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Temporal segregation in spawning between native Yellowstone cutthroat trout and introduced rainbow trout

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Abstract

Hybridisation with introduced taxa poses a threat to native fish populations. Mechanisms of reproductive isolation can limit or prevent hybridisation between closely related species. Understanding how these mechanisms interact between the same species across geographically distinct occurrences of secondary contact, and how regional factors influence them, can inform our understanding of hybridisation as a threat and management actions to mitigate this threat. We used data collected on adult fish migration timing and approximate emergence timing of subsequent juvenile fish paired with genomic data to assess whether temporal isolation in the timing of spawning exists between Yellowstone cutthroat trout, rainbow trout and hybrids in the North Fork Shoshone River drainage in northwest Wyoming. We found evidence that Yellowstone cutthroat trout spawn, on average, two to four weeks later than rainbow trout and hybrids and two environmental covariates related to water temperature and discharge were associated with differences in spawning migration timing. Despite statistical support for Yellowstone cutthroat trout spawning later, disproportionately high numbers of rainbow trout and hybrids, paired with extended spawning seasons, lead to substantial overlap between all genotypes. Our results provide further evidence of temporal segregation in the timing of spawning as a mechanism of reproductive isolation between closely related species, but substantial spawning overlap suggests temporal segregation alone will not be enough to curtail hybridisation in conservation populations.

KEYWORDS
hybridisation, rainbow trout, reproductive isolation, Yellowstone cutthroat trout

1 | INTRODUCTION

Hybridisation with introduced taxa poses one of the greatest threats to global freshwater fish biodiversity (Allendorf et al., 2001; Perry et al., 2002; Seehausen, 2006). Human-mediated non-native fish introductions, as well as the expansion of already established non-native populations, can expose native fish populations to secondary contact with closely related species, facilitating hybridisation

(Gozlan et al., 2010). Native freshwater fish populations can face reduced fitness (Muhlfeld, Kalinowski, et al., 2009), loss of locally adapted genes (McGinnity et al., 2009) and potential extirpation (Rhymer & Simberloff, 1996) as a result of hybridisation. However, species that come into contact after evolving in allopatry may have partial or complete barriers to gene flow, thus hindering or preventing hybridisation (Coyne & Orr, 1998; Dobzhansky, 1937). Barriers to gene flow may include factors linked to mate choice (Peterson

et al., 2007), reproductive compatibility (Wolstenholme, 2004), or fitness and viability of offspring (Fraser et al., 2008), and multiple mechanisms may work synergistically to reproductively isolate species (Matsubayashi & Katakura, 2009). Varying levels of genetic relatedness between populations of interest, genetic mechanisms of reproductive isolation and interactions with environmental factors may lead to inconsistent hybridisation outcomes from secondary contact between the same species across geographic locations (Mandeville et al., 2017; Matsubayashi & Katakura, 2009).

Developing a better understanding of how mechanisms of reproductive isolation interact with environmental conditions across different populations can inform management actions aimed at limiting hybridisation as a conservation threat. One popular management action to limit hybridisation is isolating native populations upstream of a natural or human-made barrier to eliminate secondary contact with introduced fish (Fausch et al., 2009; Host, 2003; Novinger & Rahel, 2003). In populations where native fish migrate extended distances between habitat types to spawn, native fish must be selectively passed around isolation barriers (High, 2010). Leveraging naturally occurring mechanisms of reproductive isolation, such as temporal variation in the timing of spawning, can allow selective passage over barriers to become more efficient and cost-effective. Isolation management is a popular management tool to protect and enhance native cutthroat trout (*Oncorhynchus clarkii*) populations in North America (Cook et al., 2010; Novinger & Rahel, 2003), and better knowledge of trout hybridisation interactions can inform when isolation management is necessary and how it may be best used.

Cutthroat trout evolved across an expansive geographic area in North America within subdrainages of the Columbia, Mississippi and Colorado Rivers. This large native range has allowed the 14 currently recognised subspecies of cutthroat trout to evolve a variety of life history traits across different habitat types (Behnke, 1992). One of the most pressing conservation threats facing native cutthroat trout populations is hybridisation with introduced rainbow trout (*Oncorhynchus mykiss*) (Docker et al., 2003; Gresswell, 2009; Kruse et al., 2000; Yau & Taylor, 2013). Occurrences of secondary contact between cutthroat trout and rainbow trout exist across the expansive native range of cutthroat trout and have led to varying hybridisation outcomes with outcomes in part related to climactic interactions, natural barriers to fish migration and historical management actions (i.e., fish stocking history, cutthroat trout conservation initiatives) (Docker et al., 2003; Muhlfeld et al., 2017; Yau & Taylor, 2013; Young et al., 2016). Previous research studying reproductive isolation between cutthroat trout and rainbow trout identified differences in the timing of spawning (temporal segregation) and differential habitat preference (spatial segregation) as two potential mechanisms of reproductive isolation limiting hybridisation between the two species (DeRito et al., 2010; Henderson et al., 2000; Muhlfeld, Kalinowski, et al., 2009). Studying how consistent these mechanisms of reproductive isolation are across populations, and identifying what environmental drivers influence them can inform future management actions aimed at limiting hybridisation threats to cutthroat trout.

Our research sought to determine whether temporal segregation in the timing of spawning exists between a subspecies of cutthroat trout, Yellowstone cutthroat trout (*O. c. bouvieri*), and rainbow trout in a highly introgressed lacustrine–adfluvial (migrating from lakes and reservoirs into lotic systems to spawn; Gresswell, 2009) population in northwest Wyoming. We hypothesised that Yellowstone cutthroat trout would spawn later as previous research with fluvial (migrating from streams and rivers into tributaries to spawn; Gresswell, 2009) Yellowstone cutthroat trout and rainbow trout found that Yellowstone cutthroat trout spawn 3–9 weeks later than rainbow trout (DeRito et al., 2010; Henderson et al., 2000). We collected data on the timing of adult fish spawning in conjunction with a novel approach integrating genomic analyses and length of emigrating juveniles (Mandeville et al., 2019). Additionally, we sought to determine whether temporal segregation in the timing of spawning was driven by species-specific responses to environmental covariates that drive spawning migrations. By identifying whether temporal segregation in spawning exists between Yellowstone cutthroat trout and rainbow trout in the North Fork Shoshone River drainage, and what environmental drivers may facilitate it, we can better inform our understanding of hybridisation and future management actions designed to mitigate hybridisation threats.

2 | METHODS

2.1 | Study system

This study was conducted in the North Fork Shoshone River drainage, part of the upper Missouri River drainage. The North Fork Shoshone River originates in the Absaroka Mountains and flows through approximately 79 km of public and private land before entering Buffalo Bill Reservoir near Cody, Wyoming, USA. Since its creation in 1910, Buffalo Bill Reservoir was stocked with a variety of trout species and now sustains self-reproducing populations of native Yellowstone cutthroat trout, non-native rainbow trout, Yellowstone cutthroat trout × rainbow trout hybrids (hereafter hybrids) and others (Kent, 1973; Nordberg et al., 2021). The North Fork Shoshone River basin provides spawning habitat for lacustrine–adfluvial populations of trout that migrate out of Buffalo Bill Reservoir each spring to spawn in the river and its tributaries. Highly variable seasonal flows and an unstable geomorphology in the North Fork Shoshone River drainage create conditions where a lacustrine–adfluvial life history is advantageous, and thus, most trout in the drainage move seasonally between the North Fork Shoshone River drainage and Buffalo Bill Reservoir (Wyoming Game and Fish Department, 2017). The native population of Yellowstone cutthroat trout has faced substantial declines related to interactions between non-native trout species, including hybridisation with rainbow trout, and as a result, Yellowstone cutthroat trout only account for approximately 20% of all trout in the system (Wyoming Game and Fish Department, 2017). Mandeville et al. (2019) showed that although multiple tributaries to the North Fork Shoshone River had evidence of Yellowstone

cutthroat trout spawning, genetically unadmixed Yellowstone cutthroat trout individuals were rare. We selected the two tributaries to the North Fork Shoshone River, Trout Creek and Middle Creek, that had the highest proportion of Yellowstone cutthroat trout ancestry among sampled juvenile fish as study sites.

2.2 | Adult fish sampling

To determine the timing of spawning, we used hoop nets to sample adult *Oncorhynchus* trout as they migrated into Middle Creek to spawn during the 2019 and 2020 spawning seasons. We considered the date we captured fish in our traps as their entry date into the spawning tributary. Hoop nets have been used previously to effectively sample migratory spawning fish in tributaries to the North Fork Shoshone River (J. Burckhardt, Wyoming Game and Fish Department, personal communication). Two styles of hoop nets were used for adult fish sampling; both styles were approximately 4.5 m long with seven hoops and two tapered throats on the second and fourth hoops with 13- to 20-cm openings. Some nets contained circular hoops that were approximately 0.91 m in diameter and were constructed of a 3.8-cm knotted nylon mesh. The second style of net was a D-frame style with D-frames that measured 0.91 m across the base, 0.76 m high at the apex and 1.94 m across the arc with 2.38-cm knotless nylon netting. Nets did not use a lead or wing to guide fish into trap openings. All hoop nets were oriented with openings downstream to capture adult fish as they migrated upstream in search of suitable spawning habitat. Hoop nets were fished at different locations on Middle Creek from the confluence with the North Fork Shoshone River to approximately 300 m upstream of the confluence.

Adult fish collected in traps were processed streamside and released directly above their point of capture. Before handling, fish were anaesthetised in a 25 mg/L Aquis-S 20E solution for two to four minutes. The date a fish was captured in the trap was recorded as its entry date into the spawning tributary. We measured each fish for total length to the closest millimetre (mm), and the adipose fin was clipped from each fish and stored in 100% ethanol for DNA extraction. Fish were assigned as male or female using morphometric characteristics such as coloration, jaw shape and gamete production.

2.3 | Juvenile fish collection

We collected emigrating juvenile fish starting in late summer and continuing through the fall at two tributaries to the North Fork Shoshone River, Trout Creek and Middle Creek, to further evaluate the timing of spawning. In the North Fork Shoshone River drainage, juvenile *Oncorhynchus* typically migrate out of their natal tributaries between August and October of the same year they are spawned (Kent, 1984). We sampled juvenile fish near the confluence of study tributaries during multiple sampling events each year. Biweekly electrofishing for juvenile fish was conducted on Trout Creek and Middle

Creek from mid-August until the first week of October during 2019. During 2020, juvenile fish were sampled over four sampling events at Middle Creek between August and October.

Juvenile fish were collected using a single backpack electrofisher (Smith Root, Model LR-24). Backpack electrofishing sampling was conducted across three transects at the study tributary near its confluence with the North Fork Shoshone River. At each transect, we electrofished upstream for 600 s collecting all fish we encountered. Age-0 fish (<75 mm in total length) were measured for total length to the closest millimetre, and a fin clip was removed from the lower caudal lobe and stored in 100% ethanol. All juvenile fish were released after fin clips were collected. We collected a minimum of 45 fish during a sampling event.

2.4 | DNA extraction and sequencing

All adult fish tissue samples were selected for DNA extraction and subsequent sequencing. A temporally and spatially balanced subset of juvenile tissue samples ($n = 572$ for Trout Creek in 2019, $n = 143$ for Middle Creek in 2019 and $n = 153$ for Middle Creek in 2020) were selected for DNA extraction and sequencing. We extracted genomic DNA from fin tissue using Qiagen DNeasy Blood and Tissue kits and a QIAcube robot according to the manufacturer's instructions (Qiagen, Inc.). Reduced complexity genomic libraries were prepared for high-throughput DNA sequencing using genotype-by-sequencing protocol outlined in Parchman et al. (2012). DNA was initially fragmented using restriction enzymes EcoRI and MseI, and fragments from each individual fish's DNA were ligated to a unique 8–10 base pair nucleotide barcode. Prior to sequencing, each library was size-selected using BluePippin (Sage Science) to retain only fragments of 350–450 base pairs in length. Following the ligation of the identification barcodes, individual samples were multiplexed and amplified by PCR. Between 192 and 248 individuals were pooled per library, and each library was sequenced on one Illumina HiSeq 4000 lane to produce approximately 1.5 billion 150 single-end base pair reads. DNA sequencing of all 11 libraries was completed at the Genomics and Cell Characterization Core Facility at the University of Oregon.

We assigned reads to individual fish by parsing unique barcodes. We then aligned reads from each individual fish to the rainbow trout genome (Pearse et al., 2019, accession GCA_002163495.1) using the bwa-mem algorithm (Li, 2013). We used samtools mpileup to identify variant sites and used BCFtools to call genotypes (Danecek et al., 2021). We filtered the initial set of variants to include only biallelic SNPs (single nucleotide polymorphisms). We additionally used vcftools (Danecek et al., 2011) to retain loci with data in at least 50% of individuals and loci with a minor allele frequency of 0.03 or greater. Individuals missing data for at least 70% of retained sites were removed from the data set.

To identify the ancestry of individual fish, we used entropy, a hierarchical Bayesian model that allows for the incorporation of genotype uncertainty into ancestry estimation (Gompert et al., 2014;

Shastry et al., 2021). For each individual fish, we estimated q, proportion of Yellowstone cutthroat trout ancestry, for a $k = 2$ model assuming two genetic clusters (i.e., Yellowstone cutthroat trout and rainbow trout). Three replicate chains were run, and the convergence of each chain was assessed by examination of trace plots. Fish with a proportion of Yellowstone cutthroat trout ancestry (q) <0.1 were classified as rainbow trout, and those with proportions of Yellowstone cutthroat trout ancestry >0.9 were classified as unadmixed Yellowstone cutthroat trout. All fish with proportions of Yellowstone cutthroat trout ancestry >0.1 and <0.9 were classified as hybrids. Although these cut-offs are approximate, they follow those used in previous work in this system (Mandeville et al., 2019) and also follow theory-informed work on hybrid ancestry estimation (Lindtke et al., 2014).

2.5 | Water temperature and discharge

We collected data on water temperature and stream discharge throughout the spawning season to examine whether environmental factors influence fish migration timing in the drainage. We deployed HOBO Pendant MX Water Temperature Loggers to measure water temperature at 15-min intervals from early April through mid-October in 2019 and 2020. Temperature loggers were deployed near the confluence of the study streams and the North Fork Shoshone River in PVC housing. Daily mean temperature was calculated for each study tributary. We also calculated a metric of the proportion of stream temperature measurements in a day that exceeded 6°C (measurements every 15 min = 96 total stream temperature measurements in a day). This 6°C threshold has been cited as a threshold temperature for spawning migrations of lacustrine–adfluvial Yellowstone cutthroat trout in Yellowstone Lake (Ertel et al., 2017).

We collected discharge data from the North Fork Shoshone River from U.S. Geological Survey (USGS) gage 06279940 near Wapiti, Wyoming (USGS, 2022). We used discharge data to determine the centre timing of flow mass, which is the date at which half of the annual flow mass has been exceeded (i.e., the median) and is considered to be a valuable metric to understand the flow regime of a system, including timing of snowmelt runoff (Regonda et al., 2005; Wenger et al., 2010). It is well documented that cutthroat trout most often spawn on the descending arm of the hydrograph as discharge declines related to seasonal snowmelt runoff (Bennett et al., 2014; DeRito et al., 2010; Schmetterling, 2001; Thurow & King, 1994). R code used to calculate the centre timing of flow mass for the North Fork Shoshone River was taken from Heim (2019).

2.6 | Data analysis

We used multiple approaches to evaluate how Yellowstone cutthroat trout ancestry affects the timing of spawning. We first evaluated differences in adult entry date by comparing across our three genotype groups (Yellowstone cutthroat trout, rainbow trout and hybrids) and with a linear mixed model that included the proportion of Yellowstone cutthroat trout ancestry as a continuous variable. We tested for significant differences in entry date between male and female fish across our three genotype groups with Wilcoxon ranked-sum tests. We used a Kruskal–Wallis test to evaluate whether there were differences in tributary entry timing between our three genotype groups. Dunn's test of multiple comparisons with a Bonferroni adjustment was used to identify where significant differences existed between genotype groups (Dunn, 1964). We limited these analyses to Middle Creek fish in 2020 due to small adult fish sample sizes in 2019. We modelled entry date against ancestry in a Bayesian framework using a linear mixed model with Middle Creek data from 2019 and 2020. The proportion of Yellowstone cutthroat trout ancestry (q), fish length, fish sex and an interaction between q and fish sex were included as fixed effects in the model. Fish length was scaled and centred. Year was included as a random effect in the model. Prior probability distributions were designed to be minimally informative and were centred around frequentist coefficient estimates. Posterior probability distributions from the model were sampled using the No-U-Turn Hamiltonian Monte Carlo sampler implemented in STAN (Carpenter et al., 2017). The R package brms (Bürkner, 2017, 2018) was used to interface with STAN via R v4.0.3 (R Core Team, 2020). Model convergence was assessed by verifying that the trace plots showed well-mixed chains, and posterior predictive checks were used to determine that model predictions reasonably corresponded with the observed data.

Tributary entry timing may not be representative of actual timing of spawning, so we also evaluated the length of juvenile fish across the season of juvenile outmigration for both Middle Creek (2019 and 2020) and Trout Creek (2019) as the length of a juvenile fish provides insight into when parental individuals spawned. By comparing the length of juvenile fish at their date of sampling with their proportion of Yellowstone cutthroat trout ancestry, we can evaluate how Yellowstone cutthroat trout ancestry affects the timing of spawning. This analysis rests on several assumptions. We assume that across our observed range of Yellowstone cutthroat trout ancestry values for juvenile fish, (1) eggs have similar developmental timing (degree days from eggs being deposited until hatching); (2) juvenile fish hatch from eggs at a similar size; and (3) juvenile fish growth (in this case, fish length) during the early stages of their lives does not correlate with ancestry. If these assumptions are met, which we believe to be the case in our system, longer juvenile fish hatched from eggs at an earlier date and were therefore produced by parental individuals spawning at an earlier date compared with shorter juvenile fish.

We modelled juvenile fish length against the proportion of Yellowstone cutthroat trout ancestry through time in a Bayesian framework using a linear mixed model. The model included all juvenile fish data from Trout Creek in 2019 and Middle Creek in 2019 and 2020. The proportion of Yellowstone cutthroat trout ancestry (q), Julian day of fish capture and an interaction between q and Julian day were included as fixed effects in the model. Julian day was scaled and

FIGURE 1 Histogram showing the distribution of the proportion of Yellowstone cutthroat trout (YSC) ancestry (q) for adult fish captured entering Middle Creek in 2019 and 2020. Two vertical dashed lines in each panel denote q cut-offs used to assign fish as rainbow trout (red dash; $q < 0.1$) and Yellowstone cutthroat trout (blue dash; $q > 0.9$); fish with q values in between were assigned as Yellowstone cutthroat trout × rainbow trout hybrids
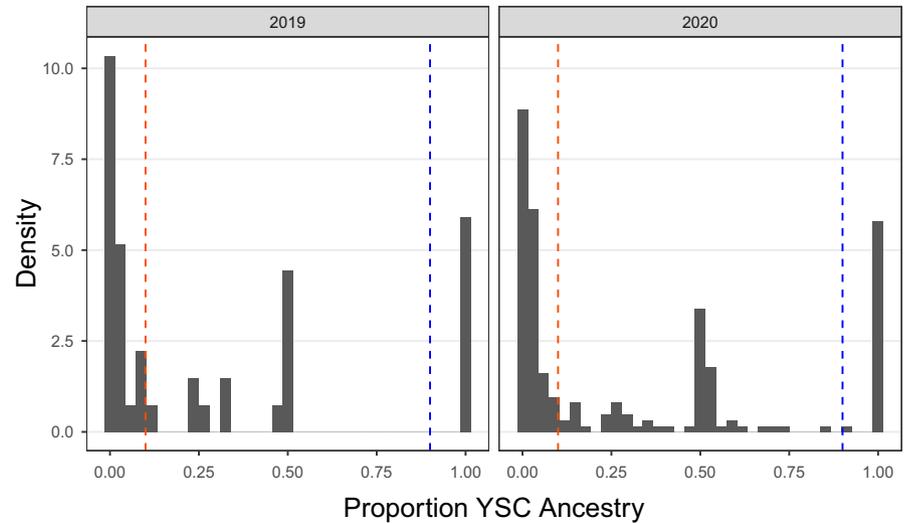
centred. Both site and year were included as random effects. Methods used to run the model are the same as outlined in the section above.

To evaluate how the proportion of Yellowstone cutthroat trout ancestry (q) of a fish entering a tributary was influenced by environmental covariates on the date the fish was captured entering the tributary, we used a generalised linear mixed model with a beta-distribution (i.e., beta regression) in a Bayesian framework. We included the proportion of stream temperature measurements over six degrees Celsius (Over6Degree) and the number of days from the centre timing of flow mass in the North Fork Shoshone River (CenterFlow) as fixed effects in the model, and the proportion of Yellowstone cutthroat trout ancestry was the response. The second covariate, number of days from the centre timing of flow mass, is an integer ranging from −44 to 45 describing the number of days before or after the recorded centre timing of flow mass on the North Fork Shoshone River. Values for both Over6Degree and CenterFlow are in relation to the date the adult fish entered Middle Creek. We also included fish sex and interactions between both fish sex and proportion of stream temperature measurements over six degrees Celsius and fish sex and number of days from centre timing of flow mass as fixed effects. An interaction term between the proportion of stream temperature measurements over six degrees Celsius and the number of days from centre timing of flow mass was also included in the model as a fixed effect. Number of days from centre timing of flow mass was scaled but not centred. Year was included in the model as a random effect. Methods used to run the model are the same as outlined for linear mixed-effects models in the previous section, with the only difference being our prior probabilities were uninformed in this model.

3 | RESULTS

3.1 | Adult fish sampling

We captured 47 adult fish in-migrating into Middle Creek in 2019 during hoop net sampling between 22 April 2019 and 9 July 2019 (3 female fish and 44 male fish). On Middle Creek in 2020, we captured 211 adult fish in hoop nets from 18 April 2020 to 4 July 2020 (87 female fish and 124 male fish). Total fish numbers were lower on Middle Creek during 2019 than on Middle Creek 2020 due to lower sampling effort in 2019.

Our genotyping included a total of 1226 fish from 2019 and 570 fish from 2020. This included 618 individuals from 2019 for the present study (46 adults, 572 juveniles) and 364 individuals from 2020 for the present study (211 adults and 153 juveniles), as well as additional individuals from both years that were used in a concurrent study (see Rosenthal, 2021). Furthermore, 133 individuals were removed from further analysis due to high amounts of missing data. After filtering out sites with >50% missing data and sites with a minor allele frequency of <0.03, the final SNP set for 2019 samples contained 71,221 SNPs, and the SNP set for the 2020 data contained 56,914 SNPs. Of the 46 adult fish with ancestry estimations from 2019, 25 were estimated to be rainbow trout, 13 were hybrids, and eight were classified as unadmixed Yellowstone cutthroat trout. For 2020 adult fish with ancestry estimations, 106 were assigned to rainbow trout, 68 were hybrids, and 37 were classified as unadmixed Yellowstone cutthroat trout (Figure 1). Entropy results were highly precise for both years with very low error in ancestry estimates. For 2019 samples, the average 95% credible interval width (an estimate of error for our entropy results) on q estimates was 0.00652. For 2020 samples, the average 95% credible interval width on q estimates was 0.00498.

3.2 | Tributary entry timing

During the 2020 spawning season, when our sample size was the largest, both male and female Yellowstone cutthroat trout entered Middle Creek later than all other sex–genotype combinations (male rainbow trout, female rainbow trout, male hybrids, female hybrids; Table 1; Figure 2). Tributary entry date for male vs. female fish was significantly different for rainbow trout ($W = 1667$, $p = .0004$) and hybrid ($W = 740.5$, $p = .0303$) genotype groups, but we observed no significant difference between male and female Yellowstone

cutthroat trout entry dates (*W* = 173.5, *p* = .1923; Figure S1). Differences in entry date between genotype groups were significant for both male (*p* = .0004) and female fish (*p* = .0002; Table 1). Female Yellowstone cutthroat trout entered spawning tributaries on average 16 days later (mean entry date = 19 June 2020) than female rainbow trout (mean entry date = 3 June 2020) and 12 days later than female hybrids (mean entry date = 7 June 2020). A similar pattern was observed in male fish: male Yellowstone cutthroat trout entered spawning tributaries 27 days later (mean entry date = 14 June 2020) than male rainbow trout (mean entry date = 18 May 2020) and 16 days later than male hybrids (mean entry date = 29 May 2020), though differences were only significant between male rainbow trout and male Yellowstone cutthroat trout and male rainbow trout and male hybrids (Table 1). Yellowstone cutthroat trout also entered spawning tributaries during a narrower window of time (smaller standard deviation in entry timing; Table 1) compared with rainbow trout and hybrids across both fish sexes. Although Yellowstone cutthroat trout entered spawning tributaries later than other genotypes, extended migration runs for rainbow trout and hybrids paired with higher counts of rainbow trout and hybrid genotypes (Table 1) led to substantial temporal overlap in tributary in-migration across the genotype groups (Figure 2).

Results from our entry date model show that the proportion of Yellowstone cutthroat trout ancestry (*q*) had a strong positive effect on the Julian entry date of fish into Middle Creek, meaning fish with higher proportions of Yellowstone cutthroat trout ancestry were predicted to enter the spawning tributary later (Table 2; Figure S2). Fish length was also a significant negative predictor of fish entry timing, meaning longer fish enter spawning tributaries earlier (Table 2; Figure S2). Fish sex overlapped zero at 95% credible intervals, so it was not considered significant in our model. Our model converged on the posterior probability distribution as shown by the Gelman–Rubin R statistic, which was 1.0 for each estimated parameter. The effective sample size for each parameter was over 1000.

Results from our juvenile length model provide evidence that Yellowstone cutthroat trout not only enter spawning tributaries later but also spawn later than rainbow trout and hybrids. The proportion of Yellowstone cutthroat trout ancestry (*q*) was a significant negative predictor of size for juvenile fish sampled in Middle Creek and Trout Creek (Table 3; Figures 3 and S3). Smaller individuals with higher proportions of Yellowstone cutthroat trout ancestry suggest later timing of spawning for adult fish with higher proportions of Yellowstone cutthroat trout ancestry. The interaction coefficient between the proportion of Yellowstone cutthroat trout ancestry and date had no significant effect on fish length, which provides evidence that juvenile fish are growing at similar rates through the fall regardless of ancestry (Table 3). We determined that our model converged using the same criteria outlined in the previous section.

3.3 | Environmental drivers of tributary entry date

We found evidence for two environmental covariates driving differences in spawning migration timing between Yellowstone cutthroat trout and rainbow trout and hybrids. Both the proportion of stream temperature measurements over six degrees Celsius and the number of days from centre timing of flow mass were related to the ancestry of fish migrating into Middle Creek. Both variables had significant positive effects on the proportion of Yellowstone cutthroat trout ancestry of a fish captured entering Middle Creek (Table 4). While rainbow trout and hybrids entered Middle Creek across a range of discharge values, Yellowstone cutthroat trout

TABLE 1 Summary of fish count, mean entry date, median entry date and results of multiple comparisons for difference in entry date for genotype groups (Yellowstone cutthroat trout—YSC; rainbow trout—RBT; and Yellowstone cutthroat trout×rainbow trout hybrid—RXC) into Middle Creek during 2020 for both male and female adult fish

Sex	Genotype	Count	Mean entry	Median entry	Std error	Dunn's post hoc comparison
Female fish	YSC	27	171 (06-19-2020)	173 (06-21-2020)	8.96	RBT: 0.0001* RXC: 0.0040*
	RBT	31	155 (06-03-2020)	158 (06-06-2020)	17.7	YSC: 0.0001* RXC: 0.5081
	RXC	29	159 (06-07-2020)	165 (06-13-2020)	16.8	YSC: 0.0040* RBT: 0.5081
Male fish	YSC	10	166 (06-14-2020)	168 (06-16-2020)	13.6	RBT: 0.0007* RXC: 0.0711
	RBT	75	139 (05-18-2020)	136 (05-15-2020)	22.7	YSC: 0.0007* RXC: 0.0243*
	RXC	39	150 (05-29-2020)	146 (05-25-2020)	20.0	YSC: 0.0711 RBT: 0.0243*

Note: Mean and median entry dates are shown as both Julian day and calendar date (in parentheses; month-day-year). Std error shows the standard error in Julian days of entry date timing for each genotype and fish sex combination. Statistically significant results from Dunn's post hoc test are designated by an asterisk.

FIGURE 2 Density plots showing the relative distribution of entry dates for each genotype group (Yellowstone cutthroat trout—YSC; rainbow trout—RBT; and Yellowstone cutthroat trout×rainbow trout hybrid—RXC) split by fish sex for adult fish captured entering Middle Creek in 2020. Dashed vertical lines represent mean entry date for each genotype group by sex
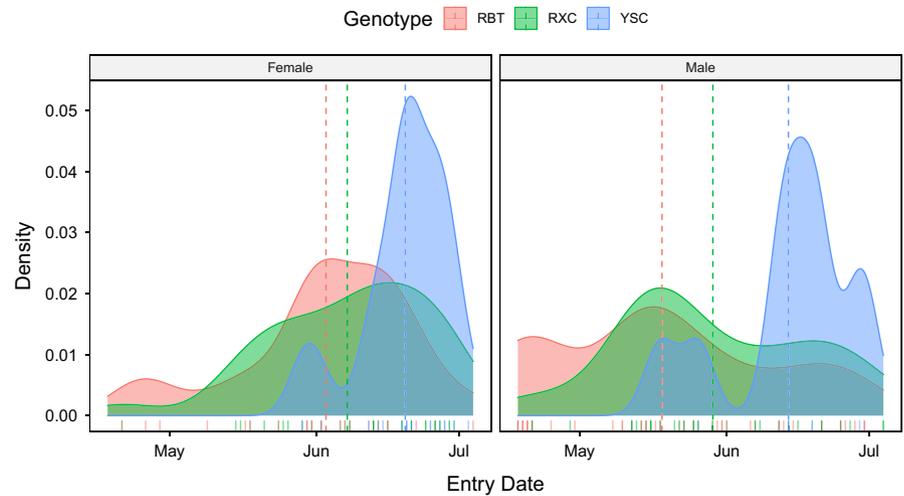

TABLE 2 Summary of model coefficients and 95% credible intervals for linear mixed-effects model of adult fish entry date into Middle Creek during the 2019 and 2020 field seasons

Variable	Effect level	Coefficient	Lower 95% CI	Upper 95% CI
Intercept	Population	143.89	137.80	149.46
q	Population	26.92	19.27	34.60
Fish length	Population	−4.15	−6.56	−1.65
Fish sex (male)	Population	−2.51	−5.28	0.17
q:Fish sex (male)	Population	−5.25	−15.00	4.53
Year	Group	2.46	0.09	6.63

Note: Parameters with 95% credible intervals that do not overlap zero were considered significant. The fish length covariate was centred and scaled.

TABLE 3 Summary of model coefficients and 95% credible intervals for linear mixed-effects model of juvenile fish length during the 2019 and 2020 field seasons

Variable	Effect level	Coefficient	Lower 95% CI	Upper 95% CI
Intercept	Population	43.76	39.29	47.88
Julian day	Population	4.55	3.87	5.24
q	Population	−8.45	−10.08	−6.80
Julian day:q	Population	0.24	−1.46	1.95
Site	Group	1.48	0.05	5.04
Year	Group	1.42	0.04	5.11

Note: Parameters with 95% credible intervals that do not overlap zero were considered significant. The Julian day covariate was centred and scaled.

focused tributary entry on dates after the recorded centre timing of flow mass (Figure 4). Fish entering Middle Creek were likely to have a higher proportion of Yellowstone cutthroat trout ancestry (*q*) on days with more temperature measurements above six degrees Celsius and on days after the recorded centre timing of flow

mass. No interaction coefficients were significant, suggesting that effect sizes do not vary by fish sex (Table 4). We determined that our model converged using the same criteria outlined in the section above.

4 | DISCUSSION

Understanding the mechanisms that limit hybridisation between closely related species, and the regional factors that affect these mechanisms, can help promote the conservation of vulnerable freshwater fishes by informing management actions that may enhance reproductive isolation between the species. Our research provides evidence that differences in spawning time act as a partial barrier to reproduction between Yellowstone cutthroat trout and rainbow trout during instances of secondary contact. We found Yellowstone cutthroat trout entered a spawning tributary to the North Fork Shoshone River, on average, two to four weeks later than rainbow trout and Yellowstone cutthroat trout×rainbow trout hybrids, which is similar to previous research demonstrating that Yellowstone cutthroat trout spawn on average three weeks later than rainbow trout in parts of the Snake River drainage (Henderson et al., 2000) and five to nine weeks later in parts of the Yellowstone River drainage (DeRito et al., 2010). Despite evidence for temporal segregation in the timing of spawning, there was considerable overlap in the range of spawning dates, highlighting that this reproductive barrier may be more porous than previously thought and creating challenges for management efforts to reduce hybridisation.

4.1 | Temporal segregation in timing of spawning

Previous research examining temporal segregation in the timing of spawning tracked adult trout throughout the spawning season and attempted to document spawning behaviour. In contrast, our analysis relied on tributary entry date as a proxy for spawning and used a novel approach integrating juvenile length and ancestry data. As
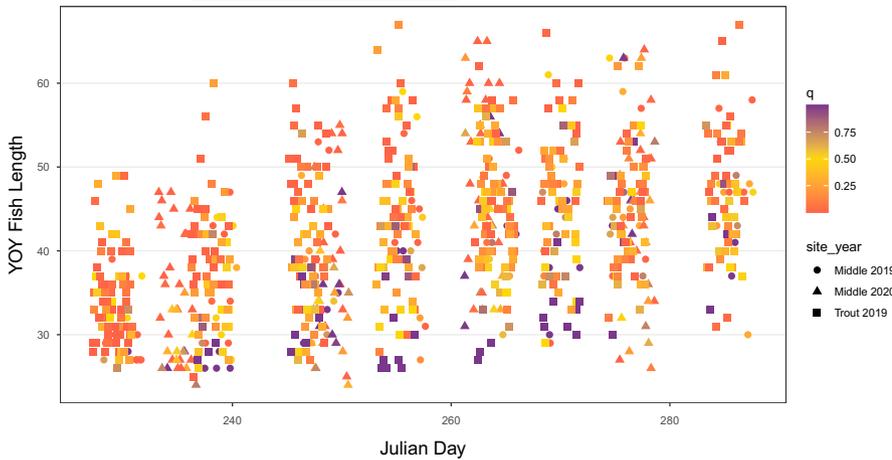
FIGURE 3 Scatter plot of juvenile fish length in millimetres through time (Julian day) with points coloured on a scale to represent the proportion of Yellowstone cutthroat trout ancestry (*q*). Point shapes denote what site and year the sample was collected

TABLE 4 Summary of model coefficients and 95% credible intervals for linear mixed-effects model of proportion of Yellowstone cutthroat trout ancestry (*q*) for adult fish entering Middle Creek during 2019 and 2020

Variable	Effect level	Coefficient	Lower 95% CI	Upper 95% CI
Intercept	Population	−0.50	−2.11	1.25
Over6Degree	Population	1.79	0.20	3.37
Fish sex (male)	Population	0.25	−0.45	0.93
CenterFlow	Population	0.76	0.17	1.33
Over6Degree:Fish sex (male)	Population	−1.54	−3.21	0.16
CenterFlow:Fish sex (male)	Population	−0.24	−0.70	0.22
CenterFlow:Over6Degree	Population	−0.20	−1.20	0.82
Year	Group	1.19	0.14	4.06

Note: Parameters with 95% credible intervals that do not overlap zero were considered significant. The centre timing of flow mass covariate (CenterFlow) was scaled but not centred.
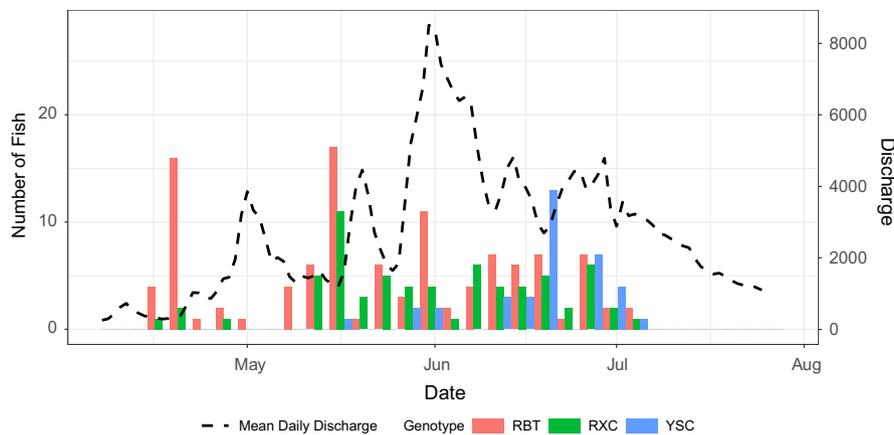
FIGURE 4 Histogram of number of adult fish caught each day entering Middle Creek during the 2020 field season. Colours of histogram bars represent fish genotype (Yellowstone cutthroat trout—YSC; rainbow trout—RBT; and Yellowstone cutthroat trout × rainbow trout hybrid—RXC). The dashed line denotes mean daily discharge (in cubic feet per second) for the North Fork Shoshone River measured at USGS gage 06279940 near Wapiti, Wyoming, and its values are represented by the secondary *y*-axis on the right side of the plot

direct observation of spawning behaviour was not feasible in our system, we collected data on the ancestry and length of juvenile progeny to provide further evidence for later spawning times for Yellowstone cutthroat trout in the drainage. Across all juvenile fish sampling at two tributaries to the North Fork Shoshone River (mid-August through early October), juvenile fish with higher proportions of Yellowstone cutthroat trout ancestry were, on average, smaller in size compared to fish with lower proportions of cutthroat trout ancestry (Figure 3). Using fish length as an approximation for age (number of days since hatching from egg), we infer that smaller fish hatched from eggs at a later date.

These conclusions rely on multiple assumptions about the relationship between juvenile fish length and adult timing of spawning across our range of observed Yellowstone cutthroat

trout ancestry. Previous research shows developmental timing for eggs is similar for Yellowstone cutthroat trout, rainbow trout and hybrid individuals (Ferguson et al., 1985), meaning that across ranges of ancestry the timing between when adult fish spawn and when juvenile fish emerge is similar. Egg developmental timing for salmonids is also related to water temperature (Ballard, 1973; Gresswell, 2011), and while water temperature varied during our juvenile sampling, increasing water temperatures from early July until mid-September would have led to potential earlier emergence for juvenile fish spawned later in the spawning season and would not confound our juvenile results. Previous research suggests growth rates between ancestry groups can differ and shows that juvenile Yellowstone cutthroat trout are at a competitive feeding disadvantage compared to rainbow trout and hybrids and exhibit slower growth rates in sympatry with rainbow trout (Seiler & Keeley, 2007, 2009). Additionally, research by Strait et al. (2021) showed that in a population of hybridised Yellowstone cutthroat trout and rainbow trout, increased rainbow trout admixture led to increased growth in warmer conditions, although this research did not include age-0 fish. We address the possibility that fish with higher proportions of rainbow trout ancestry grow faster by including an interaction term in our model between proportion of Yellowstone cutthroat trout ancestry (q) and Julian day, to understand whether the relationship between ancestry (q) and total fish length changes through time. Our interaction term between Yellowstone cutthroat trout ancestry and date had no significant effect, suggesting juvenile fish growth rates were not related to fish ancestry in our study system, although the absence of a significant result here does not entirely preclude the possibility juvenile fish growth is affected by ancestry in our system. Finally, previous work shows that larger female salmonids produce larger eggs (Kamler, 2005) and that juvenile fish length at time of hatching is positively related to egg size (Pepin et al., 1997). While the average size of rainbow trout females we sampled in Middle Creek was larger than that of Yellowstone cutthroat trout and hybrid females, the average size difference was only 17 mm in length and would likely not result in a measurable difference in egg size or juvenile fish size at emergence. Our results suggest that in systems where directly observing fish spawning is challenging, juvenile length in combination with genomics can be a powerful tool for estimating the timing of spawning of different genotypes.

We found that in addition to effects of genotype, migration timing was also sex- and size-dependent, with male fish and longer fish entering spawning tributaries earlier. Similar patterns have been documented in other populations of spawning Yellowstone cutthroat trout (Henderson et al., 2000; Thurow & King, 1994). Surprisingly, our adult fish sampling produced sex ratios skewed towards male fish (Middle Creek 2019 (14.67:1, $n = 47$) and 2020 (1.48:1, $n = 226$)), which is uncommon for lacustrine–adfluvial trout populations (Gresswell et al., 1997; Thurow et al., 1988). This may be related to our hoop net sampling method (Lamansky et al., 2009) and can be explained by male fish being more mobile during the spawning season in an attempt to find multiple spawning partners

(Keenleyside & Dupuis, 1988). In 2019, we attempted to use a rigid picket weir on Trout Creek to be able to capture all migrating trout, but high flows eventually overwhelmed the structure. However, prior to losing the weir, we observed a male: female sex ratio of 0.38:1 (164 male fish and 433 female fish), which is more consistent with previous research.

4.2 | Environmental drivers of spawning migration

We found evidence for two environmental factors driving differences in spawning time between Yellowstone cutthroat trout and rainbow trout. Both the proportion of stream water temperature measurements above six degrees Celsius and the number of days from the centre timing of flow mass had a positive relationship with the proportion of Yellowstone cutthroat trout ancestry of adult trout migrating into Middle Creek, with temperature having the stronger effect. In other words, an adult trout captured migrating into Middle Creek was more likely to have a higher proportion of Yellowstone cutthroat trout ancestry on days when the stream temperature stayed above six degrees Celsius for a longer duration, and on days after the centre timing of flow mass. These environmental covariates have been shown to drive spawning migrations for cutthroat trout in other systems (Bennett et al., 2014; DeRito et al., 2010; Schmetterling, 2001; Thurow & King, 1994). Spawning migrations of lacustrine–adfluvial Yellowstone cutthroat trout in nearby Yellowstone Lake increase sharply when water temperatures increase above six degrees Celsius (Ertel et al., 2017).

Cutthroat trout spawning after the centre timing of flow mass has been well documented (Bennett et al., 2014; DeRito et al., 2010; Muhlfeld, McMahon, et al., 2009; Schmetterling, 2001; Thurow & King, 1994). By spawning on the descending arm of the hydrograph (after peak discharge and the centre timing of flow mass), it is hypothesised that fish eggs are less likely to be scoured by high flows (Elwood & Waters, 1969) and emerging fry may have better survival (Fausch et al., 2001). This selective force is particularly prominent in the American intermountain west, the native range of cutthroat trout, where winter precipitation in the form of snow translates to high spring runoff when snow begins to melt in late spring/early summer. The difference in the timing of spawning between species likely reflects different flow regimes in the native ranges of cutthroat trout and rainbow trout; it has been demonstrated that the success of introduced rainbow trout is related to the seasonal hydrology patterns of the system where they are introduced (Fausch et al., 2001). In other systems, the relationship between timing of spawning migration and centre timing of flow mass is cited as the main factor leading to differences in the timing of spawning between cutthroat and rainbow trout (DeRito et al., 2010; Henderson et al., 2000).

While our model results indicate a significant positive relationship with centre timing of flow mass, the weak nature of the relationship is likely related to an extended spawning season by rainbow trout and hybrids in our system. While most male rainbow trout and hybrids entered Middle Creek before the centre timing of flow mass,

the spawning season of female rainbow trout and hybrids extended into the descending arm of the hydrograph (Figure 4). Rainbow trout show considerable variation in their timing of spawning with populations of coastal rainbow trout often spawning in early spring well before the descending arm of the hydrograph (Behnke, 1992), while populations of rainbow trout in the Kootenai River drainage in Montana begin spawning between 10 days and two weeks after the peak in discharge (Holecek & Walters, 2007; Muhlfeld, 2002). While the specific hatchery strain of rainbow trout stocked may be related to when fish spawn, introduced fish may also develop local adaptations through time and the timing of spawning in *Oncorhynchus* spp. has also been shown to be highly heritable (Abadía-Cardoso et al., 2013; Quinn et al., 2002). Quinn et al. (2000) showed that timing of spawning migrations and breeding by Chinook salmon (*Oncorhynchus tshawytscha*) were able to evolve under selective pressures over the course of a relatively short temporal window (approximately 30 generations). As stocking of rainbow trout in the North Fork Shoshone River first began in the early 1900s (Nordberg et al., 2021), it is possible that rainbow trout in the drainage have expanded the timing of their spawning related to regional selective pressures through time.

We examined alternate environmental covariates and combinations of covariates during exploratory analyses for fish migration analysis including daily hours of sunlight and alternate temperature formulations. Overall, we were only able to weakly explain adult fish migration timing with environmental factors (Table 4). This could be due to extended spawning migrations by fish over a range of environmental conditions, or the exclusion of important explanatory drivers of migration from our models. Many attempts to correlate Atlantic and Pacific salmon spawning migrations to environmental covariates have produced weak relationships where salmon movement patterns have no obvious relationship to environmental cues (Lilja & Romakkaniemi, 2003; Trépanier et al., 1996). Weak relationships between environmental covariates and temporal spawning segregation in the drainage may limit the ability of fish managers to optimise resource allocation related to selective passage efforts in the drainage.

4.3 | Additional mechanisms of reproductive isolation

In addition to temporal segregation in the timing of spawning, spatial isolation, in the form of spawning habitat segregation across multiple potential scales, could be an additional mechanism of reproductive isolation. Spatial isolation has been seen between rainbow trout and westslope cutthroat trout (*O. c. lewisi*) (Muhlfeld, McMahon, et al., 2009; Rasmussen et al., 2010). Previous research in the North Fork Shoshone River drainage found no isolated headwater groups of Yellowstone cutthroat trout (Kruse et al., 2000), and additional National Park Service sampling in the headwaters of Middle Creek in 2020 found no resident population of spawning Yellowstone cutthroat trout (B. Ertel, National Park Service,

personal communication). Additional forms of pre- and postmating reproductive isolation may also be occurring between the two species in the drainage. Recent research studying hybridisation outcomes between Yellowstone cutthroat trout and rainbow trout in the North Fork Shoshone River drainage hypothesises that both assortative mating and increased reproductive fitness for native female Yellowstone cutthroat trout may act as mechanisms of reproductive isolation in the drainage (Rosenthal, 2021).

4.4 | Broader implications

We provide evidence that similar mechanisms, in this case, temporal segregation, can maintain reproductive isolation across occurrences of secondary contact of the same species. While the same mechanism may exist, the degree of segregation may be different, suggesting the strength of reproductive isolation will vary across occurrences of secondary contact between the same two species. This adds to a body of literature showing the importance of considering drainage-specific ecology and the history of management actions when studying hybridisation between cutthroat trout and rainbow trout (Loxterman et al., 2014; Muhlfeld et al., 2017). In our system, we find a high degree of temporal overlap between when Yellowstone cutthroat trout and rainbow trout are spawning highlighting the substantial challenges of managing to minimise hybridisation.

Overall, our ability to predict fish migration timing into spawning tributaries using environmental covariates was low. This further complicates attempts to maximise the efficiency of selective passage of fish over isolation barriers. If rainbow trout and hybrids could be assumed to make all spawning migrations before the centre timing of flow mass or before water temperatures reached a determined threshold, less time and resources would be necessary to undertake isolation management. Furthermore, global climate change may increase stream temperature and lead to earlier centre timing of flow mass causing Yellowstone cutthroat trout to spawn earlier, thus increasing temporal spawning overlap with rainbow trout (Isaak et al., 2010; Stewart et al., 2005).

Studies that combine surveys of fish spawning ecology with genomic data provide an opportunity to study how mechanisms of reproductive isolation act in closely related species (Quinn et al., 2000; Turbek et al., 2018). In systems where economically and ecologically important species are threatened by hybridisation with introduced species, gaining insight on what regional factors promote or inhibit hybridisation can inform management decisions (High, 2010). Additionally, by studying how a previously documented mechanism of reproductive isolation varies across populations we can better understand how hybridising species interact across different occurrences of secondary contact (Mandeville et al., 2017). Our research highlights the importance of considering regional factors and historical management actions when informing management actions related to hybridisation disturbances.

CONFLICT OF INTEREST

The authors of this manuscript have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Dryad at https://datadryad.org/stash/share/GJV1PS5xfa67let DetMHicv4l61WpO3bMSu7Bvwu60o.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.