THERE AND BACK AGAIN: USING WHOLE GENOME SEQUENCING TO IDENTIFY THE GENES ASSOCIATED WITH MIGRATION PATTERNS IN RAINBOW TROUT

by

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ABSTRACT

Migration, which is defined as the seasonal movement for survival or reproductive advantage such as access to resources, is a behavioral phenomenon exhibited by many species including the salmonid *Oncorhynchus mykiss*. More commonly known as rainbow trout, *O. mykiss* exists in two life histories: migrants (steelhead trout), and residents (rainbow trout). While there are many factors that contribute to this variation in migration behavior, one of the reasons is their genetic makeup since there is an apparent correlation between the migratory behavior of parents and their offspring. The primary objective of this research project is to identify single nucleotide polymorphisms (SNPs), or genetic differences, which are associated with migratory behavior in rainbow trout. To that end, I used whole genome sequence data from five migrant and five resident rainbow trout. These data were aligned to the trout genome and used to locate genetic differences between the two migratory types. Quantitative PCR (DMAS-qPCR) approaches were used to validate the SNPs and genotype them in a larger set of twenty-five migratory steelhead. Research findings exhibited that Sashin Lake is producing smolts (young migratory steelhead) that are successfully returning to the lake and reproducing at the end of their life cycle. Additionally, while there was not a significant difference seen in terms of marine survival between the sexes, females were more likely to migrate compared to their male counterparts due to the reproductive advantage and greater access to resources that migration offers. This data will support future studies observing trout migratory behavior with larger sample sizes and from different generations and settings and will benefit conservation studies regarding population decline in migratory species.

Introduction

Many different animal species undertake seasonal movements between different areas for feeding and breeding (Airolam et al., 2019). This phenomenon, known as migration, is undertaken by these species because it gives them access to a wider range of habitats and allows them to take advantage of seasonal resources that are not available at breeding grounds (Kurowski et al., 2017). Gaining access to more resources is evolutionarily beneficial to migrant individuals within a partially migratory species because it translates to a larger body size and therefore a higher fecundity (reproductive output) compared to nonmigratory individuals (Nawaz et al., 2021). One such species is the *Oncorhynchus mykiss*, or rainbow trout (Hale et al., 2013; Barfuss, 2021).

Rainbow trout exist in two ecotypes: resident rainbow trout and migratory steelhead trout. Rainbow trout do not exhibit migratory behavior and live their entire lives in freshwater streams, while steelhead trout are anadromous in that they migrate to saltwater oceans for 2-4 years of their life and only return to freshwater streams to reproduce (Narum et al., 2018; Figure 1). Migratory steelhead undergo a hormonally driven process called smoltification which increases their salt tolerance, alters their metabolism, changes their physical appearance, impacts their schooling behavior, all of which increase their

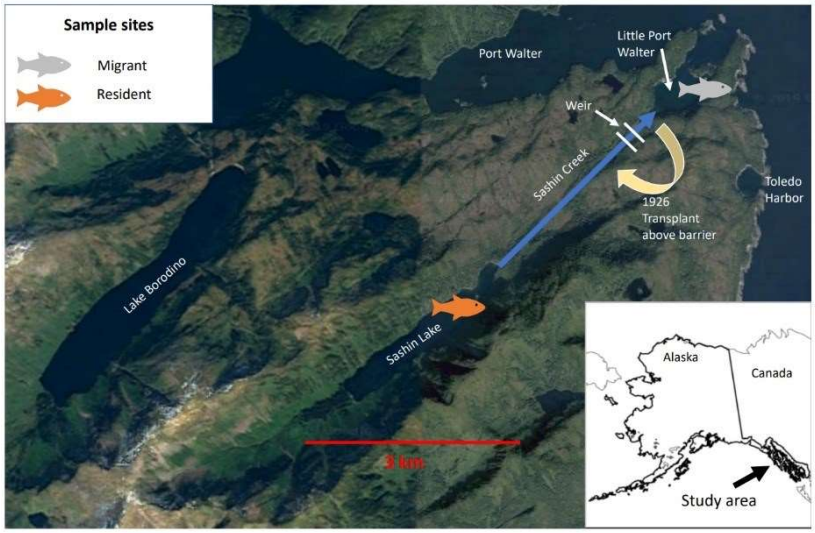

Figure 1: Map of water bodies in Southeast Alaska containing the sites of migratory steelhead and resident rainbow trout *(Clare, 2021).*

probability of surviving at sea (Björnsson et al., n.d.). Similarities between parents and offspring in migratory behavior suggest a strong heritable component to migratory behavior. In addition to genetics, the environment often plays a role in phenotypic expression of behaviors and traits and can impact migratory behavior through factors including but not limited to epigenetic modification (Baerwald et al., 2016). Nonetheless, migration is generally heritable through polygenic inheritance in that multiple alleles can impact this migratory behavior and the physiological changes associated with it (Weinstein et al., 2018).

Resident and migrant fish populations from Southeast Alaska have been studied extensively for the genetic components that contribute to the presence or absence of their migratory physiology and behavior (Pilkington, 2020). Because of the availability of data, and the clearly visible morphological differences between resident and migrant trout (Pilkington, 2020), the *Oncorhynchus mykiss* serves as a scientifically relevant candidate for continuing this research and learning more about the genetic basis behind migratory and resident patterns. The Sashin system, an established and frequently used site of sample collection, is located on Barnoff Island in Southeast Alaska and consists of a populated lake separated from the stream leading into the ocean by barrier waterfalls (Thrower et al., 2004). These waterfalls ensure that fish that undergo smoltification and leave the lake cannot enter back and must spawn at the stream and contribute to the anadromous stream population. This means that regardless of whether or not the smolts came from migrant or resident parents, they will end up contributing to the stream population which primarily consists of the declining migratory population. This one-way route is thought to place a strong selective pressure against migratory behavior. While most organisms retain the ecotype of their parents due to the largely heritable component to migratory behavior, a

conversion rate from lake to stream populations may help counter the decline in migratory rainbow trout populations. It is ideal to study this at the Sashin System due to easy data collection since the lake and stream are connected, giving migrants only one route to the ocean and no way back. This also sheds light on trout migratory behavior and the cost-benefit dynamic of migrating in that migration allows for access to marine resources, which positively impacts fecundity, but also comes with a greater risk.

The purpose of my research is to analyze the whole genomes of both the resident and migrant trout varieties and look for single nucleotide polymorphisms (mutations/differences) between them to help identify the genes associated with migratory physiology and behavior. Because migratory steelhead trout populations are experiencing a disproportionate decline in numbers largely due to human activities (Kendall et al., 2017), data on the genetic component of migratory behavior may help identify the origin of trout upon sequencing and can be used to track if lakes are producing migrants that survive long enough to spawn to mitigate the loss in migratory populations. This may contribute to conservationist efforts of declining migratory populations by giving the scientific community a better understanding of the genetics behind their behavioral patterns.

Methods

In this research project, DNA from five adult migrant and five adult resident rainbow trout had their whole genomes sequenced and aligned to the known rainbow trout genome. Genetic differences between the migrant and resident trout in the form of SNPs were then located, genotyped, and interpreted from these samples using specialized bioinformatical software.

Following this, eight SNP loci that had been previously researched by Barfuss (2021) in leaving smolt were studied in twenty-five returning migrant samples over a two-year period to determine the origin of the steelhead migrant trout and confirm the validity and continuity of data across multiple years.

Samples

Two sets of samples were used over the course of this research study. The first set of samples used for whole genome sequencing and subsequent analysis came from the non-lethal acquisition of fin clips from five migrant steelheads from Sashin Creek and five resident rainbow trout from Sashin Lake in 2020. The second set of samples consisted of twelve migrant trout that returned to spawn in 2019, and thirteen migrant trout that returned to spawn in 2020. Returning migratory trout at the end of their life cycle were collected from the weir, or fish-trapping dam (Figure 1), sampled, and weighed before being released to their intended destination at Sashin Creek so that they could spawn. Sexually mature resident trout samples were collected using hook-and-wire traps in Sashin Lake and were similarly sampled, measured, and fin-clipped non-lethally. The fin clips were stored in 70% ethanol at -20°C and served as the source of trout DNA for subsequent analysis.

Extraction

The DNA from fin clips was extracted using Qiagen DNeasy Blood and Tissue DNA extraction kit and provided protocol. The concentration of extracted DNA was measured using a Thermo Scientific NanoDrop 1000 machine and subsequently diluted to a standard concentration of 25 ng/µL.

Bioinformatics

The five resident and five migratory trout samples were sent to Novogene, a globally recognized genomic service corporation, to have their whole genome sequenced in a controlled and more accurate fashion than available with the equipment at the Texas Christian University lab. The genomes were aligned to a reference Rainbow Trout genome (Pearse et al., 2019) using the bwa-mem software (Li H., 2013). The SAMtools software (Danecek et al., 2021) was used to convert alignment data in the sam format into bam files. These files were then analyzed for single nucleotide polymorphisms using the ANGSD software (Korneliussen et al., 2014) with parameters that filter out low quality data as indicated by a p-value is greater than 0.00001, a minimum base quality score less than 20, or a mapped quality score (measuring sequence alignment to the genome) less than 20 to help find SNPs with a higher confidence in quality level.

Primer Design

Primers serve as a starting point for DNA polymerase elongating a new DNA strand based off of the template from an existing DNA strand, and both a forward and reverse primer are needed to amplify both strands. Primers were designed using Primer3Web and NCBI's Primer BLAST. Because of a relatively recent whole genome duplication within the salmonid lineage, approximately 25% of the *O. mykiss* genome exhibits tetrasomic inheritance and are therefore not ideal spots for primers to bind, and this was taken account into primer design (Campbell et al., 2019).

DMAS-qPCR

Following extraction, double-mismatch allele-specific quantitative Polymerase Chain Reaction (DMAS-qPCR) was used to amplify the DNA and record the concentration of DNA in real time. For this, two forward primers that correspond to the SNP alleles were used with a common reverse primer. The forward primers were designed to bind directly onto the SNP and were altered to be incompatible to the sequence, which helps distinguish between the two genotypes better. For instance, primer compatible to the migrant version of the SNP would bind better to DNA from a migrant sample and would take fewer rounds of amplification to reach the fluorescence threshold (Cq). For each SNP locus assay, 10µL was made using 5ng/µL of DNA and 0.5ng/µL of forward and reverse primer each, and SYBR Green MasterMix, which contains the DNA polymerase Taq, deoxynucleotides, and $MgCl_2$ (an essential cofactor of the Taq polymerase). It also contains a fluorescent label that fluoresces bright at a higher concentration of amplified DNA, making it a good proxy for DNA concentration. Each sample was assayed with both the migrant primer and the resident primer in all three trials conducted. Upon DNA amplification through DMAS-qPCR, the primer needing a fewer number of cycles needed to pass the fluorescence threshold (Cq) indicated a better fit of the appropriate forward primer, which helped characterize the sample as a migrant or resident. The difference in the number of cycles it takes to pass this threshold can point towards whether a trout is homozygous for a certain allele (migrant vs resident version), or if the trout is heterozygous (when both primers bind equally well). Amplification through DMAS-qPCR in the StepOnePlus Real-Time PCR System at a 95°C denaturing temperature and 72°C elongation temperature. The ideal primer annealing temperature varied per locus and was individually set in the DMAS-qPCR system as

listed below (Table 1). The DMAS-qPCR machine also produced a melt curve at the end of amplification to assess for sample purity.

SNP Loci	Chr 1	Chr 20	TSC 22	PAAL	GAL-R1	GCOAD	Puromycin	Methyl-26
Annealing Temperature	59.5°C	62°C	58°C	59°C	60°C	58.5°C	59°C	60°C

Table 1. Annealing temperatures used in the DMAS-qPCR system for each of the 8 SNP Loci.

Sex Typing

The 25 trout were sex-typed using the procedure described by Brunelli et al., (2008), where 2.5 ng/µL of DNA was amplified using PCR, purified using Exo-Sap, and sequenced. Males were identified by the presence of Y-chromosome polymorphisms, and the rest were identified as female due to lacking the Y-chromosome and any associated genes.

Analysis

Following DMAS-qPCR, the number of SNPs that aligned with the migratory SNP was assessed. On a graph, the data for each individual SNP exhibited consistent results as well as clustering that correlated to an SNP that was homozygous for stream migrants, homozygous for lake residents, or admixed (with alleles from both the lake and the stream) (Figure 1; Figure 2). All loci were also analyzed by sex (Figure 3) and for Hardy-Weinberg equilibrium, which they were found to be in. Being in Hardy-Weinberg equilibrium signifies that these loci were reliable markers for population origin as they are not actively undergoing selection due to environmental pressures so parental origin and migratory behavior may be attributed to genetics (Figure 4).

Results

DNA from 5 adult steelhead and 5 rainbow trout were extracted and sent for whole genome sequencing at Novogene and used to confirm the rainbow trout SNPs previously identified by Barfuss (2021). Because the migrant allele could not be determined from the genome sequences, 25 migrants that returned to spawn in 2019 and 2020 (including the 5 steelhead originally tested) were extracted were genotyped through DMAS-qPCR with two versions of the forward primer at the same 8 SNP loci (Barfuss, 2021). Results showed that 17 out of the 25, or 68%, of the returning steelhead exhibited a majority of stream SNPs, which supports the correlation between SNP and migratory behavior found by Barfuss in 2021 (Figure 2). SNP data for the trout migration patterns was also analyzed by sex, showing that 76% of the steelhead returning from their migration were female (Figure 3).

The loci were also tested for whether or not they were in Hardy-Weinberg Equilibrium and were found to be in it, which means that selection is likely not operating directly on the alleles between the lake and stream-produced steelhead. This is important because if they were not in Hardy-Weinberg Equilibrium, any differences in migratory behavior could not be attributed to genetics due to there being an environmental/selective component, but no SNPs had to be eliminated as good markers for studying migratory behavioral patterns between parent and offspring since all eight loci were found to be in Hardy-Weinberg Equilibrium.

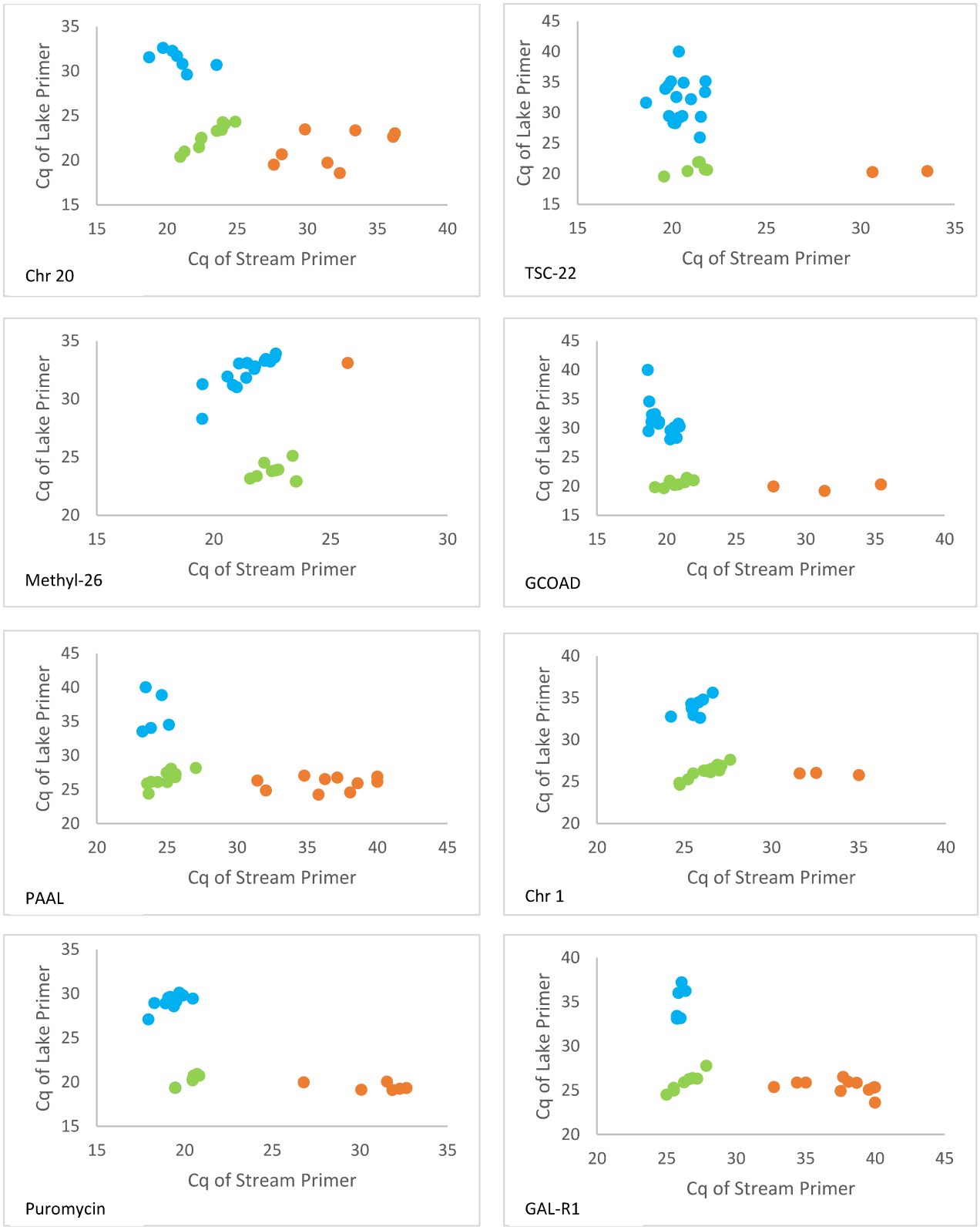

Figure 1: Discrimination plots from DMAS-qPCR at 8 SNP's of returning steelhead trout. Clustering, donated by the 3 colors, is indicative of the 3 genotypes. The legend applies to all 8 graphs.

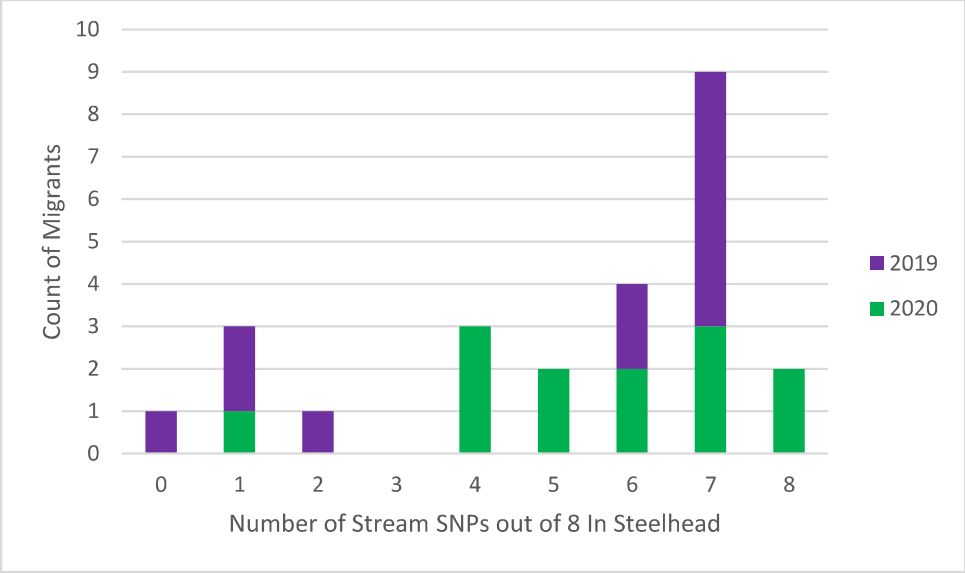

Figure 2: Count of migratory steelhead exhibiting SNPs corresponding to migratory behavior based on DMAS-qPCR genotyping of SNPs. 17 of the 25 trout contain a majority of stream SNP's, at 15 of the 25 trout contain at least 6 of the 8 SNP's associated with the migrant steelhead in streams.

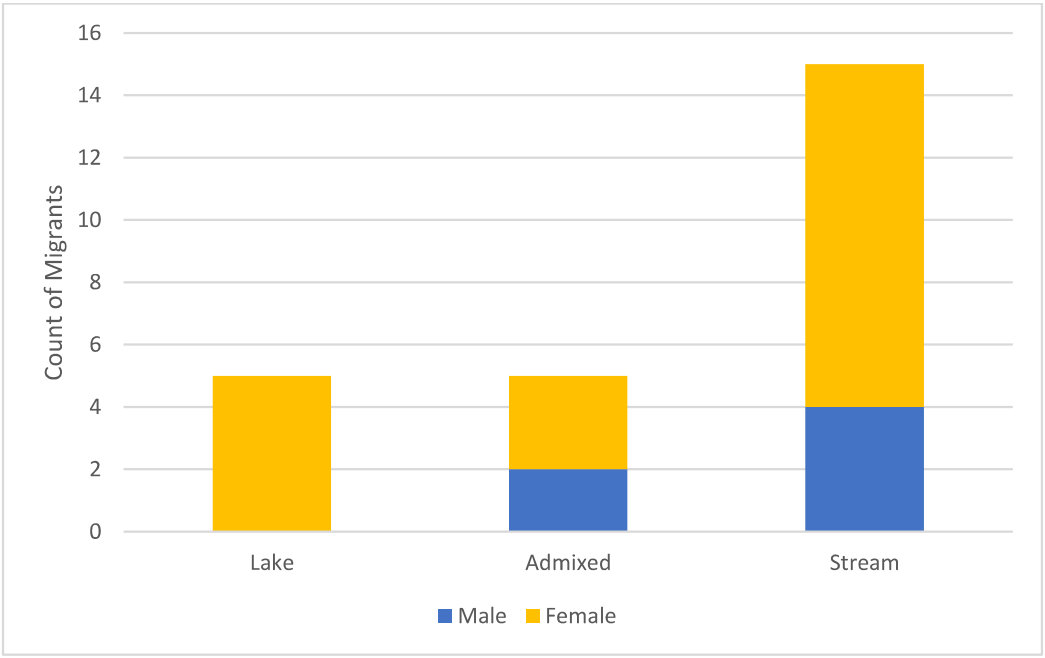

Figure 3: Count of migratory steelhead with a majority of SNPs associated with migrant trout, separated by sex. 19 of the 25 steelhead, or 76% of them, were females.

Locus	AA	Aa	aa	Observed Heterozygosity	Test for Significance
Chr 20	7	10	8	0.400	not significant; p = 0.3204
Methyl-26	16	9	0	0.360	not significant; p = 0.2724
PAAL	11	9	5	0.360	not significant; p = 0.2380
Puromycin	10	9	6	0.360	not significant; p = 0.1912
TSC-22	16	7	2	0.280	not significant; p = 0.3572
GCOAD	14	8	3	0.320	not significant; p = 0.3022
Chr 1	3	13	9	0.520	not significant; p = 0.6046
GAL-R1	11	8	6	0.320	not significant; p = 0.0956

Table 2: Statistical analysis for Hardy-Weinberg Equilibrium at the 8 loci tested. All SNPs were found to be in equilibrium, meaning that selection is not acting upon them.

Discussion

The purpose of my research was to identify single nucleotide polymorphisms that can distinguish migratory and rainbow trout and can help explain the difference in their migratory behavior. This research is of interest to the broader scientific community since it can support conservationist efforts to understand how genetics impacts the migratory phenotype as many migratory populations are declining. Since residents living in lakes can and do produce migrants, this loss in migrant populations may be countered and can result in increases of migratory populations. Prior research conducted by Barfuss (2021) suggests that up to 44.4% of smolts migrating from

Sashin Creek to the ocean had origins in Sashin Lake, strongly indicating that the lake is a source of migratory smolts. My research took this investigation a step further by inquiring if these migrant fish successfully returned to complete their life cycle and spawn; my data suggests that this is the case.

Upon graphing the discrimination plots from the data acquired through DMAS-qPCR at the 8 SNP loci, a clear degree of separation was found between the genotypes (as depicted by the clustering of data points.) The method used by Barfuss (2021) found similar patterns, suggesting that the method worked just as well for returning steelhead as it did outmigrating smolts. 15 of the 25 steelhead assayed had at least six migratory versions of the SNP (out of eight total SNPs tested), meaning that 60% of the returning steelhead are from anadromous parents from the creek. The remaining 40% were found to be an even split between originating from the lake and of admixed origin (with alleles from both the lake and the stream).

The latter result is interesting as it confirms the two migratory ecotypes can and do interbreed. This may be because residents from the lake can move downstream to the creek but do not undergo smoltification and migrate to the ocean. Although the number of residents living in the creek is thought to be low (due to a lack of consistent food) a small number of sexually mature residents could be enough to explain the occurrence of admixed samples. Alternatively, the admixed individuals could stem from returning steelhead that originated from the two different populations, i.e., one parent had origins to Sashin Creek whereas the other from Sashin Lake. Although we cannot determine with 100% accuracy the origin of the admixed individuals, their presence suggests strongly that the creek contains fish from both populations. Nonetheless, these

SNPs clearly can be used to determine the trout's origin and migratory status, which can support conservation efforts. As all eight SNPs were found to be in Hardy-Weinberg Equilibrium, these loci are not undergoing any selection and is not disqualified as a good indicator for the population since any differences in data will not be confounded with impacts from natural forces of selection.

The data also pointed towards a sex-specific bias in trout migration, both with respect to leaving the creek (i.e. after smoltification) and with returning to spawn. A total of 19 of the 25 migrants (76%) were genotyped as female. Barfuss (2021) found a similar ratio at 78% female smolts out of a sample size of 50. This similar ratio between leaving and returning organisms suggests that males that have completed smoltification are just as adapted to surviving their ocean migrations as females, but rather that fewer males migrate out to begin with. Of those that migrate out, males and females are comparable in terms of marine survival. Evolutionarily, this makes sense because female gametes are more costly and therefore females have more to gain by migrating, since they have access to a larger repository with resources that will allow the females to grow larger, which translates to higher fecundity (Nawaz et al., 2021). Moreover, other populations of rainbow trout also note a higher proportion of females than males both with respect to outmigrating smolts and returning steelhead (Sloat & Reeves, 2013).

A majority of the Sashin steelhead came from Sashin Creek, which fits the known high heritability of migration in this population. However, the data altogether suggests that the lake is producing smolts which not only undergo the smoltification process but are surviving at sea and, as steelheads, are successfully returning to spawn. This is important for conservation of *O.*

mykiss since declining migratory populations can be replenished in part by lake populations producing smolts that successfully migrate, reproduce, and pass on their migratory behavior.

Essentially, migratory populations are declining at a much faster rate than seen in residential populations, and this is true of not only salmonids such as *O. mykiss*, but also many other species. This research shows that alleles seem to segregate based on origin and that there exists a relatively straightforward way to genotype them, which means that this research's methodology and findings can be applied to future studies regarding conservation of declining migratory populations of salmonids and, perhaps upon further research, of other migratory species, because as this research shows, residents can be used to produce migratory offspring that add back to the migratory population and slow down their population decline.

Future studies could further enhance our understanding of rainbow trout migratory behavior by testing a greater sample size over more geographic locations and for a wider timeframe for both leaving smolts and returning steelhead. Because environmental factors may vary year-to-year and by location, exploring the role of the environment and genetics in species' behaviors and survival plays a major role in determining life history. Furthermore, prior studies suggest highly population-specific control of migration, meaning that the SNP loci used in this study may not be segregating similarly between anadromous and resident populations of rainbow trout in different locations (Clare 2021). Future studies exploring these trends could further support the role of genetics as well as the environment in migratory behavior and can contribute to the development of informed efforts to conserve *O. mykiss* populations.

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