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Gene Expression Life History Markers in a Hatchery and a Wild Population of Young-of-the-Year Oncorhynchus mykiss

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Gene Expression Life History Markers in a Hatchery and a Wild Population of

Young-of-the-Year *Oncorhynchus mykiss*

by

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ABSTRACT

Life history within a single species can vary significantly. Many of these differences are associated with varying environmental conditions. Understanding what environmental conditions cue alternate life histories within a single species has been researched extensively. In salmonid fishes, more than almost any other group, varying environmental conditions give rise to individuals within species that take markedly different life history trajectories.

Oncorhynchus mykiss is a species of salmonid native to the Pacific Northwest region of North America. This species has two life history forms, anadromous and resident. The anadromous form spends a portion of its life in ocean while the resident life history form completes its entire life history in freshwater. Until the decision to migrate and morphological changes associated with smoltification occur, the two life history variants of this species are indistinguishable from each other. This ambiguity in juvenile *O. mykiss* morphology presents challenges for conservation managers charged with protecting and increasing threatened *O. mykiss* populations around the Pacific Northwest because conservation efforts cannot be evaluated until juvenile fish make the decision to migrate.

In this study microarray gene expression analysis was used to profile gene expression in juvenile populations of wild and hatchery *O. mykiss* to identify gene expression variation associated with alternate life history variants. This analysis identified 8 DNA sequences present in both brain and gill tissues that differ in expression in rainbow trout and steelhead hatchery stocks. Differential expression as quantified by microarray technology was validated with quantitative real-time PCR. Lastly, the expression of these putative life history markers was preliminarily evaluated in a wild population of *O. mykiss* at sample locations in the South Fork John Day River Basin, Oregon with known ratios of juvenile anadromous and resident fish.

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INTRODUCTION

Salmonid fishes are a dominant part of marine and freshwater food webs in the northern Pacific Ocean and the river systems that drain into it. The reason for their great success is debatable, but very likely depends on their ability to exploit both freshwater and marine habitats, a trait that is rather rare amongst the fishes. The ability to exploit marine resources offers great advantages under certain circumstances, supporting large population sizes, increased growth rate, and increased adult size at reproduction (Gross et al., 1988; Hendry and Stearns, 2003; Jonsson and Jonsson, 1993). In addition, escape to the marine environment may allow populations to persist in times when freshwater habitat conditions are poor or lost due to ecological changes such as natural disasters. Alternately, in times when marine conditions are limiting, freshwater forms may sustain populations. Thus, it is not surprising that many salmonid species have evolved life history strategies that include migrating anadromous forms that exploit marine resources prior to reproduction and non-migrating resident forms that complete their entire lifecycle in freshwater. Importantly, these life history variants appear to be indistinguishable genetically (Docker and Heath, 2003) and thus it is hypothesized that differential gene expression must underlie these observed patterns of life history diversity. Gene expression is known to be highly sensitive to varying environmental conditions in many organisms (Aubin-Horth et al., 2005) and vast differences in gene expression levels can lead to markedly different life history strategies between conspecific individuals associated with changes both morphologically and behaviorally (Evans and Wheeler,

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2001; Stearns, 1989). In this thesis, I evaluate differential gene expression in young-of-the-year hatchery stocks of *Oncorhynchus mykiss*, resident: rainbow trout/anadromous: steelhead, known to differ in their migratory behavior, in an attempt to identify gene expression markers that are unique to anadromous and resident forms in salmonids.

The Origin of Pacific Salmonids

The genus *Oncorhynchus* is made up of seven salmonid species native to the Northern Pacific Ocean region of the Northern Hemisphere ranging from Northern Mexico on the North American side of the Pacific Ocean to South Korea and Southern Japan on the Asiatic side of the Pacific Ocean and are commonly referred to as "Pacific Salmon" (Quinn, 2005). These seven species include Chum (*O. keta*), Chinook (*O. tshawytscha*), Sockeye (*O. nerka*), Coho (*O. kisutch*), Pink (*O. gorbuscha*), Steelhead (*O. mykiss*) and Coastal Cutthroat (*O. clarki*).

All seven species of *Oncorhynchus* are anadromous, spending part of their life history in the ocean but all spawning in freshwater. This obligate freshwater stage of salmonid life history has been argued to suggest the salmonid family has a freshwater evolutionary origin rather than seawater origin (Allendorf and Thorgaard, 1984). Three of the seven species, *O. mykiss*, *O. clarki,* and *O. nerka* have significant resident populations; populations that complete their entire life history completely in freshwater (these populations are commonly referred to as "trout") (Quinn, 2005).

Sequence differences in genes from *Salmo salar* (Atlantic salmon), *O. mykiss* and *O. nerka* suggest an estimated divergence between the genus *Salmo* and *Oncorhynchus* in

the early Miocene period (approximately 20 MYA)(Devlin, 1993). Fossil evidence of

Oncorhynchus is limited but the fossils that have been found date the radiation of Pacific

salmonids between 20 and 6 MYA (McPhail, 1997; Stearly and R., 1993).

Oncorhynchus and *Salmo* differ greatly in that *Oncorhynchus* had a major radiation event

producing the seven extant species we see today as well as several other that have since

gone extinct (i.e. the Sabertooth salmon, *O. rastrosus*) while a major radiation event in

Salmo never occurred. Fossil evidence suggests by 6 MYA all seven extant

Oncorhynchus species had evolved to their current form (Stearly and R., 1993).

Life History Variation in Salmonid Fishes

Life history amongst the salmonid fishes has been extensively studied as a model

to better understand how traits are inherited and maintained within and between

populations. Salmonid life history typically takes two forms, resident and anadromous

and is largely related to migration. The typical resident life history form is spawned in

freshwater where it grows and completes its life cycle. The anadromous form is spawned

in freshwater where it remains for many months to several years after which it begins

morphological and physiological changes associated with a process known as

smoltification in preparation for life in the ocean. (Hendry and Stearns, 2003)

Timing of smoltification varies between different species of salmonids. Some

species such as *Oncorhynchus keta* (Chum salmon) have fry that migrate to the ocean

within a few weeks of hatching (Quinn, 2005). Others like *Oncorhynchus mykiss*

(Rainbow trout/steelhead) and *Oncorhynchus kisutch* (Coho salmon) have juveniles that

reside for one to five years in freshwater before migrating to the sea (Quinn, 2005). Prior to seaward migration and smoltification, juvenile anadromous salmonids are termed parr. Parr are typically bottom dwelling pelagic fish that establish territories that they defend aggressively. Parr are indistinguishable in appearance from resident populations of the same species. Once parr establish territories they maintain their position in the stream by actively swimming against the current. A study done by Kutty and Saunders (1973) showed that Atlantic salmon parr (*Salmo salar*) placed in a swim chamber would swim against the current to exhaustion and collapse, while smolting fish placed in the same swim chamber and under the same conditions ceased swimming and showed no signs of fatigue or exhaustion. This suggests that a smolting fish will allow the current of a stream to carry them downstream and this refusal to swim could be seen as a characteristic of seaward migration. Behavioral changes are typically the first obvious cues that a fish has begun smoltification. Smolting fish typically abandon pelagic feeding territories, cease swimming, and express a marked decrease in aggression (Hoar, 1976). This decrease in aggression has been related to the observation that out-migrating fish tend to aggregate and eventually form schools for downstream migration.

Once in the ocean, anadromous fish experience rapid and substantial growth before returning to freshwater to spawn several years later (Hendry and Stearns, 2003).

Among salmonids there exists degrees of anadromy ranging from species that are purely anadromous to those that are optionally anadromous (Hoar, 1976). Recent studies comparing the anadromous and resident (non-anadromy) life history forms of many salmonids species have suggested there may be genetic factors linked to life history,

specifically related to smoltification (Narum et al., 2011; Nichols et al., 2008; Thrower et al., 2004). However, there has never been a study on gene expression associated with alternate life history variants within salmonids on fish prior to when the changes associated with smoltification, migration, or residency can be observed.

Pacific Salmonids Impacts on the Ecosystems Pacific Northwest

Ecologically Pacific salmonids play an important role in both oceanic and terrestrial ecosystems. In the Pacific ocean salmonids are an important food source, especially for many marine mammals (Pauly et al., 1998), and sea birds (Roth et al., 2007). Recent studies, done over the last decade have shown that returning anadromous salmonids also play a critical role in the nitrogen and carbon cycles in both the fresh water stream systems as well as the forests across the Pacific Northwest.   It has also been shown that growth rates of resident population of salmonids in streams systems in Alaska were directly related to the nutrients anadromous salmonids carcasses brought to the stream systems from the oceans after the anadromous fish had spawned and died (Wipfil et al., 2003). As well as effects of animal communities salmonids have significant impacts on terrestrial plant ecosystems. An example of this is a study done by Helfield and Naiman (2001)that found Riparian forest near streams and rivers derive 22-24% of the foliar Nitrogen direct from spawning salmon. Ocean derived nitrogen brought to the freshwater ecosystems from spawning adult salmon has also been shown to be important in maintaining rearing grounds for juvenile salmonids. (Gresh et al., 2000) showed that only 6-7% of the historic ocean derived nitrogen and phosphorus is

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reach Pacific Northwest streams. Without the input of large runs of anadromous fish,

salmonid recovery efforts may be in vain.

The Life History of *Oncorhynchus mykiss*

An abundant and wide-spread species of salmonid native to the Pacific Northwest

Region of North America, *Oncorhynchus mykiss,* is classified as optionally anadromous

(Hoar, 1976). Within *O. mykiss* it is known that both the anadromous and resident life

history forms can arise from parents of either life history trajectory (Christie et al., 2011;

Thrower et al., 2004; Zimmerman and Reeves, 2000). A recent study done by Christie et

al. (2011) tracked gene flow in a population of *O. mykiss* in the Hood River, Oregon. This

showed that in the wild anadromous population of steelhead, nearly 20% of the alleles

originated from resident rainbow trout parents (Christie et al., 2011). Other studies done

by (Doucett et al., 1999; Zimmerman and Reeves, 2002) used evidence from

strontium/calcium (Sr/Ca) ratios in otoliths to identify anadromous and non-anadromous

individuals of *O. mykiss* in the Deschutes River, Oregon. Strontium is more common in

salt water than in freshwater. Thus Sr/Ca ratios can be used to determine whether or not

an individual's mother was anadromous or non-anadromous due to maternal provisioning

of these minerals into developing oocytes during oogenesis. Sr/Ca ratios indicate that in

the Deschutes River, the anadromous steelhead sampled from the river had both

anadromous and resident mothers (Zimmerman and Reeves, 2000). In addition, Sr/Ca

ratios also suggest that resident *O. mykiss* in the Deschutes River had both anadromous

and non-anadromous mothers (Zimmerman and Reeves, 2000). This study illustrates the

lack of a purely genetic explanation for anadromy in *O. mykiss* from the Deschutes River, and instead implicates a non-genetic mechanism in determining life history variation in this species such as phenotypic plasticity associated with varying environmental cues. In addition, other studies show that the life history trajectory of *O. mykiss* is influenced by physiologic, environmental as well as genetic factors that play an important role in the decision to remain resident in freshwater or initiate changes required for smoltification and anadromy (Metcalfe et al., 1988; Nichols et al., 2008; Thrower et al., 2004).

While the genetic factors influencing life history are still poorly understood, several recent studies have focused on the genetic factors associated with smoltification at the population level using single nucleotide polymorphisms or SNPs (Narum et al., 2011) and quantitative trait loci (QTL) analyses (Nichols et al., 2008).

It is important to note that a recent study done by Brown et al. (2006) found intraspecific variation in *O. mykiss* mitochondrial genomes that suggests phylogenetic divide between coastal and inland populations. This phylogenetic difference between inland and coastal *O. mykiss* population is not well understood.

Very little is known concerning the mechanisms that determine alternative life history strategies in this species, and currently no reliable biomarkers are available for identifying alternative trajectories in young-of-the-year fish prior to the obvious changes associated with smoltification at an individual level. In addition, it is not clear at what point during early life history development the decision to become anadromous or resident is made in *O. mykiss*. Understanding when life history decisions are made, and identifying the gene expression pathways that underlie this decision will play an

important role in better understanding how genetic and environmental factors interact to influence life history. This knowledge will not only inform scientists on the basic biology of salmonid species, but will also provide a powerful tool to inform resource managers concerned with the conservation of alternative life history forms in threatened *O. mykiss* populations.

Oncorhynchus mykiss in the John Day River Basin, Oregon

The John Day River is the second largest undammed river system in the American West and the largest free flowing river system in the continental United States to have an un-supplemented, wild, anadromous *Oncorhynchus mykiss* (steelhead) population (Carmichael and Talyor, 2010). This makes this population important due to its high degree of genetic diversity that has been relatively unaffected by genetic influences of hatchery fish. However, wild populations of *O. mykiss* have dramatically declined from historic levels in the Columbia River basin due to many factors, including overfishing, poor ocean conditions, habitat destruction, hydropower development and hatchery practices (Raymond, 1988). These declines led to the 1999 Endangered Species Act (ESA) listing of an environmentally significant unit (ESU) of steelhead from the Middle Columbia River as "threatened" (Federal Register, Vol. 64, No. 57, March 25, 1999, p. 14517),(Good and Waples). This ESU includes a wild population of summer-run steelhead in the South Fork John Day River Basin (SFJD).

Summer-run steelhead in the SFJD are characterized by adults returning to the main stem Columbia River from the Pacific Ocean in late-summer or early-fall and

holding in the major river system over winter until spring when they migrate up the tributaries of the SFJD to spawn when river and stream flows increase. *Oncorhynchus mykiss* populations in the Columbia River Basin can spend from 1-5 years in freshwater before migrating; however migration generally occurs at year 2 or 3 (Peven et al., 1994). The SFJD population is iteroparous (spawning more than once per generation) and optionally anadromous, and thus is composed of both a migrant anadromous (steelhead) and a resident form (rainbow trout) that are morphologically indistinguishable as juveniles.

Previous research has identified anadromous/resident life history ratios in several tributaries of the SFJD basin through mark-recapture experiments. In this study two closely spaced tributaries (Figure 1) were found to have differences in the proportion of out-migrating juvenile fish (assumed to be anadromous). In 2004, Murderers Creek was shown to have a larger proportion of out migrant fish (13.7%) compared to Black Canyon Creek (3.1%) (Tattam, 2006). The following year (2005) 11.6% of tagged juvenile *O. mykiss* emigrated downstream from Murderers Creek compared to only 3.2% in Black Canyon Creek (Tattam, 2006). These two tributaries differ in their physical characteristics. Lower Murderers creek headwaters originate in the Malheur National Forest. Murderers Creek's elevation ranges from 900 to 2000m and has a length of approximately 30 km. The stream system primarily flows through open high desert grasslands and open range cattle pasture but at its higher elevations the landscape is dominated by Ponderosa and Douglas fir forests. Black Canyon Creek headwaters originate in the Black Canyon Wilderness Area inside the Ochoco National Forest. Black

Canyon Creek's elevation ranges from 900 to 1600m and has a stream length of approximately 15 km. All 15 km of BCC are dominated by high gradient flow and most of the stream system flows in deep canyons dominated by scrub brush and ponderosa and Douglas fir forests. Stream temperatures in Murderers Creek range from 0°C in the winter to 26°C in the summer, while temperatures in Black Canyon Creek held around 1°C in the winter and rarely reach over 20°C in the summer (Tattam, 2006). Despite these physical differences, the two stream systems enter the South Fork John Day River approximately 3.7 km from each other (Figure 1). These closely spaced tributaries with differing ratios of out-migrating fish create an excellent system for investigating possible differences in life history variation in a natural system.

Hatchery Production of Oncorhynchus mykiss in the Columbia River Basin

The culturing of *O. mykiss* in hatchery environments has its beginning in the early 20th century at first as an attempt to create faster growing sport fish with high survival rates but later on as a tool to supplement declining wild *O. mykiss* populations (Service, 2010). As wild populations declined, limitations were put in place on the catch of wild fish resulting in a higher demand and therefore a greater production of hatchery fish throughout the region. Today, 70-80% of the salmon and steelhead caught in the Pacific Northwest originated from a hatchery (Service, 2010). As the numbers of hatchery *O. mykiss* released into the region's rivers and streams increased, concern of the effects of hatchery released fish on wild indigenous fish populations started to grow. Research suggests that hatchery fish are having direct and negative impacts on wild fish

populations. For instance a study done by (McMichael et al., 1997) showed a significant correlation between the occurrence of residualized hatchery *O. mykiss* (anadromous released fish that do not migrate but instead remaining at their release site as resident fish) and negative growth rates in wild indigenous *O. mykiss.*

The *O. mykiss* anadromous life history form is the primary target for hatchery release programs in river and streams within the Columbia River Basin. Like most anadromous salmonids, *O. mykiss* exhibits "homing" during migration. (Quinn, 1993). They return to their natal rivers and streams to spawn. However, not all *O. mykiss* (wild and/or hatchery) return to their natal stream to spawn; these fish are termed "strays". Strays may migrate back to a nearby tributary or as far away as other river basins. For example, a study focusing on the occurrence of hatchery strays in Oregon coastal rivers found that 22% of fish sampled in 5 streams without hatchery *O. mykiss* releases were actually hatchery strays (Schroeder et al., 2001). These strays can have significant impact on the genetic make-up of protected wild *O. mykiss* populations especially when interbreeding between wild and hatchery fish occurs. Hatchery *O. mykiss* tend to have lower reproductive success than that of wild *O. mykiss* populations (Chilcote et al., 1986) as well as higher mortality rates. Hatchery x wild spawning pairs have been shown to produced 63% fewer recruits compared to wild x wild spawning pairs (Chilcote, 2003).

Despite the negative impacts of hatchery fish on wild populations, and the artificial rearing environment they create, hatchery fish provide an opportunity to perform "common garden" experiments in a much more controlled environment than can be accomplished in natural settings. In this study, we compared individuals from resident

and anadromous hatchery stocks from Mossy Rock Hatchery, Washington. Mossy Rock

Hatchery water is fed to the facility from an underground spring providing a clean water

source with stable temperature of 10°C year-round.

A complete history of the origin of resident hatchery stock in the Pacific

Northwest is partial at best. Of all the resident rainbow trout hatchery lines available, the

line selected for this study (Goldendale Rainbow trout, GDT) has one of the better

records of development. Goldendale Rainbow trout are fall spawning. The line began at

Goldendale Hatchery in 1938. Today's Goldendale Rainbow trout line was established in

1948 and is a cross between Meader and Yakima rainbow trout lines (Kinunen and

Moring, 1976). The anadromous Steelhead line used in this study is derived from wild

fall run of Kalama River brood stock (ESA Listed) that has been long established at the

Kalama Hatchery, Washington. The returning Kalama steelhead are spawned at the

Kalama hatchery and incubated until the eggs reached the "eyed-up" stage of

development before being transported to Mossy Rock Hatchery for hatching and rearing.

Conservation management of <u>Oncorhynchus</u> <u>mykiss</u> in the SFJD

A major issue facing conservation of the SFJD *O. mykiss* populations is

measuring the effectiveness of conservation strategies such as habitat restoration. The

ESA listing of the Middle Columbia River ESU specifically targets only the anadromous

form of the *O. mykiss* population. Both government and non-government agencies have

recognized the importance of this John Day River Basin steelhead population and

devoted large amounts of resources and time to habitat protection and restoration in

hopes of increasing the steelhead populations. The ability to assess if conservation

efforts are working to increase anadromous populations in restored areas could be a very

useful management tool. Importantly, being able to identify pre-smolting juveniles as

resident or anadromous would allow for more immediate impacts of management

decisions to be assessed.

Specific Aims

　　　The primary aim of this research is to identify differences in gene expression

between known young-of-the-year (YOY) resident and anadromous forms of *O. mykiss*.

I hope to use these differences in gene expression to identify gene expression markers

that can be used to identify an individual *O. mykiss* YOY as likely to be anadromous or

resident prior to any morphological, behavioral, or physiological changes associated with

preparations for migration.

　　　To achieve these aims oligonucleotide microarray gene expression and qPCR

analyses were employed to identify gene expression differences between resident and

anadromous life history variants in YOY *O. mykiss* reared under common conditions in a

hatchery environment and in a wild, threatened population of juvenile *O. mykiss* from the

South Fork John Day River Basin.

　　　Gene expression differences were analyzed in brain and gill tissues between

anadromous versus resident life history strategies by sampling juvenile, young of the year

fish from Mossy Rock Hatchery, Washington, prior to smoltification. Gill and brain

tissues have been chosen because of the important physiologic changes that occur within

these tissues prior to smoltification and subsequent migration to the sea; because of this these tissues are an obvious location to observe differences in gene expression between alternate life history forms. Mossy Rock Hatchery was chosen as a sample location because the hatchery rears both a wild Kalama run anadromous population as well as a resident population. Both stocks of fish are brought to the hatchery at the same age and are rear under common garden conditions.

Next, gene expression was analyzed in brain and gill tissues from two different tributaries of the South Fork John Day River (Oregon) known to produce different ratios of anadromous versus resident forms independent of parental affects. Working in collaboration with a team from Oregon State University and under a permit from the National Oceanographic and Atmospheric Administration (NOAA)

MATERIALS AND METHODS

Specimen Collection and Handling

Hatchery fish. Young-of-the-year *O. mykiss* were obtained from Mossy Rock hatchery (Mossyrock, Washington). Eight fish each from two stocks known to differ substantially in their tendency to migrate were sacrificed by concussion. The heads were then removed and flash-frozen in liquid nitrogen. Total time between sacrifice and freezing was less than 1 min. Fish were randomly sampled from troughs, but chosen by size from the net to control for size-specific differences. This sampling resulted in a mean mass and mean length for the rainbow trout hatchery samples of 7.5 ± 2.57g and 85.25 ± 10.54 mm (mean ± s.d.), respectively**.** The steelhead sampling resulted in a mean mass and mean length for steelhead hatchery stock of 5.25 ± 1.10g and 79.75 ± 2.95 mm (mean ± s.d). Both the mean mass (t-test, p = 0.13) and mean length (t-test, p = 0.31) were not significantly different between the two stocks. The resident stock (MRHR) was a Goldendale Rainbow stock that had been reared for many generations at Mossy Rock hatchery for production of rainbow trout to support stocking efforts. The anadromous stock (MRHS) was produced from wild-origin Steelhead parents captured in the Kalama River for rearing in a hatchery environment for wild population supplementation.

Wild Fish. Young-of-the-year fish were sampled from two tributaries of the South Fork John Day River Basin (SFJD) that have been previously shown to produce differing proportions of anadromous and resident life history variants of juvenile *O. mykiss* (Tattam, 2006). These sites were Lowers Murderer Creek (LMC, anadromous

biased) and Black Canyon Creek (BCC, resident biased). In a recent study Murderers

Creek was shown to have juvenile growth rates significantly higher than Black Canyon

Creek and Murderers Creek was shown to produce more migrant (assumed anadromous)

O. mykiss as compared to Black Canyon Creek (Tattam, 2006).

Fish were captured by hook-and-line sampling at the BCC site, and by snorkel

herding into seine nets at the LMC site. In both locations fish were sacrificed and whole

heads were frozen as described above for the hatchery fish.

Tissue Sampled

Gill and brain tissue were sampled for both physiologic and practical reasons.

First, many of the changes associated with smoltification occur in these two tissue types.

Second, due to the limitation of lethal sampling of an ESA-listed species and the need to

share fish among several investigators, only heads were available for sampling. The size

class and weight class of hatchery fish chosen for this study were selected due to their

similar size to YOY wild fish sampled in the South Fork John Day River basin research

site.

RNA Extraction

RNA was extracted from brain and gill tissue by dissecting samples in the lab

from frozen heads. Each frozen sample was homogenized in Trizol reagent and the RNA

was extracted following the following the manufacture's protocol (Invitrogen, Carlsbad,

CA, USA.). Care was taken to ensure that the tissues did not thaw prior to being

submerged in Trizol reagent. Total RNA concentration and RNA purity were quantified

using a Tecan Infinite 200 Pro Nanoquant plate reader (Mannedorf, Switzerland). RNA

integrity was also verified by running 1 μg of total RNA on a 1% agarose gel. All

samples exhibited clear ribosomal RNA bands and a smear of high molecular weight

RNA.

Microarray Probe Preparation

Differences in gene expression were quantified using an Agilent 44K

oligonucleotide array developed for Atlantic Salmon, *Salmo salar*, and commonly used to

profile gene expression in salmonid fishes (www.agilent.com, Release Date 06/08).

Probes were generated for each sample using 200 ng of total RNA and the Agilent One-

color spike mix kit according to the manufacturer's instructions. This kit provides 10

positive controls that act as quality control for the amplification and labeling steps

preceding hybridization. Cyanine 3-CTP (Cy3) labeling dye was incorporated directly

into the cRNA during the amplification process. Fluoresecently-labeled cRNA was then

purified using RNeasy Minispin columns Qiagen (Valencia, CA, USA) following the

Qiagen protocol. Purified cRNA was eluted into a final volume of 30 μl RNAase-free

H_2O. cRNA yield was determined by measuring absorbance at 260 nm and labeling

efficiency was calculated from total yield and total Cy3 fluorescence (550/570 nm

excitation/emission) according to Agilent's protocols using a Tecan Infinite Pro

Nanoquant plate reader. Fluorescent-labeled probes with amplification yields greater

than 1.65 µg with specific activity greater than 6.0 pmol Cy3/µg cRNA were used for microarray hybridizations.

Microarray Hybridization

cRNA samples were hybridized following Agilent's hybridization protocols. 1.65 µg of cRNA was processed with 25x fragmentation buffer to fragment the cRNA into optimal sizes for hybridization. Each sample was fragmented for exactly 30 min at 60.0°C. Samples were then hybridized to the Agilent 4 x 44k oligonucleotide microarrays using the Agilent SureHyb chamber at 65.0°C for 17 hrs.

Microarray Analysis

Each microarray was scanned using a Molecular Devices (Sunnyville, CA, USA) Axon GenePix 4000B Microarray scanner at 5 µm resolution with an average of two scans per line. Images were acquired using the companion GenePix Pro 7 software. Images were then analyzed using Agilent Feature Extractor software following the software manufacture's protocols (version 9.5.3.1). Extracted data was exported to Agilent Genespring GX software (Ver 10) for expression analysis. Quality control analysis was performed on the results for all hybridizations, and one gill sample had to be excluded from analysis based on not meeting minimum standards. Genes that were differentially expressed in resident vs. anadromous life history variants were identified using a one-way ANOVA and the Benjamin Hochberg FDR correction for multiple comparisons (total $p < 0.05$). Further filtering of the data analysis by fold-change in gene

expression was sometimes performed to highlight genes with high levels of gene expression variation. A clustering analysis was performed on the genes found to be differentially expressed using non-averaged samples and a hierarchical clustering algorithm with a Pearson centered distance metric and a centroid linkage rule. Clusters were based on both entities and condition (i.e. gene probe and stock). Venn diagrams were constructed using the entity lists generated from the one-way ANOVA statistical test comparing differentially expressed genes in both tissue types. Genes differentially expressed in both tissue types were deemed candidate life history markers. The full sequence for each candidate life history marker was downloaded from GenBank using the nucleotide Blast tool (http://blast.ncbi.nlm.nih.gov/) using the accession numbers for each maker found in the microarray annotation file provided by Agilent.

Quantitative Real-time Polymerase Chain Reaction (qPCR)

Microarray data were validated using qPCR for probes that appeared to be possible markers for life history variation in *O. mykiss*. PCR primers were designed using the National Center for Biotechnology Information Primer-Blast website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on the accession number for each probe found in the Agilent *S. Salar* microarray annotation file. Information about each primer set is shown in Tables 1-9. Primers were selected for product lengths between 75 and 150 bp. Optimal annealing temperatures (Table 10) were calculated using New England Biolabs (Ipswich, MA, USA) Tm calculator (http://www.neb.com/nebecomm/tech_reference/tmcalc/default.asp#.T-1WprVDx8G)

which accounts for the annealing temperatures for each individual primer set within the buffers and reagents associated with their PCR assay. Single-stranded cDNA was synthesized using the Thermo Scientific Fermentas (Glen Burnie, Maryland) first strand cDNA synthesis kit according to the manufacturer's instructions. Standard PCR reactions were performed using New England Biolabs reagents on each primer set to optimize reaction protocols. PCR product purity was checked electrophoretically on a 2% agarose gel. Finalized PCR programs used for qPCR quantification of each probe are reported in Tables 2 and 3. qPCR reactions were run on a Bio-Rad (Hercules, CA, USA) MiniOpticon real time PCR System using QuantiTect SYBR green PCR Master mix (Qiagen – Catalog#204143). Data were collected and C_t values were calculated with the companion Opticon Monitor software (version 3.1.32) (See Figures 13-21). Comparative quantification was used using the $\Delta\Delta C_t$ method (Invitrogen, 2012). (Fold Change = $_{Eff}Sample^{\Delta Ct\ Sample}/_{Eff}Normalizer^{\Delta Ct\ Norm}$) (See Table 10) using β-actin as the reference for each comparison.

RESULTS

Gene expression patterns of hatchery stocks

      Microarray analysis revealed 154 sequences differentially expressed in the brain tissue and 137 sequences differentially expressed in gill tissue between hatchery rainbow trout stocks and hatchery-reared wild steelhead stocks. Heat maps generated from the hierarchical clustering analysis of differentially expressed sequences illustrate a clear relationship between gene expression and stock type as seen in Figures 2 and 3. Of the 15 differentially expressed sequences common to gill and brain tissues (Fig 4 and 5), 8 sequences had known accession numbers and full sequences were obtained from GenBank (Table 1). These 8 sequences were chosen as putative life history markers for resident versus anadromous life history variants in YOY *O. mykiss*. Three of the eight life history markers are known genes, proteasome subunit beta type-9b, cd83 protein, and duodenase-1 (Table 1).   Little is known about the role these genes have on alternate life history pathways but it is clear from the microarray data that the genes are alternately expressed between the two different life history types. Gene Ontology interrogation of the data resulted in no enrichment of differentially expressed genes for any specific pathway. However, this negative result is likely due mainly to the limited annotation of the *S. salar* genome and associated microarray probes represented on the microarray.

Quantitative real-time PCR Validation

Primer sets for each accession number resulted in verified PCR protocols that yielded single products of the appropriate size (Tables 2-10) as verified on 2% agarose gels. These verified PCR protocols were then used to validate the microarray data using qPCR for each gene expression marker. The Results for these qPCR runs are presented in Fig 7-15)

There was a significant positive correlation between the qPCR and microarray expression data for all eight markers. ($r = 0.72$, $p < 0.001$; Fig 16). These data validate the microarray gene expression data generated in this study by demonstrating a direct correlation between qPCR data and microarray data as seen in Figure 16. Linear regression analysis yields a slope close to 1.0, suggesting a nearly 1:1 relationship between the two methods of determining gene expression (slope $= 0.93 \pm 0.24$, $p < 0.002$).

The most significant differentially expressed marker in both brain and gill tissue was marker BT045730. For this marker, microarray analysis yielded a 8.02-fold up regulation in rainbow trout brain, while qPCR analysis using C_t values yielded a 16.28-fold up regulation in the same sample. For the same marker in gill tissue the microarray analysis yielded an 8.40-fold up regulation, while qPCR yielded a 14.27-fold up regulation. Thus, the microarray and qPCR results are consistent in both direction and magnitude of differential expression. These data indicate with some certainty that these markers were differentially expressed between the two life history stocks reared under identical hatchery conditions.

Microarray analysis of gene expression in wild fish

The microarray analysis comparing LMC, BCC, MRHR to MRHS in brain tissue

revealed 154 differentially expressed genes (Fig. 3). For gill tissue, 137 genes were

differentially expressed (Fig. 2). Cluster analysis on these differentially expressed

sequences in brain and gill tissue sampled from all sample locations (MRHS vs. MRHR

vs. LMC vs. BCC) reveal expression patterns that are unique in wild compared to

hatchery fish suggesting a large component of differential gene expression due to

environmental an ecological differences between the wild and hatchery stocks. (Fig. 2-3).

However, despite all of this variation, two of the eight potential life history markers

identified as differentially expressed in hatchery stocks were also differentially expressed

in the wild fish. These two markers' accession numbers are: BT045730, and BT047975.

DISCUSSION

The complexity of life history variants observed in extant populations of *O. mykiss* are very likely the result of selective pressures that vary over time and in intensity alternately favoring increased survival in marine and freshwater environments. Thus, the life history matrix of these fish remains flexible and able to adapt to favorable ocean or freshwater conditions. While this is clearly of evolutionary advantage, it almost certainly adds layers of complexity to the natural fluctuations in population size that are observed even in wild and undisturbed populations of fish. When human manipulations of populations and degradation of the environment (both freshwater and marine) are added to the mix, determining the causes behind population declines and expansions can be very difficult. In addition, without knowledge of the environmental factors that regulate life history decisions and the molecular pathways that are altered in resident versus anadromous variants, it is extremely difficult to predict changes in fish health or abundance over time and in response to changes in the environment. Thus, as a first step towards identifying the molecular pathways that are involved in determining life history variation in *O. mykiss* I have used gene expression analysis to identify genes that are differentially expressed in stocks of fish that are known to be primarily resident or anadromous, but were reared under identical conditions. This "common garden" experimental design provides the best conditions for identifying genes that are differentially expressed due to life history trajectory, and not local environmental conditions. It is our hope that these genes will be useful for two distinct purposes – first,

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of empirical value, and second, for the practical value for the management of threatened populations. First, I hope to identify the molecular pathways that regulate life history trajectories in this species, and the window of development in which life history trajectory is determined. This information will be invaluable in understanding how environmental and genetic factors work together to determine complex life history decisions. Second, it is hoped that the life history markers identified in this gene expression study could be used to develop molecular markers that can predict life history trajectory using a non-lethal sampling protocol in pre-smolting juveniles from both wild populations and hatchery stocks. This information can be used to determine areas where resident and anadromous variants are highly successful, which is information sorely needed in preparing management strategies for protecting endangered populations of anadromous *O. mykiss* in the Columbia River Basin.

There are many factors that have been identified as influencing life history decisions in *O. mykiss* and other populations of salmonids but the three widely accepted indicators of life history are growth-rate (Hutchings and Jones, 1998), (Metcalfe et al., 1999) movement/migration (Beckman et al., 1998) and survival (Holtby et al., 1990). These three indicators are interdependent and culminate in an individual's "decision" to mature in freshwater (resident fish) or migrate to sea (anadromous fish). These factors can be measured over long periods of time. However, these indicators are all large-scale traits that integrate changes in many physiological systems, and likely represent the downstream effects of the actual mechanistic causes that drive the changes associated with life history variation. In this thesis, I argue that gene expression changes prior to the

expression of the above mentioned life history indicators and that by focusing on the role

that these genes play in regulating cellular and organismal physiology, we can not only

quickly and easily identify a juvenile *O. mykiss's* likely life history in a very short period

of time, but we can also ultimately understand how and perhaps why the life history

decision has been made.

In this study over 150 genes have been identified that are differentially

expressed in resident versus anadromous hatchery stocks of *O. mykiss*. Of the 8

identified sequences selected as putative life history markers in hatchery stocks, several

of these sequences are also differently expressed between South Fork John Day River

sample sites. At this point, there is no cohesive story that can be told based on the genes

that have been identified. This is not a failure of the methodologies used, but rather is a

symptom of our general lack of knowledge of gene function for the genes that are

differentially expressed. The major focus of this study was to primarily identify genes

associated with alternate life histories in juvenile *O. mykiss* but preliminary research

shows the functions of the eight identified life history genes in this study are, at this point,

poorly understood. However, the association with three major physiologic processes

(proteasome activity, metabolism, digestion and immune response) has been established

for all but one of these markers (Accession # EG823395).

Markers NM_001123714, BT047975 and BT058447 have been shown associated

with proteasome function (Murray et al., 1999). BT050202, BT045730, NM_001141601

have been shown to be associated with metabolisms and specifically markers BT050202

(gene ID/name: ddn1, Duodenase-1) is a protease which has both trypsin-like and

chymotrypsin-like activities (Pletnev et al., 2000). Finally, BT045799 (GeneID/name: CD83, CD83 Protein) have been shown to be involved in immune response. Specifically CD83 is a protein in the family of immunoglobulin superfamily of receptors. It is thought to be involved in the regulation of antigen presentation and may also be involved in binding to dendritic cells to inhibit the cell's maturation (Chen et al., 2011). How these genes affect life history decisions in *O. mykiss* are unknown at this point.

Additional studies will be required to better understand the role these genes have in life history decisions. Many of the other 150 genes that are differentially expressed are also of unknown identity and or function. It will take many years to identify and determine the function of these genes, and hopefully after a great deal of effort we will be able to reconstruct a mechanistic picture of the molecular differences between a fish that expresses as a resident or anadromous life history variant. These genes are the first clues to unraveling a likely complex interaction of gene expression that leads to the differences in cellular and organismal traits we associate with both life history variants.

From a practical perspective, these differentially expressed genes represent putative biomarkers for anadromous and resident life history variants of *O. mykiss* in the two hatchery populations in this study. Importantly, these markers are expressed far prior to the morphological and physiological changes that are associated with smoltification. Identifying these markers in non-lethal tissue would make it possible to use them to identify life history trajectory in YOY wild *O. mykiss* populations. This will make possible the ability to look for changes in the ratio of anadromous to resident forms in specific stream reaches and in response to specific alterations in the environment, such as

fluctuations in temperature or changes in fish density, food quality and availability. These life history markers may also play an important role for conservation managers when evaluating habitat restoration projects. Life history markers could allow fish to be examined soon after these events which will minimize loss of the signal due to migration of fish out of an area, and it will allow a greater deal of certainty to be developed as to cause and effect relationships in migratory decisions by individual fish. In the future we may not have to wait for fish to smolt and migrate in order to determine the effects of habitat alterations on the ratio of anadromous versus resident forms.

The expression analysis of the two Mossy Rock Hatchery stocks (rainbow trout/steelhead) demonstrate there is an expression level difference between these two long establish hatchery stocks. Microarray data and qPCR data are closely correlated and both support the same conclusion of differential expression in the two stocks.

From a hatchery perspective the ability to identify the life history of juvenile fish prior to their release into local rivers and streams could alleviate many of the problems associated with residual and stray fish. The common garden conditions of Mossy Rock hatchery would be an ideal place to test whether these anadromy markers do fully predict a juvenile *O. mykiss* life history. Expression analysis of these markers could be performed on fin clips that are produced as a normal part of the PIT-tagging efforts throughout the Columbia River basin. The migration of these fish could then be easily tracked through existing PIT tag monitoring stations at all major dams and by placing new PIT tag monitoring stations at strategic sites along the fish's migration route. These

data could be used to validate the identified gene expression markers in fish released into natural waterways.

The sample size of this study is relatively low and was limited by the need to lethally sample an ESA listed population of fish. The future application of these markers for the management of wild populations will require additional studies to not only validate the results found in this study in broader populations of *O. mykiss*, but also to validate that these markers can be identified in non-lethal tissue samples. Preliminary data suggested at least one of the identified life history markers (BT045730) is expressed and can be quantified in a non-lethal tissue sample (fin clip). The ability to use fin-clips to identify future migratory behavior could provide higher resolution data on specific reaches of stream systems and provide information on how management practices are affecting the ratio of anadromous and resident forms of *O. mykiss*. It would provide a powerful tool to help in habitat restoration monitoring of ESA and non-ESA population of *O. mykiss*.

Migration behavior in *O. mykiss* from the South Fork John Day River basin has been monitored using Passive Integrated Transponders (PIT) Tags (Tattam, 2006). This study, although limited in scope and sample size illustrated differing ratios of migrant and resident juvenile *O. mykiss* in stream systems within the basin. In the future, it would be fantastic to compare the anadromous/resident ratios from tributaries with known differences in migration bias with the putative gene expression markers identified in this study. Non-lethal sampling and PIT tagging of a large number of individuals in a natural setting such as this would be an excellent way to validate the life history markers from

this study. In fact, this is the logical next step in evaluating the effectiveness of these markers.

The ability to identify what life history strategy (anadromous or resident) young of the year *O. mykiss* are expressing before there are morphological differences between individual fish has never been achieved. This study is the first to identify life history markers present in multiple tissues types that may be useful tools for understanding the mechanisms that induce life history variation, as well as a way to evaluate life history variation in this species. Further research into using these markers for verification via non-lethal sampling (blood samples or fin clips) is the next logical step in furthering this research. In addition, evaluating how early in development these differences in gene expression can be traced will help better understand the timing of the decision to follow a specific life history trajectory.
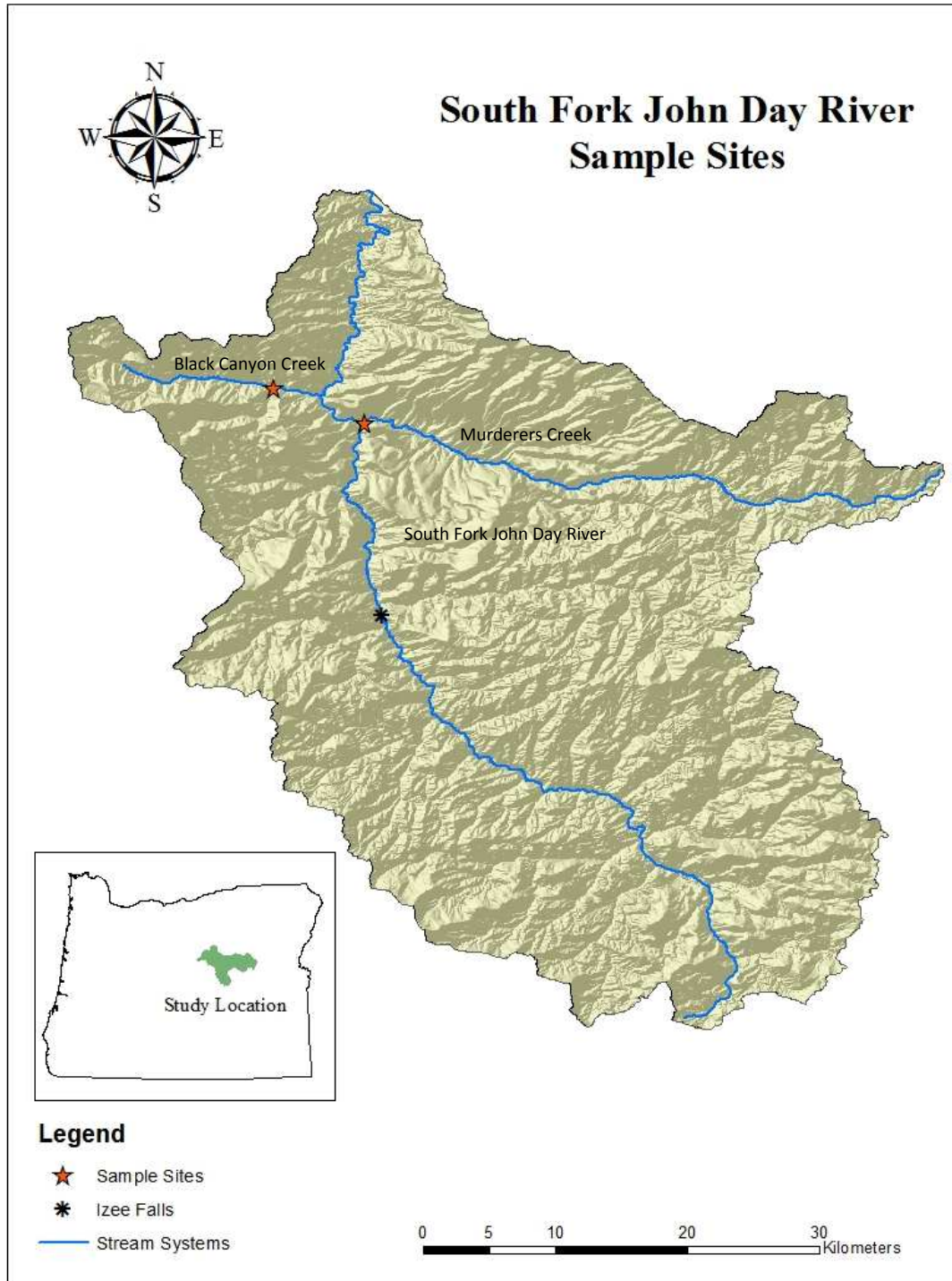
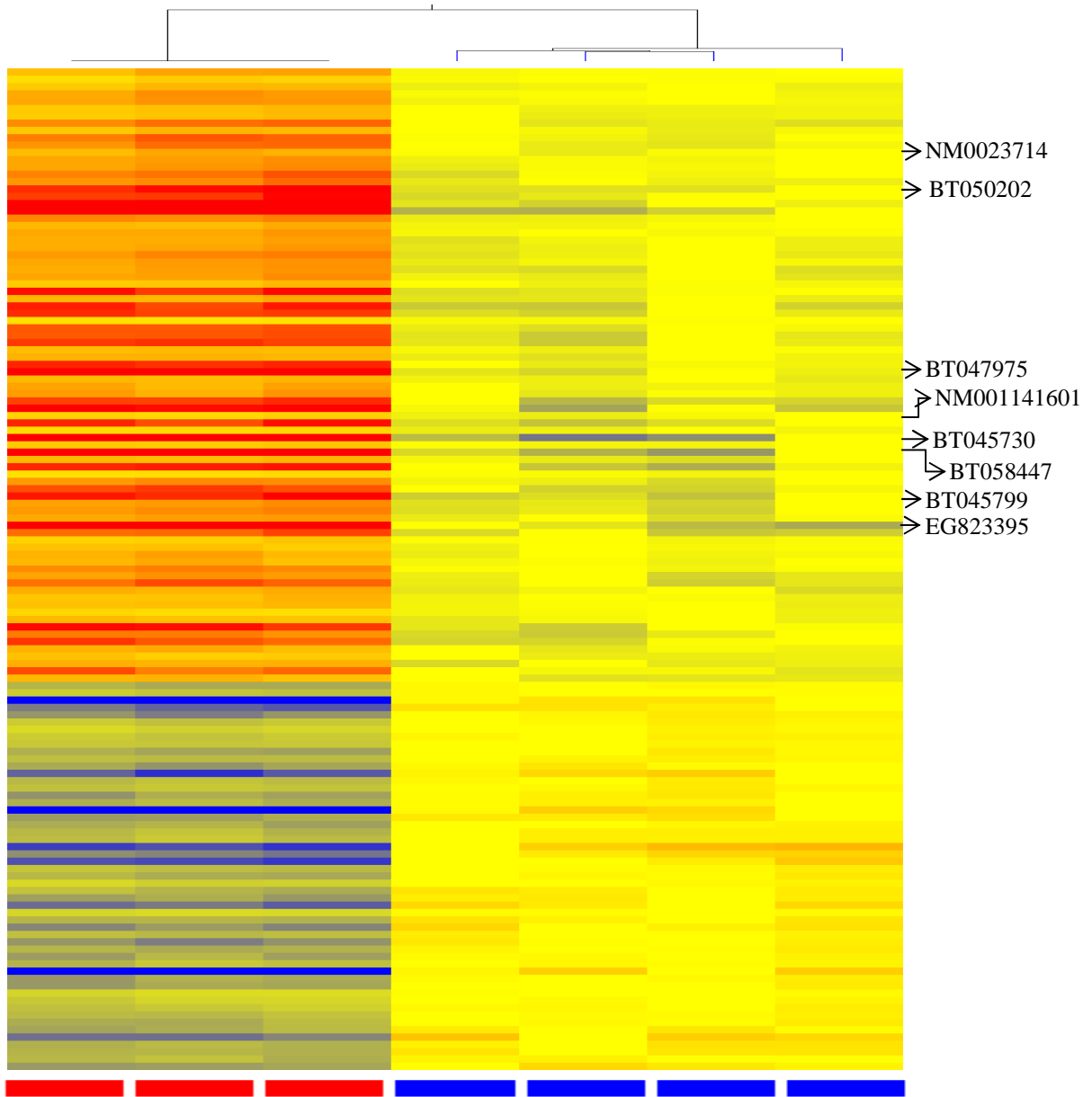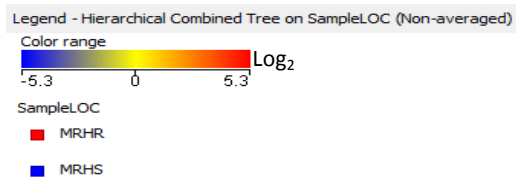**Figure 1. Map of the South Fork John Day Sample Locations**

Figure 2. Heat Map of differentially expressed gene in gill tissue between hatchery rainbow trout and steelhead stocks.

Cluster analysis (Pearson's Centered) of all genes differentially expressed in gill tissues between young-of-the-year hatchery steelhead (MRHS) and hatchery rainbow trout (MRHR) stocks. There were 137 differentially expressed genes identified using t-tests and the Benjamini-Hochberg correction for multiple comparisons (total p<0.05).
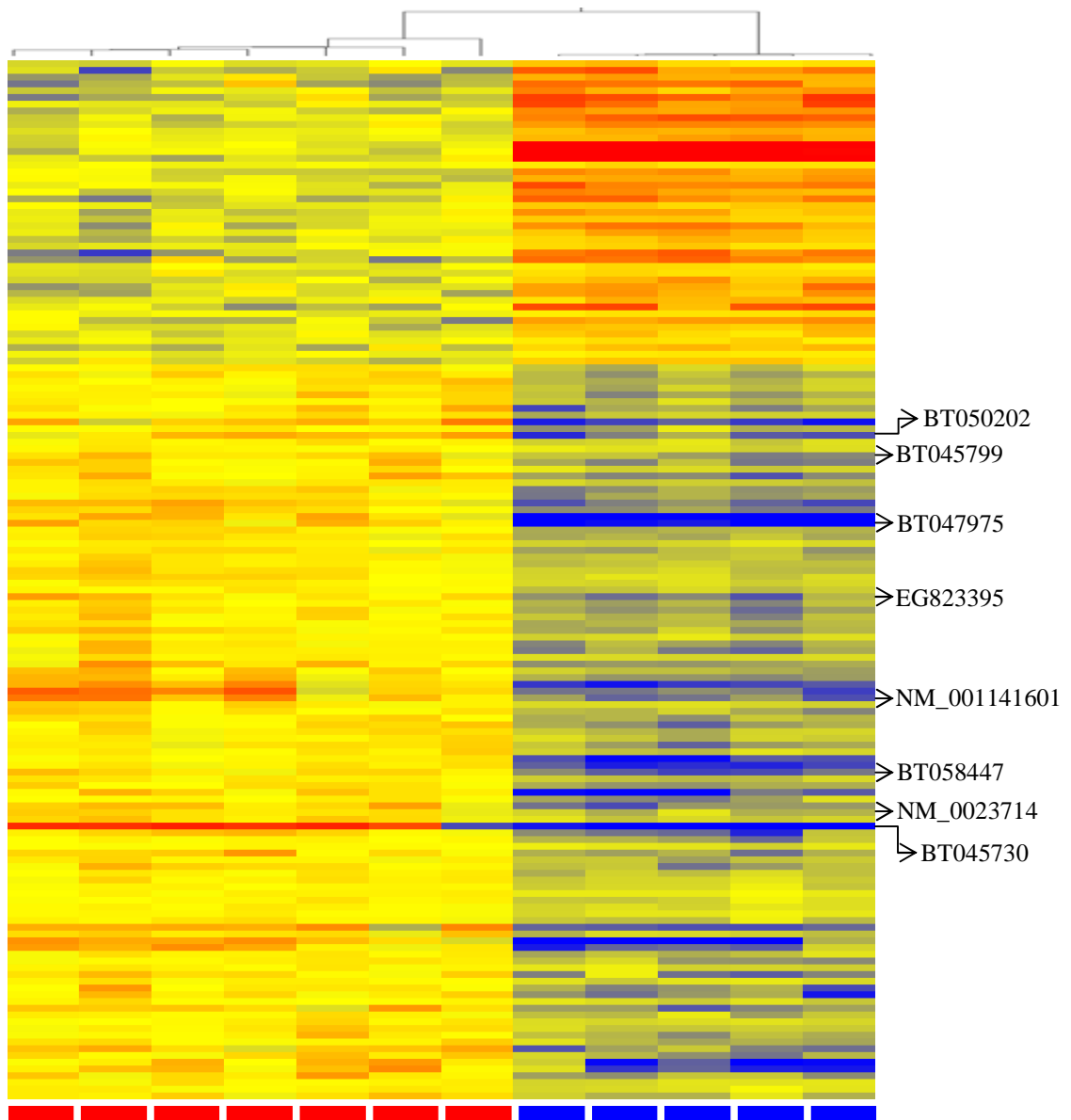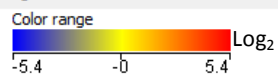
Figure 3. Heat Map of differentially expressed gene in brain tissue between hatchery rainbow trout and steelhead stocks. Cluster analysis (Pearson's centered) of all genes differentially expressed in brain tissue between young-of-the-year hatchery steelhead (MRHS) and hatchery rainbow trout (MRHR) stocks. There were 154 differentially expressed genes identified using t-tests and the Benjamini-Hochberg correction for multiple comparisons (total p<0.05).
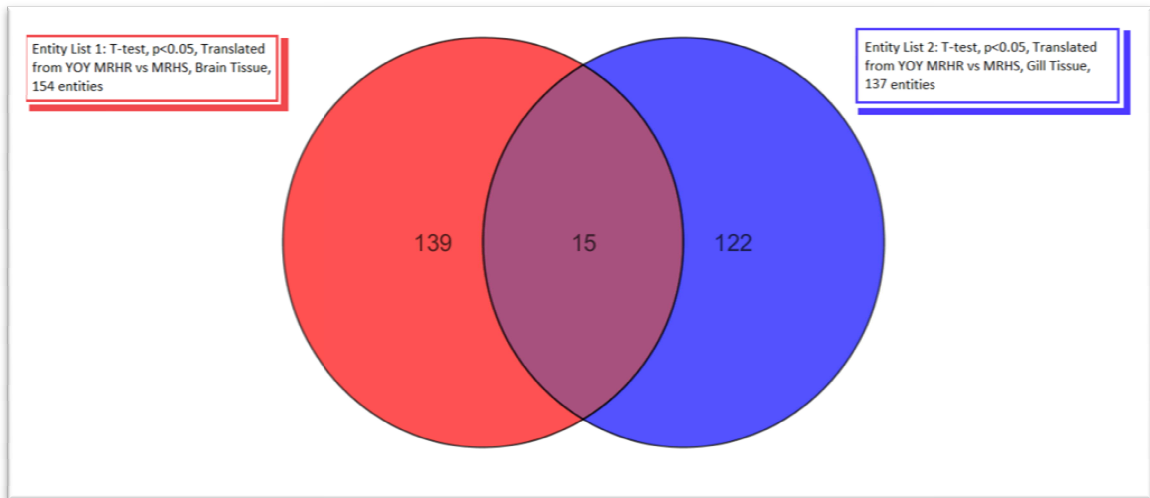
Figure 4. Venn diagram of genes differentially expressed in gill and brain tissue from young-of-the-year Mossy Rock Hatchery stocks.
(MRHS: Mossy Rock Hatchery steelhead, MRHR: Mossy Rock Hatchery rainbow Trout). 15 genes were differentially expressed in both brain and gill tissues and have been chosen as putative life history markers for anadromous versus resident forms of *O. mykiss*.
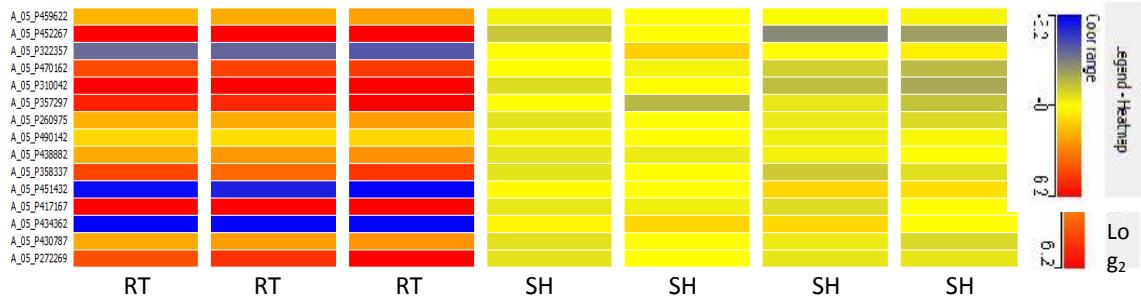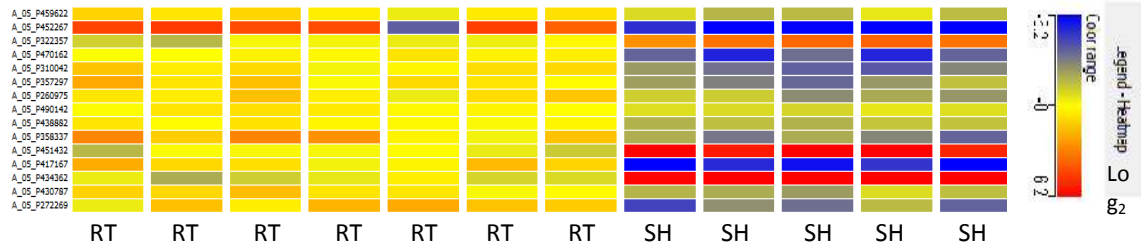
Figures 5. Heat map of gene expression for the 15 putative life history markers identified from the Mossy Rock Hatchery analysis.

In this heat map of expression levels for the 15 putative gene expression markers for alternative life history trajectories common to both brain (Figure 4) and gill tissues in MRHR (RT) and MRHS (SH) stocks.
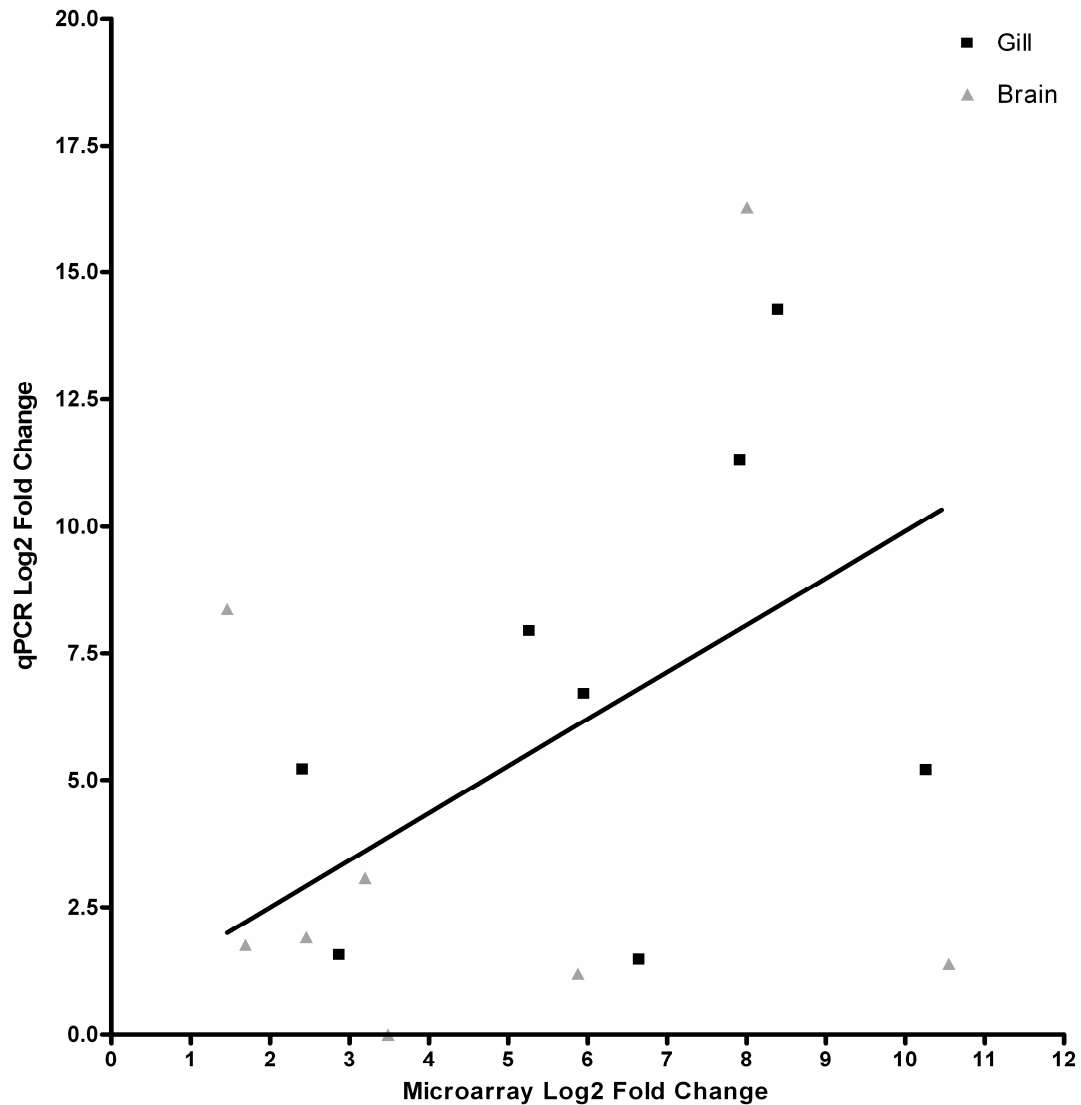
Figure 6. Graph and correlations analysis of qPCR and microarray gene expression data for the 8 selected life history markers.
A comparison of log2 fold change in gene expression using a microarray gene expression analysis and qPCR. A Pearson's correlation analysis was done comparing qPCR and Microarray data resulting in a significant r^2 value of 0.7119 (p-value<0.0010) These data verify the validity of the microarray gene expression for the life history markers selected for all but one marker in one tissue (NM_0023714, in brain tissue). Linear regression modeling produced a slope value of 0.9263 ± 0.2442 and an r^2 value of 0.5069 (p-value<0.0020

>BT045730

>BT047975
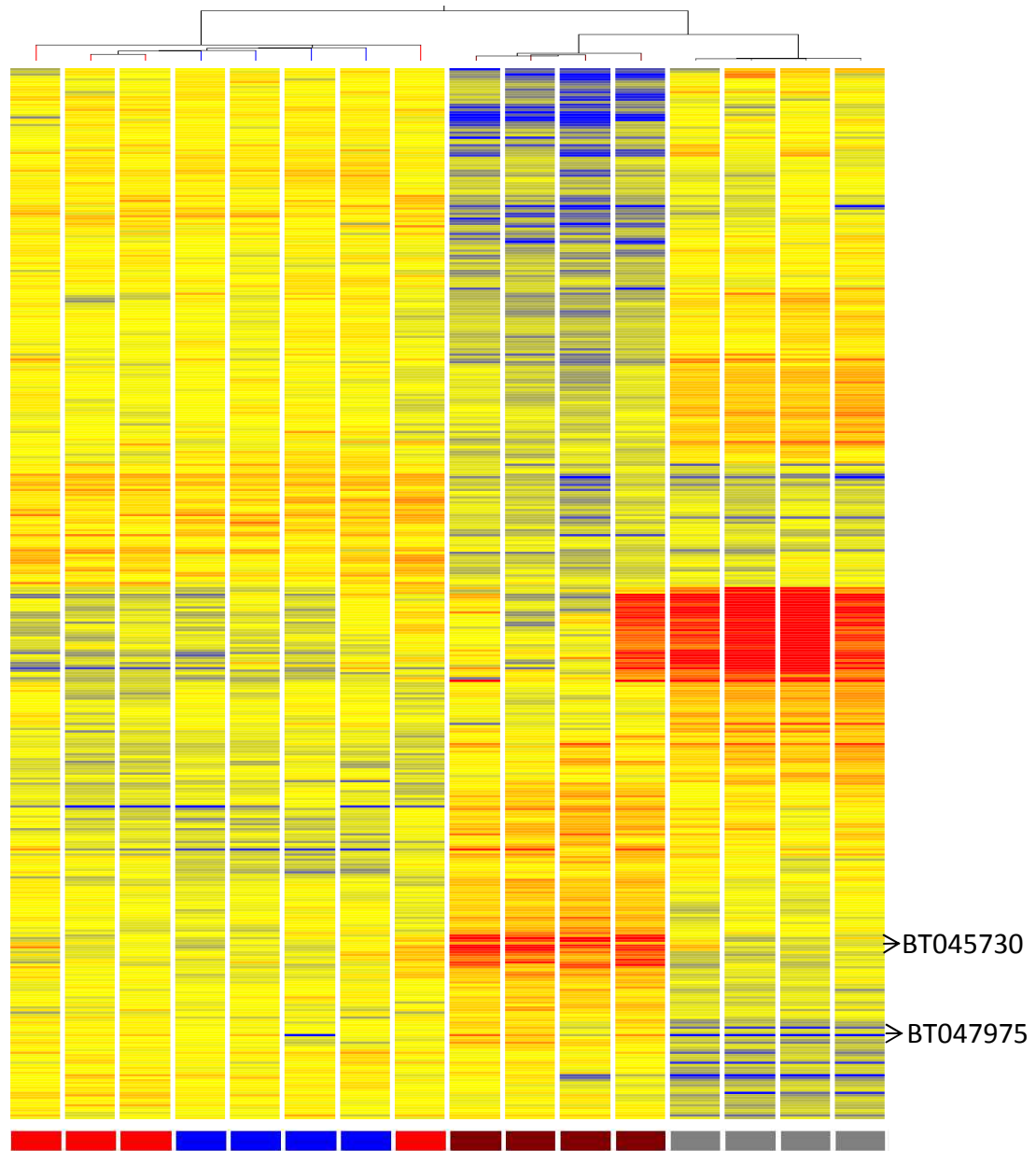
Legend - Hierarchical Combined Tree on SampleLOC (Non-averaged)

Color range

Log 2

-5   -0   5
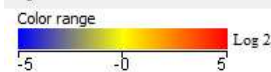
SampleLOC

■ BCC

■ LMC

■ MRHR

■ MRHS

Figure 7. Heat map diagram comparing gene expression in gill tissues from South Fork John Day River basin samples sites and Mossy Rock Hatchery Samples.
This heat map diagram compares gene expression in gill tissue from MRHS, MRHR, LMC and BCC. All samples were references to MRHS (the control wild origin hatchery Kalama steelhead stock). There were 375 differentially expressed genes identified using t-tests and the Benjamini-Hochberg correction for multiple comparisons (total p<0.05). Two of the 8 life history markers identified in hatchery samples were differentially expressed in this analysis

Figure 8. Heat map diagram comparing gene expression in brain tissues from South Fork John Day River basin samples sites and Mossy Rock Hatchery stocks. This heat map diagram compares gene expression in brain tissue from MRHS, MRHR, LMC, and BCC. All samples were references to MRHS (the control wild origin hatchery Kalama steelhead stock). There were 200 differentially expressed genes identified using t-tests and the Benjamini-Hochberg correction for multiple comparisons (total p<0.01). One of the 8 life history markers identified in hatchery samples were differentially expressed in this analysis

Figure 9. qPCR data for primer set BT045799
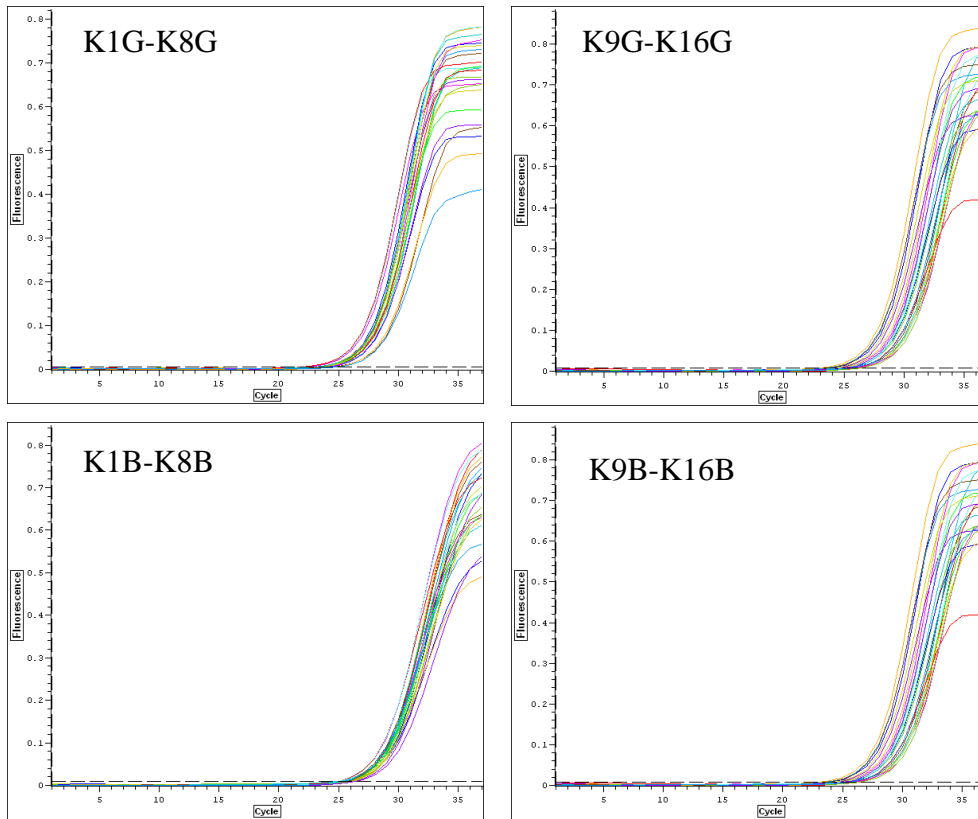
Figure 10. qPCR data for primer set BT045730

Figure 11. qPCR data for primer set BT050202

Figure 12. qPCR data for primer set EG823395
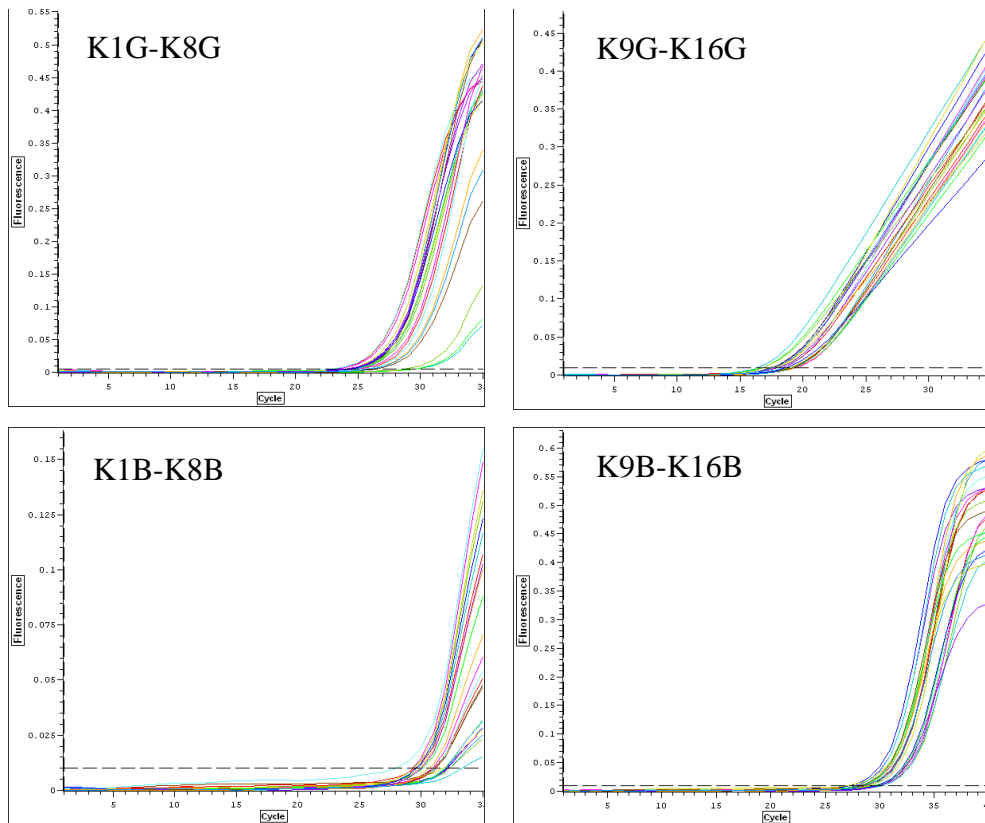
Figure 13. qPCR data for primer set BT047975
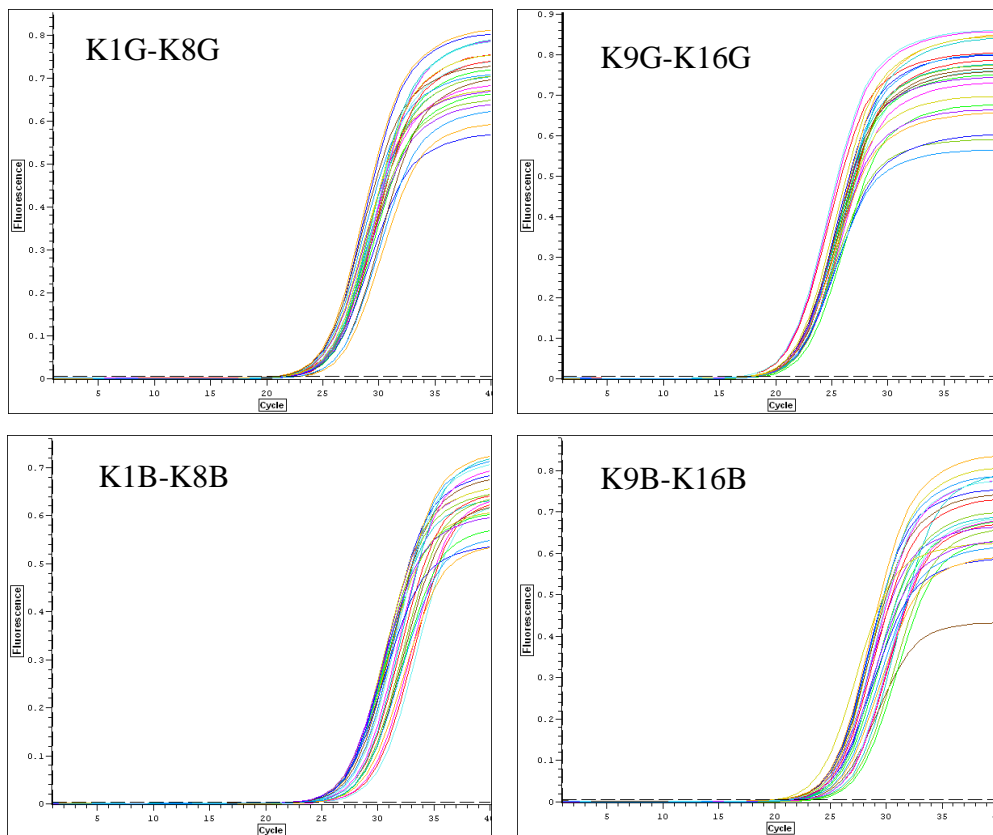
Figure 14. qPCR data for primer set ß -Actin
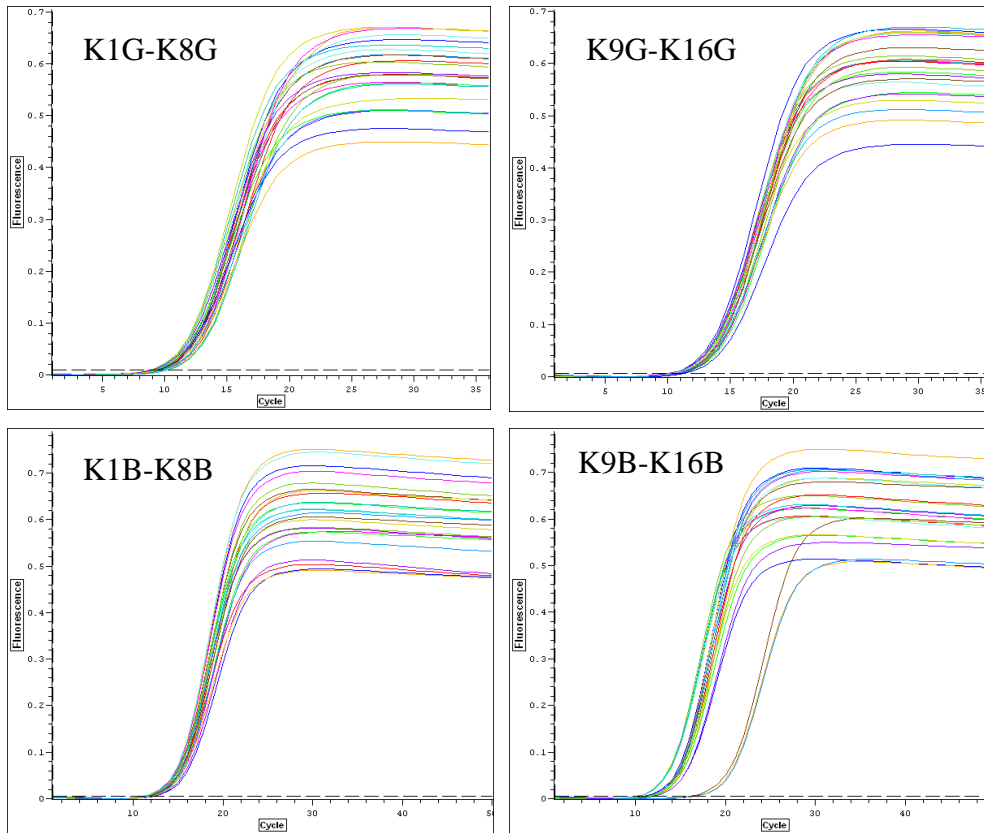
Figure 15. qPCR data for primer set BT058447
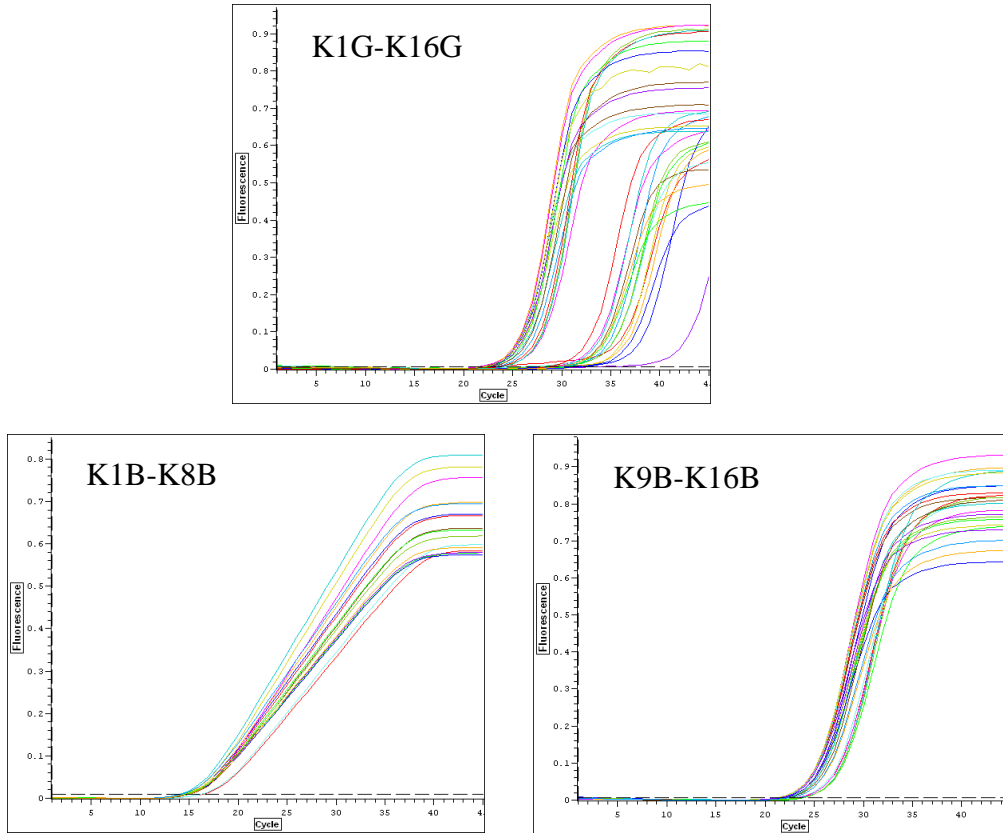
Figure 16. qPCR data for primer set NM_0023714

K1G-K16G

K5B-K8B, K14B-K16B
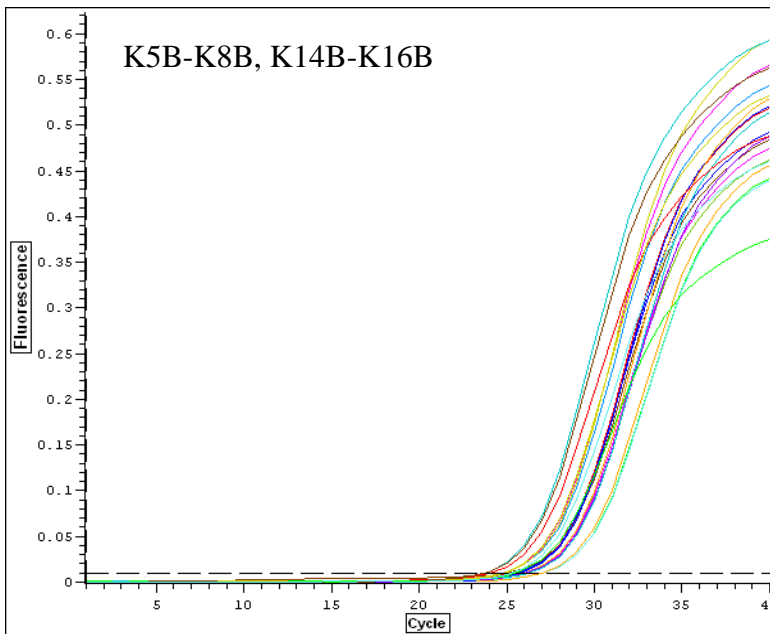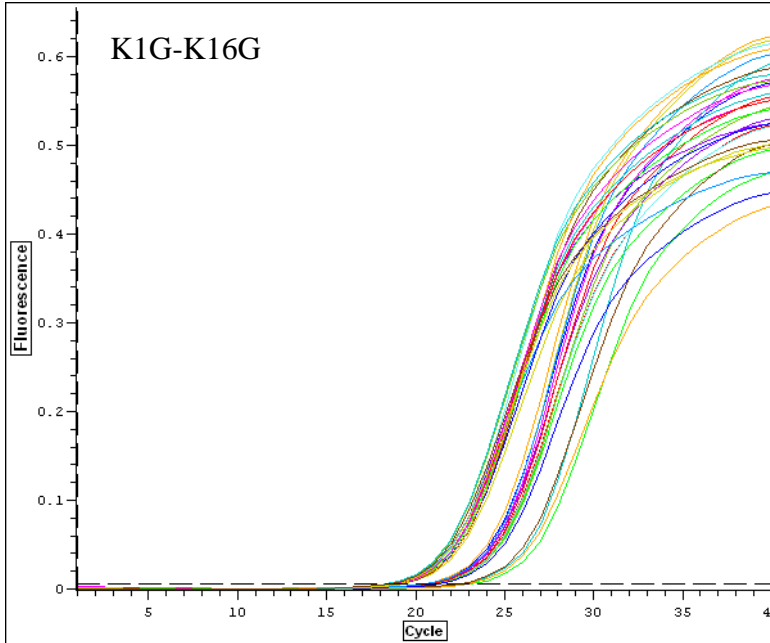
Figure 17. qPCR data for primer set NM_001141601

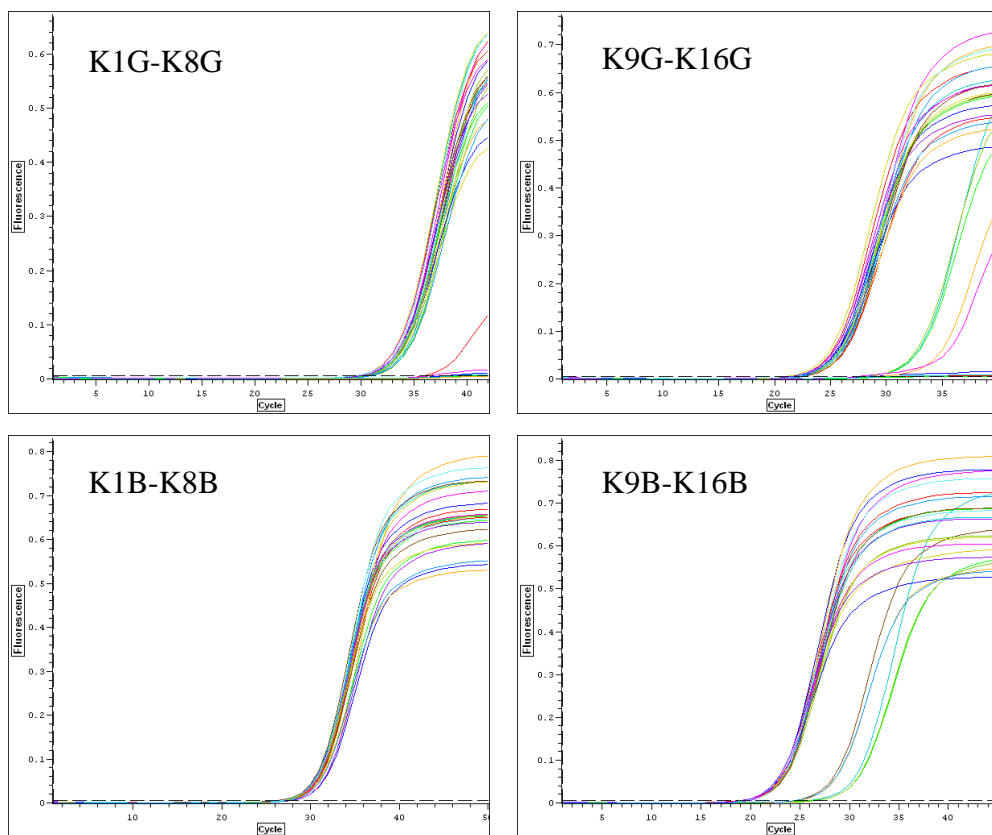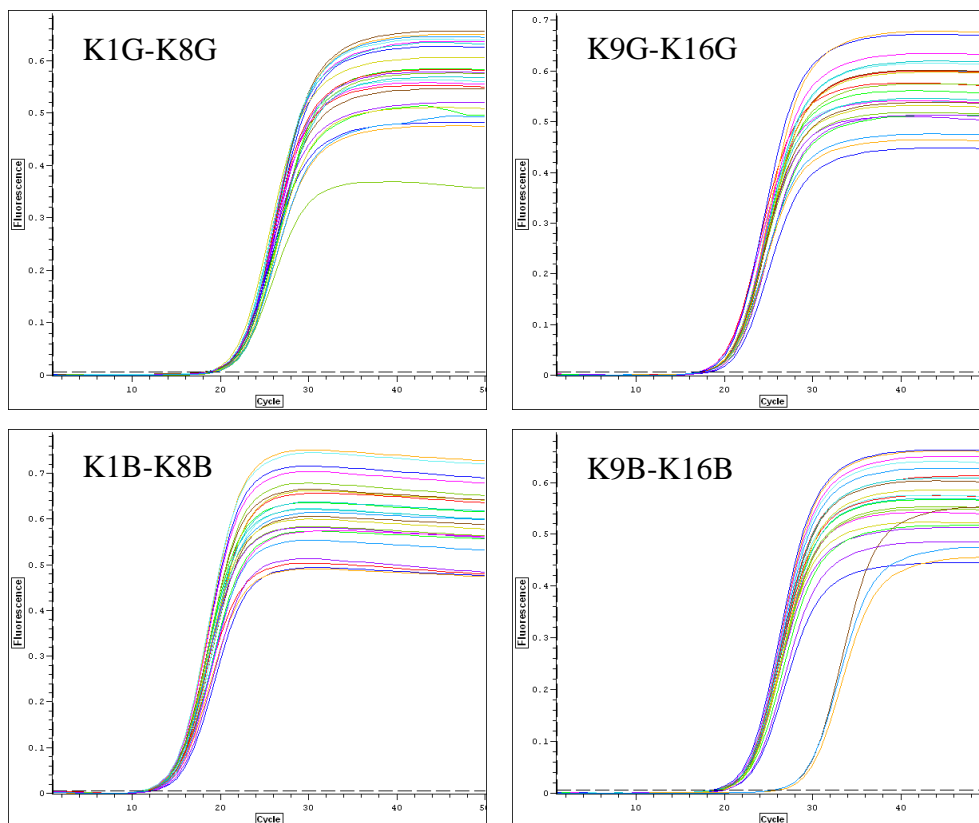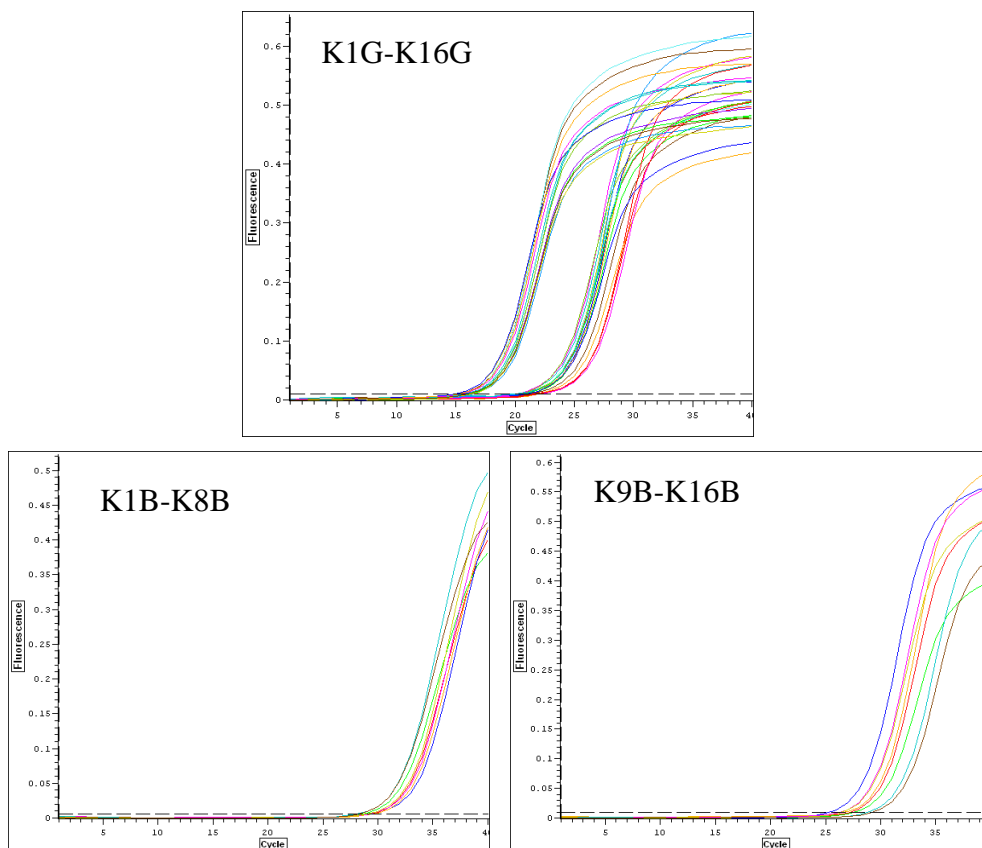| ProbeID	PrimaryAccession	GeneSymbol	GeneName	Description
A_05_P459622	NM_001123714	psmb9-b	proteasome subunit beta type	Salmo salar proteasome subunit beta type-9b (psmb9-b), mRNA [NM_001123714]
A_05_P452267	BT045730			Salmo salar clone ssal-rgf-529-363 Serine incorporator 1 putative mRNA, complete cds [BT045730]
A_05_P310042	BT058447			Salmo salar clone Contig835 Proteasome subunit beta type-8 precursor putative mRNA, complete cds [BT058447]
A_05_P357297	EG823395			EST_ssal_evd_25036 ssalevd thymus Salmo salar cDNA Salmo salar cDNA clone ssal_evd_532_214_fwd 3', mRNA sequence [EG823395]
A_05_P260975	BT045799	cd83	cd83 protein	Salmo salar clone ssal-rgf-531-267 CD83 antigen precursor putative mRNA, complete cds [BT045799]
A_05_P358337	NM_001141601	ddn1	Duodenase-1	Salmo salar Duodenase-1 (ddn1), mRNA [NM_001141601]
A_05_P417167	BT047975			Salmo salar clone ssal-eve-542-161 Proteasome subunit beta type-7 precursor putative mRNA, complete cds [BT047975]
A_05_P272269	BT050202			Salmo salar clone ssal-eve-515-010 Carbonyl reductase 1 putative mRNA, complete cds [BT050202]

Table 1. Eight putative life history markers identified in the Mossy Rock Hatchery gene analysis study. Of the 15 putative life history markers identified as differentially expressed in both brain and gill tissue 8 are described List of the identities and Genbank accession information for these 8 genetic markers are listed above.

Table 2. Life history marker information: BT045730
Accession Number: BT045730
Agilent Probe ID: A_05_P452267
Gene ID: N/A
Protocol: 1: 30sec@94.0°C; 35: 30sec@94.0°C, 30sec@68.0°C
Primer Forward: 5-GAAGACGGCGAGAACCGGGC
Primer Reverse: 5'-TCATAGCTGGCGTCGGGGCT
Product Length: 143bp

Full Sequence
```
agatcgtaaa cagacgagaa gcatccatat ttcttcccgg agaagtcgta tttcagaaaa
acaccatggg ggccgttctt ggactgtgct ccatggcgag ttggatcccc tgcctgtgtg
gcactgcccc ctgcctgctg tgcagatgct gccccagtgg gaataactcc actgtcaccc
gacttatcta tgccttcttc ctccttctgg gtgtgggcat cgcatgcatc atgctaatgc
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caatcccagg tgtggagggt catgtgaact gtgatgtctt ggtcggctac aaggctgtgt
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tttggttcta cataggaatg gctggagctt tttgcttcat cctgatccag ctggtccttc
tgattgactt tgcccactcc tggaatgagt cctgggtgga gaaaatggag gagggcaact
ctcgctgctg gtatgcagct ctgctgtctg ctacaaccat caactacatc ctgtcccttg
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tcgactgatg agctaaagaa ttagaaggga gcgcaatcat ttgctcatga ttgtgactat
tcaaattgta taaggtgaac aagctctgtg tgtgtattcc ctgtggataa cccaccctttt
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tgcaggggttc cagatattag ctaacattat cctaggttca taagtgagca tgatttacca
tttgcatgat attataggct aatctacaat catgcaaatc ttaatgttgc tgtgatttcc
ttttcagaat atctgccccg attgctaacc tgcacatctt gtaatagctc aaataagacc
ttaagtttta tgtaccttgt gttacttatt atttgcacta gttatcttct aaaggtaaaa
tactgatgtt caaacagtgg ataagttatt ttgtagatgc tttagcataa tttaaggtgt
attaagtgtt tctagcagac aagatacatg tcagaaaatg tcaactccca tttctccaga
atagtttgcc tttcgatatg tattaaagtg cattgcaaac cataaaaaaa aaaaaaaaaa
aaaaaaaag a
```

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Table 3. Life history marker information: NM_001123714
Accession Number: NM_001123714
Agilent Probe ID: A_05_P459622
Gene Symbol: psmb9-b
Gene Name: proteasome subunit beta type-9b
Protocol: 1: 30sec@94.0°C; 33: 30sec@94.0°C, 30sec@58.0°C, 30sec@72.0°C
Primer Forward: 5'-TGAGGCGTCGTTTGGTGTTTCT
Primer Reverse: 5'-CTGTGCAGTCCGCACGGTGT
Product Length: 82bp

Full Sequence:
```
ggctccttgc tcaaatatat ttaagagttg attcttaata caacagtaca gttgaatagt
aatcgttcgt gtatgtccta aatgaggcgt cgtttggtgt tttctttttc ttttcatttc
gcgttgaatc attaggcttc tcacacaccg tgcggactgc acaggtatca acgcgaccag
ccacaaaact aaagtgcttc aatatgttag aagaatcatc agagccaggg tggctatccg
aagaagtaaa aactgggacc accatcattg ctattgagtt tgatggaggt gtggtgctgg
gctctgactc tcgagtgtct gctggggaga ctgtggtgaa ccgggtgatg aacaagctct
ctctcctcca tgacaagatc tactgcgccc tgtcaggctc ggctgcagac gcccagacca
tcgctgagat ggtcaactac cagctggatg tgcacagcat tgaggttgga gaggatcctc
aagttcgttc agctgccact ctggtgaaaa acatctccta caagtacaaa gaagagctgt
cagcacatct cattgttgcc gggtgggaca agagaggagg gggacaggtg tatgtgaccc
tgaatggcct gttgtccaga caaccctttg cggtcggggg ctccggaagc gcctacgtct
atgggtttgt tgatgcagag taccggaagg ccatgagcaa agaggactgc caacagtttg
ttgtcaacac actttcattg gccatgagtc gagatggttc cagcggaggt gtggcctacc
ttgtcactat tgatgaaaag ggtgcagagg agaaatgcat cctgggcaac gagttgccca
ctttttatga tcagtgaaaa ctgggttgtg aaaacagtaa caatgaggtt gtcgtagtta
tgaaacagtg agtggacata tggccacctt acgaacaaac agtactatta aatctacaca
ggatatagaa tgtttggata ggggtatgaa tcatgctggc acaatgtcaa atattctcac
tacacatgcc attttcaaaa ttgttatgcc ctctgttgaa aacaactgat tttattttgg
atagccaggg acctcaggct ggactgtgtc ttcgggtgat tcacaagtaa attaaacttt
ccttggataa aa
```

Table 4. Life history marker information: BT050202
Accession Number: BT050202
Agilent Probe ID: A_05_P272269
Gene ID: N/A
Protocol: 1: 30sec@95.0 ^{o}C; 35: 30sec@95.0 ^{o}C, 30sec@68.0 ^{o}C
Primer Forward: 5'-CCGCTGCAGCCCTGAACTCC
Primer Reverse: 5'-CTGCCTGGGCCTCCTGGACA
Product Length: 101bp

Full Sequence
```
gggggaatgt tacggaagcc agcaagtagc ctgaagcgtg tcaacctcag tggtttgaag
ccagttctgt aacaatactg ttcgacacgt caagagtacc cccactgtaa agtatgccaa
aagttgcact ggtgactggt tccaataagg ggattggatt tgcaattgtg cggtcgcttt
gcaagcaatt caatggggat gtttcctca gtagccggga tgctggccgt ggaacagcgg
ctgtggagag cctgaattct gaagggctga aacccctctt ccaacagctt gacatcaacg
acccagaaag tgtgcgcgcg gcccgagatt tcttcaatga gaaatatggt ggccttgatg
tgctcattaa caatgctggg attgccttta aaaacgctga tactacaccc tttggaaccc
aagctgaggt gactctcaaa actaacttct ttgccacaag agacatgtgc aatgagtttc
tccccatcat caaaccagga gggaggtgg tgaacgtgtc tagtgttatg agctccatcg
ccctgaaccg ctgcagccct gaactccagg cccggttccg cagcaatgac atcacagagg
aggagctggt ggggctgatg gagagatttg tccaggaggc ccaggcaggg gcgcactccc
aggggggctg gcccgacaca gcctacggtg tgtccaaaac aggcctcacc gtgctctcca
ggatccatgc ccgcaagctg gggcatgaga gaccagctga tcagatcctt ctgaatgcgt
gctgcccggg ctgggtgagg accgatatgg ctgggcccaa cgccaccaag tcacctgacg
agggtgccat cacccccgtt tacctggccc tgcttcctgc ggggctggg gagccgcagg
gacagtttgt gatggacaag aaggtccatc cgtggtgagg attaggtttt tgagtaatgt
gtgtattctg gaggggttta cagagtctgg ctgttcttat ctgtgggaat gagggttaaa
tgcactgtat acattggaaa ggacaacatc acaggaaaat tcaggaatgt tgattttggt
acaaaaatgt gtaaacccag gtgtatttt gtgggtttat ccaatctcac tctaatagtg
tttttcaggt caatggaatt aaccatgttt ttagcaccta tgtatgatat tgtatttatt
caacatttta caagctgaac ttattttcac acaatcaggt ggggctctgt ttagcaaaaa
atagttttgc ctgttatttt ggcattgtgt cacatatcag tttgcaaaca atgtaatttc
attaaaatca tttagttaat aaagctgcat ac
```

Table 5. Life history marker information: BT058447
Accession Number: BT058447
Agilent Probe ID: A_05_P310042
Gene ID: N/A
Protocol: 1: 30sec@94.0; 35: 30sec@94.0, 30sec@56.0
Primer Forward: 5'-GACAGCGGCTACCGATATGA
Primer Reverse: 5'-AAAAGCGGTGGTACAGGTCG
Product Length: 181bp

Full Sequence
```
ttggccagag catctgcagg atcttacgtc agcactcaga tgtttaagaa ggtgatcgag
ataaacccct ttctacttgg gacaatgtca ggcagtgctg ccgactgtgt ctattgggag
agagtcttgg ccaaggagtg cgggatatca gattgggtta aagaggacgg attcaacgcg
gaacgagcgt cggtggataa agtcaatcac ttcaaattcg ctgctcagac accagagttg
gcggtacccg ttggagttga tccagcagag tttcttaggc ctctggttga cagtgaagaa
ggggtggatg gggtgaagat taacctggag catggcacta ctactctggc tttcaagttc
cagcatgggg tgatggtggc tgttgactcc agagcatctg caggatctta cgtcagcact
cagatgttta agaaggtgat cgagataaac ccctttctac ttgggacaat gtcaggcagt
gctgccgact gtgtctattg ggagagagtc ttggccaagg agtgcaggat atacaatctg
aggaacaagg agaggatctc agtatctgct gcctctaagc tccttgccaa catggtggtc
aactacagag ggatgggtct ctccatgggc acaatgatat gtggctggga caagaagggt
cctgggctgt attacgttga tgacaatggt ctgagactgt gtggaaacat gttctccact
gggtctggta acacctacgc ctatggtgtg atggacagcg gctaccgata tgacttgtct
gttccagaag cctatgattt ggcacagagg gccatctttc atgcaacaca cagagatgct
tactctgggg gaacagtcaa catgtatcac atgagagaga ctggttggat caaagtttct
caagaggatg ttggcgacct gtaccaccgc ttttacaatg acaagaagtg aatctggccc
gttgtaacta tattcacagt cacatcaata ccatacaata catcatgaac atagtcaaat
aagtatatca aatcataagg atcatggggc catgaccttc tctcaggcaa ccatatggca
gtcactg
```

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Table 6. Life history marker information: EG823395
Accession Number: EG823395
Agilent Probe ID: A_05_P357297
Gene ID: N/A
Protocol: 1: 30sec@94.0; 35: 30sec@94.0, 30sec@57.0, 30sec@72.0
Primer Forward: 5'-AAGACGGAATCTGGGAGTGG
Primer Reverse: 5'-AATGGGTCTGATGCCGTCTG
Product Length: 123bp

Full Sequence
```
ggggacttca tgaagtacaa cttggcctca catcgctata aaacgtcagc tgtatctcca
cttgtagaca ttcaactcca aatccccgac tcaacaggca gggcacccct aaagggagta
acatctggca tctcatggga agtcgatgtt gaaaagatga tgaacacctt ggcaagaaag
gcccagcaga ggaggtatta ggtgaacacc ttgacaagaa aggccctgca gaggaggtat
taggtgaaca ccttgacaag aaaggcccag cagaggaggt attaggtgaa caccttggca
agaaaggccc agcagaggag gtattaggtg aacaccttgg caagaaaggc ccagcagagg
aggtattagg tgaacacctt ggcaagaaag cccagcaga ggaggtcaga aggcatgcag
agcaatgggt gcagatgatg ttattataaa cagcagagaa gagcagatat ttatccatgg
atttaagaag aatgtctgga ttggtacata taaaaaagac ggaatctggg agtggattgg
cataacacta tttaaaccta cctattggat ggaaggggaa ccaaataaca tgcatgacga
gtgtgttgag atctcacaga cggcatcaga cccattgaag agttggaagt ttgcaccatg
tgctattcca aaatactggg tgtgtgaaaa accgttcacc ccgtagtcta attataatag
ctaaatgtaa ttccctatct tatagagttg aactggtttt ccctgtgtta ggctgaacct
gtttttgttt ctatttgtaa ctacattgaa aga
```

Table 7. Life history marker information: BT045799
Accession Number: BT045799
Agilent Probe ID: A_05_P260975
Gene Symbol: cd83
Gene Name: cd83 protein
Protocol: 1: 30sec@94.0; 30: 30sec@94.0, 30sec@57.0
Primer Forward: 5'-CGTCTGCATTCTAGCTGCCT
Primer Reverse: 5'-AGCTTGTACCACCTCACTGC
Product Length: 153bp

Full Sequence
```
tctcgatcat cgtcttcatt gctgtagttc cacaaatatg tttttttcaac tcgtctgcat
tctagctgcc tccgtgcaat gtgggctcac acagaatatg cctacccagg aagtgaagtc
aatttgtgga gaggactcaa ttctgaaatg taaagcaata tgtaagcgtg gggtccagta
ccgggcagtg aggtggtaca agctaggtga ggagccctct gataaggagt ctggtctatt
gatgaagagg ctatcaccta aaagcaccac cctatggtac gccggtctgg agcgggaagt
ggaacttttg gctgatgatt ctttcgatat cttgctgccc aatgtaacgg ctgttgatag
cgggaggtac aagtgtctcc tggcagcacc tgtaggagag cagaaccagg aggggcaggt
tcacctcaga gtgacaggtt gccttgagtc tacagaccaa tcagaagaaa gggataccat
cctagttctt tccattgtgg ggcttgtggc ggcattgctg atattcacca tcagctatgt
catcctaagg aatatgttat tgcaaaggag taagaagtat ccacaagaac cacttctaga
tgcacccctt gagaagaaag atgtaatgtt gatttacact ccggggccaa actggtccag
acagggttcc ataaaacatg tctgtgtgtg agagcctgtt ctgctcattg tatacttgaa
tgtgaagagc aaatgcagag aaagactagg gcacatttga agctgagcaa cgagtctatc
acgctgacca caccgcttgt gttatgtgcg tgagtgtggc aaaataaatg tacacatacc
tgttattcaa caatttcatc caaactgctc gcgcgcatca acgagcgcct gcgtagcaag
gctctaaaat agacgcttta tgcgctgcaa gtccactttc tcccatctcc tcattgggtt
ttcagagcat atacccacat gggtgattga aagatgaact gcgatccaca ctctagccca
gttggtggtg gtaatgcacc ttaaagttgg ttgtcaaacg ccatataaag tcagaagaag
actgacggag gagagattac tagaaacaaa ctaagtttac cctttttatct gtggattaat
tgtcagagta gaggaccttg tgtatttcag gtaaaataac aacccaatac ttatactttc
caggagaaga gttcaccact agagcagatg gaggcagaca accctctggg ttagaactaa
gatgtttagg cttttcaaag aaatgaaaca gaaacagtag cctacaccgt ttcactcagg
cagtcaaaca gcagtacaaa gtcaggctag aacagcagtt ctccagcaat gattgctcgt
ctgcagcatt cacacgcctg tgatgcgcaa gcagcagggc ctggtgaagg acccacaat
ctaagcccca agatctaacc tcaataagga gcttcaggaa ggggtgcagg aatggcctac
gatgaggggt cattctggag ggttgctcca tggcaacgtg ggggttaata tcatttacat
ttgagtcaac ccagaattga agtcgacatt gactttgaac tgggaaatga cccatgtgta
cagaactgat cttttagaat tgcctaattc aattcctgta ctgactggta ttgaaataaa
attgattcaa ttgcctccaa gcaaaatgaa agtgggcttt ggtgaaagt aattggcaga
aaatctatca ggttttttaaa atattatcag caattctgtg gacacattga aaataaagaa
gccttgtaaa taaaaaaaaa aaaaaaaaaa aaaaaaaaa aaaaaaaaaa ga
```

Table 8. Life history marker information: NM_001141601
Accession Number: NM_001141601
Agilent Probe ID: A_05_P358337
Gene Symbol: ddn1
Gene Name: Duodenase-1
Protocol: 1: 30sec@94.0; 33: 30sec@94.0, 30sec@58.0, 30sec@72.0
Primer Forward: 5'-TGCCTTGCTGCTGGATGGGG
Primer Reverse: 5'-TCCCACTGCTTCTGGCAGACC
Product Length: 110bp

Full Sequence:
```
gggggctgga tcatttgaca agccctccaa acatgcacac cataaacaaa cttctactca
ctgttctcct gacctatctg ggacattcgg ttgcatttgg gggtcaaatc atcaatggga
aaaaagccaa gaggaattcc ttgcagtaca tggcctctgt gcagaacaat gagaagcaca
tctgtggagg attcctgatc actccagact ttgtgctaac agctgcacac tgtaacaaaa
gcaacttgag tgttgttctt ggtacccaca acatcaagat gggcctcggg aaagctgtca
gatacaatgt ggaacgcaaa tgcaaatcca actcatatga aaatgtaaag gatggtagtg
acatcatgct tctaaagctg tctaggaaag ttaaattaag caaatctgtg aaaaaagtga
ggcttccaac caaggataaa gtcctcaaac ccaacacaaa gtgccttgct gctggatggg
gcactacaac aacagacaga ccattcgttg acgaacttca agtggtggat gtggaagcca
ttgatctgaa ggtctgccag aagcagtggg accatgtcga ctttaatctt cctcccaatg
tcatctgtgc aggtggatac ccgaccaaca aaggcacatg tcaggggat tctggtggtc
ctttggtgtg caatggagag gcagtgggca tcgtctcctt caacatgaat ggaaactgtg
cctatcccaa cgtgcccaat gtttacactc agatttccaa gtacttaccc tggataaagg
aggtcatcaa caagcagtca tgttagaatg gatactttgt ttgctgcaaa attatgcctt
tggctattac acataactga tgatattact gattctttag tttgctgtta aactgtctaa
tgcattacca tgtactgtaa gggaaataaa tgtgttctta atcatg
```

Table 9. Life history marker information: BT047975
Accession Number: BT047975
Agilent Probe ID: A_05_P417167
Gene Symbol: N/A
Gene Name: N/A
Protocol: 1: 30sec@94.0; 40: 30sec@94.0, 30sec@58.0
Primer Forward: 5'-ACGTGTCGTGATGGCAGTAA
Primer Reverse: 5'-AATCCCCAAAGCAGCCAAGT
Product Length: 190bp

Full Sequence
```
gggggagatc gagaatattg cgacctcgtg cgacgtgcaa agggttgtag ggaagtcaga
atcttacaat tgagtgcaaa ataacccttt tcaggtatgg cgctatcaaa tgttctcgaa
atacctacat cgggatttaa tttcaagaac gtcgccagaa atgttgctct ggagggcctg
cttgagggag gacataccaa gacacttaag cctatgaaga ctgggaccac catagctgga
ctagtgtgca aggagggagt ggtactggga gcagacacgc gggccacctc cggtgaagtg
gtggctgata agatgtgtgc caagatccac tacatctccc ccaatatata ctgctgtggt
gcagggactg cagcggatac agagaagacc accgacctgc tctcctccaa cctcaccatc
ttttctatga acagcggcag gaacccacgt gtcgtgatgg cagtaaacat actgcaggac
atgctattca ggtaccgggg ccagatAggg gccagtctaa tcctaggagg ggtggactgt
accggtaatc acctctacac agtggggccc tatgggagca tagacaatgt gcaatacctt
gcaatggggt ctggcgactt ggctgctttg gggattctgg aggacaggtt caaacctaat
atggagatgg aggaggctaa ggagctggtc cgggatgcca tccactctgg catcatgagt
gacctgggct caggaaacaa catagatatc tgtgtcatca ctaaacaggg ggtggactac
atcaggccgt accaggagtc agagtacaaa gacaagaggc ggaagagata caagtatggc
acaggtacaa cgtctatttt gacagagaaa atagtccctc tggagctgga ggtggtgcag
aagacagtcc agcggatgga tactgcctga acctgaccac tctgaaacga cagagaggct
ggagacagtc acacagaggc taatcacaat attcagacat aaaataaatg aatacaagac
acctgataat gacaagactt gactactagg tgacactaca atgcaaagcc ataaaatcat
tgtgacataa agatcttagt tgaatgaact gtagtcagcg atacattttg aacaggtaat
aggcctaggc tagtgggtga aatattgtaa agtgattgat tgaataaaaa gtctacattc tt
```

Cₜ VALUE BRAIN	Beta-Actin	BT045799	BT050202	BT058447	NM_001141601	BT047975	NM_0023714	BT045730	EG823395
K1	11.43	25.49	21.38	15.70	29.36	23.83	27.25	27.22	25.10
K2	11.03	25.27	20.46	14.08	28.74	22.14	25.95	26.97	23.46
K3	10.99	26.22	20.83	14.11	29.47	22.27	27.55	27.09	27.28
K4	10.99	25.45	15.90	13.93	29.60	22.26	27.24	27.14	26.54
K5	11.02	25.05	21.10	14.28	29.24	23.20	23.93	27.22	24.70
K6	11.24	25.69	20.55	13.80	28.16	23.66	26.12	27.35	28.54
K7	11.84	26.11	20.98	14.06	29.09	22.47	25.08	27.96	27.23
K8	11.25	25.54	20.74	14.32	28.32	23.71	25.13	27.60	25.48
K9	11.22	19.23	22.05	12.75	26.30	22.60	27.08	17.77	26.06
K10	11.45	19.27	21.07	12.15	26.60	21.74	26.16	17.98	25.77
K11	11.11	17.79	20.05	11.90	24.44	20.79	26.45	17.97	24.37
K12	11.21	19.29	20.34	11.50	25.25	20.72	28.29	17.89	25.50
K13	11.34	18.35	21.24	11.76	25.30	21.23	-	18.11	24.93
K14	11.23	16.68	21.74	13.12	28.12	23.26	23.90	24.17	25.10
K15	11.83	18.24	21.18	11.57	25.53	21.36	22.94	17.89	25.58
K16	11.34	17.67	21.63	12.19	28.58	22.25	24.28	23.13	25.99
Cₜ VALUE Gill									
K1	11.57	22.43	18.56	23.69	19.59	21.79	21.05	30.02	22.43
K2	12.11	23.68	19.11	27.28	19.69	22.06	21.17	30.69	23.68
K3	11.72	23.54	18.80	30.79	24.40	21.36	20.68	30.34	23.54
K4	11.92	23.08	18.47	29.23	19.50	21.23	20.68	30.35	23.08
K5	11.68	23.69	18.49	28.62	20.08	22.22	20.83	29.71	23.69
K6	11.49	23.50	18.86	29.63	19.30	22.11	22.95	30.07	23.50
K7	11.65	24.04	18.45	31.26	19.63	22.08	21.06	29.96	24.04
K8	12.25	24.86	19.06	29.52	20.61	23.02	22.68	30.47	24.86
K9	10.25	22.43	16.89	23.53	21.16	18.34	18.85	-	24.39
K10	10.14	23.68	17.24	22.28	14.73	18.05	18.87	-	24.60
K11	10.15	23.54	17.21	21.97	13.79	17.98	19.17	11.43	22.86
K12	10.67	23.08	17.45	21.56	14.00	17.79	19.04	11.54	24.50
K13	10.53	23.69	17.43	21.66	14.26	16.91	18.88	11.72	23.57
K14	10.92	23.50	17.71	23.71	14.82	18.41	18.39	11.62	21.92
K15	10.92	24.04	17.97	22.00	15.09	17.92	18.75	12.19	23.48
K16	10.64	24.86	17.45	22.98	15.08	18.02	18.58	16.66	22.93

Table 10. Average Ct values for the 8 life history markers identified in this study for the Mossy Rock Hatchery samples. This table contains the average Cₜ values for the 8 life history markers identified in the Mossy Rock Hatchery Steelhead (MRHS) and Mossy Rock Hatchery Rainbow Trout(MRHR) compiled from qPCR data for the 8 life history markers.

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