

Habitat use and hybridisation between the Rocky Mountain sculpin (*Cottus* sp.) and slimy sculpin (*Cottus cognatus*)

Tyana Rudolfson^{1,*} | Jonathan L. W. Ruppert^{1,2,*}  | Eric B. Taylor³ |
Corey S. Davis⁴ | Douglas A. Watkinson⁵ | Mark S. Poesch¹ 

¹Department of Renewable Resources, University of Alberta, Edmonton, Alberta, Canada

²Toronto and Region Conservation Authority (TRCA), Vaughan, Ontario, Canada

³Department of Zoology, Biodiversity Research Centre, Beaty Biodiversity Museum, University of British Columbia, Vancouver, British Columbia, Canada

⁴Molecular Biology Service Unit (MBSU), Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada

⁵Fisheries and Oceans Canada, Central & Arctic Region, Freshwater Institute, Winnipeg, Manitoba, Canada

Correspondence

Jonathan L. W. Ruppert, Toronto and Region Conservation Authority (TRCA), Vaughan, ON, Canada.
Email: jonathan.ruppert@trca.on.ca

Funding information

Fisheries and Oceans Canada

Abstract

1. Anthropogenic factors such as land-use change, pollution, and climate change can cause fragmentation and reduce the amount of habitat by altering preferred conditions. This process can also bring about novel species interactions and, in some cases, create or alter levels of hybridisation between closely related species. We assessed the threat of hybridisation to the persistence of the Rocky Mountain sculpin (*Cottus* sp.) and the slimy sculpin (*Cottus cognatus*) in the Flathead River, British Columbia, Canada.
2. Individuals collected from 95 sites provided 731 genetic samples that were genotyped at 10 polymorphic microsatellite loci and mitochondrial cytochrome C oxidase sequences, to assess: (a) if there are differences in the distribution of Rocky Mountain sculpin between contemporary and historical (35 years ago) records, (b) if hybridisation is symmetrical in terms of sex-specific parental contributions, and (c) if habitat preferences contribute to the distribution of pure parental and hybrid populations.
3. We identified three hybrid locations and that Rocky Mountain sculpin have an altitudinal distribution (1,200–1,902 m) that exceeds the range limit reported 35 years ago (1,200–1,372 m). Additionally, hybrid mating appears to involve similar proportions of parents of both sexes from each species. Lastly, elevation, turbidity, and sediment type are significant factors predicting the presence of parental species. Further, elevation and water conductivity are significant factors to predicting hybrid presence.
4. The contrasting associations of parental species with different habitat types appear to influence the extent and distribution of hybridisation. Our results suggest that Rocky Mountain sculpin (a species of conservation concern) do not appear to be at risk due to hybridisation, but that this is a system in flux. Given the importance of environmental conditions to hybridisation, there is a need to quantify how environmental changes may disrupt current hybridisation and potential population viability.

KEYWORDS

aquatic conservation, hybrid zone, mixed-effects models, species-at-risk, symmetric mating

*The authors Tyana Rudolfson and Jonathan L. W. Ruppert are share first authorship.

1 | INTRODUCTION

Globally, declining biodiversity is a major conservation concern (McCauley et al., 2015; Sala et al., 2000). The effects of climate change, land use change, pollution, biological invasions, and over-exploitation are considered to be some of the main drivers of species loss (Dudgeon et al., 2006; Sala et al., 2000; Vörösmarty et al., 2010). Additionally, the combined impact of several stressors can be additive, antagonistic, or synergistic, in their influence on the persistence of species (Jenkins, 2003; Vörösmarty et al., 2010). Altogether, these drivers can produce unsuitable habitat that lowers population viability and necessitates species to disperse or face extirpation (Fausch, Torgersen, Baxter, & Li, 2002; Olden et al., 2010). This process, which can influence the distribution of a species, might lead to novel species interactions or alter existing interactions that may accelerate population declines (Seehausen, Takimoto, Roy, & Jokela, 2008). For instance, shifts in the distribution of related species may influence the extent and distribution of hybridisation between species leading to actual or predicted declines in one or both species (Chen, Hill, Ohlemuller, Roy, & Thomas, 2011; Chunco, 2014; Kelly, Whiteley, & Tallmon, 2010; Seehausen, 2006; Seehausen et al., 2008).

Freshwater ecosystems are often characterised by high levels of fish species diversity and endemism, because of the inherent low connectivity of many waterbodies across terrestrial landscapes and the hierarchy of dendritic fluvial networks (Hughes, Huey, & Schmidt, 2013; Olden et al., 2010). Given ongoing alterations to freshwater ecosystems due to human activities (Dudgeon et al., 2006; Vörösmarty et al., 2010), many freshwater fish species that may have otherwise been historically separated are now interacting, which can result in hybridisation (Nolte, Freyhof, Stemshorn, & Tautz, 2005; Yau & Taylor, 2013). Specifically, hybridisation between fish species of conservation concern and other native species or exotic species that are expanding their ranges, is a major concern in many ecosystems (e.g. Allendorf et al., 2004; Muhlfeld et al., 2009; Seehausen et al., 2008). Thus, understanding the prevalence, location, and future trajectory of hybridisation may be beneficial to conservation management plans that aim to preserve biodiversity and the population viability of species-at-risk (Allendorf, Leary, Spruell, & Wenburg, 2001; Kuussaari et al., 2009; Olden, Poff, Douglas, Douglas, & Fausch, 2004; Pfennig, Kelly, & Pierce, 2016; Young et al., 2016).

Freshwater hybrid zones in dendritic networks often represent ecological transition zones where hybrids are either bounded by parental ranges or hybrids out-compete and exclude parental types (Arnold, 1997). A common example of how this can occur is secondary contact, where species that were formerly isolated in distinct refugia during the last glacial maximum now come into contact upon postglacial dispersal (Hewitt, 1996). Where hybridisation occurs following secondary contact, hybrid zones may persist under conditions where there is hybrid selective disadvantage or advantage. This results from either the continual dispersal of parental types into the contact zone or by hybrids having higher fitness in ecologically intermediate habitats, respectively (Arnold, 1997; Barton

& Hewitt, 1985). Thus, the geographic distribution of hybridisation may be related to the habitat preferences of the parental species and/or hybrids (Barton & Hewitt, 1985; Moore, 1977). With ongoing alterations to the configuration and amount of suitable habitat for parental and hybrid genotype classes, the spatial characterisation of hybrid zones is necessary to formulate conservation management plans (e.g. Rasmussen, Robinson, Hontela, & Heath, 2012).

The Rocky Mountain sculpin (*Cottus* sp.; hereafter referred to as RMS) is a cryptically-shaded, small-bodied, benthic fish that is found in only two river basins in Canada, including one river in southern Alberta and one river basin in British Columbia. RMS is considered to be a new taxon (Neely, 2002) awaiting formal taxonomic description and has a broader distribution within the U.S.A. compared to Canada (Figure 1a). At various times, it has been considered to be part of the *Cottus bairdii* complex, a form of *Cottus confusus* or perhaps *Cottus punctulatus* (see discussion in McPhail, 2007). The restricted distribution within Canada and sedentary nature of the RMS make it particularly susceptible to anthropogenic impacts (Bailey, 1952; Ruppert et al., 2017), which include land-use change (road construction and sediment run-off), flow augmentation (irrigation), and climate variability (drought) (COSEWIC, 2005, 2010). As a result of these impacts, RMS in Alberta have been listed as Threatened, while those in British Columbia are listed as Special Concern under Canada's *Species-at-Risk Act* (Canada G.O., 2012; COSEWIC, 2005, 2010).

There are several examples of sculpin (*Cottidae*) species forming hybrid zones (e.g. Nolte, Freyhof, et al., 2005; Stemshorn, Reed, Nolte, & Tautz, 2011), including hybridisation between RMS and slimy sculpin (*Cottus cognatus*; hereafter referred to as SS), in parts of the Flathead River in Montana (COSEWIC 2010; Zimmerman & Wooten, 1981). In contrast to RMS, SS are widely distributed across Canada (Scott & Crossman, 1998) and are currently of no conservation concern (Figure 1a). Previous work by Hughes and Peden (1984) in the Flathead River (from sampling in 1979 and 1981) showed that RMS (referred to as *C. confusus*) and SS coexist, where RMS are restricted to below 1372 m in elevation. Further, surface water velocity and instream temperature at collection sites were measured and appeared to have no influence on the distribution of parental species (Hughes & Peden, 1984). Thus, a broader characterisation of associations with environmental conditions, beyond water velocity and instream temperature, is needed to understand what conditions contribute to the formation of hybrid zones.

Here, we investigate hybridisation between RMS and SS in the Flathead River, BC, using genetic and environmental surveys over the summer of 2014 and 2015 to address three objectives. First, we assessed the spatial extent of hybrid zones and whether changes in RMS distribution have occurred in the last 35 years. Second, we assessed the symmetry of hybridisation (i.e. if male SS always mate with female RMS or vice versa). Finally, we assessed what environmental factors are most important to the spatial distribution of pure parental and hybrid genotype classes. In other words, we assessed the habitat suitability of all genotype classes, which influences the

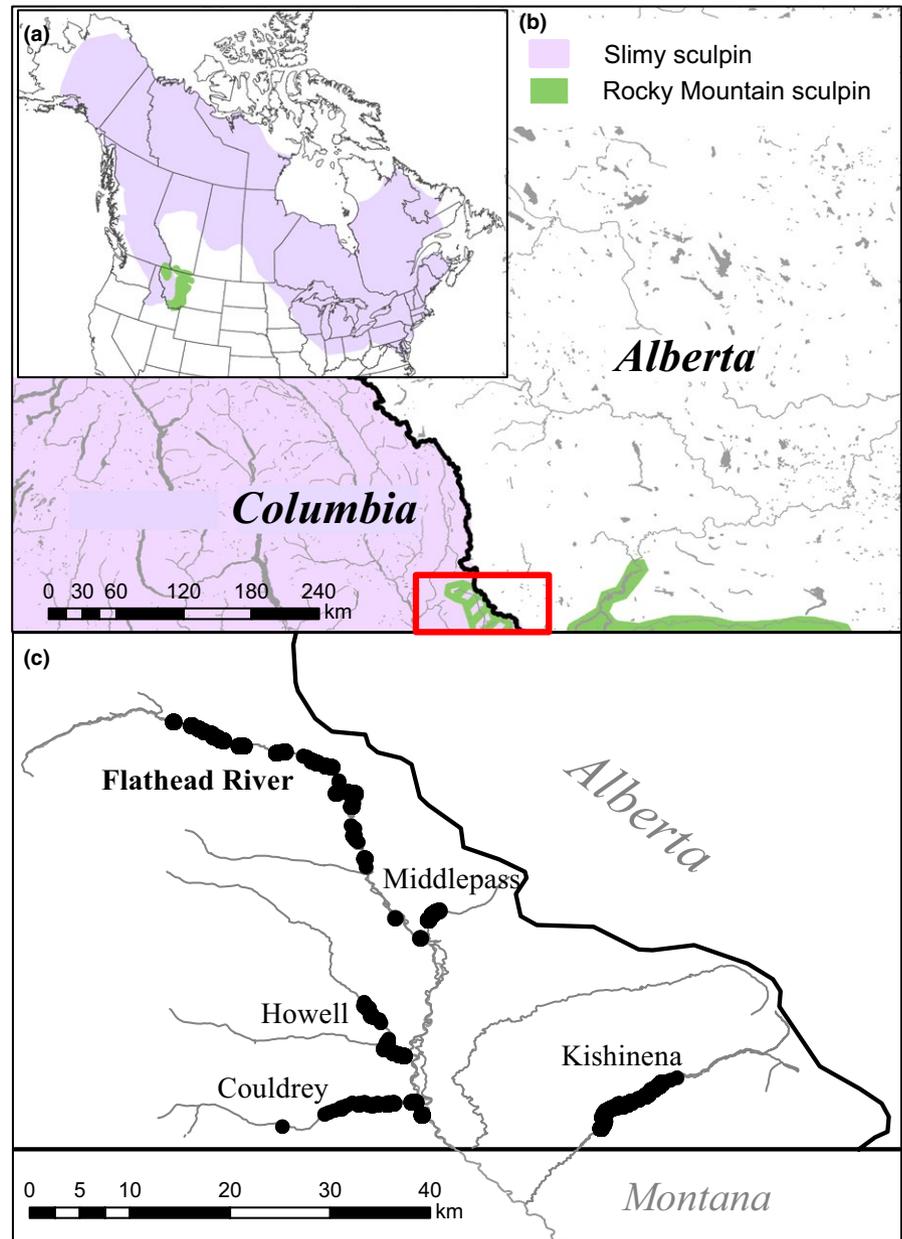


FIGURE 1 The distribution of Rocky Mountain sculpin (RMS) and slimy sculpin (SS) within (a) North America and (b) Canada. RMS distribution overlaps with SS in the Flathead Drainage in south-eastern British Columbia (outlined with box and hash; ranges modified from Scott & Crossman, 1998 and COSEWIC, 2010). (c) Specific sample locations ($n = 95$) in the Flathead River drainage were chosen based on areas that would possibly have hybrid zones based on findings by Hughes and Peden (1984)

interactions between each genotype class. Understanding what environmental factors influence the formation and maintenance of hybrid zones can inform predictions about how alterations to such environmental factors might impact the future trajectory of hybridisation, potential evolutionary interactions, and its implications for the persistence of parental species.

2 | METHODS

2.1 | Study area

The Flathead River in south-eastern British Columbia (Columbia River drainage), flows southward into Montana, which contains most of the catchment area (Figure 1a). The Flathead River drainage in British Columbia has a geology that consists mainly of limestone

and argillite, with deposits of coal and gold (Hughes & Peden, 1984; Ross, 1959). It is relatively uninfluenced by human development, yet it has been impacted by coal mining, logging, and associated road development (COSEWIC, 2010). The Flathead River and tributaries are the only known drainage in Canada where the ranges of RMS and SS overlap (Scott & Crossman, 1998; Figure 1). However, differences exist in the spatial distribution of species in the Flathead River; SS tend to be found in upstream, higher elevation locations, while RMS tend to be more abundant in downstream, lower elevation sites (Adams, Schmetterling, & Neely, 2015; Hughes & Peden, 1984; Zimmerman & Wooten, 1981). Otherwise, both RMS and SS have very similar morphology and life history characteristics, and hybridisation between the two has been previously reported, but not well characterised (McPhail, 2007; Rudolfson, Watkinson, & Poesch, 2018).

2.2 | Genetic and environmental data collection

Tissue samples (non-lethal pelvic fin clips stored in 95% ethanol) were collected for use in genetic analyses to identify RMS, SS and hybrid individuals from thirty 1-m² electrofished quadrats within 300 m length sites over the course of the summer of 2014 and 2015 (June–August). A total of 95 sites were sampled across Kishinena, Couldrey, Middlepass, and Howell creeks along with the upper Flathead River (Figure 1b). The placement of each quadrat was randomised (by both depth [10–60 cm] and distance [1–20 m] along the transect), where sampling in each quadrat lasted for 10 s using a Smith-Root LR-24 backpack electrofisher and dip nets. During the 10-s sampling period, bottom substrate was gently moved and shuffled to free sculpin that could be lodged amongst rocks and bottom debris.

We selected sites by using visual field identification to ensure our sampling effort included both RMS and SS. Preliminary work by Hughes and Peden (1984) determined that RMS can be differentiated from SS in the field using median occipital pore counts (~87% accuracy). Median occipital pore counts were used along with other characteristics, such as the presence of head papillae, anal fin ray counts, and head length:width ratio (Hughes & Peden, 1984; Rudolfesen et al., 2018). Habitat characteristics at the end of each site were recorded (within 2 hr of fish being captured), including elevation using Garmin Backroad GPS Maps software, turbidity using a LaMotte™ 2020we Portable Turbidity Meter, conductivity, and dissolved oxygen using a YSI Prp Plus multiparameter instrument (Supporting Information Table S1). At each quadrat, substrate size was quantified using the Wentworth Scale (Wentworth, 1922) and water velocity (m/s) was measured with a handheld SonTek Flowtracker®. To track the summer water temperature, Hoskin Scientific HOB0 Tidbit v2 Temperature Data Loggers were cemented to boulders in each creek using waterproof epoxy. Temperature data were logged hourly.

2.3 | Genotyping

The DNeasy 96 Blood and Tissue Kit (Qiagen) was used to isolate total genomic DNA from fin clips. Fifteen sculpin microsatellite primer sequences were identified from previous studies: Cba from Fiumera, Porter, Grossman, and Avise (2002); Cgo from Englbrecht, Largiader, Hanfling, and Tautz (1999); Cott from Nolte, Stemshorn, and Tautz (2005); and Cco from Fujishin, Barker, Huff, and Miller (2009)). We initially tested loci using a subset of individuals ($n = 8$) from across the distribution of the two species. One locus was monomorphic and three failed to amplify and were removed from further analysis. Altogether, individuals were genotyped at 11 polymorphic microsatellite loci using protocols detailed by Ruppert et al. (2017).

A subset of individuals was used to generate mitochondrial cytochrome C oxidase sequence to examine the directionality of hybridisation. Given that mtDNA is maternally inherited, the mtDNA haplotype of hybrids will reflect which species was the mother. We used 10 individuals each from both parental, first generation hybrid,

second generation hybrid and backcross generations 1 and 2 genotypic classes as identified by NEWHYBRIDS (Anderson & Thompson, 2002) for COI sequencing. The COI region was amplified in 25 μ l reactions containing 0.2 mM each dNTP, 1 \times polymerase chain reaction (PCR) buffer, 0.42 μ M each primer (FishF1 and Fish R1; Ward, Zemplak, Innes, Last, & Hebert, 2005). 40 ng template DNA, and 1.5 U iProof HiFi DNA polymerase (BioRad). PCR products were purified using Nucleomag size exclusion beads (Macherey Nagal) at a 0.7 to 1 ratio. Purified PCR products were sequenced bi-directionally using BigDye 3.1 (Applied Biosystems) and resolved on a 3730 DNA Analyzer (Applied Biosystems). Sequences were edited and aligned in SeqMan (DNA Star). Multiple sequence alignments were generated with MUSCLE implemented in Geneious v10 (Biomatters). A neighbour joining tree was generated in Geneious using the HKY distance model and 1,000 bootstrap replicates were performed to generate a 50% consensus tree. Assignments of either RMS or SS mtDNA in hybrids were tested using the binomial test in R software, to determine if overall ratios of mtDNA in hybrids deviated significantly from the expectation of symmetrical hybridisation (i.e. the expectation that the same number of hybrid individuals have RMS and SS mtDNA) (R Development Core Team, 2017). Due the small sample sizes of hybrid genotypes ($n \leq 10$), we did not test for asymmetry at finer genotype classifications.

2.4 | Genetic diversity analysis

Although 11 polymorphic microsatellite loci were initially genotyped, only 10 were suitable to be used in subsequent analysis. Loci were omitted if (1) there were many non-typed individuals (more than five), (2) a locus was fixed (major allele frequency >0.95 ; GenAIEx 6.5; Peakall & Smouse, 2012), and (3) the locus had a mean estimated null allele frequency >0.1 as determined using ML-NULL (Kalinowski & Taper, 2006; Peakall & Smouse, 2012). Using these criteria, one locus was omitted (Cco02) as there were many non-typed individuals ($>10\%$), and one individual sculpin was removed because it had many non-typed loci (>5).

Tests for linkage disequilibrium between all pairs of loci were performed using FSTAT v2.9.3.2 (Goudet, 1995). Deviations from Hardy–Weinberg Equilibrium (HWE) were tested for using the Excel GenAIEx 6.5 (Peakall & Smouse, 2012). Finally, diversity measures (number of alleles, observed heterozygosity, unbiased expected heterozygosity, and fixation rate) were calculated using the Excel GenAIEx 6.5 (Peakall & Smouse, 2012). Diversity measures were calculated separately for RMS and SS.

2.5 | Genetic differentiation and hybrid identification

Species were identified using STRUCTURE 2.3.4 (Falush, Stephens, & Pritchard, 2003; Pritchard, Stephens, & Donnelly, 2000), following the protocol outlined in Cullingham, James, Cooke, and Coltman (2012). Allele frequencies and admixture in the model were assumed to be correlated, as genetic samples came from a spatially connected

system (Falush et al., 2003). It was also assumed that there were no known priors for the number of genetic populations, therefore values of K were assessed from 1 to 10 and we used 500,000 Monte Carlo Markov Chain (MCMC) generations after a burn-in of 50,000 generations. Each K was replicated with MCMC sampling 10 times. Finally, the optimal value of K was assessed by reviewing both the mean \ln probability of K and Evanno method (Evanno, Regnaut, & Goudet, 2005).

The STRUCTURE model suggested that the best value for K was two, using the mean \ln probability and Evanno method (Supporting Information Figure S1). Results across replicates were summarised using CLUMPP v1.1.2 (Jakobsson & Rosenberg, 2007). Ancestry values (Q -values) from STRUCTURE were then used to classify individuals, where values ≥ 0.90 were classified as RMS, ≤ 0.10 indicated SS, and individuals between >0.10 and <0.90 indicated hybrids (Figure 2). Based on STRUCTURE assignments, we used classifications of 50 individuals classified as RMS ($Q \geq 0.90$) and 50 individuals classified as SS ($Q \leq 0.10$) that were from sites with no identified hybrids (i.e. all of the fish with $Q < 0.10$ or $Q > 0.90$) as reference populations for subsequent NEWHYBRIDS analysis. NEWHYBRIDS probabilistically classifies individuals based on their 10 locus genotypes into six genotypic classes: the two parental species, first (F1) and second (F2) generation hybrids and backcrosses to one or other parental species (BC 1 and BC2, respectively) (Anderson & Thompson, 2002). The NEWHYBRIDS model was built using the *genepopedit*, *paralellnewhybrid*, and *hybriddetective* packages in R software (Stanley, Jeffery, Wringe, Dibacco, & Bradbury, 2017; Wringe, Stanley, Jeffery, Anderson, & Bradbury, 2017a,b). For our NEWHYBRIDS model, we also used a burn-in of 50,000 generations and 500,000 MCMC generations to produce

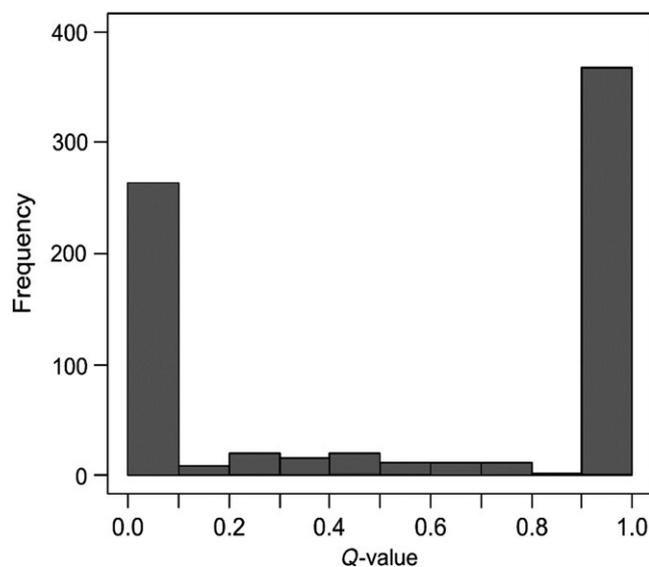


FIGURE 2 Frequency distribution of Q -values from the final STRUCTURE model ($K = 2$). Q -values represent the assignment of genotype classes where values between 0 and 0.1 represent slimy sculpin, 0.1 and 0.9 represent hybrids, and 0.9 and 1 represent Rocky Mountain sculpin

assignments of pure RMS, SS and hybrids across five simulated data sets.

2.6 | Contemporary distribution and logistic mixed-effects models

We summarised the distribution of RMS, SS and hybrids in waterbodies visually by mapping assignments geographically and using kernel density analysis along elevations where sampling was conducted. Kernel density analysis was conducted in tributaries where all genotype classes were found in order to visualise the densities of each genotype class by elevation in relation to each other. This included the upper Flathead River as well as Kishinena and Howell creeks. For the kernel density analysis, we used a bandwidth of 15 m with a Gaussian kernel using the *density* function in R software (R Development Core Team, 2017).

We developed three logistic mixed-effects models with ancestry values (Q -values) from STRUCTURE classifications as our response variable (Figure 2) to determine what environmental factors are important to the presence of RMS, SS, and hybrids (Figure 3). Site nested within waterbody was designated as a random effect, as environmental differences among the various reaches are expected and sites within reaches probably have similar environmental characteristics. Moreover, accounting for site level differences reduces issues that may arise due to pseudoreplication. Covariables used in the analysis included: water temperature ($^{\circ}\text{C}$), elevation (m), turbidity (NTU), sample depth (cm), flow velocity (m/s), dissolved oxygen (mg/L), conductivity ($\mu\text{S}/\text{cm}$), and the percentage of each substrate within 1 m^2 (small gravel, cobble, large gravel, boulder; Supporting Information Table S1). We included substrate in our analysis, because unembedded rocky material is considered to be commonly used by sculpin species for both protection from predators and for the construction of nests (Bailey, 1952; Scott & Crossman, 1998). Turbidity was also included as a covariate because it is considered one of the biggest threats to water quality in the Flathead River (COSEWIC, 2010) and it has the potential (as suspended sediment) to embed substrate and cause habitat loss. Other habitat requirements of sculpin species are thought to be cool, shallow, moving water; therefore, elevation (a proxy for temperature), water velocity, and depth were also used in our analysis (COSEWIC, 2010). Lastly, conductivity and dissolved oxygen were included as measures of water quality that can impact fish physiology (Fialho, Oliveira, Tejerina-Garro, & De Mérona, 2008).

Individuals genotyped at 62 sites having complete environmental data were included in the logistic regression models (Only 538 individuals; 206 RMS, 255 SS, and 77 hybrids). Thus, 193 individuals at 33 sites were not included in the final model analysis in order to maximise the number of environmental factors included in our analysis.

Prior to constructing logistic mixed-effects models, correlation analysis of the variables was conducted to identify collinearity and reduce the possibility of Type II errors (Zuur, Ieno, & Elphick, 2010). Water temperature was found to be correlated with elevation ($r = 0.6$) and cobble was correlated with large gravel ($r = 0.6$).

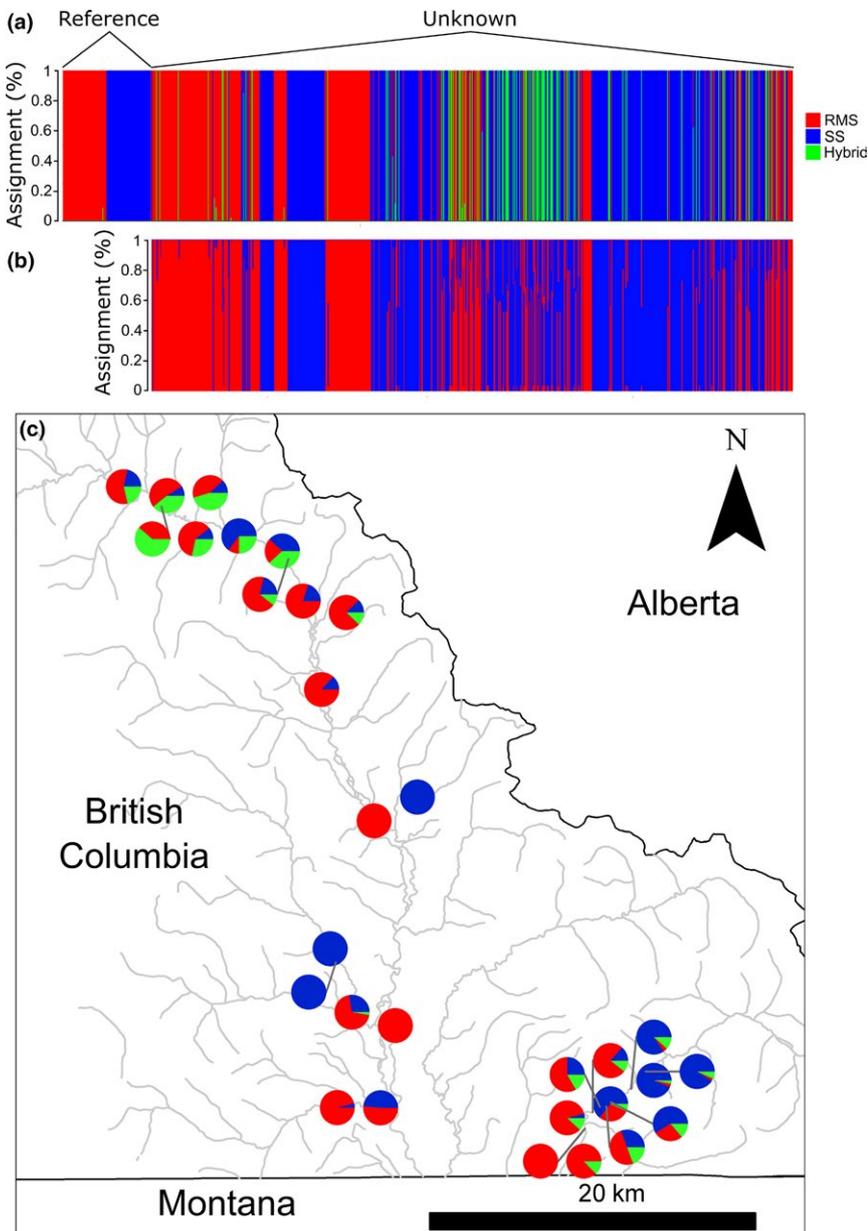


FIGURE 3 Probable genotype assignments from (a) NEWHYBRIDS and (b) STRUCTURE models. The first 100 individuals in the NEWHYBRIDS output (a) are reference individuals for Rocky Mountain sculpin (RMS) and slimy sculpin (SS). The hybrid class includes F1, F2, BC1 and BC2 genotypes. (c) STRUCTURE assignments for the 731 individuals mapped geographically across 30 locations that have been pooled for visualisation (from sites shown in Figure 1). Black bars denote historical upstream distribution limit for RMS from sampling by Hughes and Peden (1984) in 1979 and 1981

Therefore, temperature and cobble were not included in the final analysis. Covariables were standardised and centered into z-scores as they were in different units and on different scales. To select the best fitting reduced model, backward selection was conducted for each genotype class (RMS, SS, and hybrids) with the *lme4* package in R software (Bates, Maechler, Bolker, & Walker, 2015; R Development Core Team, 2017). Model selection and evaluation was conducted using the AICc value. The model with the lowest AICc value was chosen as the final reduced model (Burnham & Anderson, 2004).

Reduced models for each genotype class (RMS, SS, and hybrid) were assessed for performance using cross-validation and area under the curve (AUC) values (Fielding & Bell, 1997). Using AUC values from receiver operating characteristic (ROC) curves is a common approach to determine how well logistic models perform (Allouche, Tsoar, & Kadmon, 2006; Phillips, Anderson, & Schapire, 2006), where AUC

values of 0.5–0.7 are considered to be low model accuracy, 0.7–0.9 are considered useful models, and an AUC > 0.9 is considered to be high model accuracy (Manel, Williams, & Ormerod, 2001). For this study, model performance was assessed for each model using 100 cross-validation runs, where 70% of the data were randomly selected on each run to fit the model (training data set) and the remaining 30% were used to validate the fit of the model (testing data set; Fielding & Bell, 1997; Cullingham et al., 2012). Here, the AUC value provided a diagnostic for model performance (by assessing the number of true versus false, presence and absences). We summarised the results of this validation analysis using mean AUC values with 95% confidence intervals, while also providing these results visually for each cross-validation run in an ROC plot. This was conducted in R software (R Development Core Team, 2017) using the *vegan*, *lme4*, *ROCR*, and *caret* packages (Bates et al., 2015; Kuhn et al., 2017; Oksanen et al., 2016; Sing, Sander, Beerwinkler, & Lengauer, 2005).

To investigate whether the abundance of parental and hybrid genotypes at sites also varied in relation to environmental conditions, we analysed the abundance of each RMS, SS, and hybrid across 62 sites where environmental data were available using redundancy analysis (RDA) (Legendre & Legendre, 2012). Here, abundances were Hellinger transformed to make abundance data more amenable to Euclidean based analysis and environmental variables were also standardised and centered into z-scores, as they were in different units and on different scales (Legendre & Gallagher, 2001). The same variables used in the logistic mixed-effects models were also used here, as all covariables had variance inflation factors <2.2, indicating very little collinearity between covariables (Legendre & Legendre, 2012). Lastly, we tested for the significance of the model, axes of variation and covariables included in the final RDA using permutational analysis (Anderson, 2001; Anderson & Walsh, 2013). This was conducted using the *vegan* package in R software (Oksanen et al., 2016).

3 | RESULTS

3.1 | Genetic diversity

In total, 732 sculpins were captured, where a range of 1–20 sculpins were present in 610 of the 2,850 quadrats sampled across 95 sites. We then genotyped 731 individuals across 10 microsatellite loci. We did not find that any loci had a mean estimated null allele frequency >0.1. However, many loci for RMS and SS were not in HWE due to heterozygote deficiency (Table 1). Given that there is evidence to support that RMS and SS may have limited dispersal and fine spatial scale genetic differentiation (Ruppert et al., 2017), it would be expected that further substructure exists within these species groups. This would contribute to Hardy–Weinberg disequilibrium and to the high fixation index values across loci (Table 1). As we are not interested in population level structure

(which tends to show HWE in these species; Ruppert et al., 2017), all loci were included in the analysis. Also, we found that there was no evidence of any linkage disequilibrium across loci. Lastly, allelic diversity measures were higher for RMS in both the number of alleles and heterozygosity (Table 1).

3.2 | Identifying hybrids and contemporary distribution

Of the 731 sculpin sampled, our STRUCTURE model assigned 367 (50%) as RMS, 263 (36%) as SS, and 101 (14%) as hybrids (Figures 2 and 3). The final assignments used from STRUCTURE produced a U-shaped distribution of Q-values across all samples collected suggesting a bimodal hybrid zone structure (Figure 2). Comparisons between STRUCTURE and NEWHYBRIDS model assignments showed high concordance (99%) with only seven individuals classified differently between models (Figure 3a,b). Moreover, the NEWHYBRIDS model demonstrated a high mean assignment probability for genotype classes RMS (98.8%), SS (99.2%), and pooled hybrid classes (97.4%) with values >0.9 using the 10 microsatellite loci (Supporting Information Figure S2). The mean assignment probability, however, was lower for finer classifications of genotype classes F1 (98.2%), F2 (78.8%), BC1 (15.2%), and BC2 (19.2%) with values >0.9 using the 10 microsatellite loci (Supporting Information Figure S3).

The highest sculpin densities were found in Kishinena Creek and the upper Flathead River, with means of 14 and 7.5 individuals per site, respectively. Hybrids were detected in two tributaries, (Kishinena and Howell creeks) and the upper Flathead River (Figure 3c). Specifically, hybrids were most abundant along a 6-km section of Kishinena Creek and a 24-km section of the upper Flathead River (Figure 3c). Middlepass Creek contained only SS, while in Couldrey Creek only RMS and SS were found (Figure 3c). Both in the upper Flathead River and Kishinena Creek, there were no

TABLE 1 Genetic diversity measures for the 10 microsatellite loci that were typed for all individuals, Rocky Mountain sculpin (RMS) and slimy sculpin (SS) from the Flathead River. Shown are the number of individuals typed at each locus (N), number of alleles (N_a), observed heterozygosity (H_o), unbiased expected heterozygosity (UH_e), and fixation index (F_{IS}). Bold values indicate loci out of Hardy–Weinberg equilibrium for parental genotypes. All values were calculated using GenAEx 6.5

Locus	N	All				RMS				SS			
		N_a	H_o	UH_e	F_{IS}	N_a	H_o	UH_e	F_{IS}	N_a	H_o	UH_e	F_{IS}
Cba42	731	6	0.316	0.630	0.498	6	0.411	0.606	0.320	5	0.167	0.495	0.661
Cgo114	731	4	0.276	0.345	0.200	4	0.322	0.383	0.160	4	0.179	0.258	0.307
Cco13	731	3	0.093	0.496	0.812	3	0.123	0.406	0.698	3	0.034	0.440	0.922
Cco15	731	8	0.356	0.661	0.462	7	0.463	0.685	0.323	7	0.186	0.479	0.610
Cco17	730	14	0.518	0.753	0.312	11	0.591	0.778	0.239	10	0.384	0.605	0.364
CottES10	729	6	0.115	0.503	0.771	5	0.142	0.414	0.657	6	0.069	0.461	0.850
Cott130	730	5	0.158	0.196	0.196	3	0.115	0.137	0.161	4	0.236	0.290	0.185
Cott687	728	14	0.242	0.575	0.579	11	0.298	0.506	0.411	9	0.160	0.502	0.681
CottES19	731	4	0.008	0.038	0.782	3	0.011	0.069	0.841	2	0.004	0.004	–0.002
Cgo310	727	8	0.314	0.619	0.493	8	0.349	0.550	0.365	6	0.245	0.565	0.565
Average				0.482	0.510			0.453	0.417			0.410	0.514

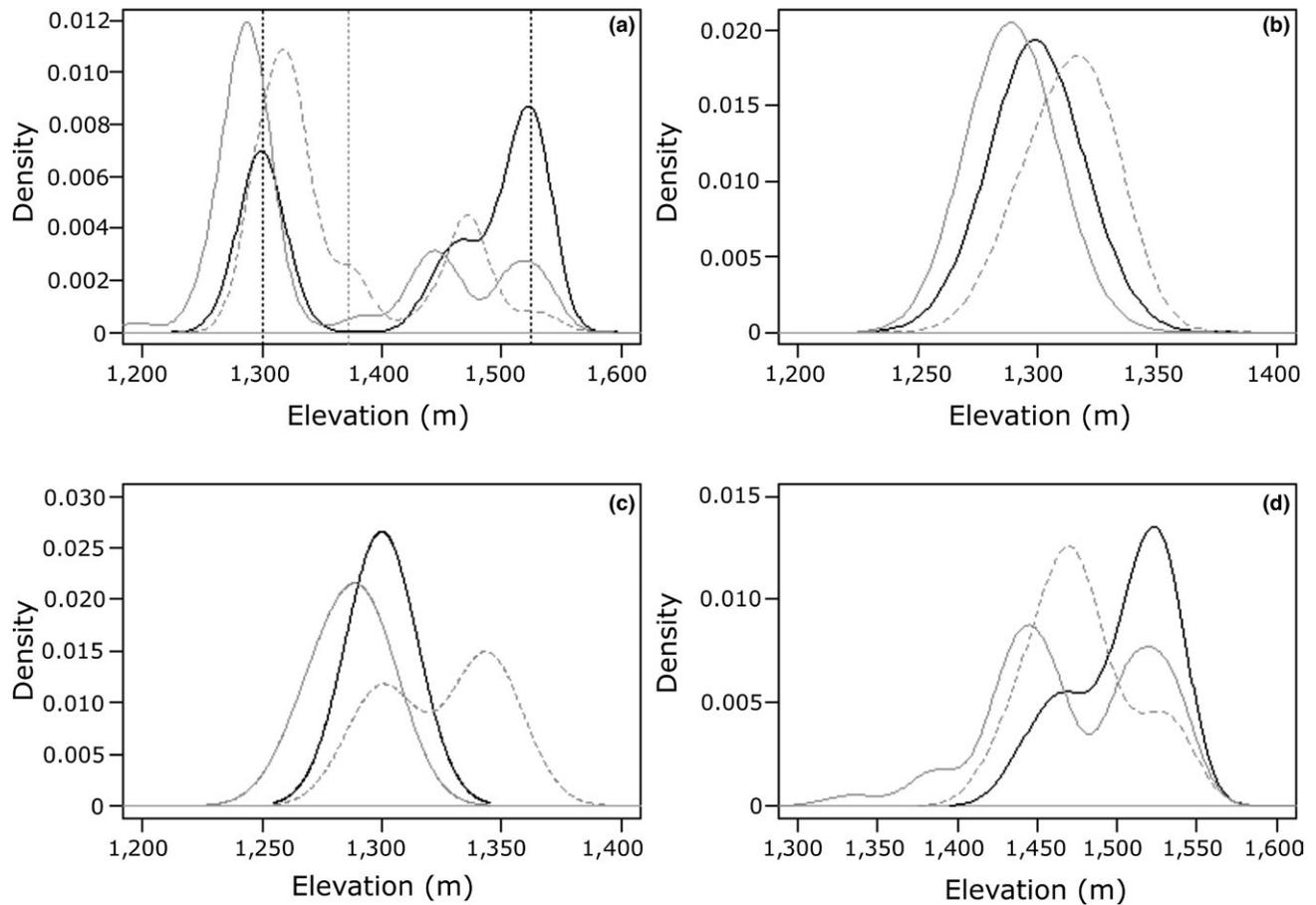


FIGURE 4 The probability density function (density) for a given elevation using kernel density estimation of Rocky Mountain sculpin (solid grey), slimy sculpin (dashed grey), and hybrid (solid black) genotype abundances by elevation for (a) all sites, (b) Kishinena Creek, (c) Howell Creek, and (d) upper Flathead River. Also shown is probability density function of hybrids (dashed grey line) and the upper range limit of Rocky Mountain sculpin reported in Hughes and Peden (1984; dashed black line). NB: the highest elevation sites from upper Flathead River (1,879 m) and Kishinena Creek (1,902 m) were omitted for presentation

spatially distinct areas where only SS exist, but in the lower Flathead River there appears to only be RMS (Figure 3c).

Distributions of RMS, SS, and hybrids differed by waterbody where all three coexist (Figure 4). The likelihood of RMS density was highest well above the historical elevation limit (1,372 m; Figure 4a). The highest probability that RMS could be found was around 1,355 m in elevation with a range that included the entire elevation of sampled (1,200–1,902 m). Correspondingly, both SS and hybrids had higher probabilities of being found at higher elevations than RMS. In both Kishinena and Howell creeks, SS probabilities peaked higher upstream than hybrids, while hybrids tended to have the highest probabilities at intermediate elevations to both RMS and SS (Figure 4b,c). Of note, hybrids only occurred where the distribution of RMS and SS overlapped with the highest probability of hybrids being present corresponding with this overlap between RMS and SS (Figure 4). Less clear were the probability of densities in the upper Flathead River (Figure 4d), where all have distributions much higher in elevation and do not follow the sequence in density seen in Kishinena and Howell creeks. In the upper Flathead River, peak hybrid probability was associated with higher elevations, but also higher

average temperatures (mean \pm 95% confidence intervals) in the upper Flathead River (1,525 m, $13.3 \pm 1.3^\circ\text{C}$) compared to their peaks in Kishinena and Howell creeks (1,300 m, $10.0 \pm 0.7^\circ\text{C}$ and $8.4 \pm 0.3^\circ\text{C}$, respectively). Moreover, at no point in the upper Flathead River were higher probability densities of only SS found, indicating that the full distribution gradient was likely not sampled by comparison to Kishinena and Howell creeks.

3.3 | Symmetry in hybrid spawning

Of the 60 individuals genotyped for mtDNA, six hybrids did not produce a high-quality sequence and were omitted (Table 2). For the remaining 34 hybrid individuals, overall ratios of RMS and SS mtDNA (RMS, $n = 14$ and SS, $n = 20$) did not deviate significantly from the expectation of being evenly mixed ($p = 0.39$ and $p = 0.39$, respectively). However, some hybrid genotypes (F1 and BC1) appear to have surplus of SS mtDNA, suggesting that there could be some asymmetry (Table 2). Despite this result, the small sample sizes (F1, $n = 8$ and BC1, $n = 7$, respectively) limit us from exploring this further. Thus, we find no evidence of asymmetric mating between RMS and SS, between hybrid classes or in backcrosses.

TABLE 2 Hybrid genotypes, as classified by NEWHYBRIDS, which were typed for Rocky Mountain sculpin (RMS) and slimy sculpin (SS) mitochondrial DNA (mtDNA). Shown are the overall sample sizes for each genotype with mtDNA classifications

Genotype	n	mtDNA	
		RMS	SS
F1	8	2	6
F2	10	5	5
BC_RMS	7	2	5
BC_SS	9	5	4
Total	34	14	20

3.4 | Predictive environmental variables

Over 100 cross-validation runs, model performance was considered to be good (0.7–0.9) or excellent (>0.9) using mean AUC values ($\pm 95\%$ confidence intervals; Supporting Information Figure S4). In particular, both reduced RMS and SS models had the best fit (0.87 ± 0.005 and 0.91 ± 0.004 , respectively), whereas the performance of the reduced logistic hybrid model performed slightly worse (0.77 ± 0.01 ; Supporting Information Figure S4). Although the AUC values were good (0.7–0.9) for the hybrid model, the poorer performance may reflect the reduced power of the model, because only 77 hybrid individuals, of 538 total samples, were used to create the regression model.

The logistic mixed-effects models indicated that multiple habitat features are important in determining the probability of the presence of parental and hybrid sculpins. Both elevation and turbidity were the most consistent at being included and significant in the reduced models (Table 3). Other covariables selected in the reduced models included conductivity, large gravel, and small gravel (Table 3). Most of the covariables in the reduced models had a significant effect on the probability of parental and hybrid presence (Table 3), with the exception of small gravel. The probability of RMS presence was significantly related to lower elevation areas (Table 3 and Supporting Information Table S1). Further, the probability of RMS presence was also significantly associated with relatively higher levels of turbidity (Table 3 and Supporting Information Table S1). In contrast, the probability of SS presence was significantly associated with relatively lower turbidity, yet relatively higher amounts of large gravel (Table 3 and Supporting Information Table S1). Turbidity had the largest coefficients in both RMS and SS models indicating that this factor appears to have the largest impact on the probability of RMS and SS presence (Table 3). Lastly, higher elevations with lower conductivity were significantly related to the probability of hybrid presence (Table 3 and Supporting Information Table S1).

The RDA provided similar results to the logistic mixed-effects models (Figure 5). As the results are the similar, the addition of the abundance of each genotype (RMS, SS, and hybrids) in the RDA appears to not alter genotype-environmental associations

TABLE 3 Coefficient estimates and standard errors of variables retained in the reduced logistic mixed-effects models for Rocky Mountain sculpin (RMS), slimy sculpin (SS), and hybrids (HYB) in the Flathead drainage. Bolded values denote p -values of significance ($<.05$)

Variables	Model		
	RMS	SS	HYB
Elevation	-1.25 ± 0.45		1.22 ± 0.22
Turbidity	1.55 ± 0.45	-1.65 ± 0.49	
Conductivity			-0.70 ± 0.22
Small gravel			-0.23 ± 0.15
Large gravel		0.31 ± 0.15	

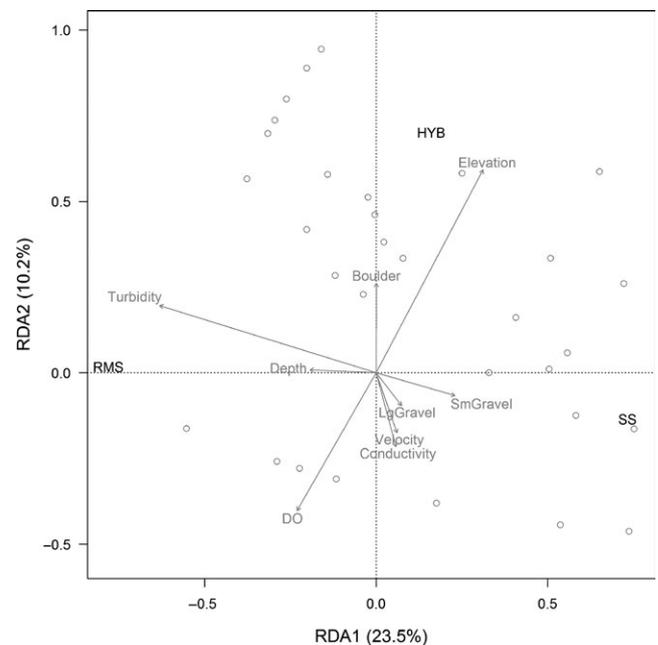


FIGURE 5 Redundancy analysis of abundances of genotypes (RMS, Rocky Mountain sculpin; SS, slimy sculpin; HYB, hybrid) found across 62 sites sampled for sculpin and environmental conditions. The overall model, first axis of variation and two covariates (elevation and turbidity) are significant ($p < .01$) using permutational tests. Abbreviations are used for some covariates (LgGravel, large gravel; SmGravel, small gravel; DO, dissolved oxygen); Components one and two of the Redundancy Analysis (RDA) are shown with the variation explained by each

(Figure 5). Specifically, we found that the overall model, first axis of variation, and two covariates (elevation and turbidity) are significant ($p < .01$) by permutational analysis (Figure 5). Specifically, RMS and SS were found at opposite points in the ordination biplot, along the significant first axis of variation, implying that higher abundances of RMS and SS are found at opposite points along environmental gradients sampled in the Flathead drainage (Figure 5). This is a similar result to the logistic mixed-effects models (Table 3).

4 | DISCUSSION

Contemporary stressors and alterations in freshwater ecosystems that are commonly linked to human activities can alter instream conditions and influence the geographic extent and position of hybrid zones (Muhlfeld et al., 2009; Seehausen, 2006; Seehausen et al., 2008). Specifically, given the persistence of hybrid individuals in the Flathead River drainage and ongoing alterations to instream water conditions, there is a need to understand how hybridisation between RMS and SS may be linked to changing environmental conditions (Seehausen et al., 2008). As a first step toward improving our understanding of underlying mechanisms that maintain hybrid genotypes, there is a requirement to (1) identify genotype classes (especially for these morphologically similar species) and determine the distribution of each in order to also assess whether distributions are changing, (2) determine if there is symmetry in hybridisation, and (3) identify what environmental conditions are related to the presence of each genotype class. Here, we provide insight into the processes that maintain hybridisation between RMS and SS and indicate how these processes might be changing to aid in conservation management programs for these species, especially the RMS, a species-at-risk.

4.1 | Habitat use and hybridisation

Hybrids can persist by having a competitive advantage over parental species or, if parental species have a competitive advantage, they may both continually disperse into an area that represents an *ecotonal zone* (Arnold, 1997). Although we did not determine if hybrid or parental genotypes have a competitive advantage, our results suggest that there are particular habitats used by RMS and SS, which play a role in promoting hybrid persistence (Barton & Hewitt, 1985). In particular, we found spatially discrete hybrid zones in the Flathead River drainage that are linked to different instream conditions that influence the distribution of RMS, SS, and hybrids. Altogether the elevation, turbidity, conductivity, and sediment at sites were identified as significant environmental variables. This corroborates previous work by finding that SS presence is related to upstream environmental conditions, whereas RMS presence is associated with environmental conditions found in downstream locations (COSEWIC, 2010; Hughes & Peden, 1984). Further, where we find overlap in the distribution between RMS and SS, we also observe that hybrids are present. This suggests that the range overlap (or ecotones) between RMS and SS is probably the process by which hybrid zones are maintained within the Flathead drainage.

Elevation is associated with a set of environmental variables (Lookingbill & Urban, 2003) and all three reduced models support that elevation and its associated conditions (e.g. turbidity) are important factors in the presence of RMS, SS, and hybrid genotypes. Higher elevations associated with SS presence validate previous work that SS are generally less common in relatively warmer water temperatures (Kowalski, Schubauer, Scott, & Spotila, 1978; Otto & Rice, 1977). In contrast, our models suggest that the RMS prefer

relatively warmer temperatures. Further supporting this difference in habitat related to temperature are increases in the average summer water temperature of the Flathead River, which has occurred since the 1980s (Muhlfeld et al., 2014). This change in temperature contributed to the incidence and location of hybridisation between westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) and non-native rainbow trout (*Oncorhynchus mykiss*) (Muhlfeld et al., 2014). Additionally, elevation could be related to changes in hydrology, which has also been shown to play a key role between cutthroat trout and non-native rainbow trout hybridisation and it can be important to within-species phenological variation (Muhlfeld et al., 2014; Rudolfesen et al., 2018). Changes in hydrology may have also contributed to alterations in RMS distribution, as RMS appear to have expanded their distribution to higher elevations: they are now found 530 m higher than in the early 1980s (Hughes & Peden, 1984). However, this study and Hughes and Peden (1984) differed in the type of survey (genetic and morphological, respectively), effort (sites; $n = 95$ and $n = 24$, respectively), range of sampling (approximately 1,200–1,900 m and 1,200–1,500 m, respectively) and the number of water bodies sampled ($n = 5$ and $n = 10$, respectively). These methodological differences could contribute to the observed alterations in RMS range limit, however, morphology has a high accuracy of identification between RMS and SS (~87%), close to half of the sampled elevation range (approximately 1,200–1,500 m) in the 1980s excluded RMS (only found up to 1,372 m), and more water-bodies had been sampled by Hughes and Peden (1984). Thus, the estimated distribution of RMS in the 1980s appears to be a robust comparison to contemporary distributions.

Turbidity and conductivity are also important environmental factors related to the presence of RMS, SS, and hybrids. Turbidity is a measure of the total suspended solids within water, whereas conductivity is related to the amount of dissolved salts and other inorganic compounds within water, which is influenced by geology and the source of water (ground versus surface) (Lewis, Harwood, Zyla, Ganshorn, & Hatfield, 2013). In particular, the magnitude of the turbidity coefficient in our models suggests that turbidity may have the largest impact of all the covariates for RMS and SS presence. RMS appear to be relatively more tolerant of downstream turbidity, which can be associated with anthropogenic siltation that increases turbidity through the creation and use of roads along with the use of all-terrain vehicles (COSEWIC, 2010). Lastly, substrate type was also important, but to a relatively lesser degree for the presence of any of the genotype classes. Thus, habitat use between RMS and SS appears to be related to elevation, turbidity, and substrate, which follows environmental differences between upstream and downstream sites.

A comprehensive understanding of the dynamics of hybridisation between species requires a characterisation of any directionality that may be associated with hybrid spawning events (e.g. Metcalf, Siegle, & Martin, 2008). Here, we found that there was reciprocal exchange of RMS and SS mtDNA and there appears to be no overall bias in the directionality of hybridisation across all hybrid genotypes (F1, F2, BC1 and BC2). The contribution of parental SS and RMS mtDNA may be equal across all hybrid genotypes, but we also

found weak evidence for asymmetry with some hybrid genotypes (F1 and BC1). To provide more conclusive results, other factors need to be investigated more thoroughly alongside mtDNA assignments, including the densities of both RMS and SS (i.e. the expectation that there are equal contributions of RMS and SS mtDNA may not be correct; e.g. Taylor & Hebert, 1993). Thus, there does not seem to be asymmetry in hybrid spawning events (e.g. male RMS do not always mate with female SS or vice versa), but this process could vary spatially or temporally with RMS and SS densities.

4.2 | Conservation implications

Given that both RMS and SS would be direct competitors for similar habitat (cold, rocky streams) and resources (benthic invertebrates), it is important to understand the process of hybridisation to determine the prospect of long-term species persistence (Allendorf et al., 2001; Kuussaari et al., 2009; Olden et al., 2004; Pfennig et al., 2016). The contemporary distribution of RMS and current trend of expansion into higher elevations suggest that RMS may benefit from changing environmental conditions. However, many factors such as turbidity, conductivity, and perhaps temperature could be important to their presence and ongoing changes to their distribution can potentially bring about changes in hybridisation with SS as observed in other taxa (Chen et al., 2011; Chunco, 2014; Seehausen, 2006; Seehausen et al., 2008). Such scenarios with rare and threatened species, generally present an increased risk to extinction through introgression (Rhymer & Simberloff, 1996; Taylor & Piercey, 2018).

Given the differences between RMS and SS in their association with environmental conditions, contemporary threats to RMS via hybridisation or displacement by SS appear to be limited for several reasons. Firstly, the range of RMS in the Flathead River appears to have expanded higher in elevation by approximately 530 m over the last 35 years (Hughes & Peden, 1984). Secondly, our logistic mixed-effects model suggests that SS are found in areas with low turbidity and higher elevations that have lower water temperatures. Given the increasing prevalence of human activities in the Flathead River drainage that are associated with increased sedimentation (COSEWIC, 2010), conditions may become less favourable for SS compared to RMS. However, it should be noted that much higher levels of sedimentation could eventually become averse to RMS. Finally, we find that SS are found in cooler waters than RMS, suggesting that differences in habitat use between RMS and SS may constrain hybridisation. However, an association with water temperature may present a risk as projected climatic change scenarios suggest further range expansion in RMS may be possible and could subsequently alter hybridisation between RMS and SS (e.g. Muhlfeld et al., 2014).

Although we showed associations between environmental factors and RMS or SS presence in the Flathead River, the presence of hybrid genotypes produced a slightly poorer fitting reduced model. This may be due to several reasons, including: (1) the relatively low prevalence of hybrids in our study, (2) parental range overlap may be a main driver of hybrid presence, (3) the inclusion of all hybrid genotypes (F1, F2, BC1, and BC2) might increase variability in this group,

and/or (4) there are other environmental conditions not considered alongside the impact of multiple stressors that may be interactive.

Prevalence can influence overall model performance, which will affect whether environmental factors (or covariates) are well resolved or not (Manel et al., 2001). In this study, 77 out of 538 individuals (~15%) were hybrids, and we found a good mean performance of the reduced model across 100 cross-validation runs (AUC = 0.77; Figure 5). However, the lower AUC value for the hybrid model may be because parental species presence plays a key role in maintaining hybridisation (i.e. parental types may have a competitive advantage), but without formally testing for competitive advantages it is still not clear how hybrids are maintained within this system (Arnold, 1997; Barton & Hewitt, 1985). Another explanation is that there could be many associations represented in this pooled group of diverse genotypes (i.e. F1, F2, BC1, and BC2; e.g. Hamilton & Miller, 2016), this can make it difficult to resolve patterns for hybrids as a whole. Finer genotypic resolution may help to resolve relationships better, but this would require more hybrid samples and a better resolution of markers given the relatively low assignment success of F2 and backcrossed (BC1 and BC2) genotypes by our NEWHYBRIDS model (79% and 15%, 19%; Figure S3). Finally, notwithstanding our results, there may also be interactions among the factors we explored or factors not considered in this study (e.g. food availability, habitat amount, and connectivity) that may influence presence of RMS, SS, and hybrids (Angermeier, 1995; Pringle, 2003). Improving knowledge of the impact of multiple stressors could better explain the differences in species presences and future trends, as important factors can interact additively, synergistically, or antagonistically to effect species (Crain, Kroeker, & Halpern, 2008; Folt, Chen, Moore, & Burnaford, 1999).

Altogether, this study provides a clear overview of the processes contributing to RMS and SS hybridisation. We showed that differences in habitat use constrain hybridisation between the two species, specifically related to elevation, turbidity, conductivity, and sediment. Where hybridisation occurs, the distribution of RMS and SS overlap. Additionally, our results suggest that RMS are currently not at risk of decreased population viability via hybridisation, but this is also a process in flux (RMS are moving to higher elevations), which requires ongoing monitoring and management to maintain species persistence.

ACKNOWLEDGMENTS

Funding for this project was provided by Fisheries and Oceans Canada, Species at Risk Program (D.W., M.P.), and NSERC Discovery Grant (M.P.). Thank you to Brendan Wringe for R software support and advice. Additionally, thanks to Christopher Smith, Elashia Young, Kenton Neufeld, Paul Fafard, Doug Leroux, Colin Kovachik, Kyle Hamilton, Hedin Nelson-Chorney, and Bryan Maitland for assisting with field collection. Field safety and support was provided by Dr Garth Mowat and Joe Caravetta. The procedures for this project were conducted under an approved British Columbia Fish Collection Permits CB14-149123 and CB15-171090.

CONFLICTS OF INTEREST

The authors declare no conflict of interests

ORCID

Jonathan L. W. Ruppert  <http://orcid.org/0000-0001-6973-107X>

Mark S. Poesch  <http://orcid.org/0000-0001-7452-8180>

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How to cite this article: Rudolfesen T, Ruppert JLW, Taylor EB, Davis CS, Watkinson DA, Poesch MS. Habitat use and hybridisation between the Rocky Mountain sculpin (*Cottus* sp.) and slimy sculpin (*Cottus cognatus*). *Freshw Biol.* 2018;00:1–14. <https://doi.org/10.1111/fwb.13225>