DETERMINING IF GENETIC MARKERS ASSOCIATED WITH LIFE HISTORY DEVELOPMENT OF RAINBOW TROUT ARE SHARED ACROSS FRESHWATER SYSTEMS

By

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ABSTRACT

Oncorhynchus mykiss, commonly known as rainbow trout, exhibit partial migratory behavior where some individuals in a population will opt to migrate, whereas others remain resident. Consequently, there are two ecotypes of *O. mykiss*: the non-migratory rainbow trout (also known as residents) and the migratory steelhead (also known as migrants). Previous evidence generated from our lab demonstrated that various loci in the rainbow trout genome segregate between resident rainbow trout and migrant steelhead trout in the Sashin creek system of Alaska. A unique feature of the Sashin system is that a series of waterfalls separate the lake and stream, thereby inhibiting gene flow between the migratory stream individuals and resident lake individuals. However, it is still unknown whether these same genetic markers also segregate between behaviors in other freshwater systems. Therefore, the goal of my research project is to use DMAS-qPCR to genotype known migrant and resident individuals from Little Sheep Creek, Oregon. This population is geographically separated from the Sashin Creek watershed and differs from Sashin in that both life histories can and do interbreed. This research found the single genetic marker GCOAD also significantly segregated according to life history; however, the alleles associated with resident versus migrant in the Sashin system have an opposite association in Little Sheep Creek. This finding underscores the complexity of the genetic factors influencing the expressed phenotype, supporting the idea that each population's genetic markers are mostly locally shared.

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Introduction

Migration is broadly understood as the seasonal movement of animals between habitats that alternately offer favorable or less favorable conditions. This adaptive behavior enables species to optimize their survival and reproductive success by exploiting spatially and temporally variable resources (Dingle and Drake 2007). The evolutionary advantages of migration are particularly evident in environments where resources fluctuate. Migratory species often experience enhanced access to food and optimal breeding sites, which can lead to increased body size and reproductive output, thereby boosting individual fitness. However, migration is not without its risks. The energy demands of long-distance travel, along with increased exposure to predators and environmental stressors during migration, can be substantial. The propensity for migration in animals is influenced by both genetic and environmental factors (Dingle 1991). While some traits associated with migration are heritable, environmental factors such as temperature and food availability play crucial roles in triggering migratory behavior (Lucas and Baras 2001).

Salmonid fish exhibit a form of migration in which a fish migrates to the ocean and then returns to its natal freshwater location for spawning. Fish that exhibit this form of migration are known as anadromous fish. However, not all fish within a population are anadromous with some individuals remaining resident (i.e., the species exhibits partial migration) (Quinn and Myers 2004). In *O. mykiss*, the phenotypes for residents versus migrants are visibly different, despite being the same species, serving as an excellent model species to study migration. The migratory fish undergo a process called smoltification in which they become adapted to seawater (Hendry et al. 2004). Some of these changes include alterations to their body shape to become more streamlined for ocean swimming, a shift in osmoregulatory function to tolerate saltwater, and

hormonal adjustments that affect metabolism and behavior (Björnsson et al. 2011). These smolts migrate to and mature in the ocean, developing into the large steelhead trout (Hendry et al. 2004). The parr who do not undergo smoltification mature into the resident rainbow trout, characterized by their smaller size, colorful stripe, and abundance of dots in comparison to their steelhead counterparts. While resident populations tend to mate with and produce predominantly resident rainbows and anadromous populations tend to mate with and produce predominantly anadromous, migrant steelhead, it is both possible for offspring to have the alternate morphology from their parents or for these two morphologies to interbreed (Thrower and Joyce 2004; Ruzycki et al. 2009). Some evidence suggests that the determinant of each individual's life history is polygenic, and the contribution of each gene involved is relatively small (Clare et al., Dingle 1991). Nevertheless, environmental to the genetic predisposition, such as local famines, has been found to trigger a higher occurrence of migratory offspring in freshwater resident fish (Lucas and Baras 2001).

Migrant smolts risk a much higher rate of predation to reach ocean waters. Mortality rates often exceed 90% (Thrower et al. 2004). However, the benefits for migration to seawater can be substantial, with steelhead growing to an average of 30 to 40 pounds, while even over 50 pounds is not too uncommon ("Rainbow Trout and Steelhead | National Wildlife Federation"). These large sizes enable a much greater fecundity (Kendall et al. 2014). Resident rainbow trout benefit from a much greater chance of survival at the cost of lower nutrition. They have more opportunities to mate, but their sizes average only 5 to 9 pounds (Fleming and Reynolds 2004).

Anthropogenic effects have drastically altered selection pressures of each life history. Habitat loss, river fragmentation (due to human constructed damns), and climate change have all been shown to adversely affect migratory salmonids (McClure et al. 2008; Keefer et al. 2008;

Wade et al. 2013). While nearly all salmonid populations have been negatively affected by these changes, migratory populations are affected more severely than resident populations (Hardesty-Moore et al. 2018; Thompson et al. 2019). Climate change, in particular, may exert selective pressure favoring thermal resilience. Steelhead trout, adapted to colder marine environments, face increased migration blockages and mortality when river temperatures rise to 21 – 24 °C (McMillan). Consequently, O. mykiss are designated as threatened in ten systems along the West Coast and endangered in one (National Oceanic and Atmospheric Administration, 2016). Whereas, resident rainbow trout are not experiencing the same level of decline (Blair et al. 2013). Because of this discrepancy, addressing the disproportionate decline of migratory populations is an ongoing area of conservation.

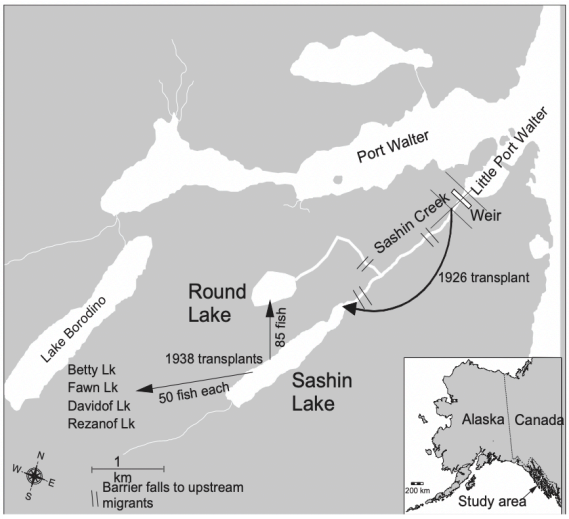

Figure 1Map of Sashin Creek System, Alaska

The Sashin Creek system, located on Barnoff Island in Southeast Alaska, is an established research site due its distinctive separation of Sashin Lake and Sashin Creek by two natural barrier waterfalls and a staffed weir that allows researchers to sample and tag fish migrating to the ocean and returning for spawning (Thrower et al. 2004, Figure 1). In 1926, a small number of steelhead trout from Sashin Creek were transplanted to the fish barren Sashin Lake. The waterfalls provide a one-sided genetic flow barrier, as the resulting resident rainbow population thrived and migratory steelhead smolts leave downstream and do not return to the lake (Thrower et al. 2004). The advantages for studying the genetic basis of migration are two-fold: First, the waterfalls prevent gene flow between migrants and residents in the lake and secondly, the short time scale since the transplant means any alleles

associated with residency should have been rapidly selected for (Thrower et al. 2004). Recent data from the Hale lab has discovered eight single nucleotide polymorphisms (SNPs), serving as genetic markers of life history. Alleles at these loci are associated with either resident or migrant life history. Furthermore, heterozygosity of these alleles in Sashin creek fish indicate some extent of successful migrants with Sashin Lake descent. Thus, Sashin lake looks likely to be used to supplement decreasing migrant populations returning to Sashin Creek. recent Hale lab research indicates a decreased fitness in migrant offspring of these residents (Brown 2022).

It is unknown if the eight genetic markers are universally shared or population specific. Thus, I turned my attention to Little Sheep Creek, Oregon. This freshwater creek is 1,770 km away (calculated using onmicalculator.net) from Sashin and is more representative of the majority of O. mykiss populations as a) it is located far inland, and b) both life histories co-exist (Berntson 2011). There are also multiple connecting tributaries to the major river pathways to the ocean, increasing the likelihood of gene flow between multiple separate O. mykiss populations (Berntson 2011). It also has a staffed weir, again ensuring reliable data collection (WALLOWA HATCHERY PROGRAM MANAGEMENT PLAN 2023). The main objective of this thesis is to determine if the same genetic markers identified in the Sashin system can be used to determine life history in Little Sheep Creek. If the eight loci do segregate, they may be linked or involved in the genetic mechanism for regulating partial migration.

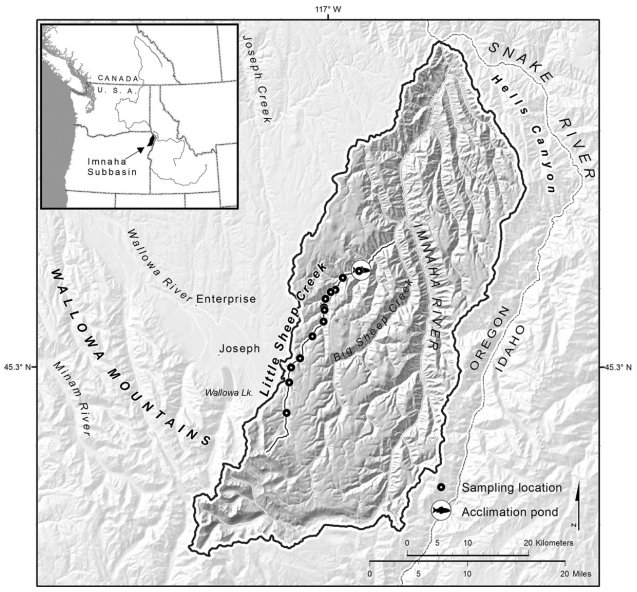

Figure 2 Map of Little Sheep Creek, Oregon

Methods

Fin clip samples were collected from mature rainbow and steelhead trout. DNA was extracted from fin clips using Qiagen's DNeasy kit according to the manufacturer's protocol. Nanodrop was used to quantify the DNA and samples were then diluted to a standard concentration of 50.0 ng/µL for assay use.

Using the previously identified SNPs that distinguish between phenotype in Sashin, we employed the same double-mismatch allele-specific qPCR (DMAS-qPCR) technique as described in Barfuss 2021 and outlined in Lefever et al. (2019). The DMAS-qPCR procedure utilizes a universal reverse primer paired with two allele-specific forward primers—one for the migrant allele and one for the resident allele. These primers are designed such that the 3' end targets the SNP site, and a deliberate mismatch is introduced three bases before the 3' end, enhancing the specificity of allele recognition (Barfuss 2021). Therefore, the primer corresponding to the accurate SNP allele anneals more efficiently to the DNA, facilitating more effective amplification. Fluorescence detection was achieved using SYBR Green reagent, where the reagent fluoresces when binding to DNA in the double-helix structure. As each sample underwent 40 replication cycles with both primers simultaneously, qPCR analysis uses comparison in cycle quantification (Cq), or average number of cycles to reach a locus-specific fluorescent threshold, to determine the fish sample's genotype at that locus. Faster annealing primers to the DNA correlated with faster amplification and quicker attainment of the fluorescence threshold in fewer qPCR cycles. Differences in average Cq values between the two reactions indicated genotypes, with homozygous individuals displaying significantly lower average Cq values for their respective SNP allele, whereas heterozygous individuals showed comparable Cq values for both primers. Cq thresholds for each locus were pre-calibrated using

data from assays on samples with established genotypes from the Sashin water system (Barfuss 2021).

The qPCR assays were conducted using an Applied Biosystems StepOnePlus Real-Time PCR System with SYBR Select reagents, maintaining a DNA concentration of 5.0 ng/μL and a primer concentration of 0.5 ng/μL in a total reaction volume of 10μL. Assays were performed in triplicate, with outlier data points, defined by a standard deviation exceeding 0.5, excluded from analysis unless no clear outlier was identified, in which case the sample was retested.

The average Cq's of the Migrant Allele forward primer were then plotted against the Cq's of the Resident Allele forward primer (Figure 3). It was expected that the graph would produce three distinct clusters, one for each genotype: homozygotes for the resident allele, homozygotes for the migrant allele, and heterozygotes. Plots were constructed using Microsoft Excel. Each sample's genotype at each locus was determined (Table 1). The frequency of each allele was counted and used to perform a Chi-Square Test of Independence and Fisher's Exact Test (Table 2). Genotypes (i.e. Sashin homozygous resident, heterozygous, homozygous migrant) were counted and used to perform a Hardy-Weinberg Test of Equilibrium in order to test for selection pressures (Table 3). All calculations use a p value of ≤ 0.05 to determine significance. All calculations were performed in R.

Results

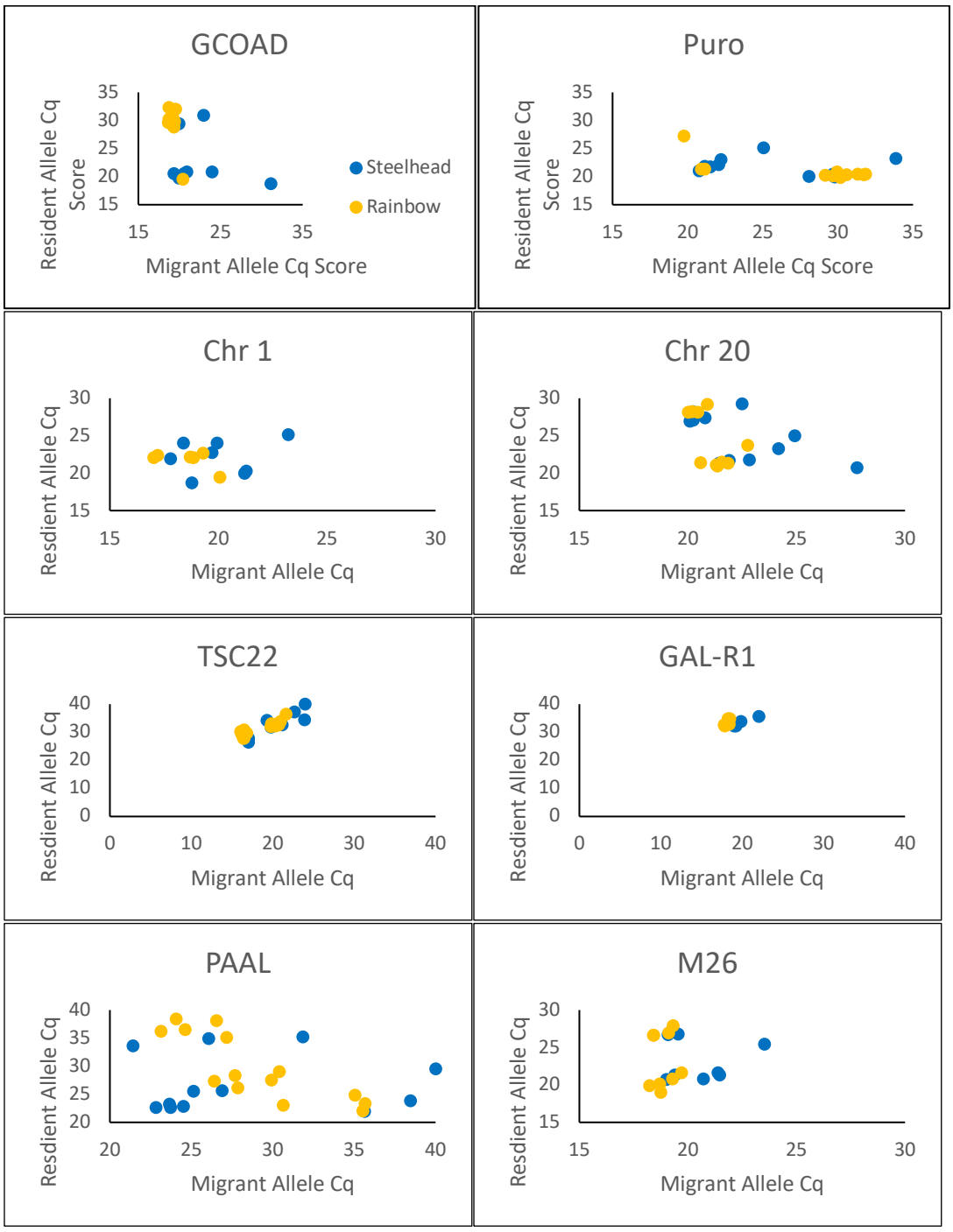

Figure 3: Discrimination plots from qPCR at 8 SNP's of Little Sheep Creek trout. Clustering indicates a shared genotype of alleles determined by Barfuss 2021. The legend applies to all 8 graphs.

Known Migrants	Chr1	Chr20	TSC-22	PAAL	GAL-R1	GCOAD	Puro	M26
90		TC	AA	GT	CC	TT	AG	
132	TT	TT	AA	TT	CC	TG	AG	TC
154	CC	CC	AA	GG	CC	TT	AG	TT
157	TC	TC	AA	GG	CC	GG	GG	TC
166	TT	TC	AA	GT	CC	TT	AG	TC
175	TT	TT	AA	TT	CC	TG	AG	TC
74		TT		GT			GG	
56		TT		GG			GG	
82		TC		GT			GG	
161	TT	CC	AA	TT		GG	AG	TC
189	TC	TC	AA	GT		TG	GG	TT
190	TT	TT	AA	GT		TG	GG	TC

Known Residents	Chr1	Chr20	TSC-22	PAAL	GAL-R1	GCOAD	Puro	M26
19	TT	TT	AA	GG	CC	TT	AA	TC
35	TT	TT	AA	GG	CC	TT	GG	TT
116	TT	TC	AA	TT	CC	TT	GG	TT
88	TC	TT	AA	GG	CC	TT	AG	TC
117		TT	AA	TT	CC	TT		
129		TT	AA	GT	CC			
13	TT	TT	AA	GG		TT	GG	TC
34		TC	AA	GT			GG	
42	TT	TC	AA	GT		TT	AG	TC
49		TC	AA	TT		TT	GG	
57		TC	AA	TT		TT	GG	TT
58		CC	AA	GT		TT	GG	TT
119		CC	AA	GG		TG	GG	TC
167		TC	AA	GT			GG	

Table 1: Genotyping of trout from Little Sheep Creek based on DMAS-qPCR findings. Blue indicates alleles associated with Sashin migrants. Orange indicates alleles associated with Sashin residents. Green indicates equal association of life history.

Locus	Steelhead Resident Allele	Steelhead Migrant Allele	Rainbow Resident Allele	Rainbow Migrant Allele	Chi-squared p-value	Fisher's Exact Test p-value
Chr 1	4.0	12.0	1.0	11.0	0.5215	0.3553
Chro 20	9.0	15.0	10.0	18.0	1.0	1.0
TSC-22	0.0	18.0	0.0	28.0	n/a	1.0
PAAL	12.0	12.0	13.0	15.0	1.0	1.0
GAL-R1	0.0	12.0	0.0	12.0	n/a	1.0

GCOAD	8.0	10.0	1.0	21.0	0.0086	0.0055
Puro	18.0	6.0	20.0	4.0	0.7223	0.7238
M26	6.0	10.0	5.0	13.0	0.8122	0.7166

Table 2: Frequency table of alleles. Statistical analysis of allele segregation according to life history was performed using chi squared test and Fisher's exact test. Significant p value: ≤0.05

	Steelhead				**Rainbow**			
Locus	**Homozygous Resident**	**Heterozygous**	**Homozygous Migrant**	**Hardy-Weinberg p value**	**Homozygous Resident**	**Heterozygous**	**Homozygous Migrant**	**Hardy-Weinberg p value**
Chr 1	1.0	2.0	5.0	0.346	0.0	1.0	5.0	0.824
Chr 20	2.0	5.0	5.0	0.700	2.0	6.0	6.0	0.803
TSC-22	0.0	0.0	9.0	NaN	0.0	0.0	14.0	NaN
PAAL	3.0	6.0	3.0	1.000	4.0	5.0	5.0	0.291
GAL-R1	0.0	0.0	6.0	NaN	0.0	0.0	6.0	NaN
GCOAD	2.0	4.0	3.0	0.764	0.0	1.0	10.0	0.875
Puro	6.0	6.0	0.0	0.248	9.0	2.0	1.0	0.166
M26	0.0	6.0	2.0	0.090	0.0	5.0	4.0	0.249

Table 3: Genotype frequency table of each loci. Existence of selection was tested for by Hardy-Weinberg equilibrium. Significant HWE p value: ≤ 0.05.

Discussion

The data demonstrates that the GCOAD locus shows significant segregation between migratory and resident phenotypes in Little Sheep Creek, distinct from patterns observed in the Sashin Creek population (both using a chi-squared test and a Fisher's exact test). GCOAD is a locus in a noncoding region of the genome. It is unlikely that it has a specific function relating to the determination of life history. However, it is possible that this locus is linked to a region more

involved in this mechanism. This suggests that while some genetic markers like GCOAD may be consistently involved in influencing life history traits across populations, the specific role and effect of these markers can vary considerably. This variability could be attributed to different environmental pressures or genetic backgrounds across the two sites. Therefore, while GCOAD may serve as a reliable marker for determining life history, its applicability and influence might not be universal, underscoring the need for localized studies when considering conservation and management strategies.

In contrast to the GCOAD locus, other genetic markers did not demonstrate significant segregation between the migratory and resident phenotypes within Little Sheep Creek. This lack of segregation across multiple loci supports that the genetic underpinnings of migration and residency are likely influenced by a complex suite of population specific genes, further evidence of each contributing subtly to the phenotypes observed through environmental mediation (Clare et al.). Moreover, the absence of clear genetic differentiation for these traits across the populations studied may indicate an entirely separate pathway may be determining phenotype according to each life history. As the phenotype is likely polygenic, the complexity of environmental interaction, interconnected genes, and additive effects could produce multiple pathways towards the same expression.

Using DMAS-qPCR, proved effective in differentiating alleles associated with life history variations among trout. The clarity of clustering in cycle quantification values affirms the reliability of this technique in distinguishing genetic variations relevant to phenotypic traits. This is a relatively new technique for genotyping specific SNPs of interest and the data generated in my thesis confirms that it is a reliable and methodologically easy way to obtain genotypes from multiple loci. When the genotypes of these loci are statistically analyzed, there is a potential

selection bias towards the Sashin migrant allele observed in Little Sheep Creek resident population, though not supported by Hardy-Weinberg equilibrium. This observation, coupled with the limited sample size, points to the need for caution in interpreting these results without further validation through increased sample sizes and expanded geographic sampling.

There are several areas for future research. Firstly, my sample size was low and genotyping more samples would be useful especially with regard to confirming GCOAD's segregation between phenotypes. Additionally, investigating these genetic markers in different populations could also provide valuable insights into the segregation between alleles and phenotype in other populations of O. mykiss. As the samples only came from 2016 and 2017, long-term studies would further enhance understanding of how genetic and environmental factors interact over multiple generations, influencing the evolutionary trajectories of migratory and resident populations.

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