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Discovery and validation of candidate smoltification gene expression biomarkers across multiple species and ecotypes of Pacific salmonids

Aimee Lee S. Houde[a,b], Oliver P. Günther[c], Jeffrey Strohm[b], Tobi J. Ming[b], Shaorong Li[b], David A. Patterson[d], Anthony P. Farrell[e], Scott G. Hinch[a], Kristina M. Miller[b,*]

[a]*Department of Forest and Conservation Sciences, University of British Columbia, Vancouver, British Columbia (BC), V6T 1Z4, Canada*
[b]*Pacific Biological Station, Fisheries and Oceans Canada, Nanaimo, BC, V9T 6N7*
[c]*Günther Analytics, 402-5775 Hampton Place, Vancouver, BC, V6T 2G6*
[d]*School of Resource and Environmental Management, Fisheries and Oceans Canada, Simon Fraser University, Burnaby, BC, V5A 1S6*
[e]*Department of Zoology and Faculty of Land and Food Systems, University of British Columbia, Vancouver, BC, V6T 1Z4*

Abstract

Early marine survival of juvenile salmon is intimately associated with their physiological condition during ocean entry and especially smoltification. Smoltification is a developmental parr–smolt transformation allowing salmon to acquire the trait of seawater tolerance in preparation for marine living. Traditionally, this developmental process has been monitored using gill Na^+/K^+-ATPase (NKA) activity or plasma hormones, but gill gene expression can be reliably used. Here, we describe the discovery of candidate genes from gill tissue for staging smoltification using comparisons of microarray studies with particular focus on the commonalities between anadromous Rainbow trout and Sockeye salmon datasets, as well as literature comparison encompassing more species. A subset of 37 candidate genes mainly from the microarray analyses was used for Taq-Man qPCR assay design and their monthly expression patterns were validated using gill samples from four groups, representing three species and two ecotypes: Coho salmon, Sockeye salmon, stream-type

*corresponding author
Email address: kristi.saunders@dfo-mpo.gc.ca (Kristina M. Miller)

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Chinook salmon, and ocean-type Chinook salmon. The best smoltification biomarkers, as measured by consistent changes across these four groups, were genes involved in ion regulation, oxygen transport, and immunity. Smoltification gene expression patterns (using the top 10 biomarkers) were confirmed by significant correlations with NKA activity and were associated with changes in body brightness, caudal fin darkness, and caudal peduncle length. We incorporate gene expression patterns of pre-smolt, smolt, and de-smolt trials from acute seawater transfers using a companion study to develop a preliminary seawater tolerance classification model for ocean-type Chinook salmon. This work demonstrates the potential of gene expression biomarkers to stage smoltification and classify juveniles as pre-smolt, smolt, or de-smolt.

Keywords: transcription, parr–smolt transformation, de-smolt, Na^+/K^+-ATPase activity, hatchery, aquaculture, body morphology, skin pigmentation

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1. Introduction

Beyond their cultural importance, salmonids can provide over a billion dollars annually to the economies of countries with recreational and commercial fisheries (e.g. Canada, Pinfold 2011). Yet, populations of several salmonid species are declining on the Pacific and Atlantic coasts, and lower early marine survival of juveniles is associated with these declines (Beamish et al. 2010, Friedland et al. 2003, Mills et al. 2013). To increase salmonid populations and augment fisheries, hatchery breeding programs are used (Fraser 2008). As well, aquaculture is used to alleviate some of the fishing pressure on wild populations (Naylor et al. 2000) and provide additional economic opportunities (Bostock et al. 2010). However, the success of both hatcheries and aquaculture is known to be limited by the physiological condition of the smolt life stage during the transition from freshwater to seawater (e.g. Chittenden et al. 2008, Stien et al. 2013). Consequently tools to measure the physiological condition of smolts are routinely used and improvements in them sought to inform culture and decisions for optimizing smolt performance.

All salmonid species begin their lives in freshwater as eggs, alevins, and fry, and then the juvenile anadromous forms become smolts to successfully outmigrate to seawater, where rapid bodily growth and increased reproduc-

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tive success are greatly improved over freshwater residence. A trade-off may be survival because of increased predation, variable prey availability, and other risks in the marine environment (Quinn 2005). The developmental process preparing salmonids for the transition from freshwater to marine habitats is termed smoltification or parr–smolt transformation, which is characterized by changes in behaviour, skin pigmentation, body morphology, and physiology (reviewed by Björnsson et al. 2011, McCormick et al. 1998, McCormick et al. 2013). Changes in behaviour include increased negative rheotaxis (i.e. downstream movement) and schooling (i.e. the loss of territorial behaviour). The schooling behaviour may lower the risks of predation in river and the early marine environment. Changes in skin pigmentation include acquiring silver skin pigmentation and dark caudal fin tips. Changes in body morphology include a more streamlined body shape, elongation of the caudal peduncle, and associated lower body condition and increased buoyancy. These changes in pigmentation and morphology may be adaptations to marine habitats as camouflage from predators and increased swimming performance in open water, respectively.

The physiological changes during smoltification are equally numerous, such as increased hemoglobin, metabolism, and seawater tolerance. Higher hemoglobin may increase the oxygen-capacity of the blood because seawater is typically lower in dissolved oxygen than freshwater (Seear et al. 2010). Higher metabolism may be to meet the increased energetic demands during smoltification and migration (Robertson and McCormick 2012). Of the physiological changes, acquired seawater tolerance may be the most important (Björnsson et al. 2011, McCormick et al. 1998, McCormick et al. 2013). Indeed, juvenile salmonids that are unprepared for increased salinity, i.e. pre-smolts that have not completed the parr–smolt transformation or de-smolts that have remained in freshwater too long and have reverted to a physiology more suited to freshwater, have greatly reduced survival and slower growth because of internal ionic and osmotic disturbances from the excess ions in seawater relative to freshwater. As a result, smoltification is regarded as a 'physiological window' for seawater tolerance, one that can narrow because of higher water temperature, which may have implications with global climate change (e.g. Bassett et al. 2018). Moreover, the physiological window may be altered in the culture relative to natural environment, with hatchery juveniles generally having lower seawater tolerance than wild juveniles (e.g. Chittenden et al. 2008, Shrimpton et al. 1994). Knowing the smolt status of juveniles in particular is critical for hatchery and aquaculture

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operations to optimize the timing the smolt release directly into seawater. Federal hatcheries guidelines in British Columbia, Canada suggest that the time of release should coincide with that of the wild migration (MacKinlay et al. 2004), but certain hatcheries may have a specific range of dates used every year. Altogether, hatcheries and aquaculture can benefit from tools that reliably measure the smolt status of salmonids for planning releases and modifying the culture environment, if necessary.

In general existing tools take advantage of known changes associated with smotification. For example, salmonids generally need to reach a critical body size prior to smoltification. Photoperiod and, to a lesser extent, temperature also drives smoltification (Björnsson et al. 2011, McCormick et al. 1998, McCormick et al. 2013). Since an increase in day length activates the light-brain-pituitary axis to release a cascade of hormones including growth hormone, insulin-like growth factor I, cortisol, and thyroid, these hormones can be monitored in plasma samples. Growth hormone and cortisol stimulate the development of gill ionocytes and their associated Na^+/K^+-ATPase (Evans et al. 2005, McCormick 1993), the activity of which can be monitored in gill samples. Thyroid hormones may be involved in the changes in behaviour and skin pigmentation, which are useful visual indicators of smoltification. All the same, smoltification research has mainly focussed on species and ecotypes that migrate to seawater after one or more years in freshwater, e.g. Coho salmon (*Oncorhynchus kisutch*), stream-type Chinook salmon (*O. tshawytscha*, see Bourret et al. 2016), Sockeye salmon (*O. nerka*), anadromous Rainbow trout (*O. mykiss*), Atlantic salmon (*Salmo salar*), and Brook trout (*Salvelinus fontinalis*). However, species and ecotypes that migrate to the ocean after less than a year in freshwater: e.g. ocean-type Chinook salmon (*O. tshawytscha*, see Bourret et al. 2016), Pink salmon (*O. gorbuscha*), and Chum salmon (*O. keta*), enter seawater at a smaller body size and may remain longer in estuaries than the other groups. In these species and ecotypes, smoltification may not depend on photoperiod and may be body size based (Clarke et al. 1992, 1994, Gallagher et al. 2013, but see Hoffnagle and Fivizzani 1998). Thus, tools to define smolt status have focussed on gill Na^+/K^+-ATPase activity and plasma hormone concentrations.

Recently, techniques for monitoring smoltification have shifted to candidate gill gene expression using quantitative PCR (qPCR) for hormones and their receptors (e.g. Hecht et al. 2014, Kiilerich et al. 2007), as well as the precursors to Na^+/K^+-ATPase (e.g. Nilsen et al. 2007, Piironen et al. 2013). In particular, the gill expression of Na^+/K^+-ATPase α-1 isoforms for

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'a' freshwater and 'b' seawater ion regulation (c.f. Richards et al. 2003, Shrimpton et al. 2005), which typically change reciprocally during smoltification, are compared. More recently, smoltification has been examined at the genomic level using microarrays (e.g. Robertson and McCormick 2012, Seear et al. 2010, Sutherland et al. 2014) which have identified gill expression patterns for the upregulation of biological functions such as ion regulation, metabolism, oxygen transport, growth, structural integrity (e.g. collagen), calcium uptake (i.e. nutrient limitation for growth), and immunity, as well as downregulation of immunity and a few ion regulation and hormones. The upregulation of innate immunity is suggested as a preparation for exposure to new pathogens in marine environments (Boulet et al. 2012), while the downregulation of anti-viral immunity (Sutherland et al. 2014) is suggested to be due to the suppression by cortisol (Lemmetyinen et al. 2013). Despite these recent advances, it is not known if expression patterns of specific genes for smoltification can be reliably applied across salmonid species and different ecotypes.

Therefore, our objective was to discover candidate genes for smoltification and validate a subset of these genes using new samples from multiple species with different ecologies. To this end, we used mapping approaches to discover candidate smoltification genes by a meta-analysis of microarray gene expression patterns across studies. In particular, we focused on a comparison between anadromous Rainbow trout (Sutherland et al. 2014) and in-house Sockeye salmon datasets, as well as mining the literature for a wider collection of salmonid studies based on gene names. We then selected a subset of candidate genes for validation. These genes were developed into TaqMan qPCR assays and tested for expected gene expression patterns using various hatchery and wild sources of gill samples from Coho salmon, Sockeye salmon, stream-type Chinook salmon, and ocean-type Chinook salmon. We used the Fluidigm BioMark™ HD platform for measuring gene expression, a high throughput microfluidics-based technology that can individually quantify 96 assays across 96 samples at once. We focused on these four groups because of their population declines in Southern British Columbia (BC), Canada and subsequent hatchery supplementation (DFO 2013, Beamish et al. 2009, Noakes et al. 2000). In particular, the Sockeye salmon were from the endangered population of Cultus Lake, BC (COSEWIC 2003).

We hypothesize that a suite of biomarkers will be consistently associated with the smoltification process across species and ecotypes, and the specific level of activation of this smoltification biomarker panel alone could predict

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smolt status. As such, the present study would mark the first step in a process by identifying biomarkers that change with monthly smolt development. Our companion study examines the gene expression associated with seawater survival using pre-smolt, smolt, and de-smolt juveniles (e.g. ocean-type Chinook salmon, Houde et al. 2018). Using the smolt status for the trials of the companion study, here we explore a preliminary seawater tolerance classification model for ocean-type Chinook salmon. We examined how the seawater tolerance changed during monthly development for ocean-type Chinook salmon in the present study.

2. Materials and Methods

2.1. Candidate smoltification gene discovery

Smoltification candidate genes for gill tissue were identified using two approaches: (1) comparisons between a Sockeye salmon (*Oncorhynchus nerka*) cGRASP 44K internal microarray dataset of the Molecular Genetics Lab, Pacific Biological Station, Nanaimo, BC and the signatures of four external cGRASP microarray studies, i.e. 44K: Sutherland et al. (2014) and 16K: Robertson and McCormick (2012), Boulet et al. (2012), Lemmetyinen et al. (2013), and (2) a literature mining of significant gene names across published studies. Statistical analyses were conducted in R 3.1.2 (R Core Team). Methods for the Sockeye salmon microarray studies are described by Miller et al. (2011, 2009). The Sockeye salmon dataset is composed of 7 parr and 8 smolt samples for 27,104 features. This dataset was filtered with a 50% threshold for missing values and imputation of missing values was performed with the mean value over available samples. The Rainbow trout (*Oncorhynchus mykiss*) dataset (Sutherland et al. 2014) was downloaded from NCBI's Gene Expression Omnibus (GEO) public repository using the *GEOquery* R package (Sean and Meltzer 2007) and the processing steps of the authors were honoured.

For the direct comparisons between the internal Sockeye salmon and external microarray datasets, first significant features that separated parr and smolt for the Sockeye salmon dataset were identified using the robust empirical Bayes method of the *limma* R package (Ritchie et al. 2015). Features with a false discovery rate (FDR) ¡ 0.05 were considered significant. Next, to identify the top 100 features that separated parr and smolt for both species, significant features of the Rainbow trout and the Sockeye salmon datasets (both 44K platforms) were combined and analyzed collectively using a sparse

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independent principal component analysis (sIPCA) with the *mixOmics* R package (Rohart et al. 2017). These 100 features were examined for overlap with the identified significant features from the Sockeye salmon robust limma analysis described above. For the remaining three datasets using the 16K platform, both the 16K and 44K features were mapped to Atlantic salmon (*Salmo salar*) gene IDs from NCBI (see below for details), enabling comparisons across platforms. Similarly, the 16K features were examined for overlap with the identified significant features for both Sockeye salmon and Rainbow trout datasets.

Mining published literature involved discovering the overlap of significant gene names across five microarray studies that used the gill tissue of salmonid fishes, i.e. the four external microarray studies and Seear et al. (2010) that used a TRAITS/SGP microarray. Given the small number of studies and that different gene subunits invariably contribute to a protein, the study tables were visually examined for overlap using generalized gene names. Names that significantly separated parr and smolt in at least two microarray studies were recorded and were organized by smoltification biological function. Additional candidate gene studies ($n = 5$) examining the expression of specific ion regulation, hormone, and hormone receptor genes for gill tissue were also considered.

2.2. qPCR assay design

A Microsoft Access relational database containing mRNA sequences of salmonids was produced for qPCR TaqMan assay design, with the objective of developing assays that were gene specific and worked across several species. The database is available from the authors. Until recently, there were limited mRNA sequence data for the genus *Oncorhynchus*, so sequences were generated for Coho (*O. kisutch*), Sockeye (*O. nerk*a), and Chinook (*O. tshawytscha*) salmon from pools of six to eight individuals per species. Samples were enriched for GRASP microarray features using SureSelectXT (Agilent) and then sequenced using IonTorrent (Thermo-Fisher) following the manufacturer kits. Akbarzadeh et al. (2018) provides greater methodological details.

Sequences were mapped to the Atlantic salmon genome using the methods described by Houde et al. (2019). The database also contained maps of microarray features to the Atlantic salmon genome, specifically GRASP (16K, 32K, and 44K), TRAITS (version 1, 2.1, and 2.2) and SIQ. Altogether, database retrieval of mRNA sequences for the three species used microarray

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features IDs, gene IDs, gene names, or official gene symbols. Available sequences of several salmonid species, i.e. from the database and additional searches of the NCBI repository, were aligned using MEGA7.0.14 (Kumar et al. 2016). Sequences of closely related genes, e.g. duplicate genes, were also included in the alignment. Sequence regions that differed among closely related genes and were conserved among species were used as template in Primer Express 3.0.1 (Thermo Fisher) using the default setting optimized for the BioMark platform. Primer and TaqMan probe combinations that mismatched in at least one base pair at the 3' end of closely related genes were preferentially selected. Another check of potential gene specificity and workability across salmonids used NCBI Primer-Blast. One or two assays were designed per candidate gene.

2.3. Assay efficiencies

Assay design efficiencies were measured using pools of cDNA samples for six to nine salmonid species. Species-specific pools contained a mix of five tissues (gill, liver, heart, kidney, and brain tissue) from several individuals, with the exception of Rainbow trout and Atlantic salmon (only gill tissue). For each species, cDNA was diluted using a five-fold serial dilutions 1 to 1/625. Following Fluidigm BioMark™ prescribed methods, target cDNA sequences were enriched using a specific target amplification (STA) method that included small concentrations of the assay primers as well as three housekeeping genes: Coil-P84, 78d16.1, and MrpL40 (Miller et al. 2017). Specifically, for each reaction, 3.76 µL 1X TaqMan PreAmp master mix (Applied Biosystems), 0.2 µM of each of the primers, and 1.24 µL of cDNA. Samples were run on a 14 cycle PCR program, with excess primers removed with EXO-SAP-IT (Affymetrix), and diluted 1 in 5 with DNA suspension buffer.

The sample dilutions were run in duplicate and most assays in singleton following the Fluidigm platform instructions. Specifically, for sample reactions, 3.0 µL 2X TaqMan mastermix (Life Technologies), 0.3 µL 20X GE sample loading reagent, and 2.7 µL STA product. For assay reactions, 3.3 µL 2X assay loading reagent, 0.7 µL DNA suspension buffer, 1.08 µL forward and reverse primers (50 µM), and 1.2 µL probe (10 µM). The PCR was 50℃ for 2 min, 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s, and then 60℃ for 1 min. Data were extracted using the Real-Time PCR Analysis Software (Fluidigm) using Ct thresholds set manually for each assay. Linear models of the Ct values by the log of the dilutions for each group-assay set were produced using the plyr R package (Wickham 2011). Efficiency values

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were calculated using $10^{-1/\text{slope} - 1}$, and assays with values between 0.9 and 1.1 were ideal.

2.4. Validation samples

Juveniles representing the four groups (three species and two ecotypes) were collected monthly between November 2015 to May 2016 which spanned the smoltification period at four Salmon Enhancement Program (SEP) hatchery facilities. Nitinat Hatchery and Quinsam Hatchery on Vancouver Island, BC for Coho salmon (*Oncorhynchus kisutch*) and Chinook salmon (ocean-type, *O. tshawytscha*) (Table 1). Inch Creek Hatchery and Chehalis Hatchery on mainland BC, respectively, for Sockeye salmon (*O. nerka*) and Chinook salmon (stream-type, *O. tshawytscha*). In addition, wild (i.e. natural-born) juvenile counterparts of Coho salmon and Sockeye salmon were collected from the hatchery-supplemented source rivers and lakes using baited traps, dip nets, seines, or downstream fences. We targeted 20–30 individuals monthly for each set and the last collection date was as close as possible to the hatchery release date.

Fish were euthanized using buffered MS-222 (300 mg L^{-1}) then measured for length (± 0.1 cm) and mass (± 0.01 g). Body condition was calculated as 100 × mass ÷ length^3 (Fulton 1904). For the months of March, April, and May, Nitinat and Quinsam hatchery and wild juveniles were also digitally photographed (Nikon Coolpix AW110) using a camera stand with a light grey background and a length scale. Photographs were examined for skin pigmentation and body morphology (detailed by Houde et al. 2015) to generate LAB colour space values for anterior, posterior, and caudal fin regions, which were subjected to a principal component analysis, as well as morphology values using 21 landmarks which were subjected to a relative warp analysis using tpsRelw32 software (Rohlf 2017). Gill tissue from the right side was then placed into a cryovial and immediately frozen with liquid nitrogen or dry ice for Na^+/K^+-ATPase activity. Gill tissue from the left side (used for gene expression) was placed into RNAlater (Ambion) for 24 h before freezing or the whole fish was placed into a Whirl-pack bag and then immediately frozen between slabs of dry ice for later gill dissection. Tissues were stored at -80℃ until used for measurements.

2.5. Gene expression

We targeted a minimum subset of eight individual fish each month for gill gene expression and measured Na^+/K^+-ATPase activity (McCormick 1993)

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in around half of these samples. For gene expression, gill tissue was homogenized in TRIzol (Ambion) and BCP reagent using stainless steel beads on a MM301 mixer mill (Retsch Inc.). RNA was extracted from the homogenate using the 'No-Spin Procedure' of MagMAX-96 Total RNA Isolation kits (Ambion) and a Biomek FXP automation workstation (Beckman-Coulter). RNA yield was quantified using the A260 value and extracts were normalized to 62.5 ng mL^{-1}. Normalized RNA was reverse transcribed to cDNA using SuperScript VILO synthesis kits (Invitrogen). Normalized RNA and cDNA were stored at -80℃ between steps.

Gene expression was quantified using the assays and samples in singleton with STA enriched cDNA and the Fluidigm platform as described above. We included additional assays for candidate genes of thermal and hypoxia stress to assess cross-reactivity with candidate smoltification genes (data available from authors). Each gene expression chip contained three housekeeping genes (i.e. Coil-P84, 78d16.1, and MrpL40, Miller et al. 2017), dilutions of a group-specific cDNA pool, and a group-specific calibrator sample. For determining the optimal normalization gene(s) from the three housekeeping (HK) candidates, gene expression of each HK was first linearly transformed (efficiency minimum textsuperscriptCt – sample Ct). Values were then used in the NormFinder R function (Andersen et al. 2004) with groupings for constituents (e.g. hatchery location) by month to identify the gene or gene pair with the lowest stability (standard deviation). Sample gene expression was normalized with the ΔΔCt method (Livak and Schmittgen 2001) using the mean (for single gene) or geometric mean (for pair of genes) and the group-specific calibrator sample. Gene expression was then log transformed: $\log_2(2^{-\Delta\Delta Ct})$.

2.6. Statistical analysis for validating genes

Candidate smoltification genes were validated using a correlation analysis based on principal components analyses (PCA) across groups and within groups. Analyses were performed using R 3.4.4 at a significance level of $\alpha = 0.05$. Across the four groups, the expression values of all freshwater monthly gill samples were placed into a single PCA. Loadings and scores were visualized using the *fviz_pca function* of the *factoextra* R package (Kassambara and Mundt 2017). The PC axis best separating earlier and later months was identified. Candidate genes were ranked as biomarkers based on the significance of Pearson correlations between each gene assay and this PC axis. A second PCA and visualization was performed using the top 10 biomarkers with p

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< 0.05. Additional Pearson correlations examined the relationships between gene expression patterns (PC1 and PC2 of the second PCA) and Na^+/K^+-ATPase activity, as well as body length, mass, condition, morphology, and skin pigmentation. The same approach was used to examine each of the four groups separately. Student's t-tests also examined gene expression differences for all 37 gene assays between freshwater and seawater samples collected at the same time in late April for Nitinat ocean-type Chinook salmon.

2.7. Seawater tolerance classification model

We conducted a companion study using ocean-type Chinook salmon, for which juveniles were exposed to salinity treatments (freshwater, brackish, and seawater) during four trials covering the smoltification period (Houde et al. 2018). We categorized each trial as either pre-smolt, smolt, or de-smolt based on fish survival over several days after acute seawater transfer for a subset of individuals. The PCA pattern for the ocean-type Chinook salmon in the present study was applied to the freshwater juveniles of the companion study. Next, the gene expression PC axis thresholds that best separated three smolt statuses were identified by the maximum of Youden's J statistic (sensitivity + specificity − 1, Youden 1950) from receiver operating characteristic (ROC) analysis using the *pROC* R package. The resulting thresholds were used to classify fish as seawater tolerant (smolt) or intolerant (pre-smolt and de-smolt). This seawater tolerance classification model was then applied to the constituents of ocean-type Chinook salmon in the present study, to examine how seawater tolerance progressed with development on a monthly basis.

3. Results

3.1. Candidate smoltification genes using microarray comparisons

Limma analysis of the Sockeye salmon dataset identified 1,296 significant features that separated parr and smolt. By comparison, the Rainbow trout dataset had a published 400 feature parr–smolt signature. Combining the Sockeye salmon and Rainbow trout signatures using sIPCA to identify the top 100 features, there were 53 upregulated and 23 downregulated features that were also significant for the Sockeye salmon limma analysis (Table S1). Upregulated features were represented by six biological functions of

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metabolism ($n = 21$), immunity (8), oxygen transport (7), growth (7), structural integrity (5), ion regulation (4), and calcium uptake (1); the ion regulation and oxygen transport functions were predominantly at the top end of fold changes. Downregulated features were represented mainly by the function of immunity (17); other functions included ion regulation (1), growth (1), repressor of circadian rhythm (1), and unknown (3). Comparisons to three additional studies using the 16K platforms identified 7 upregulated and 14 downregulated significant features for parr to smolt for both species (Table S2). Upregulated features contained the functions of immunity (4), metabolism (2), and ion regulation (1). Downregulated features all contained the function of immunity.

3.2. Candidate smoltification genes using literature mining

Across the five published microarray studies, there were 15 upregulated and 6 downregulated genes for parr-to-smolt that were significant in at least two studies (Table S3). Including the studies that examined specific genes, several of these studies found that the ion regulation genes Na^+/K^+-ATPase α-1 were significant, specifically for parr-to-smolt there was an upregulation of isoform 'b' and downregulation of isoform 'a' (e.g. Nilsen et al. 2007, Piironen et al. 2013, Stefansson et al. 2007). There was also support for parr-to-smolt upregulation of other ion regulation genes, i.e. cystic fibrosis transmembrane conductance regulator I (e.g. Nilsen et al. 2007) and $Na^+/K^+/2Cl^-$ co-transporter (e.g. Nilsen et al. 2007, Stefansson et al. 2007). Although many plasma hormones change appreciably during smoltification (e.g. McCormick et al. 2013), the majority of the associated genes were unchanged for parr-to-smolt in the five microarray studies. However, directed qPCR parr-to-smolt revealed significant upregulation of glucocorticoid (cortisol) receptor (Kiilerich et al. 2007, but see Hecht et al. 2014), growth hormone (Hecht et al. 2014) and receptor (Hecht et al. 2014, Kiilerich et al. 2007, Stefansson et al. 2007), insulin-like growth factor and receptor (Stefansson et al. 2007), and thyroid receptor beta (Hecht et al. 2014). Also, there may be a significant downregulation for parr-to-smolt of prolactin receptor (Kiilerich et al. 2007, but see Hecht et al. 2014). Hence, we included assays to these genes in our test panel.

3.3. qPCR assays of select candidate genes

A total of 45 candidate smoltification genes were selected for TaqMan qPCR design: 25 upregulated and 20 downregulated for parr to smolt (Table

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2). The majority of the candidate genes ($n = 34$) were from the microarray analyses using both Sockeye salmon and Rainbow trout; 13 of these genes were also present in the literature review. Of the 34 genes, 28 were from the 44K analysis and mainly represented the extremes of the fold changes (Table S1), and six were from the 16K analysis and represented most of the available genes for this analysis (Table S2). Another two genes (S100A4 and FKBP5) were identified as highly differentially expressed by Sutherland et al. (2014) for Rainbow trout and were added by visual inspection of Sockeye salmon boxplots. The last nine genes were from the literature mining to fill eight biological functions, i.e. ion regulation, oxygen transport, metabolism, growth, calcium uptake, structural integrity, immunity, and hormones, so there would be at least two representative genes.

For efficiency testing, two assays were designed for the top 12 upregulated and 10 downregulated genes (set 1), remaining genes had one assay design (set 2) (Table S4). Eight out of 45 assays did not pass the efficiency criteria (i.e. CD3Z, GAPDH, GlyT2, NKCC, RGS5, TYK2, S100A4, and WHRN) across species, thus leaving 20 upregulated gene assays and 17 downregulated genes (Table S5). For the genes with two assays (set 1), the assay with the highest efficiency across species was selected for further analysis with validation juvenile samples.

3.4. Validation of smoltification genes

Across all four groups, a PCA of gill expression of 37 candidate genes identified that PC2 separated earlier and later months (Figure S1). PC1 was associated with group differences. The expression of 32 genes was significantly correlated ($p < 0.05$) with PC2 (summary in Table 3; statistics in Table S6–S10; data in Appendix 1–4). The top 10 genes based on correlation significance were represented by five upregulated biomarkers and five downregulated biomarkers in smolts (Figure 1).

Within each of the four groups, PCAs of gill expression of 37 candidate genes identified that PC2 separated earlier and later months (Figure S2). PC1 was associated with different sets, i.e. hatchery or wild and source population. Coho salmon had 26 genes, Sockeye salmon had 28 genes, stream-type Chinook salmon 21 genes, and ocean-type Chinook salmon had 30 genes with expression values significantly correlated with PC2 (Table 3). Notably, ocean-type Chinook salmon had metabolic and growth genes downregulated, and eight immunity genes upregulated during smoltification, opposite the prediction. Five biomarkers, i.e. CA4, CFTR-I, HBA, HBAt, NKAa1b, were

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consistently upregulated across all groups (Figure 2). An additional four biomarkers, i.e. CCL19, CCL4, IFI44, IL12B, were consistently downregulated for Coho salmon, Sockeye salmon, and stream-type Chinook salmon, but upregulated for ocean-type Chinook salmon.

Comparing Nitinat ocean-type Chinook salmon collected at the same time in late April from freshwater and seawater (about two weeks exposure to an estuary), 13 of the 30 genes were differently expressed between environments (Table 3; Table S11; Appendix 4). Interestingly, the genes predicted to be downregulated during smoltification were first upregulated in freshwater and only downregulated in seawater.

3.5. Relationship to gill NKA activity and body variables

Smoltification biomarker panels for each of the four groups, i.e. PC1 and PC2 using the top 10 genes (Figure 2), were significantly correlated with gill Na^+/K^+-ATPase activity (Figure 3). Body length and mass were positively correlated with PC1 for each of the four groups, as expected for juveniles growing during smoltification (Figure 2, statistics in Table S12). Body condition was also correlated with PC1 for ocean-type Chinook salmon, whereas it was correlated with PC2 for Coho salmon, Sockeye salmon, and stream-type Chinook salmon. Monthly body length, mass, and condition for all groups are presented in Figure S3.

Photographs to examine for correlations with skin pigmentation and body morphology were available only for the Coho salmon and ocean-type Chinook salmon. We considered the first four principal component axes (PCs) for skin pigmentation and two relative warps axes (RWs) for body morphology. For skin pigmentation, Coho salmon and Chinook salmon PC1 (53.8 and 37.3%) and PC2 (23.1 and 31.2%) were primarily associated with the posterior and anterior region brightness, respectively. Coho salmon PC3 (10.1%) was associated with body (posterior, anterior, and caudal fin) region yellowness and PC4 (5.7%) with caudal fin darkness; these traits were PC4 (6.5%) and PC3 (19.3%) for Chinook salmon, respectively. For body morphology, we considered the RWs for truncated to streamlined body shape, i.e. Coho salmon RW2 (12.6%) and Chinook salmon RW5 (6.6%), and caudal peduncle length, i.e. RW7 (4.5 and 3.9%), because of their relationship with smoltification (Björnsson et al. 2011, McCormick et al. 1998, McCormick et al. 2013).

The smoltification biomarker PC1s for both groups were positively correlated with caudal fin darkness (Figure 2). Coho salmon PC1 also had a positive trend for posterior brightness, as well as negative correlations with

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streamlined to truncated shape and caudal peduncle length and there was a trend for body yellowness. PC2 was correlated with anterior brightness. Chinook salmon PC1 was also positively correlated with caudal peduncle length. PC2 was correlated with posterior brightness, anterior brightness, body yellowness, and streamlined to truncated shape. Monthly skin pigment and body morphology are presented in Figure S4 and Figure S5, respectively.

3.6. Seawater tolerance classification model

The initial PCA of the gill expression using 37 candidate genes for ocean-type Chinook salmon in the present study indicated a pre-smolt to smolt pattern for PC2, and suggested a smolt to de-smolt pattern for PC3 (Figure 4a). Specifically, Quinsam May juveniles separated from earlier months along PC3. De-smoltification was also suspected for Quinsam May juveniles because of a decrease in gill Na^+/K^+-ATPase activity (mean ± SE, April 5.7 ± 0.7 and May 3.9 ± 0.4 µmol ADP (mg protein)$^{-1}$ h^{-1}, Student's t-test p = 0.028). PC3 was significantly correlated with the expression of 25 genes (Table S10).

A new PCA using the top 20 biomarkers ($p < 1\times10^{-5}$ for both PC2 and PC3) maintained patterns as expected (Figure 4b), and the freshwater individuals of a companion study (Houde et al. 2018) were projected into this PCA. These freshwater individuals were assigned a smolt status at the trial-level based on the survival (over several days) of other individuals from the same trial during acute seawater transfer. By maximum of Youden's J statistic and ROC analysis, the best PC2 threshold separating pre-smolt and smolt trials was 0.01, and the best PC3 threshold separating smolt and de-smolt trials was -1.40 (Figure 5). Individuals were classified as seawater tolerant (smolt) or intolerant (pre-smolt and de-smolt) using the areas defined by the thresholds.

The classification model was applied to the unknown smolt status ocean-type Chinook salmon of the present study. Nitinat and Sarita juveniles were largely classed as seawater intolerant pre-smolt from January to March and seawater tolerant smolt in April and May (Table 4). On the other hand, Quinsam juveniles were classed as seawater intolerant pre-smolt in February, seawater tolerant smolt in March and April, and largely seawater intolerant de-smolt in May.

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4. Discussion

Comparing gill gene expression for anadromous Rainbow trout (Sutherland et al. 2014) with our internal Sockeye salmon dataset, we discovered numerous common candidate smoltification genes. A subset of 25 upregulated and 20 downregulated genes, mainly representing the fold change extremes of the 44K analysis, were selected for TaqMan qPCR assay design. Of these 45, 20 upregulated and 17 downregulated genes passed our assay efficiency criteria and were then tested on monthly gill samples of Coho salmon, Sockeye salmon, stream-type Chinook salmon, and ocean-type Chinook salmon. We identified 32 common smoltification biomarkers; however, within each group variation of the smoltification biomarkers ranged from 21 to 30 genes. Nevertheless, in all cases, the smoltification biomarkers could be reduced to the top 10 genes and still retain good separation among the monthly samples along the smoltification axis. Indeed, smoltification gene expression patterns (i.e. PC1 and PC2 of the biomarker panels using the top 10 genes for each group) were confirmed by correlations with gill Na^+/K^+-ATPase activity. We recommend the smoltification biomarkers panels using the top 10 genes for the four groups (Figure 2). For species and ecotypes not examined in the present study, we recommend the smoltification biomarker panel using the top 10 genes for the groups combined (Figure 1).

4.1. Common gill smoltification genes among groups

Across the four groups, ion regulation (carbonic anhydrase 4, CA4 and cystic fibrosis transmembrane conductance regulator I, CFTR-I, and Na^+/K^+-ATPase α-1b, NKAa1-b) and oxygen transport (hemoglobin alpha, HBAt and HBA) genes were upregulated during smoltification. Another oxygen transport gene (Rhesus blood group-associated glycoprotein, RHAG) was also upregulated for Coho salmon and Sockeye salmon. CFTR-I and NKA1a-b are important ion regulators for gill ionocytes that help remove excess chloride and sodium ions, respectively, from the body of fish in seawater (Evans et al. 2005).

Furthermore, four immunity genes (C-C motif chemokine 19, CCL19; C-C motif chemokine 4, CCL4; interferon-induced protein 44, IFI44; and interleukin-12 beta, IL12B) were downregulated during smoltification for Coho salmon, Sockeye salmon, and stream-type Chinook salmon, but upregulated for ocean-type Chinook salmon (elaborated below). Yet, these four genes had lower expression in seawater than freshwater for ocean-type

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Chinook salmon. The majority of immunity genes (300 out of 360), such as chemokines, can be downregulated during seawater acclimation, possibly because of a trade-off between the energetic costs of osmoregulation and pathogen resistance in seawater (Johansson et al. 2016). These eight genes were predominantly at the top end of upregulated and downregulated genes (based on fold change) in the 44K analysis, but were not detected in the 16K analysis. The upregulated genes and chemokines were also identified by literature mining. Four uncharacterized features showed downregulation in the 44K analysis, but limited sequence template precluded assay design. They may be worth pursuing should more sequence data become available.

The consistency of these ion regulator genes across groups suggests that the $Na^+/K^+/2C^-$ cotransporter (NKCC), also within gill ionocytes, may also be a good species-wide smoltification biomarker (see Nilsen et al. 2007, Stefansson et al. 2007). Unfortunately, our single assay for NKCC only worked for Rainbow trout, possibly because at the time we had limited sequence information; thus, we were not able to examine this gene for our target salmonids. Relative to the other ion regulators, carbonic anhydrase has received lesser research attention. Yet recently, carbonic anhydrase genes were under rapid genetic selection for osmoregulation of Rainbow trout introduced from high to low salinities (Willoughby et al. 2018). Carbonic anhydrase can be important for both acid-base and ion regulation because of the productions of H^+ and $HCO3^-$ needed for Na^+ and Cl^- exchange in gill tissue (Gilmour 2012, Havird et al. 2013). CA4 was the second most powerful single predictor of smoltification after CFTR-I using all groups.

Hemoglobin isoforms change from juvenile to adult types during smoltification of Coho salmon and Sockeye salmon (Vanstone et al. 1964). The adult type may have a higher oxygen affinity and weaker Bohr effect than the juvenile type, suggesting an adaptation to the lower oxygen tension of seawater than freshwater. Yet, Fyhn et al. (1991) found that the isoforms shifted after smoltification for stream- and ocean-type Chinook salmon, suggesting that they may be more body size dependent. However, our results, which show a contribution of hemoglobin genes to smoltification in stream-type and ocean-type Chinook salmon, suggest that hemoglobin may still have a smoltification role for Chinook salmon, although it may not be related to the isoform switching.

Our confidence in the smoltification biomarkers is strengthened by the gene expression similarities in response to higher salinity. In the companion study, we used these same candidate gene assays on juvenile ocean-type Chi-

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nook salmon exposed to freshwater (0 PSU), brackish (20 PSU), and seawater (28 or 29 PSU) for six days (Houde et al. 2018). Ion regulation genes (i.e. CA4, CFTR-I, and NKAa1-b) and an oxygen transport gene (i.e. HBA) that were upregulated during smoltification in the present study also had higher expression in brackish and seawater than freshwater in the companion study. Similarly, the four immunity genes (i.e. CCL19, CCL4, IFI44, and IL12B) that were downregulated during smoltification had lower expression in brackish and seawater than freshwater. Overall, the strongest gill smoltification biomarkers, consistent for all the Pacific salmonid groups examined, were likely in preparation for higher salinity and decreased dissolved oxygen in marine relative to freshwater environments.

4.2. Different gill smoltification genes among groups

Beyond ion regulation and oxygen transport, gene expression patterns for the remaining six upregulated biological functions were dependent on the group or did not fit the prediction based microarray or literature information. In particular, three metabolic genes (NADH dehydrogenase 1 beta subcomplex subunit 2 and 4, NDUFB2 and NDUFB4, and mitochondrial pyruvate carrier 1, MPC1) were generally upregulated for Coho salmon, Sockeye salmon, and stream-type Chinook salmon, but downregulated for ocean-type Chinook salmon. Expression of metabolic genes can be related to body growth (Salem et al. 2007), and importantly photoperiod in known to influence growth of stream-type Chinook salmon but not ocean-type Chinook salmon (Clarke et al. 1992, 1994). Conceivably, metabolic gene expression may differ as a result of photoperiod dependence, but a mechanistic link would need to be found.

Three growth genes (monocarboxylate transporter 10, SLC16A10; elongation factor 2, EEF2; and 60S ribosomal protein L31, RPL31) were also generally upregulated for Coho salmon or stream-type Chinook salmon, but downregulated for Sockeye salmon or ocean-type Chinook salmon even though these two groups also continued to grow. Thus, elongation factors and ribosomal genes may not be consistently upregulated during smoltification, e.g. downregulation of elongation factor 1B and upregulation of ribosomal proteins (Lemmetyinen et al. 2013), downregulation of ribosomal proteins (Seear et al. 2010), and mixture of up and downregulation of ribosomal proteins (Boulet et al. 2012, Robertson and McCormick 2012).

The structural integrity gene (beta actin, ACTB) did not change with smoltification for Sockeye salmon and stream-type Chinook salmon. Hecht

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et al. (2014) also found no change with ACTB for Rainbow trout. The calcium uptake gene (cytochrome P450 2K1, CYP2K1) was upregulated for Coho salmon and Sockeye salmon, but downregulated for stream- and ocean-type Chinook salmon. Another calcium uptake gene, protein S100-A4 (S100A4) had the largest parr-to-smolt difference in expression for the Rainbow trout microarray study (Sutherland et al. 2014); unfortunately, our assay for S100A4 did not work for Chinook salmon and Sockeye salmon, so this gene was not examined further. One gene each represented the structural integrity and calcium uptake biological functions. Future work should examine other structural integrity genes such as collagen, SPARC, or tropomyosin (e.g. Lemmetyinen et al. 2013, Seear et al. 2010) and develop an assay for S100A4 which works on a broader range of species to examine the consistency of regulation across species and ecotypes.

No across group support was found for any of the hormone genes and for just one of three immunity genes predicted to be upregulated during smoltification. The immunity gene FK506–binding protein 5 (FKPBP5) was upregulated for Coho salmon, Sockeye salmon, stream-type Chinook salmon, with a similar trend for ocean-type Chinook salmon. On the other hand, translocator protein (TSPO) was upregulated for Sockeye salmon and ocean-type Chinook salmon only, and c-type lectin domain family 4 member M (CLEC4M) was upregulated for stream-type Chinook salmon only. In contrast to the Sockeye salmon and ocean-type Chinook salmon examined in the present study, Atlantic salmon, Rainbow Trout, and Brook trout in previous studies (Boulet et al. 2012, Lemmetyinen et al. 2013, Seear et al. 2010, Sutherland et al. 2014) showed upregulation of c-type lectins 2 or 4M. Growth hormone receptor 1 (GHR1) was upregulated for Sockeye salmon and stream-type Chinook salmon but downregulated for ocean-type Chinook salmon. Glucocorticoid (cortisol) receptor 1 (NR3C1) was upregulated for Sockeye salmon (trend) but downregulated for stream-type Chinook salmon. Thyroid hormone receptor beta 1 (THRB1) was upregulated for both types of Chinook salmon only. Although plasma values of these hormones are well associated with smoltification across species (e.g. McCormick et al. 2013), our results can be added to other studies suggesting that the gene expression patterns of these hormones or their receptors are not necessarily in line with plasma patterns (e.g. Hecht et al. 2014, Kiilerich et al. 2007, Stefansson et al. 2007). Overall, the immunity and hormone gene expression patterns suggest that there are species and ecotype differences during smoltification, or that these genes are functioning outside of the smoltification process. Further

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studies should examine the reproducibility of these patterns across species and ecotypes.

Beyond the four immunity genes that were generally downregulated during smoltification described above, predicted downregulation of remaining gill genes depended on the group. In particular, ocean-type Chinook salmon generally displayed an upregulation of certain immunity genes that where then generally downregulated in seawater. Immunity genes appear to be downregulated during smoltification for certain species and ecotypes, e.g. Sockeye salmon, while other species and ecotypes may not have a downregulation of these genes until reaching higher salinity, e.g. ocean-type Chinook salmon. Furthermore, ion regulation Na^+/K^+-ATPase α-1a (NKAa1-a) and prolactin receptor (PRLR) were lower in seawater than freshwater for ocean-type Chinook salmon, similar to the results of Houde et al. (2018). Higher expression of both genes was associated with mortality in seawater (Houde et al. 2018), indicating that the expression should decrease for seawater acclimation (also see Flores and Shrimpton 2012).

4.3. Relationship to gill NKA activity

Elevated gill Na^+/K^+-ATPase activity has been associated with seawater survival of Atlantic salmon (e.g. Stich et al. 2015, Stich et al. 2016) and ocean-type Chinook salmon (Houde et al. 2018), as well as with the risk of predation for Rainbow trout (Kennedy et al. 2007). Similar correlations existed between log10 Na^+/K^+-ATPase (NKA) activity and the primary smoltification gene expression pattern (PC1) for Coho salmon (0.40), stream-type Chinook (0.43) salmon, and ocean-type Chinook salmon (0.43). The correlation was even stronger for Sockeye salmon (0.77), perhaps because the 44K candidate gene discovery analysis used this species. Although only moderate correlations are common between gene expression and protein activity (Kanerva et al. 2014, Schwänhausser et al. 2011), possibly because of post-transcriptional and post-translational modifications (Maier et al. 2009), changes in gene expression may be one of the first indicators of a physiological change or response (Feder and Walser 2005, Miller et al. 2017). Furthermore, high NKA activity prior to seawater entry may not be necessary if a juvenile can rapidly increase NKA activity once in seawater (Bassett et al. 2018, Madsen and Naamansen 1989). In support of this idea, we observed that ocean-type Chinook salmon smolts achieved a higher median NKA activity in seawater than in either pre-smolts or de-smolts (i.e. 10.2 vs. < 7.5 µmol ADP (mg protein)$^{-1}$ h^{-1}, Houde et al. 2018).

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4.4. Relationship to body appearance

Gill gene expression patterns were associated with skin pigmentation and body morphology changes during smoltification. Lower body condition, more streamlined body shape, elongation of caudal peduncle, increased body silvering, and darkening of caudal fin margins are commonly used indices of smoltification (Björnsson et al. 2011, McCormick et al. 1998, McCormick et al. 2013). Correspondingly, the primary smoltification gene expression pattern (PC1) for Coho salmon and ocean-type Chinook salmon was associated with caudal fin darkness, as well as a positive trend with body brightness for Coho salmon. Although the PC1 pattern for Chinook salmon was also positively associated with caudal peduncle length, it was negative for Coho salmon. As far as we are aware, this is the first study to find relationships between gene expression patterns and body appearance during smoltification. Conceivably, caudal fin darkness may be a proxy of smoltification across other species and ecotypes but we did not have photographs of stream-type Chinook salmon and Sockeye salmon to test this possibility. Further research should examine whether these patterns occur in additional species and ecotypes.

4.5. Seawater tolerance model

Our preliminary seawater tolerance classification model for ocean-type Chinook salmon incorporated the gene expression patterns of freshwater pre-smolt, smolt, and de-smolt trials from a companion study using acute seawater transfers (Houde et al. 2018). Similar to Di Cicco et al. (2018) for classifying viral disease states, we statistically identified the gene expression (PC2 and PC3) thresholds that best separated pre-smolt, smolt, and de-smolt trials to classify individuals as seawater tolerant (smolt) or intolerant (pre-smolt and de-smolt). Our preliminary model appears to detect the gain as well as the loss of seawater tolerance using smolt status. Nitinat and Sarita juveniles were seawater tolerant in April and/or May around the hatchery release times, while Quinsam juveniles that were seawater tolerant in March and April were seawater intolerant (de-smolt) around the release times in May. The de-smoltification of May Quinsam juveniles was also confirmed by lower Na^+/K^+-ATPase activity. Even so, our discovery process for the candidate genes focussed on smoltification, i.e. pre-smolt to smolt. Other genes (e.g. FKBP5, IFI44, NAMPT, and UBA1) and a longer sampling period into the summer may improve resolution between smolts and de-smolts.

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Similar seawater tolerance classification models may be produced for Coho salmon, stream-type Chinook salmon, and Sockeye salmon. Our preliminary model for ocean-type Chinook salmon used the freshwater smolt status at the level of the trial, with other individuals acutely transferred to seawater for measures of survival (Houde et al. 2018). A more direct approach of linking freshwater gene expression to seawater survival at the level of the individual would have been more powerful. For example, a small gill biopsy a few days before seawater transfer followed by a survival measure covering a few days after transfer, e.g. six days (Houde et al. 2018). Additional data are needed between individual gene expression and subsequent seawater tolerance to improve the model.

4.6. Conclusion

Ion regulation, oxygen transport, and certain immunity genes were the best gill smoltification biomarkers, with consistent changes across multiple populations samples in the four groups examined. These genes were mainly selected from the 44K microarray discovery analysis and represented the top end of upregulated or downregulated genes based on fold changes. The directional shifts in expression were also similar between exposure to freshwater and brackish or seawater (Houde et al. 2018), implying an important role for higher salinity acclimation. Smoltification gene expression patterns had significant relationships with gill Na^+/K^+-ATPase activity, as well as caudal fin darkness for both Coho salmon and ocean-type Chinook salmon. Metabolic genes were upregulated and immunity genes were downregulated for photoperiod dependent species and ecotypes, i.e. stream-type Chinook salmon, Coho salmon, and Sockeye salmon, but the opposite occurred for photoperiod independent species and ecotypes, i.e. ocean-type Chinook salmon. We have provided a preliminary seawater tolerance classification model of pre-smolt, smolt, and de-smolt for ocean-type Chinook salmon. To expand this model for classification to other species and ecotypes, additional individual-level data linking freshwater gene expression and its association to seawater survival are required. Beyond the gill smoltification biomarkers, we are also developing biomarkers predictive of other divergent stressors, e.g. general stress and imminent mortality (Evans et al. 2011, Jeffries et al. 2014, Jeffries et al. 2012, Miller et al. 2011); viral disease development (Miller et al. 2017); and salinity, thermal, and hypoxia stress (Houde et al. 2018), to support the development of a 'Salmon Fit-Chip' tool to rapidly and inexpensively assess the physiological condition of 100s to 1000s of fish.

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Table 1: Summary of samples sizes for the four groups collected from four hatcheries and their wild source counterpart. Presented is the number of juveniles of Coho salmon (*Oncorhynchus kisutch*), Sockeye salmon (*O. nerka*), Chinook salmon (stream-type, *O. tshawytscha*), and Chinook salmon (ocean-type, *O. tshawytscha*). Juveniles were collected from freshwater unless denoted by the symbol 'E' which denotes juveniles from an estuary where they were exposed to seawater for about two weeks. Digital photograph were collected for the Nitinat and Quinsam juveniles in March, April, and May. Nitinat wild Coho salmon were collected from Campass Creek, a neighbouring tributary of Nitinat River, which was smaller and thus more feasible for catching juveniles with traps than Nitinat River.

	November	December	January	February	March	Early April	Late April	May
Nitinat Hatchery								
Coho salmon (Nitinat River), age 1+								
Hatchery	20	-	20	20	20	20	-	20
Wild	30	-	30	30	30	30	-	20
Chinook salmon (Nitinat River), age 0+								
Hatchery	-	-	20	20	20	20	20, 20 E	20 E
Chinook salmon (Sarita River), age 0+								
Hatchery	-	-	20	20	20	20	-	20
Quinsam Hatchery								
Coho salmon (Quinsam River), age 1+								
Hatchery	30	-	30	30	30	30	-	30
Wild	30	-	30	35	30	29	-	30
Chinook salmon (Quinsam River), age 0+								
Hatchery	-	-	-	30	30	30	-	30
Inch Creek Hatchery								
Sockeye salmon (Cultus Lake), age 1+								
Hatchery	20	20	20	20	20	20	-	-
Wild	20	-	-	20	17	10	-	-
Chehalis Hatchery								
Chinook salmon (Chilko River), age 1+								
Hatchery	-	20	20	19	20	30	-	-
Chinook salmon (Upper Fraser Summer Red), age 1+								
Hatchery	-	20	20	20	20	20	-	-

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Table 2: Summary of the candidate smoltification biomarkers for qPCR assay design using gill tissue. Presented for each gene is the smoltification functional group, feature (probe) ID for the 44K cGRASP microarray, and Atlantic salmon (*Salmo salar*) gene ID. The symbol x indicates that the gene was significant for parr to smolt for a specified analysis. Stars represent genes that were added after visualization of Rainbow trout *(Oncorhynchus mykiss*) and Sockeye salmon (*O. nerka*) boxplots.

Gene symbol	Gene name	Functional group	Probe ID	Gene ID	44K analysis	16K analysis	Literature mining
Upregulated in smolt							
CA4	Carbonic anhydrase 4	ion regulation	C148R144	106569487	x		
CFTR-I	Cystic fibrosis transmembrane conductance regulator I	ion regulation	C161R157	100136364	x		x
NKAa1-b	Na^+/K^+-ATPase α-1b (seawater)	ion regulation	C230R144	100136390	x		x
NKCC	$Na^+/K^+/2Cl^-$ co-transporter	ion regulation	C188R143	112220018[1]	x		x
HBA	Hemoglobin subunit α	oxygen transport	C228R104	106601077	x		x
HBAt	Hemoglobin subunit α(true HBA)	oxygen transport	C109R104	100136572	x		x
RHAG	Rhesus blood group-associated glycoprotein	oxygen transport	C069R106	100136438	x		
MPC1	Mitochondrial pyruvate carrier 1-like	metabolism	C010R030	106612504	x		x
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	metabolism	C146R081	106569991	x		x
NDUFB2	NADH dehydrogenase 1 beta subcomplex subunit 2	metabolism	C037R160	106576359		x	x
NDUFB4	NADH dehydrogenase 1 beta subcomplex subunit 4	metabolism	C216R021	100196139	x		
RPL31	60S ribosomal protein L31	growth	C209R008	106582252	x		x
SLC16A10	Monocarboxylate transporter 10-like	growth	C230R050	106571314	x		
EEF2	Elongation factor 2	growth	-	100194965			x
CYP2K1	Cytochrome P450 2K1	calcium uptake	C247R082	106572755	x		x
S100A4	Protein S100-A4	calcium uptake	C153R120	100196458	x[2]		
WHRN	Whirlin	structural integrity	C105R124	106585216	x		
ACTB	Beta actin	structural integrity	-	100136352			x
TSPO	Translocator protein	immunity	C213R123	100286416	x	x	
RGS5	Regulation of G protein signalling 5	immunity	C212R121	106560296	x		
FKBP5	FK506-binding protein 5	immunity	C148R059	106565346	x[3]		
CLEC4M	C-type lectin domain family 4 member M	immunity	C010R062	106578890			x
THRB1	Thyroid hormone receptor beta 1	hormone	C139R155	100136934			x
GHR1	Growth hormone receptor 1	hormone	-	100136442			x
NR3C1	Glucocorticoid receptor 1	hormone	-	100380779			x
Downregulated in smolt							
NKAa1-a	Na^+/K^+-ATPase α-1a (freshwater)	ion regulation	C217R121	106610479			x
GlyT2	Na- and Cl-dependent glycine transporter 2	ion regulation	C017R076	106561903	x		
CCL4	C-C motif chemokine 4	immunity	C240R068	106585882	x		

Gene symbol	Gene name	Functional group	Probe ID	Gene ID	44K analysis	16K analysis	Literature mining
CCL19	C-C motif chemokine 19	immunity	C188R011	106585878	x		
IFI44	Interferon-induced protein 44	immunity	C260R153	106573916	x		
MS4A4A	Membrane-spanning 4-domains A-4A	immunity	C023R137	106605437	x		
PLK2	Serine/threonine-protein kinase PLK2	immunity	C164R090	100195918	x		x
CD3Z	T-cell surface glycoprotein CD3 zeta chain precursor	immunity	C241R010	106575734	x		
UBA1	Ubiquitin-like modifier-activating enzyme 1 X	immunity	C230R063	106566065	x		
EXO1	Exonuclease 1	immunity	C090R027	106576944	x		
NAMPT	Nicotinamide phosphoribosyltransferase	immunity	C259R043	106561705	x		
IL12B	Interleukin-12 beta	immunity	C095R005	106603888	x		
MCM4	DNA replication licensing factor MCM4-B	immunity	C124R129	106569128	x	x	x
TUBA8L2	Tubulin, alpha 8 like 2	immunity	C218R157	100194601		x	
FMNL1	Formin-like protein 1	immunity	C217R022	106601135		x	
TRA	T-cell receptor alpha	immunity	C123R016	106569062		x	
TYK2	Non-receptor tyrosine-protein kinase	immunity	C058R025	106597276		x	
WAS	Wiskott-Aldrich Syndrome protein	immunity	C052R071	106567248		x	x
RGS21	Regulator of G-protein signalling 21	immunity	C097R005	106598526			x[4]
PRLR	Prolactin receptor	hormone	-	100136497			x

[1] Unknown Atlantic salmon gene ID for $Na^+/K^+/2Cl^-$ co-transporter, so the Chinook salmon gene ID is given.

[2] Protein S100-A4 was identified as highly significant by Sutherland et al. (2014), checked visually for Sockeye salmon dataset using boxplots.

[3] FK506-binding protein 5 was identified as highly by Sutherland et al. (2014), checked visually for Sockeye salmon dataset using boxplots.

[4] Regulator of G-protein signalling was identified by literature mining, subunit 21 was identified from Sockeye salmon dataset using boxplots.

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Table 3: Summary of the gill smoltification gene expression patterns for the four groups. The expression values of the 37 candidate genes were subjected to principal component analysis (PCA) for all four groups and each group separately: Coho salmon, Sockeye salmon, stream-type Chinook salmon, and ocean-type Chinook salmon. Gene expression relationships with the main PC axis separating earlier and later months was examined. Student t-tests examined expression differences between freshwater and seawater Nitinat ocean-type Chinook salmon sampled at the same time in late April; estuary juveniles were exposed to seawater for about two weeks. Presented are the significant ($p < 0.05$) expression patterns: + for positive correlation with smoltification or higher in seawater and - for negative correlation with smoltification or lower in seawater. Trends ($p < 0.1$) are presented with a t in brackets. Pearson correlations and p-values are displayed in Table S6–S10; beanplots of the data are displayed in Appendix 1–4. Freshwater and seawater mean differences and statistics are displayed in Table S11; beanplots of the data are displayed in Appendix 4

Gene name	Assay name	All groups	Coho salmon	Sockeye salmon	Chinook salmon (s-t)	Chinook salmon (o-t)	Seawater
Upregulated in smolt (predicted)							
Beta actin	ACTB_v1		+			-	
Carbonic anhydrase 4	CA4_v1	+	+	+	+	+	+
Cystic fibrosis transmembrane conductance regulator I	CFTR.I_v1	+	+	+	+	+	+ (t)
C-type lectin domain family 4 member M	CLEC4M_v1	+		-	+	-	
Cytochrome P450 2K1	CYP2K1_v2	+	+	+	- (t)	-	
Elongation factor 2	EEF2_v1	+	+	-	+	- (t)	
FK506-binding protein 5	FKBP5_v1	+	+	+	+	+ (t)	
Growth hormone receptor 1	GHR1_v1	+		+	+	-	
Hemoglobin subunit α	HBA_v1	+	+	+	+	+	
Hemoglobin subunit α(true)	HBAt_v1	-	+	+	+	+	
Mitochondrial pyruvate carrier 1	MPC1_v1	+		+	+ (t)	-	
NADH dehydrogenase 1 beta subcomplex subunit 2	NDUFB2_v1	+	+	+		-	-
NADH dehydrogenase 1 beta subcomplex subunit 4	NDUFB4_v1	+	+	+	+ (t)	-	
Na^+/K^+-ATPase α-1b (seawater)	NKAa1.b_v2	+	+	+	+	+	
Glucorticoid receptor 1	NR3C1_v1	-		+ (t)		-	
Rhesus blood group-associated glycoprotein	RHAG_v1	+	+	+		-	-

Gene name	Assay name	All groups	Coho salmon	Sockeye salmon	Chinook salmon (s-t)	Chinook salmon (o-t)	Seawater
60S ribosomal protein L31	RPL31_v1		+	-		-	
Monocarboxylate transporter 10	SLC16A10_v1	+	+	+		-	
Thyroid hormone receptor beta 1	THRB1_v2			-	+	+	- (t)
Translocator protein	TSPO_v2	+		+		+	+
Downregulated in smolt (predicted)							
C-C motif chemokine 19	CCL19_v1	-	-	-	-	+	-
C-C motif chemokine 4	CCL4_v1	-	-	-	-	+	-
Exonuclease 1	EXO1_v1	-			-	-	
Formin-like protein 1	FMNL1_v1	-	-	-	-		
Interferon-induced protein 44	IFI44_v1	-	-	-	-	+	-
Interleukin-12 beta	IL12B_v1	-	-	-	-	+	- (t)
DNA replication licensing factor MCM4	MCM4_v1		-		-	-	
Membrane-spanning 4-domains A-4A	MS4A4A_v1	-			-		- (t)
Nicotinamide phosphoribosyltransferase	NAMPT_v1	-	-	-			-
Na^+/K^+-ATPase α-1a (freshwater)	NKAa1.a_v2	-	+	+ (t)			-
Serine/threonine-protein kinase PLK2	PLK2_v2	-				+	
Prolactin receptor	PRLR_v1	-	+	+		+	-
Regulator of G-protein signaling 21	RGS21_v1	-	+	-	-	+	+
T-cell receptor alpha	TRA_v1	-		-	-	+ (t)	
Tubulin, alpha 8 like 2	TUBA8L2_v1			-	- (t)	-	
Ubiquitin-like modifier-activating enzyme 1 X	UBA1_v1	-	-			+	-
Wiskott-Aldrich Syndrome protein	WAS_v1	-	-		-	+	-

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Table 4: Modelled seawater tolerance by monthly development for ocean-type Chinook salmon. The classification model used gene expression pattern thresholds for delineating seawater tolerant (smolt) and intolerant (pre-smolt and de-smolt). Month symbols are in chronological order of development and are the first three letters.

Month	Seawater tolerance		
Nitinat			
	pre-smolt	smolt	de-smolt
Jan	6	0	2
Feb	8	0	0
Mar	7	1	0
Apr	1	14	1
Sarita			
	pre-smolt	smolt	de-smolt
Jan	8	0	0
Feb	8	0	0
Mar	4	4	0
Apr	2	6	0
May	2	5	1
Quinsam			
	pre-smolt	smolt	de-smolt
Feb	18	4	0
Mar	0	14	0
Apr	6	22	0
May	4	2	20

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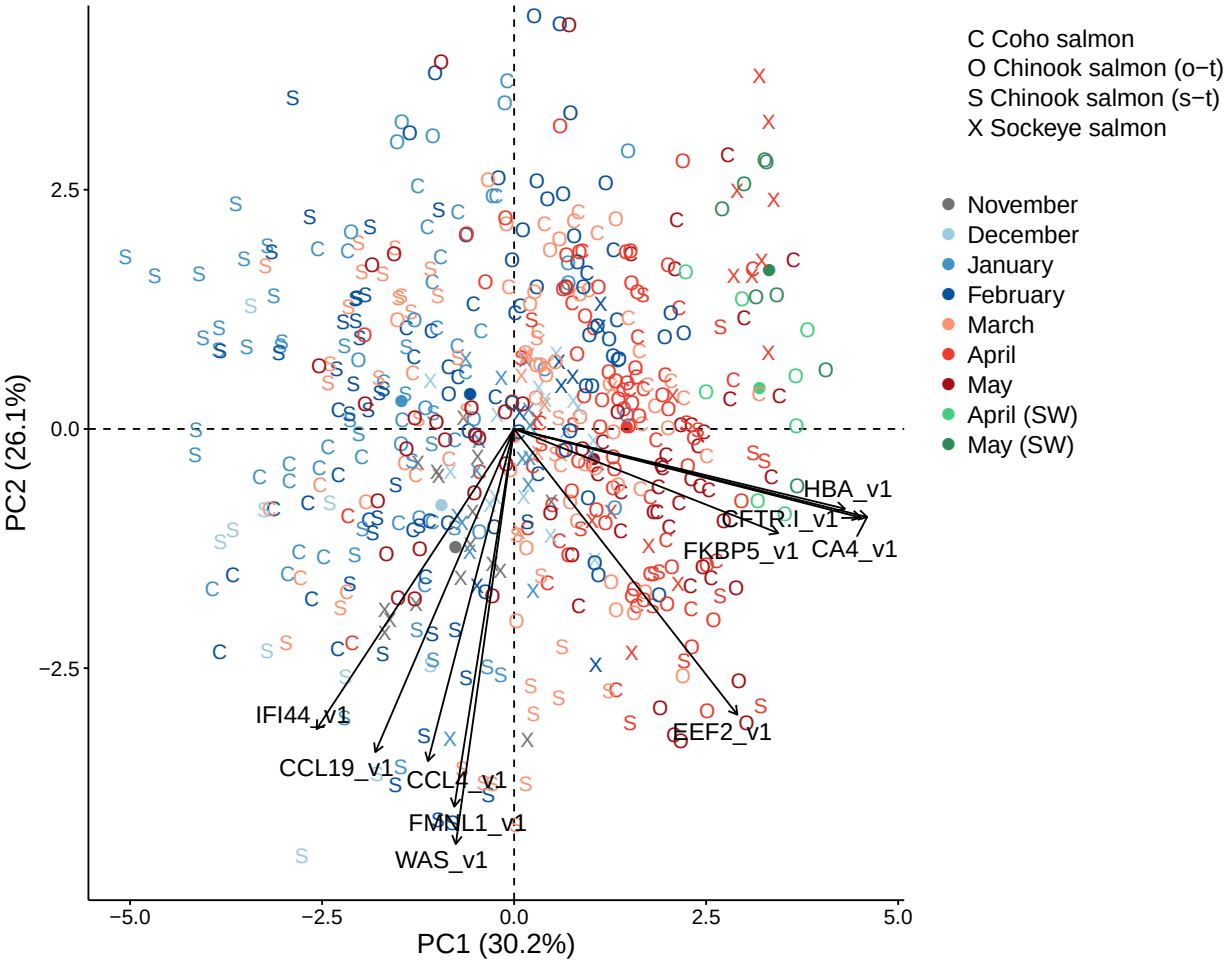

Figure 1: Canonical plots of the first two principal components of the top 10 biomarkers for smoltification using all four groups. Groups are Coho salmon (*Oncorhynchus kisutch*), Sockeye salmon (textitO. nerka), Chinook salmon (stream-type, *O. tshawytscha*), and Chinook salmon (ocean-type, *O. tshawytscha*). Percentage in brackets is the variation explained by the component. Monthly sample centroids are represented by the circle of the same colour. Black arrows represent loading vectors of the biomarkers. Legend symbol SW is for seawater and these individuals were not used in the PCA.

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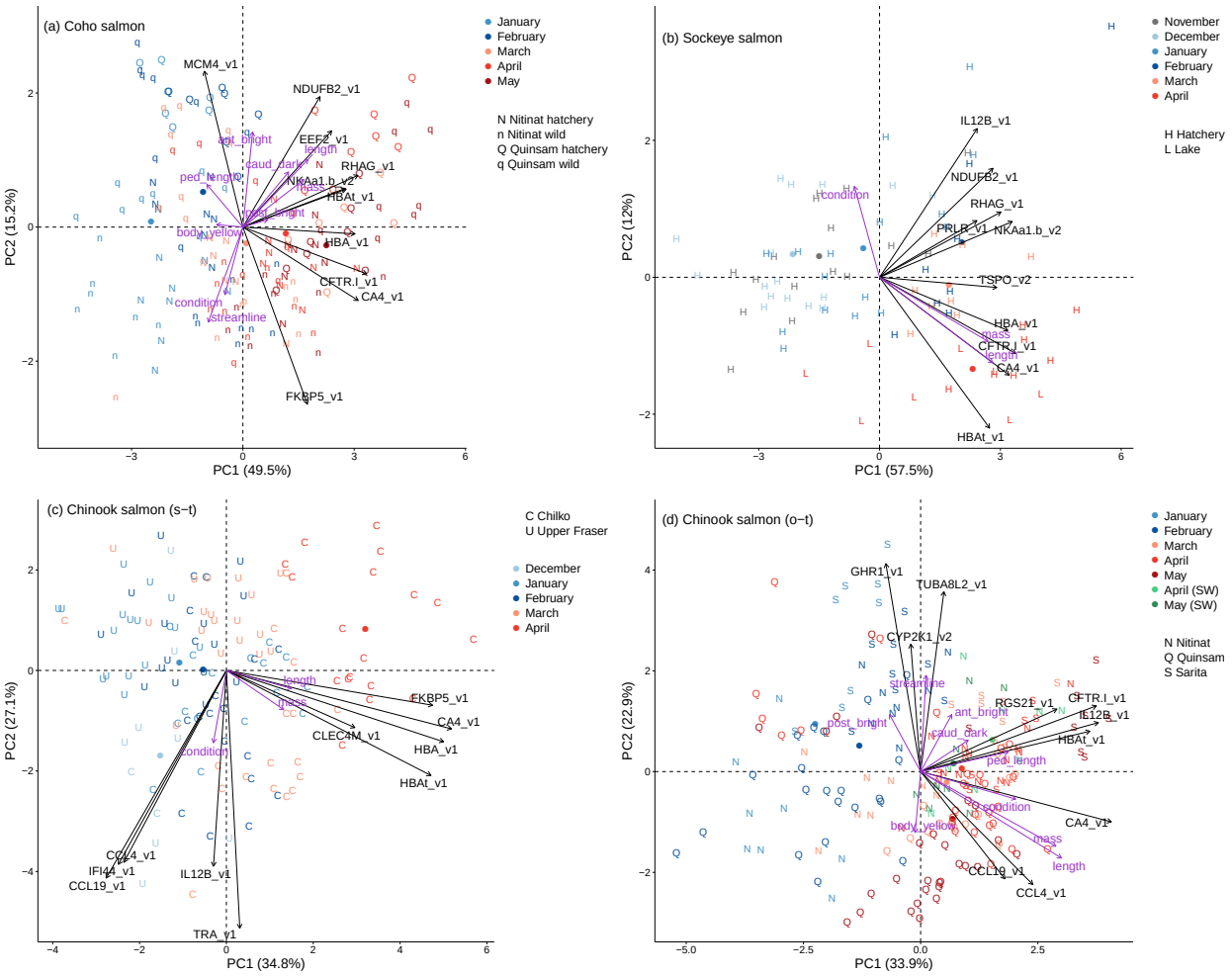

Figure 2: Canonical plots of the first two principal components of the top 10 biomarkers for smoltification using each of the four groups. (a) Coho salmon, (b) Sockeye salmon, (c) stream-type Chinook salmon, and (d) ocean-type Chinook salmon. Purple arrows represent loading vectors of the body variables. See Figure 1 legend.

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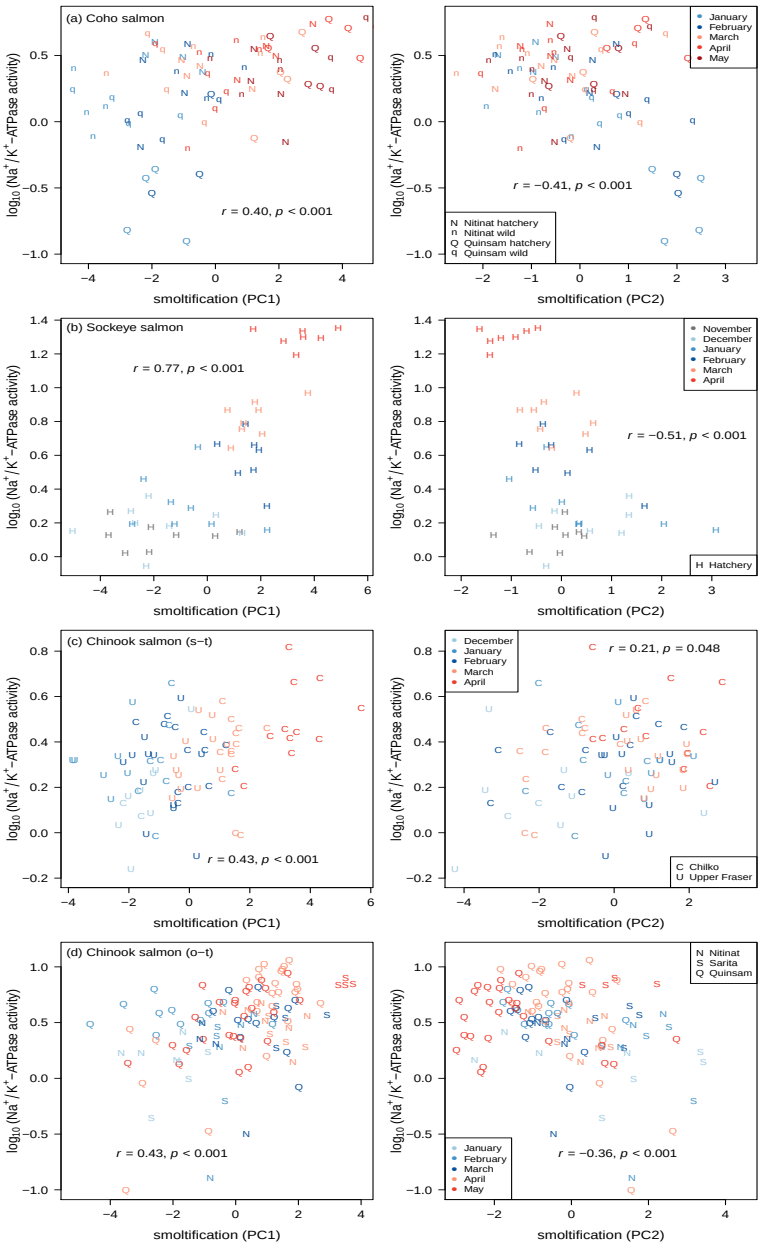

Figure 3: Relationships between smoltification gene expression patterns and Na^+/K^+-ATPase activity for the four groups. By row: (a) Coho salmon, (b) Sockeye salmon, (c) stream-type Chinook salmon, and (d) ocean-type Chinook salmon. Gene expression patterns used the top 10 biomarkers. Gill Na^+/K^+-ATPase activity units are µmol ADP (mg protein)$^{-1}$ h^{-1} , which are presented as $\log_{10}$. There were no samples for Sockeye salmon from Cultus Lake in April. Legend symbol SW is for seawater.

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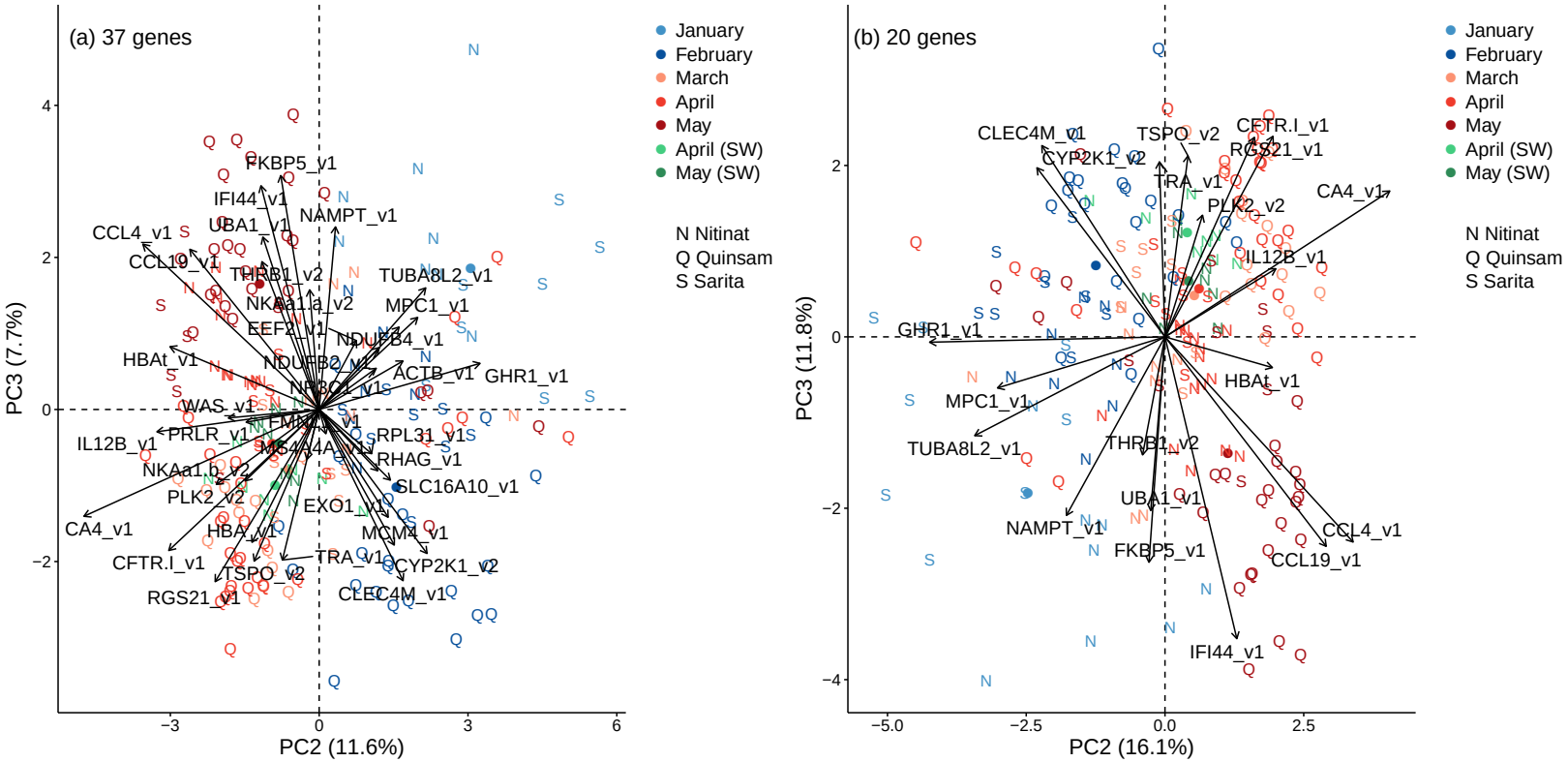

Figure 4: Canonical plots of the second and third principal components for the candidate genes using ocean-type Chinook salmon. Displayed are (a) all 37 genes and (b) the top 20 genes. See Figure 1 legend.

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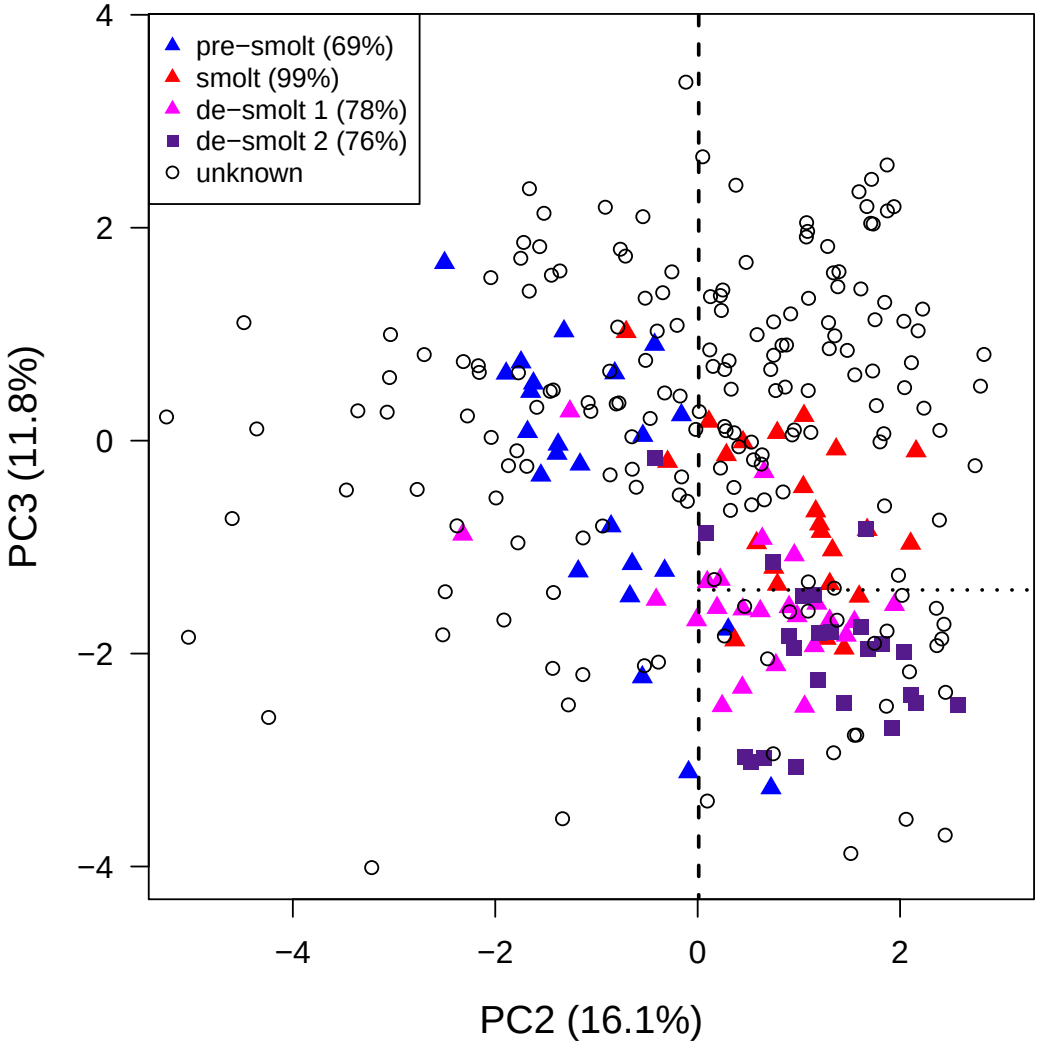

Figure 5: Seawater tolerance classification model using gene expression patterns of ocean-type Chinook salmon. Freshwater individuals with a smolt status are from the four trials of the companion study of Houde et al. (2018). Percentages for smolt statuses represent the trial seawater survival. The plot is based on the PCA using the top 20 biomarkers displayed in Figure 4b, and individuals of the companion study were projected into PC2 and PC3. Dashed lines represent the PC axis thresholds that separate (1) pre-smolt and smolt and (2) smolt and de-smolt. Thresholds were determined using Youden's J statistic and ROC analysis. Juveniles within the 'smolt' area were classified as seawater tolerant and juveniles within the 'pre-smolt' and 'de-smolt' areas were classified as not seawater tolerant.

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Supplementary Tables

Table S 1: Summary of the results from the 44K analysis using the combined Sockeye salmon and Rainbow trout datasets for gill tissue. The Rainbow trout dataset was from Sutherland et al. (2014). Sockeye salmon and Rainbow trout datasets were combined and analyzed collectively using a sparse independent principal component analysis to identify the top 100 features (i.e. Probe IDs) that separated parr and smolt. Presented are the 76 features that overlapped with the identified significant features of the separate Sockeye salmon robust limma analysis, which are ordered by fold change. Mean values are for parr and smolt of the Sockeye salmon dataset. Bold italics are gene names with Probe ID used for qPCR assay development; normal italics are gene names and Probe IDs matching to same gene ID.

Probe ID	Gene symbol	Gene name	Functional group	Mean for parr	Mean for smolt	$\log_2$ fold change	Fold change	Adjusted *p*-value
Upregulated in smolt								
C148R144	***CA4***	***Carbonic anhydrase 4***	ion regulation	-2.40	0.82	3.22	9.31	0.00
C209R008	***RPL31***	***60S ribosomal protein L31***	growth	-1.04	1.08	2.12	4.34	0.01
C228R104	***HBA***	***Hemoglobin subunit alpha***	oxygen transport	-1.66	0.32	1.98	3.96	0.00
C105R124	***WHRN***	***Whirlin***	structural integrity	-0.84	0.97	1.81	3.51	0.00
C069R106	***RHAG***	***Rhesus blood group-associated glycoprotein***	oxygen transport	-0.68	0.92	1.60	3.04	0.00
C109R104	***HBAt***	***Hemoglobin subunit alpha (true HBA)***	oxygen transport	-1.25	0.30	1.55	2.93	0.03
C011R079	*RHAG*	*Rhesus blood group-associated glycoprotein*	oxygen transport	-0.48	1.07	1.55	2.93	0.00
C212R121	***RGS5***	***Regulator of G-protein signalling 5***	immunity	-0.93	0.48	1.41	2.65	0.03
C161R157	***CFTR-I***	***Cystic fibrosis transmembrane conductance regulator I***	ion regulation	-1.65	-0.39	1.26	2.40	0.01
C230R144	***NKAa1-b***	***Na^+/K^+-ATPase α-1b (seawater)***	ion regulation	-1.22	0.04	1.26	2.39	0.01
C213R123	***TSPO***	Translocator protein	immunity	-0.99	0.10	1.09	2.13	0.00
C158R093	RGS5	Regulator of G-protein signaling 5 (gene ID 106584144)	immunity	-0.22	0.86	1.08	2.12	0.00
C230R050	***SLC16A10***	***Monocarboxylate transporter 10-like***	growth	-0.17	0.91	1.08	2.11	0.00
C146R037	CDA	Cytidine deaminase	metabolism	-1.12	-0.11	1.01	2.01	0.00
C010R030	***MPC1***	***Mitochondrial pyruvate carrier 1-like***	metabolism	-0.44	0.55	0.98	1.98	0.04
C247R082	***CYP2K1***	***Cytochrome P450 2K1***	calcium uptake	-0.40	0.51	0.91	1.88	0.04
C188R143	***NKCC***	***$Na^+/K^+/2Cl^-$ co-transporter***	ion regulation	-0.85	0.01	0.86	1.82	0.04
C111R149	SEC61G	Protein transport protein Sec61 subunit gamma	growth	-0.08	0.77	0.85	1.80	0.01
C109R066	TIMM17A	Mitochondrial import inner membrane translocase subunit Tim17-A	metabolism	-0.48	0.35	0.84	1.78	0.01
C146R081	***GAPDH***	***Glyceraldehyde-3-phosphate dehydrogenase***	metabolism	-1.07	-0.25	0.82	1.77	0.03

Probe ID	Gene symbol	Gene name	Functional group	Mean for parr	Mean for smolt	$\log_2$ fold change	Fold change	Adjusted p-value
C183R158	BCKDK	3-methyl-2-oxobutanoate dehydrogenase [lipoamide] kinase	metabolism	-0.61	0.19	0.80	1.74	0.01
C005R080	GLRX5	Glutaredoxin-related protein 5	oxygen transport	-0.52	0.24	0.77	1.70	0.02
C069R043	HIGD1A	HIG1 domain family member 1A	metabolism	0.05	0.81	0.76	1.69	0.02
C165R074	RNF222	RING finger protein 222	structural integrity	-0.62	0.13	0.75	1.68	0.03
C202R155	At5g50100	Uncharacterized protein At5g50100	growth	-0.16	0.58	0.74	1.67	0.00
C085R077	CTNS	Cystinosin	immunity	-0.09	0.64	0.72	1.65	0.02
C196R101	PSPH	Phosphoserine phosphatase	metabolism	-0.46	0.24	0.70	1.63	0.04
C221R144	COX7A2	Cytochrome c oxidase polypeptide VIIa-liver/heart	metabolism	-0.53	0.17	0.70	1.62	0.03
C213R046	PSPH	Phosphoserine phosphatase	metabolism	-0.42	0.27	0.69	1.62	0.01
C030R160	*TSPO*	*Translocator protein*	immunity	-0.71	-0.02	0.69	1.61	0.02
C010R060	MPC2	Mitochondrial pyruvate carrier 2	metabolism	-0.27	0.42	0.68	1.61	0.05
C117R042	MPC2	Mitochondrial pyruvate carrier 2	metabolism	-0.42	0.26	0.68	1.60	0.02
C156R115	MPC2	Mitochondrial pyruvate carrier 2	metabolism	-0.40	0.27	0.67	1.59	0.02
C057R083	PPT2	lysosomal thioesterase PPT2-A-like	metabolism	0.02	0.68	0.66	1.58	0.00
C111R151	HMGB3	High mobility group protein B3	immunity	0.30	0.93	0.63	1.55	0.02
C010R151	CYCS	Cytochrome c, somatic	metabolism	-0.48	0.15	0.63	1.55	0.03
C208R060	CHCHD7	Coiled-coil-helix-coiled-coil-helix domain-containing protein 7	metabolism	0.06	0.69	0.63	1.54	0.04
C142R087	ALAD	Delta-aminolevulinic acid dehydratase	oxygen transport	-0.60	0.02	0.62	1.54	0.03
C066R146	MRPS17	28S ribosomal protein S17	growth	-0.31	0.31	0.62	1.54	0.02
C167R048	BCKDK	3-methyl-2-oxobutanoate dehydrogenase [lipoamide] kinase	metabolism	0.08	0.70	0.62	1.54	0.00
C204R009	UBALD1	UBA-like domaing containing 1	immunity	-0.46	0.16	0.62	1.54	0.00
C175R095	CCDC56	Coiled-coil domain-containing protein 56	metabolism	0.04	0.64	0.61	1.52	0.02
C172R080	TOMM6	Translocase of outer mitochondrial membrane 6	immunity	-0.28	0.32	0.60	1.52	0.02
C224R050	PSMD9	26S proteasome non-ATPase regulatory subunit 9	growth	-0.21	0.38	0.60	1.51	0.03
C251R097	TMEM254	Transmembrane protein 254	structural integrity	-0.14	0.45	0.59	1.51	0.04
C216R021	***NDUFB4***	***NADH dehydrogenase 1 beta subunit 4***	metabolism	-0.22	0.37	0.59	1.51	0.03
C211R097	COX17	Cytochrome c oxidase copper chaperone	metabolism	-0.22	0.36	0.58	1.50	0.03
C238R060	MRPL37	39S ribosomal protein L37	growth	-0.18	0.35	0.53	1.44	0.04
C045R135	COX7A2L	Cytochrome c oxidase subunit VIIa-related protein	metabolism	-0.01	0.51	0.52	1.44	0.04
C101R159	TMED2	Transmembrane emp24 domain-containing protein 2 precursor	structural integrity	-0.10	0.41	0.51	1.42	0.02
C096R011	ALAD	Delta-aminolevulinic acid dehydratase	oxygen transport	-0.55	-0.07	0.48	1.40	0.01
C245R002	UQCRC2	Cytochrome b-c1 complex subunit 2	metabolism	0.03	0.47	0.44	1.36	0.05
C184R070	ARPC5L	Actin-related protein 2/3 complex subunit 5-like protein	structural integrity	-0.03	0.39	0.41	1.33	0.02

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Probe ID	Gene symbol	Gene name	Functional group	Mean for parr	Mean for smolt	$\log_2$ fold change	Fold change	Adjusted p-value
Downregulated in smolt								
C240R068	***CCL4***	***C-C motif chemokine 4***	immunity	1.19	-0.55	-1.74	-3.33	0.00
C017R076	***GlyT2***	***NA- and Cl-dependent glycine transporter 2***	ion regulation	0.84	-0.80	-1.64	-3.13	0.02
C188R011	***CCL19***	***C-C motif chemokine 19***	immunity	0.92	-0.59	-1.51	-2.86	0.00
C260R153	***IFI44***	***Interferon-induced protein 44***	immunity	0.40	-0.78	-1.18	-2.27	0.00
C124R109	At5g5010	Uncharacterized protein At5g50100	growth	0.41	-0.67	-1.09	-2.12	0.02
C023R137	***MS4A4A***	***Membrane-spanning 4-domains A-4A***	immunity	0.40	-0.58	-0.99	-1.98	0.02
C164R090	***PLK2***	***Serine/threonine-protein kinase PLK2***	immunity	0.31	-0.64	-0.95	-1.93	0.01
C241R010	***CD3Z***	***T-cell surface glycoprotein CD3 zeta chain precursor***	immunity	0.19	-0.74	-0.93	-1.90	0.01
C230R063	***UBA1***	Ubiquitin-like modifier-activating enzyme 1 X	immunity	0.49	-0.43	-0.92	-1.89	0.04
C244R027		UNKNOWN		0.29	-0.62	-0.91	-1.88	0.02
C090R027	***EXO1***	***Exonuclease 1***	immunity	0.11	-0.78	-0.89	-1.85	0.00
C245R123		UNKNOWN		0.25	-0.61	-0.86	-1.81	0.02
C259R043	***NAMPT***	***Nicotinamide phosphoribosyltransferase***	immunity	0.14	-0.62	-0.76	-1.70	0.04
C095R005	***IL12B***	***Interleukin-12 beta***	immunity	0.31	-0.43	-0.74	-1.67	0.00
C223R096	KDM5B	Lysine demethylase 5B	immunity	0.34	-0.36	-0.70	-1.62	0.04
C164R017	BHLHE40	Basic helix-loop-helix family member E40	repressor of circadian rhythm	-0.26	-0.91	-0.65	-1.57	0.03
C081R077	NFX1	Nuclear transcription factor, X-box binding 1	immunity	0.19	-0.44	-0.62	-1.54	0.03
C146R113	DTX3	Deltex E3 ubiquitin ligase 3	immunity	0.15	-0.46	-0.61	-1.52	0.02
C229R071	ASAP2	ArfGAP with SH3 domain, ankyrin repeat and PH domain 2	immunity	0.25	-0.36	-0.61	-1.52	0.01
C124R132	RIPK4	Receptor interacting serine-threonine kinase 4	immunity	0.31	-0.27	-0.58	-1.50	0.01
C057R087		Transposable element		0.05	-0.50	-0.54	-1.46	0.03
C124R129	***MCM4***	***DNA replication licensing factor MCM4-B***	immunity	-0.06	-0.55	-0.49	-1.41	0.04
C177R004	BCL10	B-cell lymphoma/leukemia 10	immunity	0.08	-0.40	-0.48	-1.40	0.04

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Table S 2: Summary of the results from the 16K signature analysis using the Sockeye salmon and Rainbow trout datasets for gill tissue. The Rainbow trout dataset was from Sutherland et al. (2014). Presented are the mapped 44K features that were significant for both Sockeye salmon and Rainbow trout, which are ordered by fold change. Bold italics are gene names with Probe ID used for qPCR assay development; normal italics are gene names and Probe IDs matching to same gene ID.

Probe ID	Gene symbol	Gene name	Functional group	16K study	Sockeye salmon		Rainbow trout	
					Log_2 fold change	*p*-value	Log_2 fold change	*p*-value
Upregulated in smolt								
C213R123	***TSPO***	***Translocator protein***	immunity	McCormick et al. 2012	1.04	<0.01	0.50	<0.01
C071R152	*TSPO*	*Translocator protein*	immunity	McCormick et al. 2012	0.70	<0.01	0.44	<0.01
C030R160	*TSPO*	*Translocator protein*	immunity	McCormick et al. 2012	0.64	<0.01	0.54	<0.01
C004R095	*TSPO*	*Translocator protein*	immunity	McCormick et al. 2012	0.61	<0.01	0.45	<0.01
C183R077	SGK3	Serine/threonine kinase 3	ion regulation	McCormick et al. 2012	0.48	<0.01	0.66	0.04
C057R039	CYCS	Cytochrome C, somatic	metabolism	Boulet et al. 2012	0.48	0.04	0.98	<0.01
C037R160	***NDUFB2***	***NADH dehydrogenase 1 beta subunit 2***	metabolism	McCormick et al. 2012	0.29	0.02	0.26	0.02
Downregulated in smolt								
C218R157	***TUBA8L2***	***Tubulin, alpha 8 like 2***	immunity	Lemmetyinen et al. 2013	-0.73	<0.01	-0.28	0.04
C217R022	***FMNL1***	***Formin-like protein 1***	immunity	Lemmetyinen et al. 2013	-0.62	<0.01	-0.30	0.01
C118R080	PBRM1	Polybromo-1	immunity	McCormick et al. 2012	-0.60	0.02	-0.29	0.01
C217R123	*TRA*	*T-cell receptor alpha*	immunity	McCormick et al. 2012	-0.58	0.05	-0.71	0.01
C123R016	***TRA***	***T-cell receptor alpha***	immunity	McCormick et al. 2012	-0.56	0.03	-0.81	<0.01
C189R144	*TRA*	*T-cell receptor alpha*	immunity	McCormick et al. 2012	-0.54	0.03	-0.74	0.01
C185R169	*TRA*	*T-cell receptor alpha*	immunity	McCormick et al. 2012	-0.52	<0.01	-0.63	0.01
C062R132	*FMNL1*	*Formin-like protein 1*	immunity	Lemmetyinen et al. 2013	-0.51	0.03	-0.39	0.03
C135R158	*FMNL1*	*Formin-like protein 1*	immunity	Lemmetyinen et al. 2013	-0.50	0.04	-0.35	0.01
C124R129	***MCM4***	***DNA replication licensing factor MCM4-B***	immunity	McCormick et al. 2012	-0.49	<0.01	-0.57	<0.01
C058R025	***TYK2***	***Non-receptor tyrosine-protein kinase TYK2***	immunity	McCormick et al. 2012	-0.46	0.01	-0.34	<0.01
C052R071	***WAS***	***Wiskott-Aldrich syndrome protein***	immunity	McCormick et al. 2012	-0.42	0.01	-0.77	<0.01
C236R165	*TYK2*	*Non-receptor tyrosine-protein kinase TYK2*	immunity	McCormick et al. 2012	-0.36	0.03	-0.42	<0.01
C131R157	*TYK2*	*Non-receptor tyrosine-protein kinase TYK2*	immunity	McCormick et al. 2012	-0.36	0.02	-0.40	<0.01

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Table S 3: Summary of the gene names associated with smoltification across published microarray studies using gill tissue. Presented are generalized gene names organized by smoltification functional group for microarray studies including Atlantic salmon (*Salmo salar*), Brook trout (*Salvelinus fontinalis*), and Rainbow trout (*Oncorhynchus mykiss*). The symbol x indicates that a gene name was significant for separating parr and smolt.

	Seear et al. (2010)	Boulet et al. (2012)	Robertson and McCormick (2012)	Lemmetyinen et al. (2013)	Sutherland et al. (2014)
species	Atlantic salmon	Brook trout	Atlantic salmon	Atlantic salmon	Rainbow trout
platform	TRAITS/SGP	cGRASP 16K	cGRASP 16K	cGRASP 16K	cGRASP 44K
Upregulated in smolt					
Ion regulation					
Na^+/K^+-ATPase α-1b (seawater)	x		x	x	x
Cystic fibrosis transmembrane conductance regulator I			x		x
Oxygen transport					
Hemoglobin (e.g. αand β)	x		x	x	
Metabolism (oxidative phosphorylation)					
NADH dehydrogenase	x	x	x		x
ATP synthase	x	x		x	x
Cytochrome c and cytochrome c oxidase	x		x	x	x
Glyceraldehyde-3-phosphate dehydrogenase		x			x
Structural integrity					
Actin (e.g. βactin, cytoplasmic actin-1)			x	x	
Collagen (e.g. α1I and α1X)	x			x	x
Myosin (e.g. tropomyosin α1)	x	x		x	
Body growth					
Elongation factor 2	x			x	
Glutamine synthetase			x		x
Ribosomal proteins (e.g. 40S, 60S)		x	x	x	x
Immunity					
C-type lectin (e.g. 2 and 4m)	x	x	x	x	x

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	Seear et al. (2010)	Boulet et al. (2012)	Robertson and McCormick (2012)	Lemmetyinen et al. (2013)	Sutherland et al. (2014)
CC Chemokine (e.g. SCYA113)	x				x
Downregulated in smolt					
Immunity					
CC chemokine (e.g. SCYA112)	x			x	x
Regulator of G-protein (e.g. 1)				x	x
Keratin (e.g. type 12, 13)	x		x	x	
Wiskott-Aldrich Syndrome protein			x		x
DNA replication licensing factor (e.g. MCM4-B)			x		x
Serine/threonine-protein phosphatase or kinase (e.g. PLK2)				x	x

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Table S 4: Summary of qPCR TaqMan assay designs for candidate smoltification genes. Presented are the forward, reverse, and TaqMan probe sequences, as well as the amplicon size. Assay names use the symbols described in Table 2 in main text. Two assays were designed for the top 12 upregulated and 10 downregulated genes (set 1), remaining genes had one assay design (set 2): v1 is version 1 and v2 is version 2.

Assay name	Forward	Reverse	Probe	Amplicon size (bp)
Upregulated in smolt				
NKAa1-b_v1	TGAAGAAGTGGTGGTTGGAGATC	GGCAGAGACAATACGCAAATCA	TGAAAGGAGGAGATAGAAT	79
NKAa1-b_v2	GCCTGGTGAAGAATCTTGAAGCT	GAGTCAGGGTTCCGGTCTTG	CCTCCACCATTTGCTCA	81
CA4_v1	GGTCATTTTGGTTTTGTACACAGTCT	CCTAGATATAGCTATCCACGTACT CACCTA	TGATACGTGGTATAGAAAAG	86
CA4_v2	CGTTACCTTGGCTCCCTGACT	CTTGAACACGGTCCAAACCA	TCCAAATTGCAACGAGG	63
CFTR-I_v1	GAGCTGTCAGAGAGGAAGTTCTCA	GCAGCGACTCTTCAACCTGAT	TGGTGCCCGAGGAC	61
CFTR-I_v2	ACGCCTGTCCAAAGATAGTGTCTA	GCAAAGCATTGCTCCATATCC	AGCGAGGATGTGGACG	72
RHAG_v1	GGTAGCTATGGCGATTGGATTT	AATGTATCGGGATCCCAAGGT	TGCTGCACTGATATC	61
NKCC_v1	GACACTACTGTGCTCTGTCCAAGAG	TCAACATGGCTAGGCCACAA	CCAAAAGATGTTGCCTGTAA	67
GAPDH_v1	GTGTCCACAGAACTTCACAGTGACT	TGGTCGTTGAGTGCAATTCC	ATCTTTGACGCCGGTGC	74
GAPDH_v2	CGGACATCCCACCAATCC	TGTTGCCTTGGAGTTCACTACAG	CACCAGAGAAAAAGAG	60
NDUFB2_v1	GCTTCTTACACGTGGACCTCAGA	GCCTGTACTGGGCCTCTATGTG	CGAAAAGCTGGTGGTGGA	77
NDUFB2_v2	TCATAGCGCTCGTTGTTGGTT	TGTTTTCTCAAAATGTCGTTCCA	TCGCCCTCTGTGTCAA	64
NDUFB4_v1	AAACAACCCGCACAGAAAAGA	GGGTTGGTGCGTGCATACA	CTTATTGAAGACCCTGCCTT	72
MPC1_v1	GCAGGCACTTTGGCACGTA	TGAGATACTCTCTGAACTCCTTGCTT	AGCTGTTGACCATCTTA	62
HBA_v1	GCCCTGGCTGACAAATACAGA	GAGCAGGAACTGGAGTCCAATG	ACCATCATGAAAGTCC	65
HBA_v2	GCTGCCCTGGCTGACAAA	GGAACTGGAGTCCAATGATGGA	ACAGATAAGACCATCATGAAA	63
HBAt_v1	TGGCTGACAAATACAGATAAGACCAT	TTGTGATAACAACAGAGCAGAAACAG	ATGAAAGTCCAAACTTG	75
ACTB_v1	GAAATCGCCGCACTGGTT	CGGCGAATCCGGCTTT	TTGACAACGGATCCGGT	58
ACTB_v2	GAAATCGCCGCACTGGTT	GACGCCCCACGATGGA	CCGGAGATGACGCGC	103
WHRN_v1	GGCTTTAGCAGGACTTTGCACTT	CATCTCCCTCCTGTTCTCTCCTT	AAAGAGGTGAAACGGAAGG	68
RPL31_v1	GAGTACACGGTCAACATCCACAA	CGAGGTGCCCTCCTCTTAAA	CGCATACATGGCGTCT	62
RPL31_v2	CGAAAGTTCGCCATGAAGGA	CCTTGTTCAGGCGAGTATCGA	TGGGAACCCCTGACGTG	64
SLC16A10_v1	ACTGCGGAGGAGCCTGAGTT	ACATGGCAGCCAGCATAACC	CACCCAGAAGGAGGATG	70
EEF2_v1	CCACCAAGATTTCCCAGATTGT	TGCAGGAATTCCCTCCTTGA	TGACACACGTAAACGC	68
CYP2K1_v1	ACCAGCCAAGACGTCACCTT	CAGACGTCAGTAGAGGTAACACATCA	CAGGGATACTTCATCAAA	73
CYP2K1_v2	GGAAGAACCTGCCCTACACTGA	GGATGGACATGGGTACAATGTTG	TGATCCATGAGACCCAAA	75
S100A4_v1	TGGCGCTGTGGACTTCAAG	AGGAAGCAGTTGCACATGCA	AGTACATCACCCTGATTGA	69
TSPO_v1	GGTGAAAACCTGGTACACCACTCT	TGGGAACACAGCATTATTTGGA	AACAAGCCGTCATGGC	65
TSPO_v2	CGATCTTCTTTGGAGCACACAA	AACAGCACCAGTCAACATCACTATC	TTGAAAATGGCACTCATAG	68

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Assay name	Forward	Reverse	Probe	Amplicon size (bp)
CLEC4M_v1	CGTGGCACTGAACTCTTATGCA	GGTACAGCCACTGTCACACTTGA	TGAGGGCTTCGATGAC	67
CLEC4M_v2	GGGCTTCGATGACATCAAGTG	ACTCCACCAGTAGCAATTCTCATG	CAGTGGCTGTACCTTT	69
RGS5_v1	CAGCATTTCTGTTTGTCACTAACCA	CCCTCAAAACCATCTACTTCTCTGA	TCACTATAATAACTAGCCTT CACAC	85
FKBP5_v1	GGGCGTTCCTCTGGGTGTA	GCATGCAGCATTCTCCTTTCT	ACAGGGCCATGGAGA	62
THRB1_v1	GGCTAAGTCAATGGCCCTAGAA	TGTCTCAAACGAATTGCCTCAA	AGTTGTGACAATCAGC	66
THRB1_v2	TGCATGGAGATCATGTCTTTACG	CGGCCATCTCCCCGTTA	AGAGTGAGACGCTGACG	82
GHR1_v1	CCGTTCCTTCTCCCAACATG	TGTACTCACCATACATCTTACCAAACC	CAGCTGTAGACATTTT	66
NR3C1_v1	TTGGACTTGCCTGGCTCTCT	GACAGGGAGGAAAGGAAAGCA	ACCTGAATGAGTTTTACGTGTC	77
Downregulated in smolt				
NKAa1-a_v1	GAACAGAACAACGTACCTATCCTCAA	TTGAGGATAGGTACGTTGTTCTGTTC	CTGTGCAACCGAGCC	79
NKAa1-a_v2	TGGAATCAAGGTTATCATGGTCACT	CCCACACCCTTGGCAATG	ATCATCCCATCACTGCGA	69
GlyT2_v1	TTCCTTGTCGTTTTCCTGTTCTC	CACCCACATTGAATGGAAAGAG	ACTTCATCTCTCCTTTCC	72
GlyT2_v2	CACTTCACTTCATCTCTCCTTTCCT	GGATGTGTTGGCTTGCTTTTC	TTTCCATTCAATGTGGGTGC	73
CCL4_v1	TCTCTTCATTGCAACAATCTGCTT	ACAGCAGTCCACGGGTACCT	CTACGCAGCAGCATT	67
CCL4_v2	GACTGCTGTCTGTCAACCACTGA	CTGCAGCAGGTAGGAGACCAT	TTTCCCTCGCCACTTT	70
CCL19_v1	ACCTGGGTTACAGACCTGATGAA	TGGTTTCGTGGCATTTCTTG	CTCATGGACCGCCTCA	61
IFI44_v1	AGGAAACTGCCCGTGACATC	GGTCATCGTCTGCTGAACGA	CCCAGGTGTACAACAGT	62
IFI44_v2	CCACTGGACTAACCCTCCATGA	TGTGTCCCTCGGGTGCAT	ACTCTGGCTATCATCAAA	63
PLK2_v1	AACAATGGCACACACATGAGTCT	CCCAGCTCGGCATAGTAGTGA	CTGGCAGATAAGAGGAC	65
PLK2_v2	CTGGGCAGCAGCTTCCA	TGGTAGCCAAAGCCATACTTGTT	TGACCAAATGGGTGGACT	68
CD3Z_v1	CCCTGCAGAGGCGATGAC	GGAGGAAGCCATCCAGAATGT	CCACTGTACGACCCTAA	65
CD3Z_v2	CCCTGCAGAGGCGATGAC	GGAGGAAGCCATCCAGAATGT	CGACCCTAAACTCTG	65
IL12B_v1	GGAGCCTCCCATGCTCTTACT	TGGCGTGGACCACTTTGAC	CCCCTCACATTCCA	57
IL12B_v2	GGGAACCAGATCCAGGTGAA	AGCGTTGTGGCAGCTGTAGTT	TGGAGGAGATGATGGGA	63
MCM4_v1	AAGCTGGAGGAGATCAGTGTGATT	TGCACATGGCTGCAGTTGA	TGAGCCTGTGCTGAAT	62
MCM4_v2	GCTGCCAAGTGCAGATGGT	GACATCAGTGCCCCAGATAACC	CAGGCAGCGGGACA	76
WAS_v1	GCAGGAGCTCTATAACCAAATGGT	CAGCAAATGCGTGGAAGAAG	TACCACAGCCCCCGAC	62
WAS_v2	GCATTCCATCCTGTATTATTTTCCA	GAGATTAACCGGATCAACCAGACT	TCTGTAACCATGGACTCAA	70
TYK2_v1	GGGAGTGGCCAAAAGTGTCTT	CGTGAAGCGCATAGATAGAGAAGTC	CGGTGTTTTCGTCTTGTAG	68
TYK2_v2	CGCATGTGATAAATACGACTTGATT	TCCCCTTGAACCAGCTGTACTAC	AGCTATACCACAAGCGTAC	70
RGS21_v1	TCCCGACTACAGCGCAGAT	TCCTCAGGGCTAAGTCGTTCA	TTCCCAATCCCCC	59
MS4A4A_v1	TGGGCAGTGTTGAAGATTCTGA	GCATGGTTCTGCCTCAAGGA	CCCCGGCTCCACCA	63
UBA1_v1	TTGAGGGCAGAGAACTACGACAT	TGCGACCCGCGATCA	CTGATCGTCACAAGAGT	67
EXO1_v1	GTGGTGGCCCCGTATGAA	CAATGCCAGCCTTGTTAAGGA	CTGATGCCCAGCTGG	58
NAMPT_v1	CCAACACACTACGCAGAAAGGA	CCTTGGTGGTCAGTGCTGTACA	TGGATCCCTTCTACCTCC	64

Assay name	Forward	Reverse	Probe	Amplicon size (bp)
TUBA8L2_v1	CCCGCGCCATCTTTGTAG	AACAGCTGGCGGTAGGTACCT	ACTGTCATTGATGAGGTGC	73
FMNL1_v1	GTTTGGATGTACTGGTGGATTACCT	GCACCCTCCAAGTCAAACGA	CTACGCCCAGTGTGAC	67
TRA_v1	TTCAGCGCACACAATGCTACT	TGGTGGCCTCTGTCTTGTTG	CGCACTTGGAATCC	58
PRLR_v1	GATGCCGGAGGGAAAAGAC	CCGACTGGCTCTTGGACTTG	TCCAAGATGTTGGCTGC	59

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Table S 5: Summary of efficiency values for the qPCR TaqMan assay designs using six to nine salmonid species. Species abbreviations: CK= Chinook salmon (*Oncorhynchus tshawytscha*), CO= Coho salmon (*O. kisutch*), SX= Sockeye salmon (*O. nerka*), PK= Pink salmon (*O. gorbuscha*), CM= Chum salmon (*O. keta*), AS= Atlantic salmon (*Salmo salar*), RT= Rainbow trout (*O. mykiss*), AC= Artic charr (*Salvelinus alpinus*), and BT= Bull trout (*Salvelinus confluentus*). Both assay designs of set 1 were tested for efficiency using six species. The best assay design of set 1 and all of set 2 single assay designs were then tested using nine species. Efficiency values between 0.8 and 1.2 were considered good. FAIL indicates no detectable amplification. See Table 2 legend in main text for additional details.

Assay name	Selected	CK	CO	SX	PK	CM	AS	RT	AC	BT
Upregulated in smolt										
NKAa1-b_v1		0.870	1.010	0.844	1.304	0.865	0.923			
NKAa1-b_v2	yes	1.156	1.044	1.073	1.051	1.122	1.068	1.086	0.987	1.067
CA4_v1	yes	0.999	1.055	1.011	0.981	1.090	1.149	0.937	FAIL	0.918
CA4_v2		0.872	1.138	0.855	0.945	0.911	FAIL			
CFTR-I_v1	yes	0.980	0.704	1.046	0.999	1.126	0.969	1.183	0.882	1.078
CFTR-I_v2		0.946	1.512	0.908	0.537	1.770	3.891			
RHAG_v1	yes	1.046	1.097	1.099	1.003	1.125	0.953	1.019	0.967	0.999
NKCC_v1		FAIL	FAIL	FAIL	FAIL	FAIL	FAIL	0.887	FAIL	FAIL
GAPDH_v1		1.069	FAIL	0.898	FAIL	0.896	FAIL			
GAPDH_v2		FAIL	FAIL	FAIL	FAIL	FAIL	FAIL			
NDUFB2_v1	yes	0.992	0.972	1.029	1.023	1.060	1.100	1.042	0.921	1.009
NDUFB2_v2		1.164	0.934	0.774	0.757	FAIL	1.352			
NDUFB4_v1	yes	1.029	1.129	1.137	1.019	1.218	0.953	1.090	0.991	1.143
MPC1_v1	yes	1.005	1.044	1.187	1.163	1.166	1.127	1.039	1.108	1.029
HBA_v1	yes	1.200	0.995	1.070	1.248	1.111	1.630	1.048	FAIL	FAIL
HBA_v2		0.851	0.849	0.862	1.099	0.802	1.042			
HBAt_v1	yes	1.081	0.946	0.997	1.142	1.187	FAIL	1.008	FAIL	FAIL
ACTB_v1	yes	1.210	1.050	1.071	1.046	1.192	1.167	1.260	1.251	1.070
ACTB_v2		0.806	1.100	0.979	1.406	1.177	1.542			
WHRN_v1		FAIL	1.385	0.825	3.648	4.090	1.025	FAIL	1.187	2.553
RPL31_v1	yes	0.993	0.974	1.003	0.983	1.009	1.342	1.072	0.913	1.000

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Assay name	Selected	CK	CO	SX	PK	CM	AS	RT	AC	BT
RPL31_v2		0.858	0.863	0.845	1.257	0.857	1.372			
SLC16A10_v1	yes	0.901	0.963	1.103	0.885	1.057	0.893	1.283	0.895	0.915
EEF2_v1	yes	0.940	1.037	1.020	0.972	1.089	1.244	1.138	1.024	1.096
CYP2K1_v1		0.834	0.855	0.872	0.702	1.275	1.409			
CYP2K1_v2	yes	0.937	0.933	1.029	0.966	0.811	1.130	0.828	0.810	1.525
S100A4_v1		FAIL	1.128	FAIL	1.491	1.428	1.755	1.691	1.432	1.188
TSPO_v1		0.835	0.850	0.844	1.139	0.829	FAIL			
TSPO_v2	yes	0.920	0.845	0.880	0.936	0.980	0.890	0.966	0.875	0.853
CLEC4M_v1	yes	1.030	0.875	0.975	1.106	1.153	1.031	1.137	1.519	FAIL
CLEC4M_v2		0.955	1.063	0.685	0.819	0.951	0.819			
RGS5_v1		1.298	0.982	1.176	1.228	1.272	1.358	1.220	1.025	1.151
FKBP5_v1	yes	0.867	0.963	1.019	0.713	0.964	0.977	0.897	0.847	0.880
THRB1_v1		0.796	0.806	0.792	1.054	0.784	0.760			
THRB1_v2	yes	0.841	0.724	0.848	0.964	0.997	FAIL	1.094	FAIL	FAIL
GHR1_v1	yes	0.937	1.160	0.958	1.081	0.945	0.967	0.978	0.845	0.935
NR3C1_v1	yes	1.082	0.993	1.046	0.937	1.028	1.106	FAIL	0.891	0.929
Downregulated in smolt										
NKAa1-a_v1		FAIL	FAIL	FAIL	FAIL	FAIL	FAIL			
NKAa1-a_v2	yes	1.160	1.042	1.064	1.013	1.162	1.151	1.153	0.988	1.033
GlyT2_v1		FAIL	FAIL	FAIL	FAIL	FAIL	FAIL			
GlyT2_v2		FAIL	FAIL	FAIL	FAIL	FAIL	FAIL			
CCL4_v1	yes	0.981	1.130	1.021	1.018	0.998	0.948	1.085	0.916	0.687
CCL4_v2		0.879	1.027	0.856	1.314	1.159	0.839			
CCL19_v1	yes	1.010	0.954	1.019	0.973	1.084	1.110	1.003	0.970	1.026
IFI44_v1	yes	1.096	1.028	1.012	1.079	1.089	1.185	1.083	1.060	1.131
IFI44_v2		0.855	0.823	0.889	0.881	1.068	0.838			
PLK2_v1		0.902	0.957	1.457	0.865	0.895	0.966			
PLK2_v2	yes	0.931	0.866	1.578	0.847	0.972	1.063	0.983	1.370	FAIL
CD3Z_v1		2.972	1.561	1.161	2.040	2.750	1.316	FAIL	1.137	0.776

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Assay name	Selected	CK	CO	SX	PK	CM	AS	RT	AC	BT
CD3Z_v2		1.321	1.054	2.097	1.256	1.147	1.308			
IL12B_v1	yes	0.967	0.931	0.846	0.881	0.805	0.885	0.976	FAIL	FAIL
IL12B_v2		0.774	FAIL	0.766	1.228	0.916	FAIL			
MCM4_v1	yes	0.903	0.942	1.013	0.862	1.139	0.913	0.934	0.848	0.924
MCM4_v2		0.855	0.828	0.933	FAIL	0.776	0.701			
WAS_v1	yes	1.012	1.028	0.969	0.955	0.999	1.251	1.049	0.870	1.003
WAS_v2		0.774	0.716	0.803	0.816	0.872	2.125			
TYK2_v1		FAIL	FAIL	FAIL	FAIL	FAIL	FAIL			
TYK2_v2		FAIL	FAIL	FAIL	FAIL	FAIL	FAIL			
RGS21_v1	yes	0.867	0.838	1.099	FAIL	FAIL	0.993	FAIL	0.872	0.991
MS4A4A_v1	yes	0.960	1.066	1.265	1.016	1.084	1.027	1.041	1.236	1.051
UBA1_v1	yes	0.937	0.854	1.049	0.984	1.064	1.080	0.983	0.896	0.885
EXO1_v1	yes	0.930	0.902	1.017	1.032	1.033	0.742	1.005	0.916	1.191
NAMPT_v1	yes	0.978	1.029	1.170	1.066	1.020	1.105	1.024	1.193	0.965
TUBA8L2_v1	yes	1.078	1.084	1.126	1.159	1.138	1.279	1.140	1.077	1.131
FMNL1_v1	yes	1.059	0.867	0.967	1.176	1.225	1.208	1.127	1.043	1.058
TRA_v1	yes	0.963	1.500	0.972	0.940	0.978	FAIL	FAIL	FAIL	FAIL
PRLR_v1	yes	0.949	1.100	0.865	1.010	0.979	1.728	0.722	0.916	FAIL

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Table S 6: Summary of the Pearson correlations between PC2 and the expression of 37 candidate smoltification genes using all four groups.

Assay name	*r*	*p*
ACTB_v1	-0.03628	0.402779
CA4_v1	0.650688	0
CCL19_v1	-0.45388	0
CCL4_v1	-0.37056	0
CFTR.I_v1	0.723941	0
CLEC4M_v1	0.343777	4.44E-16
CYP2K1_v2	0.226706	1.19E-07
EEF2_v1	0.414287	0
EXO1_v1	-0.16926	8.48E-05
FKBP5_v1	0.518222	0
FMNL1_v1	-0.3929	0
GHR1_v1	0.199101	3.54E-06
HBA_v1	0.624489	0
HBAt_v1	-0.13112	0.002398
IFI44_v1	-0.56695	0
IL12B_v1	-0.32228	2.26E-14
MCM4_v1	-0.00672	0.876895
MPC1_v1	0.147246	0.000642
MS4A4A_v1	-0.23536	3.72E-08
NAMPT_v1	-0.16426	0.000137
NDUFB2_v1	0.302671	8.92E-13
NDUFB4_v1	0.232095	5.79E-08
NKAa1.a_v2	-0.09342	0.030888
NKAa1.b_v2	0.339919	6.66E-16
NR3C1_v1	-0.14933	0.000536
PLK2_v2	-0.15146	0.000445
PRLR_v1	-0.17944	3.04E-05
RGS21_v1	-0.10182	0.018594
RHAG_v1	0.244954	9.76E-09
RPL31_v1	0.06362	0.14205
SLC16A10_v1	0.103012	0.017256
THRB1_v2	0.068314	0.114846
TRA_v1	-0.22363	1.77E-07
TSPO_v2	0.133854	0.001936
TUBA8L2_v1	-0.04732	0.275042
UBA1_v1	-0.15486	0.000328
WAS_v1	-0.4355	0

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Table S 7: Summary of the Pearson correlations between PC2 and the expression of 37 candidate smoltification genes for Coho salmon.

Assay name	*r*	*p*
ACTB_v1	-0.1777	0.026462
CA4_v1	-0.86386	0
CCL19_v1	0.243586	0.002182
CCL4_v1	0.178953	0.025403
CFTR.I_v1	-0.88924	0
CLEC4M_v1	-0.0952	0.23714
CYP2K1_v2	-0.22418	0.004903
EEF2_v1	-0.50957	1.09E-11
EXO1_v1	0.118789	0.139682
FKBP5_v1	-0.58557	8.88E-16
FMNL1_v1	0.399286	2.43E-07
GHR1_v1	-0.1223	0.128273
HBA_v1	-0.7775	0
HBAt_v1	-0.66312	0
IFI44_v1	0.377681	1.17E-06
IL12B_v1	0.232621	0.003475
MCM4_v1	0.435372	1.35E-08
MPC1_v1	-0.03564	0.658675
MS4A4A_v1	0.107597	0.181235
NAMPT_v1	0.266893	0.000757
NDUFB2_v1	-0.42869	2.36E-08
NDUFB4_v1	-0.38738	5.86E-07
NKAa1.a_v2	-0.25199	0.001507
NKAa1.b_v2	-0.68527	0
NR3C1_v1	0.053141	0.509986
PLK2_v2	0.000665	0.993427
PRLR_v1	-0.22419	0.0049
RGS21_v1	-0.23058	0.003781
RHAG_v1	-0.73892	0
RPL31_v1	-0.40186	2.00E-07
SLC16A10_v1	-0.39925	2.43E-07
THRB1_v2	-0.03892	0.629536
TRA_v1	0.107788	0.180456
TSPO_v2	-0.05689	0.480554
TUBA8L2_v1	0.054845	0.49649
UBA1_v1	0.351538	6.80E-06
WAS_v1	0.378703	1.09E-06

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Table S 8: Summary of the Pearson correlations between PC2 and the expression of 37 candidate smoltification genes for Sockeye salmon.

Assay name	*r*	*p*
ACTB_v1	0.082718	0.468612
CA4_v1	0.793261	0
CCL19_v1	-0.22605	0.045165
CCL4_v1	-0.35073	0.00153
CFTR.I_v1	0.832734	0
CLEC4M_v1	-0.38243	0.000505
CYP2K1_v2	0.327642	0.003203
EEF2_v1	-0.51509	1.19E-06
EXO1_v1	-0.02941	0.796958
FKBP5_v1	0.232226	0.039454
FMNL1_v1	-0.24315	0.030835
GHR1_v1	0.490326	4.50E-06
HBA_v1	0.813886	0
HBAt_v1	0.682975	4.10E-12
IFI44_v1	-0.46057	1.95E-05
IL12B_v1	0.592312	8.93E-09
MCM4_v1	-0.16452	0.147384
MPC1_v1	0.454375	2.60E-05
MS4A4A_v1	0.154063	0.175218
NAMPT_v1	0.244134	0.03014
NDUFB2_v1	0.621709	9.67E-10
NDUFB4_v1	0.515412	1.17E-06
NKAa1.a_v2	0.211878	0.060856
NKAa1.b_v2	0.75616	8.88E-16
NR3C1_v1	0.203574	0.071946
PLK2_v2	0.09461	0.406893
PRLR_v1	0.597026	6.35E-09
RGS21_v1	-0.41606	0.000137
RHAG_v1	0.80588	0
RPL31_v1	-0.25337	0.024257
SLC16A10_v1	0.398443	0.000276
THRB1_v2	0.245944	0.028901
TRA_v1	-0.44742	3.56E-05
TSPO_v2	0.733132	1.55E-14
TUBA8L2_v1	0.265784	0.017912
UBA1_v1	-0.0791	0.488367
WAS_v1	-0.11068	0.331505

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Table S 9: Summary of the Pearson correlations between PC2 and the expression of 37 candidate smoltification genes for stream-type Chinook salmon.

Assay name	*r*	*p*
ACTB_v1	0.063431	0.47514
CA4_v1	-0.58541	3.19E-13
CCL19_v1	0.709524	0
CCL4_v1	0.621435	4.00E-15
CFTR.I_v1	-0.24342	0.00544
CLEC4M_v1	-0.33614	9.84E-05
CYP2K1_v2	0.157624	0.074423
EEF2_v1	-0.31222	0.000316
EXO1_v1	0.321477	0.000203
FKBP5_v1	-0.60248	4.24E-14
FMNL1_v1	0.275064	0.001606
GHR1_v1	-0.22987	0.008777
HBA_v1	-0.56708	2.45E-12
HBAt_v1	-0.44971	8.97E-08
IFI44_v1	0.746626	0
IL12B_v1	0.385703	6.36E-06
MCM4_v1	0.308279	0.000379
MPC1_v1	-0.16822	0.056697
MS4A4A_v1	0.196692	0.025475
NAMPT_v1	0.132866	0.133346
NDUFB2_v1	-0.13715	0.121179
NDUFB4_v1	-0.15982	0.070426
NKAa1.a_v2	-0.09251	0.297078
NKAa1.b_v2	-0.1793	0.042038
NR3C1_v1	0.071568	0.420253
PLK2_v2	0.101785	0.251057
PRLR_v1	-0.01846	0.835538
RGS21_v1	0.224464	0.010548
RHAG_v1	-0.12244	0.166865
RPL31_v1	0.051096	0.56525
SLC16A10_v1	0.033999	0.702085
THRB1_v2	-0.2421	0.005707
TRA_v1	0.462402	3.45E-08
TSPO_v2	-0.01029	0.90786
TUBA8L2_v1	0.157099	0.075406
UBA1_v1	0.10494	0.236586
WAS_v1	0.308611	0.000373

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Table S 10: Summary of the Pearson correlations between PC2 or PC3 and the expression of 37 candidate smoltification genes for ocean-type Chinook salmon.

Assay name	r (PC2)	p (PC2)	r (PC3)	p (PC3)
ACTB_v1	0.295468	9.16E-05	0.111455	0.147902
CA4_v1	-0.82921	0	-0.24564	0.001244
CCL19_v1	-0.45581	4.21E-10	0.368436	7.68E-07
CCL4_v1	-0.62148	0	0.380255	3.14E-07
CFTR.I_v1	-0.53029	1.03E-13	-0.3243	1.60E-05
CLEC4M_v1	0.295834	8.97E-05	-0.3938	1.08E-07
CYP2K1_v2	0.380222	3.15E-07	-0.33119	1.03E-05
EEF2_v1	0.131025	0.08855	0.158466	0.039022
EXO1_v1	0.24312	0.0014	-0.24796	0.001113
FKBP5_v1	-0.13548	0.078149	0.53799	3.86E-14
FMNL1_v1	0.02361	0.759903	-0.05587	0.469299
GHR1_v1	0.567149	8.88E-16	0.106027	0.168788
HBA_v1	-0.23681	0.001876	-0.30541	5.13E-05
HBAt_v1	-0.52606	1.75E-13	0.144307	0.060452
IFI44_v1	-0.20517	0.007275	0.51451	7.12E-13
IL12B_v1	-0.57207	4.44E-16	-0.05122	0.507131
MCM4_v1	0.264691	0.000486	-0.31182	3.48E-05
MPC1_v1	0.347345	3.47E-06	0.211596	0.005607
MS4A4A_v1	-0.04324	0.575596	-0.11498	0.135411
NAMPT_v1	0.057584	0.455739	0.419977	1.19E-08
NDUFB2_v1	0.212028	0.005508	0.142497	0.063782
NDUFB4_v1	0.281835	0.000197	0.189455	0.013345
NKAa1.a_v2	-0.0336	0.663534	0.27528	0.00028
NKAa1.b_v2	-0.26095	0.000588	-0.1643	0.032274
NR3C1_v1	0.200761	0.008663	0.094512	0.22022
PLK2_v2	-0.36256	1.18E-06	-0.17241	0.024564
PRLR_v1	-0.25758	0.000696	-0.02923	0.70518
RGS21_v1	-0.36651	8.85E-07	-0.39598	9.04E-08
RHAG_v1	0.206415	0.006921	-0.14098	0.066685
RPL31_v1	0.1849	0.015784	-0.10159	0.187427
SLC16A10_v1	0.251176	0.000953	-0.16253	0.034209
THRB1_v2	-0.20179	0.008319	0.340011	5.72E-06
TRA_v1	-0.12969	0.09187	-0.34609	3.78E-06
TSPO_v2	-0.22918	0.002645	-0.3487	3.16E-06
TUBA8L2_v1	0.375566	4.50E-07	0.279671	0.000221
UBA1_v1	-0.1985	0.009462	0.396463	8.69E-08
WAS_v1	-0.32037	2.05E-05	-0.01946	0.801146

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Table S 11: Summary of the Student's t-tests examining the difference in expression of 37 candidate smoltification genes between freshwater and seawater for ocean-type Chinook salmon. Nitinat juveniles were sampled at the same time in late April in both environments. Seawater juveniles were contained within a netpen in an estuary for about two weeks prior to sampling. Presented are the mean values in freshwater and seawater; t and p-values for the mean difference; and significance coding for the difference, i.e. *** $p < 0.001$, ** $0.001 < p < 0.01$, * $0.01 < p < 0.05$, . $0.05 < p < 0.1$

Assay name	Freshwater	Seawater	t-value	p-value	Signif.
ACTB_v1	0.781499	0.614146	0.454184	0.656659	
CA4_v1	-0.16458	1.665436	-5.67612	7.47E-05	***
CCL19_v1	-1.1124	-2.10943	2.783734	0.014903	*
CCL4_v1	0.324899	-2.61555	7.859551	1.13E-05	***
CFTR.I_v1	0.367682	1.632086	-1.77163	0.107445	
CLEC4M_v1	-0.72391	-0.34098	-0.60563	0.5545	
CYP2K1_v2	0.229148	0.581438	-0.42066	0.680431	
EEF2_v1	1.00145	1.11542	-0.5718	0.576693	
EXO1_v1	0.365374	0.453229	-0.49978	0.625311	
FKBP5_v1	-0.03752	0.537554	-1.37563	0.196721	
FMNL1_v1	0.401456	0.076089	0.927794	0.369291	
GHR1_v1	0.5083	0.260142	0.467005	0.647695	
HBA_v1	1.379431	1.06952	0.756477	0.463339	
HBAt_v1	-0.64149	-1.82298	1.600008	0.132927	
IFI44_v1	0.128963	-0.9276	3.608317	0.002892	**
IL12B_v1	1.002072	0.131237	1.740927	0.103722	
MCM4_v1	0.276983	0.247993	0.131698	0.897428	
MPC1_v1	0.889465	0.567753	0.939307	0.363611	
MS4A4A_v1	0.973487	0.1996	2.029614	0.061889	.
NAMPT_v1	0.92058	-0.05864	2.503539	0.025465	*
NDUFB2_v1	1.034136	0.233148	2.567294	0.022735	*
NDUFB4_v1	0.708509	0.322051	1.252024	0.231117	
NKAa1.a_v2	1.05694	-1.17179	7.392296	1.28E-05	***
NKAa1.b_v2	0.312624	0.763602	-1.44866	0.169462	
NR3C1_v1	0.876391	0.443096	1.640321	0.123257	
PLK2_v2	1.101163	0.811895	0.877102	0.395861	
PRLR_v1	1.421913	-0.68146	6.365303	3.24E-05	***
RGS21_v1	-0.32839	1.33284	-6.60176	1.19E-05	***
RHAG_v1	1.739602	0.695542	3.181861	0.006896	**
RPL31_v1	0.646804	0.496804	0.554798	0.589087	
SLC16A10_v1	1.488335	1.127572	0.762769	0.460651	
THRB1_v2	1.371714	0.648913	1.865934	0.083299	.
TRA_v1	0.125936	0.032976	0.305942	0.764628	
TSPO_v2	1.123814	0.046151	2.58888	0.021507	*
TUBA8L2_v1	0.132761	-0.18029	1.625875	0.126282	
UBA1_v1	0.745251	-0.2679	3.094738	0.008873	**
WAS_v1	0.272223	-0.28009	2.243392	0.041572	*

Table S 12: Summary of body variable correlations with smoltification gene expression patterns for the four groups. Presented are the Pearson correlations (r) with p-values in brackets for body variables and smoltification gene expression patterns (PC1 and PC2) using Coho salmon, Sockeye salmon, stream-type Chinook salmon, and ocean-type Chinook salmon. Smoltification gene expression patterns (PC1 and PC2), using the top 10 biomarkers, for each group are displayed in Figure 2.

Body variable	Coho salmon		Sockeye salmon		Chinook salmon (s-t)		Chinook salmon (o-t)	
	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
Length	0.47 (<0.001)	0.27 (0.001)	0.72 (<0.001)	-0.32 (0.004)	0.25 (0.005)	-0.06 (0.475)	0.77 (<0.001)	-0.44 (<0.001)
Mass	0.44 (<0.001)	0.19 (0.016)	0.69 (<0.001)	-0.24 (0.032)	0.22 (0.011)	-0.13 (0.131)	0.74 (<0.001)	-0.38 (<0.001)
Condition	-0.13 (0.119)	-0.27 (0.001)	-0.16 (0.157)	0.34 (0.002)	-0.05 (0.549)	-0.24 (0.005)	0.52 (<0.001)	-0.14 (0.061)
Skin pigmentation								
Posterior brightness	0.18 (0.083)	0.03 (0.749)					-0.17 (0.082)	0.29 (0.003)
Anterior brightness	0.07 (0.480)	0.38 (<0.001)					0.17 (0.094)	0.29 (0.003)
Body yellowness	-0.19 (0.075)	0.01 (0.941)					-0.03 (0.798)	-0.31 (0.002)
Caudal fin darkness	0.33 (0.001)	0.22 (0.033)					0.26 (0.010)	0.16 (0.107)
Morphology								
Streamlined to truncated shape	-0.25 (0.017)	-0.38 (<0.001)					0.03 (0.766)	0.49 (<0.001)
Caudal peduncle length	-0.26 (0.011)	0.17 (0.108)					0.48 (<0.001)	0.10 (0.326)

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Supplementary Figures

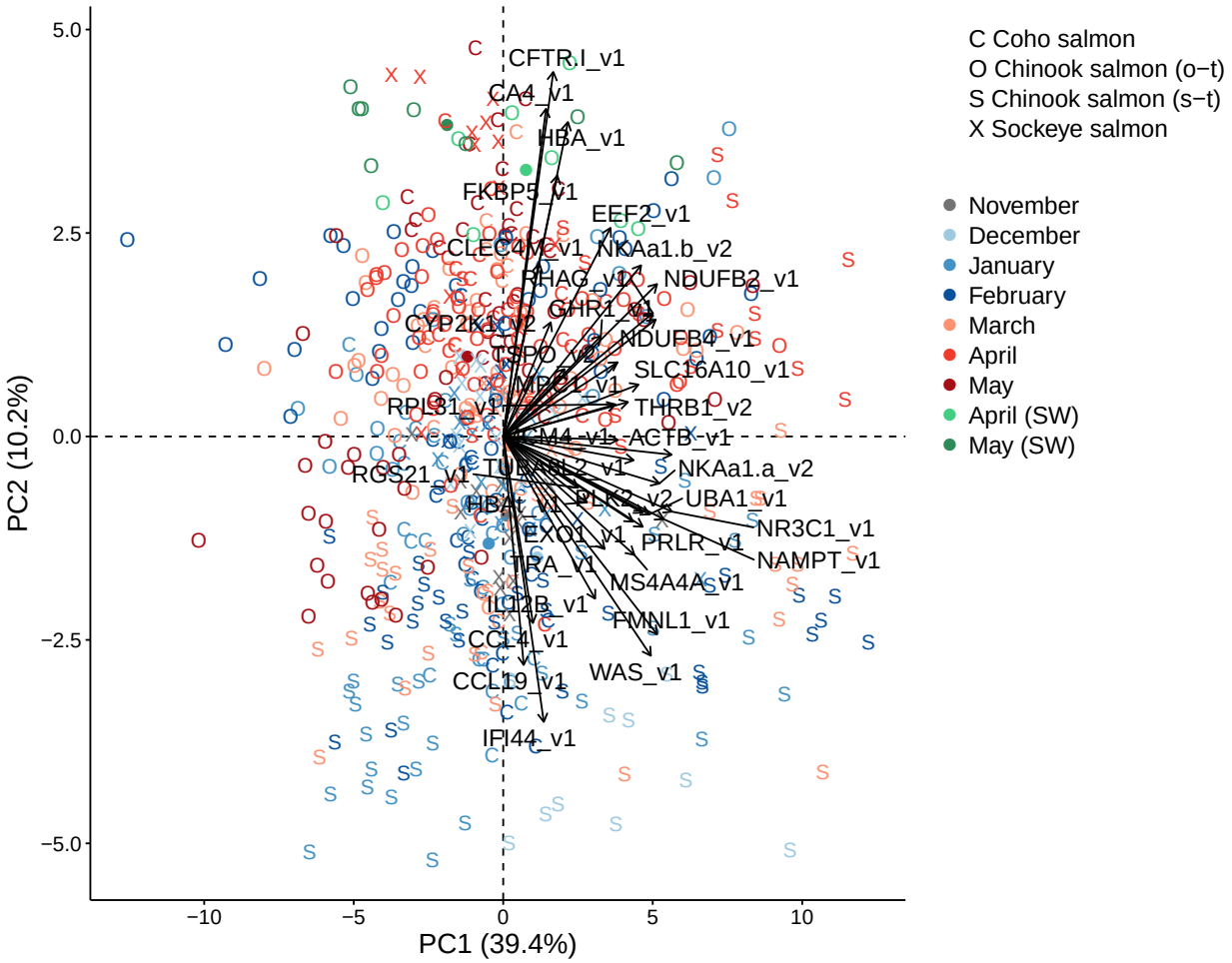

Figure S 1: Canonical plots of the first two principal components of all 37 candidate genes for smoltification using all four groups. Groups are Coho salmon (*Oncorhynchus kisutch*), Sockeye salmon, Chinook salmon (stream-type, *O. tshawytscha*), and Chinook salmon (ocean-type, *O. tshawytscha*). See Figure 1 legend.

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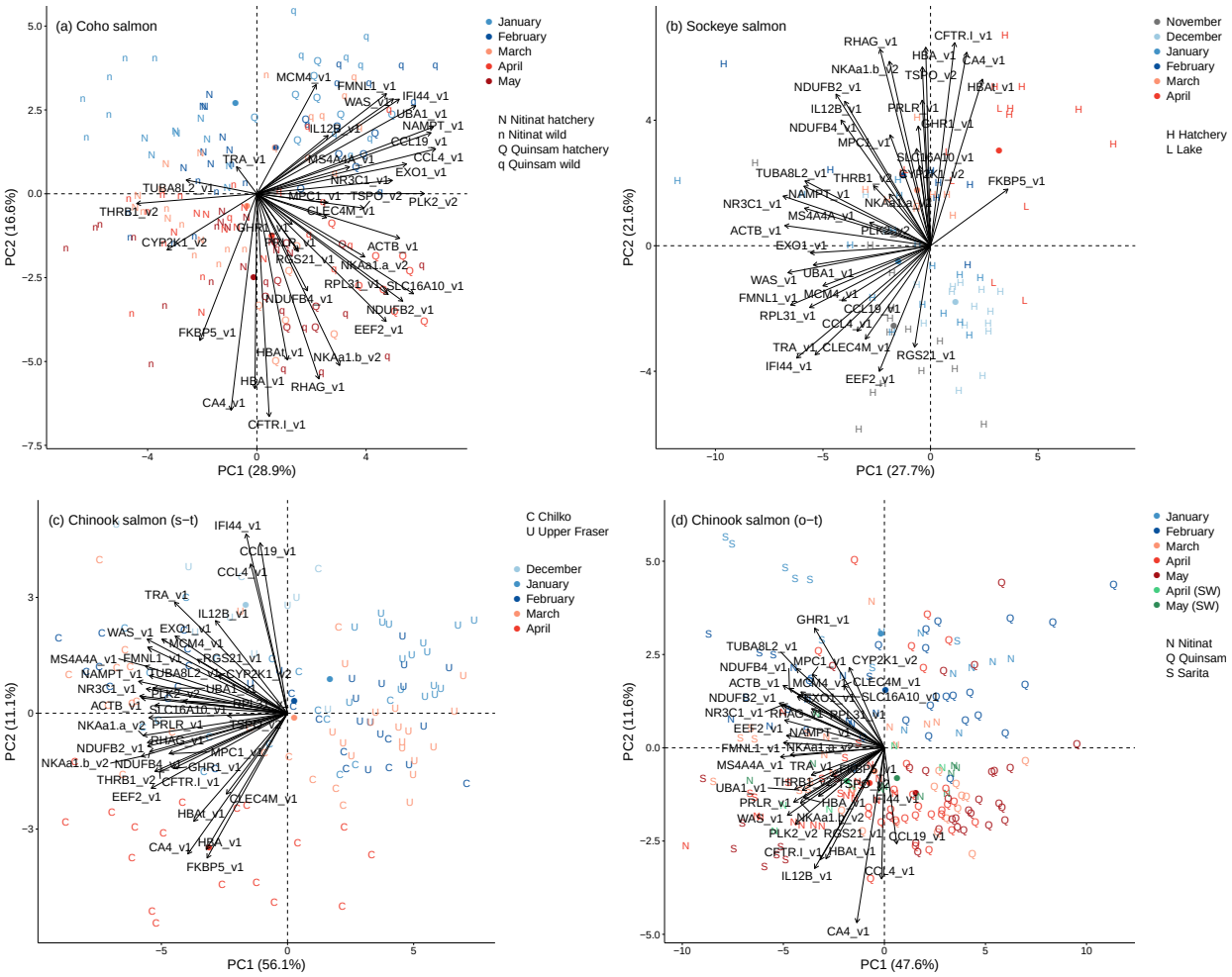

Figure S 2: Canonical plots of the first two principal components of all 37 candidate genes for smoltification using each of the four groups. (a) Coho salmon, (b) Sockeye salmon, (c) stream-type Chinook salmon, and (d) ocean-type Chinook salmon. See Figure 1 legend.

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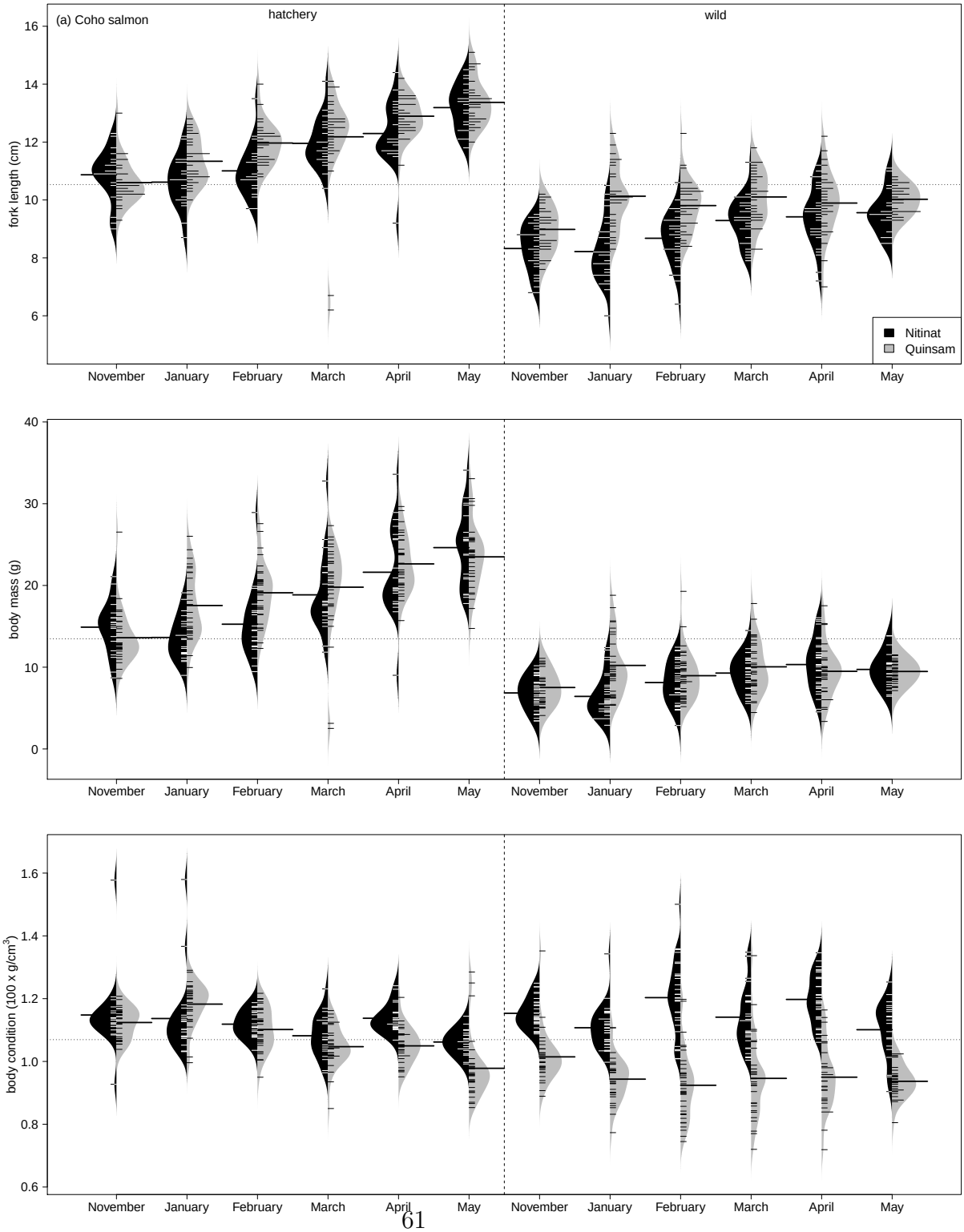

Figure S 3: Beanplots of body length, mass, and condition by monthly development for each of the four groups. (a) Coho salmon, (b) Sockeye salmon, (c) stream-type Chinook salmon, and (d) ocean-type Chinook salmon. Solid horizontal line is the mean for each bean; dashed horizontal line is the overall mean across beans. Month symbol E is early and L is late.

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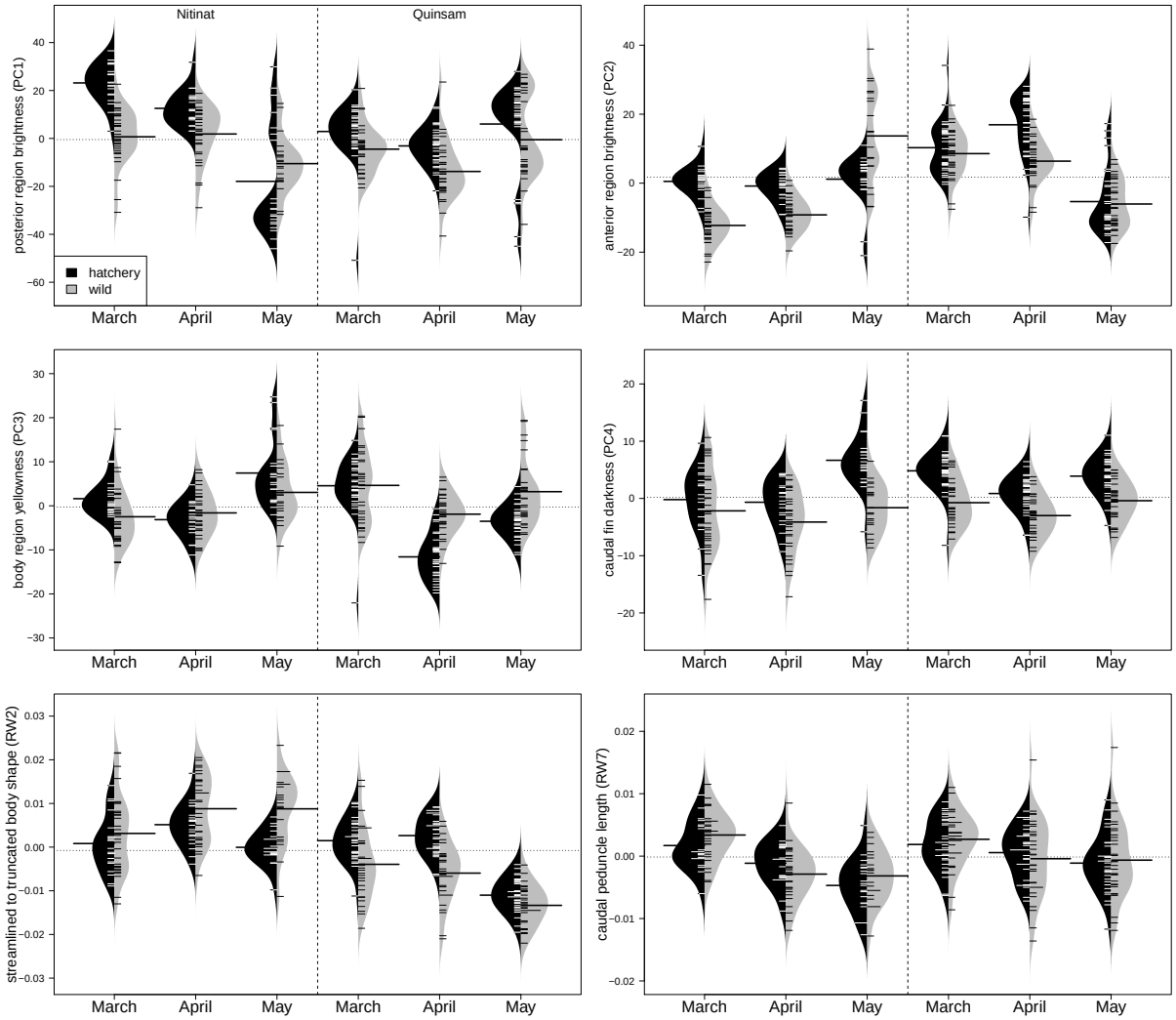

Figure S 4: Beanplots of skin pigmentation and body morphology by monthly development for Coho salmon. Solid horizontal line is the mean for each bean; dashed horizontal line is the overall mean across beans.

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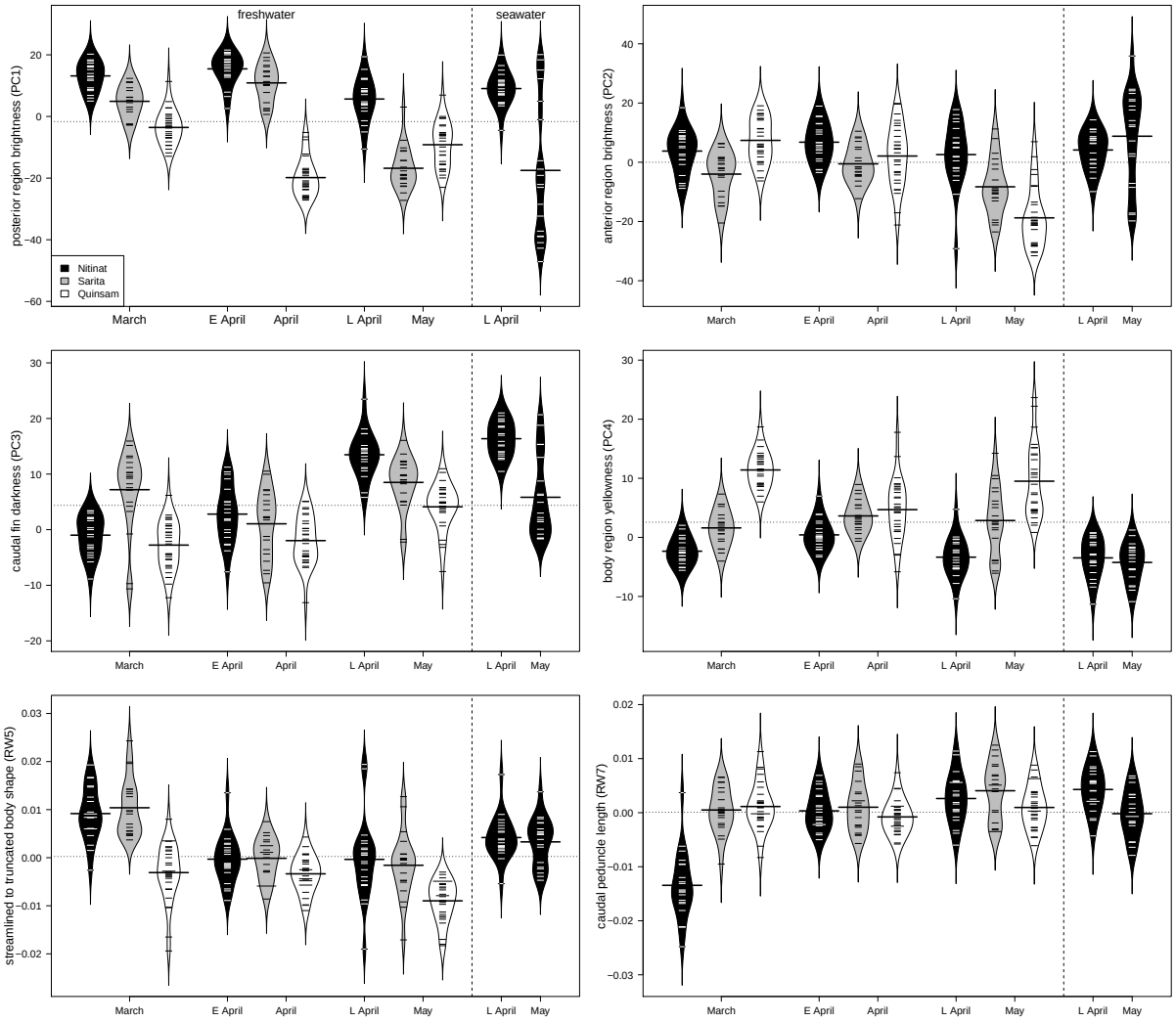

Figure S 5: Beanplots of skin pigmentation and body morphology by monthly development for ocean-type Chinook salmon. Solid horizontal line is the mean for each bean; dashed horizontal line is the overall mean across beans. Month symbol E is for early and L is for late.

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Supplementary Appendices

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Appendix 1. Beanplots of the expression of 37 candidate smotification genes by monthly development for Coho salmon. Solid horizontal line is the mean for each bean; dashed horizontal line is the overall mean across beans.

Appendix 2. Beanplots of the expression of 37 candidate smotification genes by monthly development for Sockeye salmon. Solid horizontal line is the mean for each bean; dashed horizontal line is the overall mean across beans.

Appendix 3. Beanplots of the expression of 37 candidate smotification genes by monthly development for stream-type Chinook salmon. Solid horizontal line is the mean for each bean; dashed horizontal line is the overall mean across beans.

Appendix 4. Beanplots of the expression of 37 candidate smotification genes by monthly development for ocean-type Chinook salmon. Solid horizontal line is the mean for each bean; dashed horizontal line is the overall mean across beans. Month symbol E is early and L is late.