29$), 24 had F_{IS} values not significantly different from 0, while five had significantly positive F_{IS} values associated with heterozygote deficiencies (e.g., Haiduk Lake, Smuts Creek, Fisher Creek, Spotted Wolf Creek, Supplementary Table 1). The estimated value of q_{wsct} was negatively correlated with the average (across loci) number of alleles observed; localities with high levels of admixture with rainbow trout (low q_{wsct}) tended to have a higher average number of alleles ($r_s = -0.73$, $P < 0.0001$). The simulated hybrids ($N = 100$ individuals) had a mean q_{wsct} value of 0.51 ($SD = 0.053$).

Influences on admixture values

Incorporation of all predictor variables into the RANDOMFOREST regression tree analysis resulted in a pseudo- R^2 value of 23.7 % and a mean square error (MSE) of 0.059. The variable importances (Ip) for admixture prediction ranged from a low of 0.037 for stream order to a high of 1.019 for water temperature. Model improvement ratios relative to the best predictor (1.0 = water temperature) ranged from 0.036 (stream order) to 0.654 (elevation).

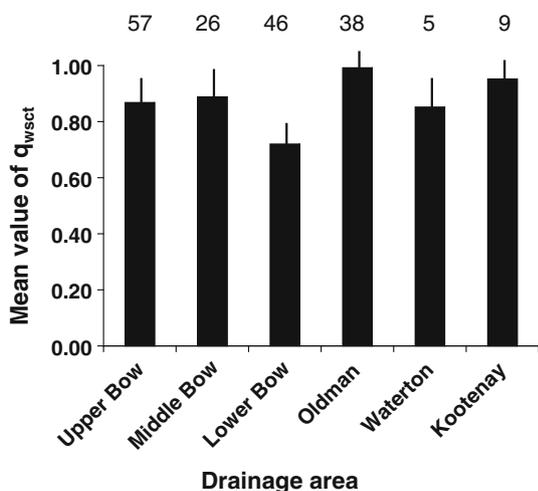


Fig. 2 Variation in mean (+SD) admixture values (q_{wsct} , 0 = rainbow trout, 1 = westslope cutthroat trout) for westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) sampled from 181 localities (those with $N \geq 10$) across seven drainage groupings in southwestern Alberta and adjacent streams tributary to the upper Kootenay River in British Columbia assayed at 10 microsatellite DNA loci

Five variables, water temperature, elevation, stockD, powerD and railD were selected for sub-model analyses based on MIRs of at least 0.3. A model incorporating just these variables produced a pseudo- $R^2 = 33.2\%$ and a $MSE = 0.052$ ($P < 0.001$). Analyses of the various sub-models (Table 2) indicated that a four variable sub-model incorporating water temperature (MIR = 1.0), elevation (MIR = 0.824), railD (MIR = 0.717) and stockD (MIR = 0.612) (but removing powerD, MIR = 0.632) had a mean pseudo- R^2 (34.4 %, $MSE = 0.051$, $P < 0.001$ from randomization test) that was not significantly worse than the full five variable sub-model, but that removing any other single variable resulted in significantly lower pseudo- R^2 values (minimum value = 26.3 % after removing temperature). This final, four variable model was used to predict admixture levels (q_{wsct}) based on 10,000 bootstrapped regression trees. The correlation between observed admixture values and those predicted by the RANDOMFOREST model was 0.61 ($df = 58$, $P < 0.001$). The distributions of observed and predicted admixture levels mirrored each other reasonably closely, but the greatest deviations were observed at very low and very high q_{wsct} levels; about 21 % of the samples had an observed q_{wsct} of ≥ 0.99 , but the RANDOMFOREST model predicted that the highest q_{wsct} was 0.96 (Fig. 3).

Partial dependence plots indicated the effect of each retained predictor variable after averaging out the effects of all other predictor variables. The response of q_{wsct} to changes in water temperature suggested a step-like response as q_{wsct} decreased abruptly between about 11 and 13 °C (Fig. 4). Conversely, q_{wsct} increased steadily as elevations rose above 1,300 m (Fig. 4). The q_{wsct} also tended to increase with increasing stockD, although somewhat more irregularly and leveled off after about 4,000 m (Fig. 4). Finally, distance to the nearest railway (RailD) was the most irregular showing variable responses across different distances (Fig. 4).

Discussion

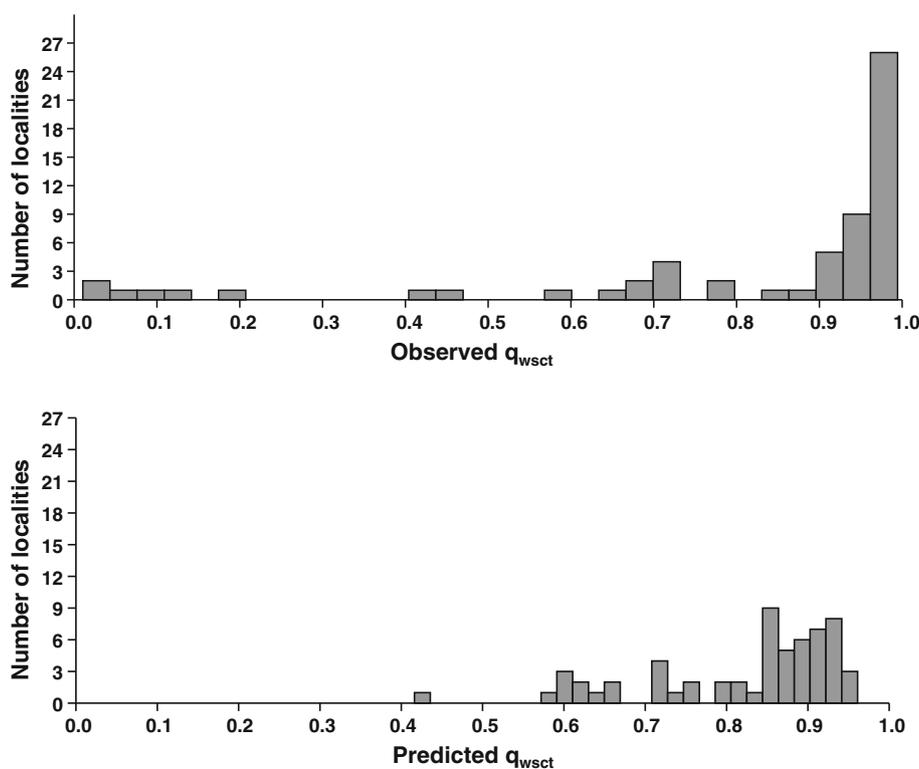
Hybridization and introgression have become important issues in the conservation of biodiversity as an appreciation developed for phenomena such as genomic extinction and the role of admixture in demographic decline (Huxel 1999; Rhymer and Simberloff 1996; Muhlfeld et al. 2009a). The study of admixture in cutthroat trout and other taxa has been widespread throughout its native range and dates back to the early 1980s (e.g., Leary et al. 1984; Allendorf and Leary 1988). All together, it has been estimated that genetically non-admixed westslope cutthroat trout may exist in as little as less than 10 % of the total historical native range (Trotter 2008). In western Alberta, westslope

Table 2 Variable importance values and model improvement ratios (MIR) for the full and final, four variable (in boldface) models determined from randomForest analysis of the relationship between admixture values between westslope cutthroat trout and rainbow trout and stream variables for 58 localities, in southwestern Alberta

Variable	Importance-full	MIR-full	Importance-final	MIR-final
Mean depth	0.1378	0.1351	NA	NA
Maximum depth	0.1511	0.1482	NA	NA
Temperature	1.0294	1.0000	1.073	1.0000
Elevation	0.6672	0.6545	0.8848	0.8247
Stream order	0.0373	0.0365	NA	NA
RoadD	0.2567	0.2498	NA	NA
PipeD	0.2000	0.1964	NA	NA
PowerD	0.3364	0.3300	NA	NA
RailD	0.3762	0.3691	0.7697	0.7172
StockI	0.0833	0.0788	NA	NA
StockN	0.1751	0.1717	NA	NA
StockY	0.2130	0.2089	NA	NA
StockD	0.3297	0.3234	0.6121	0.6122

NA not applicable

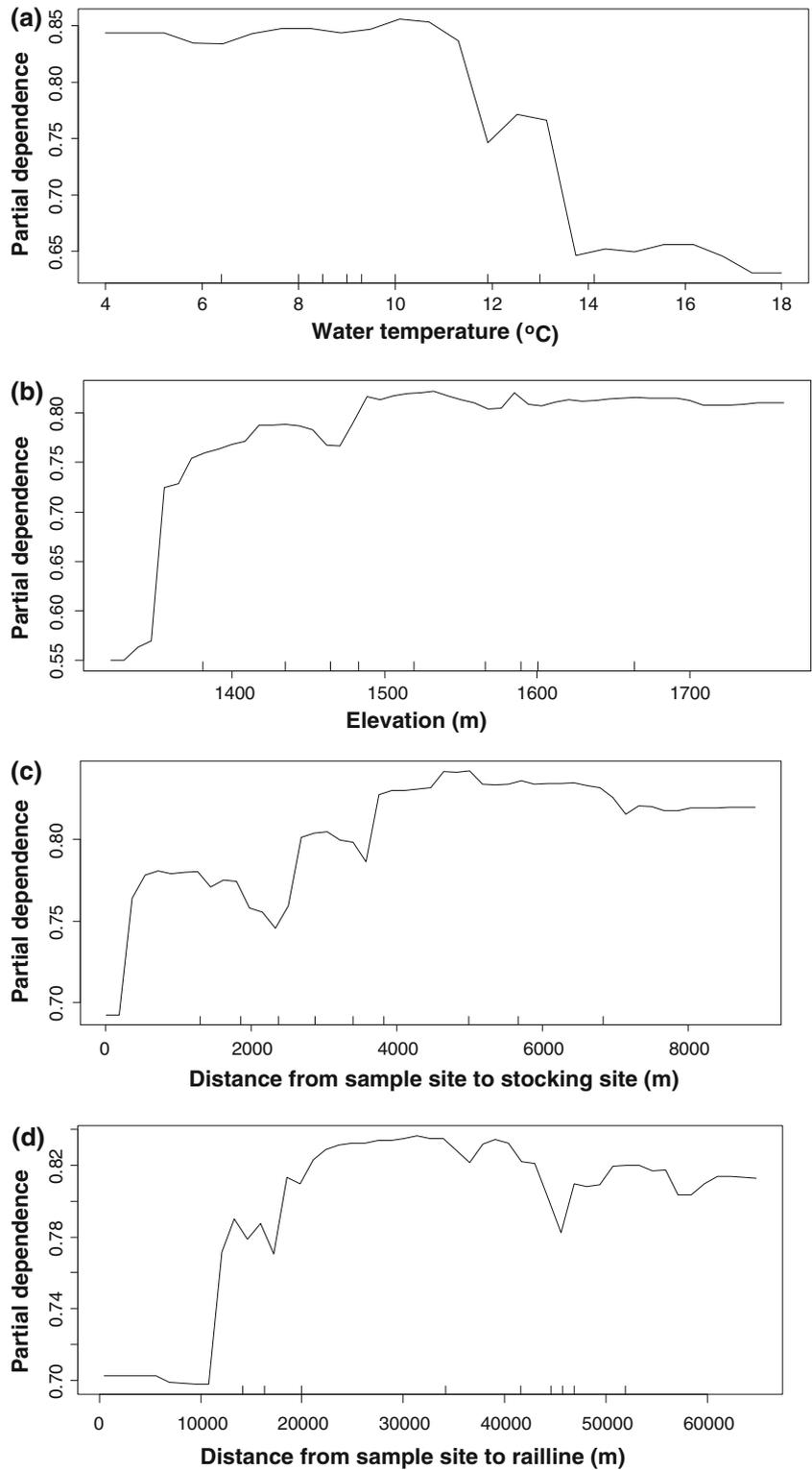
Fig. 3 Observed (*upper*) and predicted (from RANDOMFOREST derived relationships, *lower*) admixture values (q_{wsct} , 0 = rainbow trout, 1 = westslope cutthroat trout) as a function of variation in water temperature, elevation, distance from sample site to stocking site and distance from sample site to the nearest railway line for westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) sampled across 58 localities in southwestern Alberta, Canada, and assayed at 10 microsatellite DNA loci



cutthroat trout and rainbow trout hybrids had been first recognized, morphologically, as early as 1947 and were considered widespread by 1950 (Mayhood and Taylor 2011). It is now estimated that fewer than 10 % of populations of westslope cutthroat trout in western Alberta have no detectable introgression with rainbow trout and that

many of these populations are relatively isolated in head-water reaches (Mayhood and Taylor 2011). The perilous state of these populations over the medium to long term was a major factor leading to their assessment as Threatened by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC 2007).

Fig. 4 Partial dependence plots showing the relationship between **a** water temperature, **b** elevation, **c** distance from sample site to stocking site, and **d** distance from sample site to nearest railway line and admixture values (q_{wsc} , 0 = rainbow trout, 1 = westslope cutthroat trout) for westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) sampled from 58 localities in southwestern Alberta, Canada, and assayed at 10 microsatellite DNA loci



By contrast, there have been very few studies that have sought an understanding of the fate of hybrids or general explanations for spatial variation in admixture between native westslope cutthroat trout and introduced rainbow trout. Some laboratory-based (Ferguson et al. 1985; Leary

et al. 1985) and, more recently, field-based studies (Muhlfeld et al. 2009a; Rasmussen et al. 2012) have shed light on the performance and fate of hybrids and in some cases have demonstrated that even small amounts of admixture from rainbow trout can influence physiology or

reduce survival of hybrid offspring. The demonstration of real fitness costs to admixture stresses the importance of understanding what influences spatial variation in admixture in nature because understanding what factors may explain variation in admixture can aid our understanding of the biology of the hybridizing taxa, if and how admixture spreads, and what strategies might be best to deal with admixture.

Spatial variation in admixture

Our survey indicated that while some localities showed considerable admixture with rainbow trout (i.e., $q_{\text{wscet}} < 0.75$), most areas showed q_{wscet} levels of between 0.85 and 0.99. We are unaware of any study of admixture between rainbow trout and westslope cutthroat trout that is as geographically extensive as the present study, but even on smaller geographic scales a similar pattern of spatially variable admixture is usually found. For instance, Hitt et al. (2003) documented a few heavily admixed populations (i.e., >85 % genetic contribution from rainbow trout) and a majority of localities with 1–27 % admixture from rainbow trout across 42 samples from the upper Flathead River drainage of Montana. Such variation might be expected given differences in various aspects of introduction intensity and history across areas. For instance, both Rubidge et al. (2001) and Hitt et al. (2003) reported that admixture with rainbow trout decreased upstream from sources of introduction of rainbow trout. By contrast, Beetles et al. (2005) and Heath et al. (2010) reported extensive, but less structured spatial variation in the extent of natural admixture between native populations of rainbow trout and coastal cutthroat trout (*O. c. clarkii*). The finding that spatial variation in admixture does not appear to be governed simply by idiosyncrasies of specific artificial introduction programs suggests that local conditions also appear to play an important role (Aldridge and Campbell 2009).

Regardless of the geographic pattern of variation in admixture levels, our results demonstrate that admixture with rainbow trout is widespread in southwestern Alberta. There has been no comparably extensive survey in British Columbia (BC), but surveys by Rubidge et al. (2001), Taylor et al. (2003) and Rubidge and Taylor (2005) indicate that many geographic localities in the upper Kootenay River system in BC are similarly significantly admixed.

Environmental correlates of admixture

The landscape genetic approach to studies of the spatial distribution of genetic variability (e.g., Manel et al. 2003; Holderegger and Wagner 2006; Van Houdt et al. 2010; Tamkee et al. 2010) can also clearly apply to understanding how admixture between species is distributed and spreads

across a landscape. Our analyses were conducted over a series of watersheds and thus add generality to the observations made in smaller scale studies across western North America suggesting that a consistent set of physical factors influence the extent of admixture between westslope cutthroat trout and rainbow trout (e.g., Rubidge and Taylor 2005; Muhlfeld et al. 2009c; Weigel et al. 2003). Specifically, water temperature and elevation are consistently associated with admixture levels; admixture between the species is consistently lowest in high elevation, low order and cooler streams. In particular, all three of these variables tend to be intercorrelated, but water temperature was the most important factor associated with admixture in our regression tree models. The influence of water temperature observed in our study is consistent with previous studies of hybridization in westslope cutthroat and other species of salmonids that typically report a gradient of admixture with genetically pure, native trout at the highest elevations, increasing levels of admixture downstream and pure genotypes of the introduced species in the lowest reaches (Larson and Moore 1985; Paul and Post 2001; Hitt et al. 2003; Rubidge and Taylor 2005; Muhlfeld et al. 2009b; Rasmussen et al. 2010, 2012). Westslope cutthroat trout and rainbow trout exhibit very similar optimal growth temperatures (13.6 and 13.1 °C), at least as inferred by limited laboratory study, but *O. mykiss* displayed better growth at higher water temperatures and a broader range of growth at warmer temperatures (Bear et al. 2007). Rainbow trout also may be better adapted to warmer temperatures as suggested by having an upper incipient lethal temperature that is 4.7 °C greater than in westslope cutthroat trout (Bear et al. 2007). These trends in the elevational distribution of admixture and observations of different thermal characteristics of hybridizing species have led to the development of the “elevation refuge hypothesis” (Fausch 1989; Paul and Post 2001; Rasmussen et al. 2010). Here, habitats at high elevations may provide a refuge for cold water-adapted, native trout, at temperatures that introduced species find less favourable. A number of laboratory studies support this hypothesis, demonstrating a shift in growth rates and survival as water temperatures change (e.g., Reese and Harvey 2002; McMahon et al. 2007). Recent work in bull trout (*Salvelinus confluentus*) and brook trout (*S. fontinalis*) showed a competitive advantage of introduced brook trout at warm temperatures reared in sympatry with bull trout (McMahon et al. 2007), but no significant differences were observed when these species were grown in allopatry. Further, Rasmussen et al. (2010, 2012) found significant associations among admixture gradient, elevation, and difference in life history characters and metabolism between rainbow trout and westslope cutthroat trout and hybrids in the upper Oldman River, Alberta. Westslope cutthroat trout with low levels of

admixture with rainbow trout tended to predominate in headwater reaches of the upper river and were also older and grew more slowly than hybrids and pure rainbow trout which tended to predominate in lower elevation and warmer reaches. Hybrids predominated in mid-elevation reaches and exhibited intermediate metabolic rates that seem well-suited to such ecotonal habitats (Rasmussen et al. 2012).

Weigel et al. (2003) proposed one mechanism by which cold summer temperatures may impede the establishment of rainbow trout alleles. Cold summer temperatures can delay egg production and prolong incubation of early spring spawning rainbow trout compared to westslope cutthroat trout that spawn later in the season (Hubert et al. 1994; Stonecypher et al. 1994). Such a developmental delay may act to compound a decrease in growth rate at cooler water temperatures and also reduce overwinter survival of rainbow trout and hybrid fry. Indeed, Culumber et al. (2012) presented evidence that physiological adaptation to different thermal niches could explain, in large part, the elevational pattern of genotypic distribution of two species of swordtails (*Xiphophorus*) and their hybrids in southeastern Mexico.

Our analysis suggested, from the partial dependence plots, that the effect of water temperature was non linear and that a temperature of between 11 and 13 °C might represent a threshold at which the likelihood of admixture changes abruptly. This temperature range as a specific threshold must clearly be interpreted with caution because our temperature sampling was superficial and denser sampling, spatially and temporally, must be completed to see if such a threshold has any veracity. Interestingly, however, Rasmussen et al. (2010) proposed that a temperature threshold of about 7.3 °C marks a point at or below which admixture was low to absent within one tributary of the Oldman River system, Alberta. Of course, even with such more intensive sampling of water temperature, Rasmussen et al. (2010) cautioned that its extension to other streams was problematic given the many the features than can vary between streams and influence admixture (e.g., barriers). If such thermal thresholds to admixture exist, they can perhaps help to predict responses to environmental changes (e.g., long-term climate change, land use changes) that can influence stream water temperatures in terms of changes in the levels of genetic admixture, its geographic distribution, and the speed at which such changes might occur (cf. Rasmussen et al. 2010; Isaak et al. 2012). There is some evidence of spatial segregation of rainbow trout and westslope cutthroat trout and differences in growth potential as a function of water temperature (e.g., Bosek and Hubert 1992; Sloat et al. 2005; Bear et al. 2007), but McHugh and Budy (2005) reviewed several studies and suggested that, overall,

condition (temperature)-dependent competition is an unlikely explanation for salmonid zonation patterns. A better understanding of the physiological performance of these species and their hybrids across multiple life stages, particularly at low water temperatures, is needed.

Our analyses also suggest that elevation plays a role in influencing admixture levels (cf. Weigel et al. 2003; Rasmussen et al. 2012). There is a clear negative association between water temperature and elevation (this study, see also Rasmussen et al. 2010; Culumber et al. 2012), but the partial dependence plots suggest an effect of elevation in excess of that which can be accounted for by its association with water temperature. Rainbow trout tend to be more abundant in wider, lower elevation, lower gradient stream reaches (McCrimmon 1971; Gard and Flittner 1974; Paul and Post 2001; Bosek and Hubert 1992). Henderson et al. (2000) and Muhlfeld et al. (2009c) found that upstream–downstream segregation in westslope cutthroat trout and rainbow trout, respectively, may also include spawning habitats, but that they also overlapped in some tributaries. Given that gradient increases and stream width generally declines as elevation increases, the physically smaller and more energetic higher elevation habitats may be less preferred by rainbow trout regardless of water temperature and reduce the incidence of interactions and hybridization with westslope cutthroat trout. In addition, given that stocking of rainbow trout was characteristically in the lower portions of our study streams, the negative association between admixture level and elevation (and its association with water temperature) may result from non-equilibrium conditions in which invasive rainbow trout and admixture are still in the process of extending in upstream directions (Rubidge et al. 2001; Hitt et al. 2003; Boyer et al. 2008; Muhlfeld et al. 2009a, b). Our data can serve as a useful baseline to monitor changes in admixture with time and/or in response to habitat disturbances such as fires or floods (e.g., Isaak et al. 2012).

Anthropogenic correlates of admixture

A number of anthropogenic factors related to stocking operations and environmental disturbance were found to be related to admixture with rainbow trout amongst our samples. For instance, the level of admixture has been often observed to decline with distance from the point(s) of stocking (Rubidge et al. 2001; Hitt et al. 2003; Rubidge and Taylor 2005; Muhlfeld et al. 2009b). Our results are in agreement with these findings and emerged despite the likelihood that all such comparisons suffer to varying degrees from incomplete stocking histories, undocumented movement of trout by the public, failure to establish spawning populations, and migration of stocked fish from the sampling location (Moring 1993; Weigel et al. 2003).

We expected that the number of fish stocked and stocking intensity would increase propagule pressure of rainbow trout and be associated with increasing admixture levels as has been reported in other salmonid systems (Lockwood et al. 2009; Muhlfeld et al. 2009c; Marie et al. 2010, 2012). We also expected an increase number of years elapsed since the last stocking and increasing isolation (a combination of stream distance and presence of upstream migration barriers) from the stocking site would be associated with low levels of admixture (e.g., Rubidge et al. 2001; Ruzzante et al. 2004). Only the latter variable, however, was identified as an important variable in the RANDOMFOREST analysis. Two streams, Loomis Creek in the Bow River and South Castle Creek in the Oldman River had unusually high numbers of fish stocked and stocking intensities. These streams had between five and 30 times as many fish stocked (up to 3 million in South Castle Creek) and between six and 10 times the stocking intensity (up to 64 years in South Castle Creek) as the average values for all other localities (Supplementary Table 2). Despite such high stocking levels, these two streams had levels of admixture with rainbow trout that were negligible in Loomis Creek ($q_{wsct} = 0.97$) to about the average ($q_{wsct} = 0.88$) in South Castle Creek ($q_{wsct} = 0.87$). Loomis Creek had one of the coolest water temperatures (7 °C which was in the lowest 15th percentile of all streams) and was almost 5 km upstream of the stocking site which was below a migration barrier and in a different tributary. Further, the water temperature of South Castle Creek (9.2 °C) was below the average value of 10 °C for all streams. Thus if our spot water temperatures do accurately reflect cooler than average conditions in these streams, this factor combined with the location of stocking sites in terms of distance and in relation to migration barriers, and other aspects of habitat that may be unsuitable for rainbow trout may help to explain the relatively low admixture levels despite high levels of stocking of rainbow trout in some systems. More generally, these results suggest that the numbers and intensity of stocking interact with local environmental conditions to influence admixture levels (Weigel et al. 2003; Taylor et al. 2007; Muhlfeld et al. 2009b). Certainly, other cases of intraspecific salmonid supplementation programs have illustrated the idiosyncratic nature of the outcome of stocking that is dependent on more than just the numbers and intensity of non-native fish stocked (e.g., Krueger and Menzel 1979; Largiadèr and Scholl 1995; Taylor et al. 2007; Halbisen and Wilson 2009; Marie et al. 2012). Our analysis reinforces the importance of connectivity between streams (as influenced by fluvial distance and migration barriers) as a critical factor influencing admixture in many situations (Rubidge et al. 2001; Gunnell et al. 2008; Muhlfeld et al. 2009b).

The effects of human disturbance factors on the spread of non-native species and hybridization in stream fishes may be direct, from increasing access to streams through road or railway construction, or indirect, from landscape developments that influence key features such as water temperature which themselves influence dispersal of non-native species and hybridization (e.g., Dunham et al. 2003; McMahon et al. 2007; Heath et al. 2010). For instance, Muhlfeld et al. (2009b) found that the number of upstream road crossings was positively correlated with levels of admixture between native westslope cutthroat trout and invasive rainbow trout in the upper Flathead River in British Columbia and Montana. In our study systems, we found only that distance to the nearest railway appeared to influence admixture levels, but in an erratic manner. The apparent lack of an influence of roads, pipelines, and powerlines should be interpreted cautiously as they may not have represented measures of human disturbance per se particularly well. For instance, Muhlfeld et al. (2009b) found that road density did not have a significant effect in their study, but that number of upstream road crossings did. In this case, the latter variable is perhaps a more direct (and hence more sensitive) measure of potential habitat disturbance for streams. Similarly, our measures of disturbance were expressed only as distance to the nearest anthropogenic structure and did not incorporate density nor actual crossings. Interestingly, railways were historically the source of much stocking pressure (Mayhood 2000), but our analysis revealed a complex interaction between distance to the nearest railway (RailD) and admixture. This result may stem from the highly bimodal distribution of RailD values we obtained; one mode was found at about 16 km and the other at about 46 km (Supplementary Table 2). Still, we could not resolve any consistent directionality to the influence RailD and admixture. Finally, other human disturbance factors such as logging activity, recreational land use including angling activity or natural factors such as seasonal flood dynamics, and their interactions, may play roles in admixture and should be investigated (e.g., Fausch et al. 2001; Heath et al. 2010).

In summary, our analysis of variation in admixture levels between westslope cutthroat trout and rainbow trout in western Alberta further emphasizes what appears to be a basic spatial pattern; admixture levels tend to be low in cooler, low order, high elevation streams (cf. Paul and Post 2001; Rubidge et al. 2001; Weigel et al. 2003; Rubidge and Taylor 2005; Muhlfeld et al. 2009c). This pattern has led to the temperature/elevation refuge hypothesis that suggests that native population of salmonid fishes in mountainous areas may be less susceptible to invasion of non-natives if native fishes have a physiological or behavioural advantage over non-native and hybrids at cooler water temperatures (Paul and Post 2001; McMahon et al. 2007; Rasmussen

et al. 2010). In addition, the characterization of westslope cutthroat trout and rainbow trout as relatively cool-water and warm-water adapted, respectively, may be considered consistent with their evolutionary and biogeographic history given the concentration of the former in high elevation areas of the Rocky Mountains and adjacent mountain ranges. Still, the relative performance of either species at low water temperatures, a key component of the temperature refuge hypothesis, has been little explored, as has the potential role of thermal adaptation in speciation in fishes (cf. Culumber et al. 2012; Keller and Seehausen 2012). Our results and those of others (e.g., Rubidge et al. 2001; Fausch 2008; Muhlfeld et al. 2009b, c; Marie et al. 2012) clearly indicate, however, that factors other than water temperature (e.g., habitat area, stocking practices, migration barriers) can also influence admixture levels and that the influence of human disturbance factors (resource extraction, road and pipeline density and routes) will often be contingent on the local regulatory regime.

Implications for recovery planning

Our comprehensive survey of Alberta populations can focus future research to assist with recovery planning for threatened westslope cutthroat trout (COSEWIC 2007). For instance, our results are being used to help prioritize populations for conservation during the assessment of recovery potential. Populations with little to no detectable admixture may be the highest priority for conservation and perhaps serve as candidate sources of fish for recovery in more affected streams (Muhlfeld et al. 2009b; Mayhood and Taylor 2011). Second, the RANDOMFOREST analysis performed reasonably well in terms of predicting admixture levels in bootstrapped samples and could be used as a “triage” procedure to categorize localities with unknown admixture levels in terms of which are more or less likely to exhibit admixture with rainbow trout. The use of our model would, however, prove conservative because it was unable to accurately predict q_{wsct} values of >0.96 . Thus, the use of this statistical model alone would be problematic for correctly identifying genetically non-admixed populations of westslope cutthroat trout under a proposed threshold value of $q_{\text{wsct}} \geq 0.99$ (Allendorf et al. 2004). Finally, more intensive sampling of stream water temperatures through time could help better assess the role of low water temperature on relative performance of genotypes and its effects on reproductive isolation and hybridization between species. Our results also support the idea that physical stream characteristics, stocking practices, anthropogenic habitat modifications, and the local regulatory regime (e.g., within or outside protected areas) all interact to influence admixture levels in salmonid fishes in diverse ways which emphasizes the need for context-specific

solutions. Our results could also be used to alert managers to situations where stocking should not occur, e.g., in streams with conditions that might favour non-natives and/or hybrids especially if such areas show high levels of interconnectivity with other streams) or, if stocking does occur or did occur in the past, its likely consequences to native fish gene pools (e.g., relative probability of admixture). Finally, understanding what influences admixture can help suggest potential remedial actions, e.g., streams that have marginal conditions for non-natives or that were stocked only lightly may present the best cases for recovery of native fishes.

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