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ARTICLE

Transcriptome profiles relate to migration fate in hatchery steelhead (*Oncorhynchus mykiss*) smolts[1]

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Abstract: For anadromous Pacific salmonid (*Oncorhynchus* spp.) smolts, the physiological state of individuals can influence migration fate. This critical life stage is typically associated with poor survival and influences population productivity, highlighting the need to identify intrinsic factors associated with outmigration fate. To better understand and identify such factors, we combined acoustic telemetry with nonlethal gill biopsies and used high-throughput real-time quantitative polymerase chain reaction to assess how infectious agents and host gene expression profiles influence migration fate for hatchery steelhead smolts (*Oncorhynchus mykiss*). Redundancy analyses of gene expression, infectious agent loads, and body condition highlighted gene expression profiles indicative of migratory fate. Smolts never detected after release in the river had significantly elevated expression of the immune genes *Il-17D* and *RPL6*, and lower expression of the osmoregulatory gene *NKA α1b* relative to other individuals. *Flavobacterium psychrophilum* and "*Candidatus* Branchiomonas cysticola" were detected in gill samples, but neither influenced survival. We demonstrate rare evidence of gene expression profiles relating to migration fate in juvenile salmonids and highlight potential mechanisms influencing fate for hatchery steelhead smolts.

Résumé : Pour les saumoneaux de salmonidés anadromes du Pacifique (*Oncorhynchus* spp.), l'état physiologique des individus peut influencer le résultat de la migration. Cette étape clé du cycle de vie est typiquement associée à une faible survie et elle influence la productivité des populations, d'où l'importance de cerner les facteurs intrinsèques associés au résultat des migrations de sortie. Pour mieux comprendre et cerner ces facteurs, nous avons combiné la télémétrie acoustique à des biopsies de branchies non létales et utilisé l'amplification en chaîne par polymérase quantitative en temps réel et à débit élevé pour évaluer l'influence d'agents infectieux et des profils d'expression génique des hôtes sur le résultat de la migration pour des saumoneaux de truite arc-en-ciel (*Oncorhynchus mykiss*) issus d'écloseries. Des analyses de redondance de l'expression génique, des charges d'agents infectieux et de l'embonpoint font ressortir des profils d'expression génique qui prédisent le résultat de la migration. Les saumoneaux jamais détectés après leur lâcher dans la rivière présentaient une expression significativement élevée des gènes de l'immunité *Il-17D* et *RPL6* et une expression plus faible du gène de l'osmorégulation *NKA α1b* par rapport aux autres individus. *Flavobacterium psychrophilum* et "*Candidatus* Branchiomonas cysticola" ont été détectés dans les échantillons de branchies, mais ni l'un ni l'autre n'influençait la survie. Nous présentons de rares preuves d'un lien entre les profils d'expression génique et le résultat de la migration chez des salmonidés juvéniles et décrivons des mécanismes qui pourraient influencer le destin des saumoneaux de truite arc-en-ciel issus d'écloseries. [Traduit par la Rédaction]

Introduction

Large-scale migrations are an important life history component for Pacific anadromous salmonids (*Oncorhynchus* spp.). Towards the end of their freshwater residence, individuals undergo dramatic physiological changes prior to migrating to the marine environment as smolts (Groot and Margolis 1991). For smolts, the period of outmigration through freshwater and marine coastal regions can be associated with particularly poor survival (Balfry et al. 2011; Clark et al. 2016; Friedland et al. 2014; Welch et al. 2011). Declining productivity in some species and stocks (Irvine and Akenhead 2013; Peterman and Dorner 2012) has been linked to the smolt life stage (Goetz et al. 2015; Kendall et al. 2017), underscoring the need to identify factors influencing survival during this critical period. An increased understanding of processes linked to outmigration survival could be used by managers to enhance the predictive capabilities of population productivity models (Beamish and Mahnken 2001; Evans et al. 2014; Irvine and Fukuwaka 2011) and to improve conservation measures for species or stocks in decline. Studies focusing on the smolt life stage have suggested various factors that can influence survival, including predation (Berejikian et al. 2016; Hostetter et al. 2012), environmental conditions (Beamish et al. 2000; Friedland et al. 2014), and food availability (Beamish and Mahnken 2001; Hertz et al. 2016).

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Physiological condition can also play an important role in survival for smolts during outmigration (Hostetter et al. 2011; Jeffries et al. 2014). The smoltification process is energetically intensive and consists of various key physiological changes enabling fish to transition from fresh water to the marine environment (Groot and Margolis 1991; Hanson et al. 2011). Most importantly, smolts must undergo shifts in ion regulation at the gills (Stefansson et al. 2007), which if compromised could contribute to reduced estuary or early marine survival (McCormick et al. 2009). Size- or growth-related factors may also influence fate for outmigrating smolts because alterations in body size and morphology are important for entering marine systems (Beamish et al. 2004; Beckman et al. 1999). However, our current understanding of how smolt condition influences fate is lacking, as few studies have directly linked individual physiology with migration fate (but see Evans et al. 2014; Hostetter et al. 2011; Jeffries et al. 2014).

An understudied aspect of the smolt life stage is the role that disease and immune responses play on migration performance (Miller et al. 2014; but see Jeffries et al. 2014). Smolts can be exposed to infectious agents during freshwater rearing and upon entering the marine environment (Bakke and Harris 1998). Infected individuals may be less capable of successfully migrating (Jeffries et al. 2014) or at greater risk to predation along migratory pathways (Hostetter et al. 2012; Miller et al. 2014). Recent studies with outmigrating steelhead smolts (*Oncorhynchus mykiss*) in the Columbia River system have linked poor external body condition to increased levels of infectious agents and reduced survival (Evans et al. 2014; Hostetter et al. 2011). Few studies on disease in migrating populations have linked infectious agents to fate directly, because it can be particularly challenging to observe mortality in wild systems (La and Cooke 2011; Miller et al. 2014). At present, population-level monitoring for diseases in the Pacific Northwest is limited (Miller et al. 2014), particularly for species in decline, such as steelhead (Scheuerell et al. 2009; Smith and Ward 2000). Thus, a necessary step in determining the role disease plays in migrating populations will be identifying infectious agents and intrinsic factors (e.g., stress and immune responses) that are associated with individual smolt outmigration fate.

Recent advancements in transcriptomics technology (quantifying the expression levels of mRNA in a tissue sample) have vastly improved our ability to study an organism's physiology. High-throughput real-time quantitative polymerase chain reaction (HT-qRT-PCR) is a powerful and sensitive tool that allows researchers to assess tissue samples simultaneously from many individual fish against multiple assays. Assays can be chosen to target the expression of genes in the tissue and (or) assess the presence and loads of infectious agents within the sample itself (Miller et al. 2016). The resulting data can be combined with biotelemetry to identify associations among gene expression, infectious agents, and survival for individual migrating salmonids (Miller et al. 2009, 2011; Evans et al. 2011). Jeffries et al. (2014) demonstrated this approach by combining nonlethal gill biopsies with acoustic telemetry and found that infectious agents and immune gene expression profiles of sockeye salmon smolts (*Oncorhynchus nerka*) were predictive of migration fate in fresh water.

Acoustic telemetry is an effective tool for studying multiple aspects of smolt outmigration ecology. Individual movements, as well as survival and migration rates can be estimated across large distances of both freshwater and marine migration (e.g., Clark et al. 2016; Melnychuk et al. 2007; Moore et al. 2010; Welch et al. 2009, 2011). In 2015, an acoustic tagging study took place with hatchery steelhead (*O. mykiss*) smolts from the Seymour River (North Vancouver, British Columbia; Healy et al. 2017). This study used both river and marine release locations to test the hypothesis (put forth by Balfry et al. 2011) that the initial segment of the marine pathway (Burrard Inlet) was associated with poor survival for migrating smolts. The study concluded that the river and Burrard Inlet were regions of particularly poor survival (Healy et al. 2017) and thus identified regions where external and physiological factors may be particularly important determinants of migration fate.

The primary objective of the present study was to empirically investigate the relationship between the physiological condition of Seymour River steelhead smolts and outmigration fate. We collected nonlethal gill biopsies from acoustically tagged steelhead at the Seymour River Hatchery and used HT-qRT-PCR to screen for multiple infectious agents. Additionally, we assessed the expression of a suite of host genes and related gene expression profiles and infectious agents to migration fate.

Methods

Study system

The Seymour River is a regulated (i.e., dammed) river in North Vancouver, British Columbia (Fig. 1). Its watershed flows south into the marine system, Burrard Inlet, which separates the city of Vancouver from North Vancouver. The Seymour River Hatchery, located downstream of the Seymour Falls dam, produces up to 30 000 teelhead trout annually (Seymour Salmonid Society 2015; www.seymoursalmon.com). If steelhead smolts are released in the lower Seymour River (below the dam), they migrate south to Burrard Inlet, then typically to the northwest through the Salish Sea (Balfry et al. 2011; Welch et al. 2011), a semi-enclosed marine system situated between Vancouver Island and the mainland of British Columbia. Smolts then must navigate through the Discovery Islands, Johnstone Strait, and Queen Charlotte Strait, before ultimately making their way to the open ocean (Fig. 1). At present, the hatchery transports and releases most of its steelhead in salt water beyond Burrard Inlet, after an earlier acoustic telemetry study (Balfry et al. 2011) suggested this inlet may be associated with poor survival.

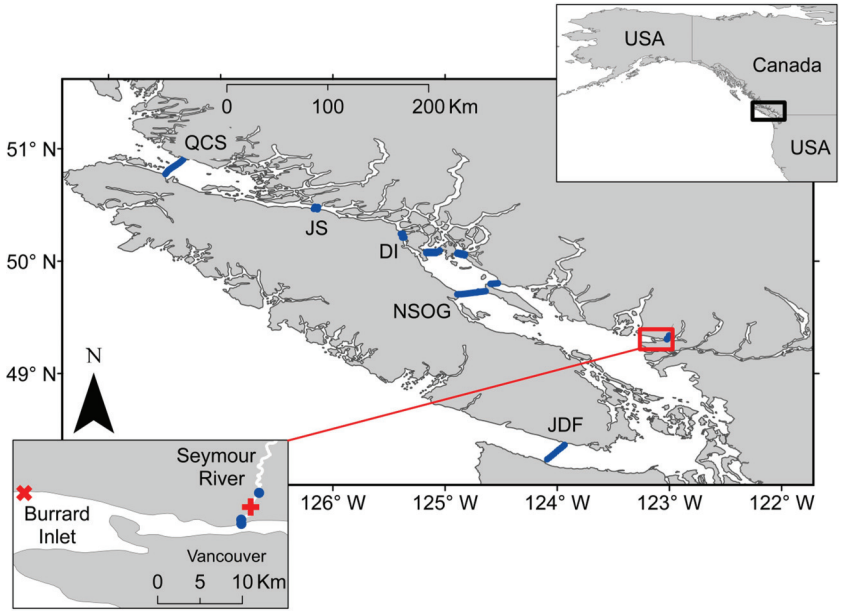
Acoustic tagging and gill sampling

Tagging took place at the Seymour River Hatchery (49°26′15.2″N, 122°58′01.1″W) between 14 and 15 May 2015 (University of British Columbia Animal Use Protocol: A15-0205). Steelhead smolts (fork length (FL) = 200.2 mm (±0.8 mm standard error, SE); mass = 77.0 g (±1.1 g SE); n = 243) were removed from hatchery rearing channels, placed in separated raceways, and starved for 24 h prior to surgeries. Following Collins et al. (2013) and Furey et al. (2016), fish were haphazardly selected, anaesthetized in a solution of buffered tricaine methanesulfonate (MS-222; 100 mg·L^{-1}; 200 mg·L^{-1} $NaHCO_3$), measured for mass and FL (total air exposure <1 min), and placed ventral side up on a V-shaped surgery trough. Water from a maintenance bath of MS-222 (50 mg·L^{-1} MS-222, 100 mg·L^{-1} $NaHCO_3$), which was oxygenated using air stones and monitored for consistent temperature, was irrigated across the gills for the duration of each surgery. A small (~8–10 mm) midventral incision was made just posterior of the pelvic fins. VEMCO V7-2L acoustic transmitters (7 mm × 18 mm, ~1.6 g in air; 69 kHz, VEMCO Ltd., Bedford, Nova Scotia; www.vemco.com) were inserted through the incision and positioned lengthwise inside the body cavity. The incision was closed using two absorbable monofilament sutures (Ethicon monocryl 5-0 monofilament; www.ethicon.com). During acoustic tagging, 164 smolts were nonlethally biopsied for gill tissue using small bone cutting forceps to remove two to three gill filaments (Jeffries et al. 2014), which were placed in RNAlater (Life Technologies, Grand Island, New York). Gill samples were stored at 4 °C for 24–48 h before being frozen at −80 °C prior to laboratory work. Following surgery, fish were placed in separate pens within a flow-through raceway (grouped by release location) and allowed to recover for at least 4 days prior to release.

Releases and telemetry infrastructure

Tagged steelhead were transported in ~1000 L tanks on trucks and released at one of two locations: (*i*) directly into salt water (marine-release) ~18 km west of the Seymour River estuary

Fig. 1. Map of the 2015 study area for Seymour steelhead (*Oncorhynchus mykiss*). Circles and lines of circles represent either individual receivers or a receiver subarray. The inset (bottom left) shows a close-up of Burrard Inlet and the Seymour River. Fish were tagged at the Seymour River Hatchery in May, then transported and released beyond Burrard Inlet ("×"; n = 160) or the lower Seymour River ("+"; n = 83). QCS = Queen Charlotte Strait subarray, JS = Johnstone Strait subarray, DI = Discovery Islands subarrays, NSOG = Northern Strait of Georgia subarray, JDF = Juan de Fuca Strait subarray.



(49°20′24.8″N, 123°13′58.2″W; FL = 199.4 mm (±1.0 mm SE), mass = 75.5 g (±1.3 g SE), n released = 160, n biopsied = 68) or (*ii*) in the lower Seymour River (river-release) (49°19′18.7″N, 123°00′50.4″W; FL = 201.7 mm (±1.7 mm SE), mass = 78.6 g (±2.3 g SE), n released = 83, n biopsied = 46); Fig. 1). These release locations (same used by Balfry et al. 2011) were selected for a parallel study using acoustic telemetry to validate the hypothesis that Burrard Inlet was a region of poor survival for migrating steelhead (i.e., Healy et al. 2017). Marine-released fish were transported and released on 19 May, and river-released fish were released over the course of 3 days (21–23 May) to maximize detections (i.e., minimize acoustic interference between tags, or "tag collisions") in the estuary. Marine-released fish were released with ~20 000 untagged conspecifics produced by the hatchery, which were transported by separate trucks. River-released fish were released with several hundred untagged hatchery conspecifics.

Steelhead were tracked by a suite of acoustic receiver subarrays (combination of Vemco VR2W, VR3, and VR4; www.vemco.com; Halifax, Nova Scotia), originally set up by the Pacific Ocean Salmon Tracking project (Welch et al. 2003), and now maintained by the Ocean Tracking Network Canada (Cooke et al. 2011) and the Pacific Salmon Foundation. Three additional receivers (Vemco VR2W) were placed in fresh water: one ~1.5 km upstream of the release site and two at the mouth of the Seymour River to monitor estuary residence time (duration between first and last detection). These fixed freshwater and marine arrays allowed us to track smolts from their point of release to the northern or southern tip of Vancouver Island (up to ~400 km in-water distance; Fig. 1).

Laboratory work

Steelhead smolt gill samples were analysed for 57 host genes and 18 infectious agents (run in duplicate) using HT-qRT-PCR on the Fluidigm BioMark HD platform (Fluidigm, San Francisco, California, USA). This technology uses microfluidics and is used in medical fields (Diercks et al. 2009; Michelet et al. 2014; Spurgeon et al. 2008), but more recently has been adopted by fisheries ecologists (Evans et al. 2011; Jeffries et al. 2014; Miller et al. 2011) and has been demonstrated as a reliable methodology for use in salmonid infectious agent studies (Miller et al. 2016). This platform allows for 96 different samples against 96 different assays to be run on a single dynamic array (Miller et al. 2016). Host gene assays were selected based on important processes related to smoltification (Beamish and Mahnken 2001; Havird et al. 2013; Nilsen et al. 2007; Stefansson et al. 2007) and immune or stress responses to potential infectious agents (e.g., Henriksen et al. 2015; Raida and Buchmann 2008) (Table 1). Infectious agent assays were selected based on pre-analyses screening of pooled gill samples (i.e., Miller et al. 2016), as well as prior knowledge of agents known to infect salmonids in the Pacific Northwest (Jeffries et al. 2014; Miller et al. 2014, 2016).

Gene expression and infectious agent detection assessments took place at the Fisheries and Oceans Canada Pacific Biological Station (Nanaimo, British Columbia). Gill RNA extraction methods followed those previously described (Bass et al. 2017; Jeffries et al. 2014; Miller et al. 2011). Gill filaments were removed from RNAlater (Life Technologies) vials and homogenized using Magmax-96 for Microarrays Kits (Ambion Inc., Austin, Texas, USA). Gill filaments were homogenized using TRI-reagent (Ambion Inc.), then 1-bromo-3-chloropropane was added to the homogenate. 100 μL aliquots of the aqueous phase were placed in 96-well plates prior to RNA extraction. RNA purity was assessed and normalized to 15 ng·μL^{-1} using the A_{260}/A_{280} method using a Biomek FXP liquid handling instrument (Beckman-Coulter, Mississauga, Ontario, Canada). Normalized RNA (0.25 μg) was used to make cDNA using VILO (SuperScript VILO MasterMix; Life Technologies) and PCR cycling at 25 °C for 5 min, 24 °C for 60 min, and 85 °C for 5 min according to the BioMark protocol.

To account for the small assay volumes used by the BioMark platform, suspended cDNA (1.25 μL) was pre-amplified with a 5 μL mix of primers corresponding to all 75 PCR assays (at a 1:20 normal PCR dilution), by cycling 15 times in a PCR machine at 95 °C for 10 min, 95 °C for 10 s, and 60 °C for 4 min, as per the Fluidigm protocol. Because variation between duplicate assays using the BioMark platform is typically minimal, host biomarkers (n = 54) were run singularly, while all infective agent assays (n = 18) were

Table 1. Primer and probe sequences corresponding to assays used in HT-qRT-PCR analyses on hatchery steelhead (*Oncorhynchus mykiss*) smolts.

Gene abbrev.	Infectious agent – host gene name	Assay class	Type–function	Primer sequence*	Efficiency
re.sal	*Renibacterium salmoninarum*	Microbe	Bacteria	F: CAACAGGGTGGTTATTCTGCTTTC R: CTATAAGAGCCACCAGCTGCAA P: CTCCAGCGCCGCAGGAGGAC	1.89
vi_sal	*Vibrio salmonicida*	Microbe	Bacteria	F: GTGTGATGACCGTTCCATATTT R: GCTATTGTCATCACTCTGTTTCTT P: TCGCTTCATGTTGTGTAATTAGGAGCGA	1.87
fl_psy	*Flavobacterium psychrophilum*	Microbe	Bacteria	F: GATCCTTATTCTCACAGTACCGTCAA R: TGTAAACTGCTTTTGCACAGGAA P: AAACACTCGGTCGTGACC	1.84
c_b_cys	"*Candidatus* Branchiomonas cysticola"	Microbe	Bacteria	F: AATACATCGGAACGTGTCTAGTG R: GCCATCAGCCGCTCATGTG P: CTCGGTCCCAGGCTTTCCTCTCCCA	1.80
ae_sal	*Aeromonas salmonicida*	Microbe	Bacteria	F: TAAAGCACTGTCTGTTACC R: GCTACTTCACCCTGATTGG P: ACATCAGCAGGCTTCAGAGTCACTG	2.03
vi_ang	*Vibrio anguillarum*	Microbe	Bacteria	F: CCGTCATGCTATCTAGAGATGTATTTGA R: CCATACGCAGCCAAAAATCA P: TCATTTCGACGAGCGTCTTGTTCAGC	1.82
ic_hof	*Ichthyophonus hoferi Sphaerothecum*	Microbe	Mesomycetozoean	F: GTCTGTACTGGTACGGCAGTTTC R: TCCCGAACTCAGTAGACACTCAA P: TAAGAGCACCCACTGCCTTCGAGAAGA	1.86
lo_sal	*Loma salmonae*	Microbe	Microsporidian	F: GGAGTCGCAGCGAAGATAGC R: CTTTTCCTCCCTTTACTCATATGCTT P: TGCCTGAAATCACGAGAGTGAGACTACCC	1.81
pa_the	*Paranucleospora theridion*	Microbe	Microsporidian	F: CGGACAGGGAGCATGGTATAG R: GGTCCAGGTTGGGTCTTGAG P: TTGGCGAAGAATGAAA	1.60
ce_sha	*Ceratonova shasta*	Microbe	Myxozoan	F: CCAGCTTGAGATTAGCTCGGTAA R: CCCCGGAACCCGAAAG P: CGAGCCAAGTTGGTCTCTCCGTGAAAAC	1.81
pa_min	*Parvicapsula minibicornis*	Microbe	Myxozoan	F: AATAGTTGTTTGTCGTGCACTCTGT R: CCGATAGGCTATCCAGTACCTAGTAAG P: TGTCCACCTAGTAAGGC	1.78
p_pse	*Parvicapsula pseudobranchia*	Microbe	Myxozoan	F: CAGCTCCAGTAGTGTATTTCA R: TTGAGCACTCTGCTTTATTCAA P: CGTATTGCTGTCTTTGACATGCAGT	2.13
cr_sal	*Cryptobia salmocidica*	Microbe	Protozoan	F: TCAGTGCCTTTCAGGACATC R: GAGGCATCCACTCCAATAGAC P: AGGAGGACATGGCAGCCTTTGTAT	1.84
ic_mul	*Ichthyophthirius multifiliis*	Microbe	Protozoan	F: AAATGGGCATACGTTTGCAAA R: AACCTGCCTGAAACACTCTAATTTTT P: ACTCGGCCTTCACTGGTTCGACTTGG	1.84
sch	Salmon (gill) chlamydia	Microbe	Virus	F: GGGTAGCCCGATATCTTCAAAGT R: CCCATGAGCCGCTCTCTCT P: TCCTTCGGGACCTTAC	1.82
vhsv	Viral hemorrhagic septicemia virus	Microbe	Virus	F: ATGAGGCAGGTGTCGGAGG R: TGTAGTAGGACTCTCCCAGCATCC P: TACGCCATCATGATGAGT	1.60
prv	Piscine reovirus	Microbe	Virus	F: TGCTAACACTCCAGGAGTCATTG R: TGAATCCGCTGCAGATGAGTA P: CGCCGGTAGCTCT	1.90
ihnv	Infectious hematopoietic necrosis virus	Microbe	Virus	F: AGAGCCAAGGCACTGTGCG R: TTCTTTGCGGCTTGGTTGA P: TGAGACTGAGCGGGACA	1.81
ATP5G3-C	ATP synthase	Host gene	Ion transport – metabolism	F: GGAACGCCACCATGAGACA R: CGCCATCCTGGGCTTTG P: AGCCCCATTGCCTC	1.81
C4B	Complement component 4B	Host gene	Immune	F: TCCAACCACATCGCATTATCC R: ATCTCTGACACCACTGACCACAA P: ATAGACAGGCTTCCC	1.83
C5	Component factor 5	Host gene	Immune	F: TGGCAAGGACTTTTTCTGCT R: AGCACAGGTATCCAGGGTTG P: CTGGCAGGGATTGCATCAAATC	1.93
C5aR	Complement component 5a receptor 1	Host gene	Immune	F: ACGCACCTTGAGGGTCATT R: CAGTGGAAACCAGCACAGG P: TTGCCGTGTCGCTGAGCTTCTT	1.92

Table 1 (*continued*).

Gene abbrev.	Infectious agent – host gene name	Assay class	Type–function	Primer sequence*	Efficiency
C7	Complement component C7 precursor	Host gene	Immune	F: ACCTCTGTCCAGCTCTGTGTC R: GATGCTGACCACATCAAACTGC P: AACTACCAGACAGTGCTG	1.80
CCT5	T-complex protein 1 subunit epsilon	Host gene	Immune	F: CCTCAGTGGGAGGTCCCTAAT R: CCCCAGGTAGTCAAAATGATCCT P: CTTCTGAAGTCATCTATCT	1.74
CD4	Cell receptor	Host gene	Immune	F: CATTAGCCTGGGTGGTCAAT R: CCCTTTCTTTGACAGGGAGA P: CAGAAGAGAGAGCTGGATGTCTCCG	1.91
CD83	Cluster of differentiation 83	Host gene	Immune	F: GATGCACCCCTTGAGAAGAA R: GAACCCTGTCTCGACCAGTT P: AATGTTGATTTACACTCTGGGGCCA	1.82
CD8α	Cluster of differentiation 8α	Host gene	Immune	F: ACACCAATGACCACAACCATAGAG R: GGGTCCACCTTTCCCACTTT P: ACCAGCTCTACAACTGCCAAGTCGTGC	1.81
CIRP	Cold inducible RNA binding protein	Host gene	Stress–osmoregulation	F: AAGCTGTGATTGTGCTCTAAAGAC R: TCCCACTTAGCATTCCATCCTTG P: CTCCTTCAGTTCTGTAATGC	NA
COMMD7	COMM domain containing protein 7	Host gene	Immune	F: CAAAGCCAGTATGGACTGTTTCAG R: TTGTTTTCTGCTGCCCCTCTA P: ACCTGATCGCCAGTAGCATGAGCATGTAC	1.80
CXCR4	C-X-C chemokine receptor type 4	Host gene	Immune–osmoregulatory	F: GGAGATCACATTGAGCAACATCA R: GCTGCTGGCTGCCATACTG P: TCCACGAAGATCCCCA	1.82
FYB	FYN-T-binding protein	Host gene	Immune	F: TGCAGATGAGCTTGTTGTCTACAG R: GCAGTAAAGATCTGCCGTTGAGA P: CTCAACGATGACATCCACAGTCTCCC	1.88
GHR	Growth hormone receptor	Host gene	Growth	F: TGGGAAGTTGAGTGCCAGACT R: CACAAGACTACTGTCCTCCGTTGA P: TGGGAGAGCCAGCCAGCCTGC	NA
GR-2	Glucocorticoid receptor 2	Host gene	Growth	F: TCCAGCAGCTATGCCAGTTCT R: TTGCCCTGGGTTGTACATGA P: AAGCTTGGTGGTGGCGCTG	NA
Hep	Hepcidin	Host gene	Immune	F: GAGGAGGTTGGAAGCATTGA R: TGACGCTTGAACCTGAAATG P: AGTCCAGTTGGGGAACATCAACAG	1.93
HSC70	Heat shock cognate 70 protein	Host gene	Stress	F: GGGTCACACAGAAGCCAAAAG R: GCGCTCTATAGCGTTGATTGGT P: AGACCAAGCCTAAACTA	1.86
HSP90	Heat shock protein 90	Host gene	Stress	F: TGGGCTACATGGCTGCCAAG R: TCCAAGGTGAACCCAGAGGAC P: AGCACCTGGAGATCAA	1.63
HTA	HIV-1 Tat interactive protein	Host gene	Immune	F: CTTGTAACAGTTCGACATGGCTTATT R: TGGTGAAGCATTTCTGTATGTCAA P: TCTGTACTGAGCATCCCCGCACATTACA	1.83
IFNα	Interferon alpha	Host gene	Immune	F: CGTCATCTGCAAAGATTGGA R: GGGCGTAGCTTCTGAAATGA P: TGCAGCACAGATGTACTGATCATCCA	1.87
IGF-1R	Insulin-like growth factor 1	Host gene	Growth	F: TGAAGAGCCACCTGAGGTCACT R: TCAGAGGTGGGAGGTTGAGACT P: CGGGCTAAAGACCCGTCCCAGTCC	1.99
IgMs	Immunoglobulin	Host gene	Immune	F: CTTGGCTTGTTGACGATGAG R: GGCTAGTGGTGTTGAATTGG P: TGGAGAGAACGAGCAGTTCAGCA	1.86
IgT	Immunoglobulin tau	Host gene	Immune	F: AGCACCAGGGTGAAACCA R: GCGGTGGGTTCAGAGTCA P: AGCAAGACGACCTCCAAAACAGAAC	2.10
Il-10	Interleukin 10	Host gene	Immune	F: CGACTTTAAATCTCCCATCGAC R: GCATTGGACGATCTCTTTCTTC P: CATCGGAAACATCTTCCACGAGCT	NA
Il-11	Interleukin 11	Host gene	Immune	F: GCAATCTCTTGCCTCCACTC R: TTGTCACGTGCTCCAGTTTC P: TCGCGGAGTGTGAAAGGCAGA	1.94
Il-15	Interleukin 15	Host gene	Immune	F: TTGGATTTTGCCCTAACTGC R: CTGCGCTCCAATAAACGAAT P: CGAACAACGCTGATGACAGGTTTTT	1.85

Table 1 (*continued*).

Gene abbrev.	Infectious agent – host gene name	Assay class	Type–function	Primer sequence*	Efficiency
Il-17D	Interleukin 17D	Host gene	Immune	F: CAACAGAAGTGCGAACGATG R: GATGCCACATCGCATAACAG P: TGGTCGAGTATCTTTCGTGTGTTTGC	1.91
Il-1β	Interleukin 1 beta	Host gene	Immune	F: AGGACAAGGACCTGCTCAACT R: CCGACTCCAACTCCAACACTA P: TTGCTGGAGAGTGCTGTGGAAGAA	1.83
Il-1R	Interleukin-1 receptor complex	Host gene	Immune	F: ATCATCCTGTCAGCCCAGAG R: TCTGGTGCAGTGGTAACTGG P: TGCATCCCCTCTACACCCCAAA	1.80
Il-8	Interleukin 8	Host gene	Immune	F: GAGCGGTCAGGAGATTTGTC R: TTGGCCAGCATCTTCTCAAT P: ATGTCAGCGCTCCGTGGGT	1.97
JUN	Transcription factor	Host gene	Immune	F: TTGTTGCTGGTGAGAAAACTCAGT R: CCTGTTGCCCTATGAATTGTCTAGT P: AGACTTGGGCTATTTAC	NA
MARCH2	*Salmo salar* E3 ubiquitin-protein ligase MARCH2	Host gene	Immune–stress	F: GCACCTGCGATAGAAGAGCAT R: GAGATGGAATCCGCAGAAGCT P: ACTTGTTTAACCATGCTGTGCGACTCTTCCT	1.85
MHC1	Major histocompatibility complex class I	Host gene	Immune	F: GCGACAGGTTTCTACCCCAGT R: TGTCAGGTGGGAGCTTTTCTG P: TGGTGTCCTGGCAGAAAGACGG	1.99
MHCII	Major histocompatibility complex class II	Host gene	Immune	F: TGCCATGCTGATGTGCAG R: GTCCCTCAGCCAGGTCACT P: CGCCTATGACTTCTACCCCAAACAAAT	1.64
MMP13	Matrix metalloproteinase-13	Host gene	Immune	F: GCCAGCGGAGCAGGAA R: AGTCACCTGGAGGCCAAAGA P: TCAGCGAGATGCAAAG	1.81
MMP25	Matrix metalloproteinase-25 precursor	Host gene	Immune	F: TGCAGTCTTTTCCCCTTGGAT R: TCCACATGTACCCACACCTACAC P: AGGATTGGCTGGAAGGT	1.75
Mx	Antiviral protein	Host gene	Immune	F: AGATGATGCTGCACCTCAAGTC R: CTGCAGCTGGGAAGCAAAC P: ATTCCCATGGTGATCCGCTACCTGG	1.82
NKA α1a	Na^+/K^+-ATPase α1a subunit	Host gene	Osmoregulatory	F: CCAGGATCACTCAATGTCACTCT R: GCTATCAAAGGCAAATGAGTTTAATATCATTGTAAAA P: ACGATTACATTATAAGGCAATACT	NA
NKA α1a	Na^+/K^+-ATPase α1a subunit	Host gene	Osmoregulatory	F: AGGAAGCCTTCCAGAACGCT R: CAATCAAACTGGAAGCCCTCA P: AATCCCCAGGCAAAGTGGCCCA	NA
NKA α1b	Na^+/K^+-ATPase α1b subunit	Host gene	Osmoregulatory	F: GCTACATCTCAACCAACAACATTACAC R: TGCAGCTGAGTGCACCAT P: ACCATTACATCCAATGAACACT	1.87
NKA α1c	Na^+/K^+-ATPase α1c subunit	Host gene	Osmoregulatory	F: AGGGAGACGTACTACTAGAAAGCAT R: CAGAACTTAAAATTCCGAGCAGCAA P: ACAACCATGCAAGAACT	1.81
NKA α3	Na^+/K^+-ATPase α3 subunit	Host gene	Osmoregulatory	F: GGAGACCAGCAGAGGAACAG R: CCCTACCAGCCCTCTGAGT P: AAGACCCAGCCTGAAATG	1.80
NKA b1	Na^+/K^+-ATPase subunit beta 1	Host gene	Osmoregulatory	F: CGTCAAGCTGAACAGGATCGT R: CCTCAGGGATGCTTTCATTGGA P: CCTTGGCCTGAAGTTG	1.80
NKCC	Na^+/K^+,$2Cl^-$ contransporter	Host gene	Osmoregulatory	F: GATGATCTGCGGCCATGTTC R: AGACCAGTAACCTGTCGAGAAAC P: CTCCAGAAGGCCCAACTT	NA
RPL6	Ribosomal protein L6	Host gene	Immune	F: CGCCACCACAACCAAGGT R: TCCTCAGCCTCTTCTTCTTGAAG P: AGATCCCCAAGACTCTGTCAGACGCCT	1.81
SAA	Serum amyloid protein A	Host gene	Immune	F: GGGAGATGATTCAGGGTTCCA R: TTACGTCCCCAGTGGTTAGC P: TCGAGGACACGAGGACTCAGCA	1.87
SAP	Serum amyloid P	Host gene	Immune	F: CAACGTCTCAAAGCCCATTT R: GCCTCGTTCTTGCTCAGAGT P: CTGCTATGACCATGTGTCAGAGGTTC	1.59
SHOP21	Salmon hyperosmotic protein 21	Host gene	Stress–immune	F: GCGGTAGTGGAGTCAGTTGGA R: GCTGCTGACGTCTCACATCAC P: CCTGTTGATGCTCAAGG	2.00

Table 1 (*concluded*).

Gene abbrev.	Infectious agent – host gene name	Assay class	Type–function	Primer sequence*	Efficiency
STAT1	Activator of transcription 1-alpha/beta	Host gene	Immune	F: TGTCACCGTCTCAGACAGATCTG R: TGTTGGTCTCTGTAAGGCAACGT P: AGTTGCTGAAAACCGG	1.75
TCRβ	T-cell receptor beta	Host gene	Immune	F: TCACCAGCAGACTGAGAGTCC R: AAGCTGACAATGCAGGTGAATC P: CCAATGAATGGCACAAACCAGAGAA	1.75
TF	Transferrin	Host gene	Immune	F: TTCACTGCTGGAAAATGTGG R: GCTGCACTGAACTGCATCAT P: TGGTCCCTGTCATGGTGGAGCA	1.72
TNF-α	Tumor necrosis factor alpha	Host gene	Immune	F: GGGGACAAACTGTGGACTGA R: GAAGTTCTTGCCCTGCTCTG P: GACCAATCGACTGACCGACGTGGA	2.10
Coil	Reference gene	Reference gene	Reference	F: GCTCATTTGAGGAGAAGGAGGATG R: CTGGCGATGCTGTTCCTGAG P: TTATCAAGCAGCAAGCC	1.82
EF1a	Elongation factor 1 alpha	Reference gene	Reference	F: CGGAACGACGGTCGATCT R: GCTCACATCGCCTGCAAGT P: CTCCTTGAGCTCGCTG	1.74
786d16.1P	Si:dkey-78d16.1 protein (*Danio rerio*)	Reference gene	Reference	F: GTCAAGACTGGAGGCTCAGAG R: GATCAAGCCCCAGAAGTGTTTG P: AAGGTGATTCCCTCGCCGTCCGA	1.81

*F: forward primer sequence (5′–3′); R: reverse primer sequence (5′–3′); P: probe sequence (FAM-5′–3′-MGB).

run in duplicate to validate positive and negative detections. Three reference genes (run in duplicate) were included on each array. Two of these reference genes are commonly used with salmonid samples on the BioMark platform (*COIL* and *78d16.1*; Miller et al. 2016; Teffer et al. 2017), and *EF1a* was also included based on prior transcriptome studies with *O. mykiss* (Gunnarsson et al. 2017; Stefansson et al. 2007). After specific target amplification, ExoSAP-IT (Affymetrix, Santa Clara, California) was used to remove unincorporated primers by PCR cycling at 37 °C for 15 min and 80 °C for 15 min, then samples were diluted 1:5 in DNA Suspension Buffer (TEKnova, Hollister, California). Artificial positive constructs (cloned DNA sequence standards corresponding to all infectious agent assays, as outlined in Miller et al. 2016) were run in a panel of six serial dilutions on each dynamic array. For host gene assays, five 3× serial dilutions of host DNA were run on each dynamic array using 1 μL from each pooled sample. These dilutions were used for the calculation of host gene assay efficiencies and to quantify loads of any detected infectious agents (Miller et al. 2016).

Two 96.96 Fluidigm BioMark dynamic arrays were loaded in preparation for qPCR (each with identical assays, but different smolt samples randomly stratified across both arrays). Sample mix (5 μL) was prepared using 1× TaqMan Universal Master Mix (Life Technologies), 20× GE Sample Loading Reagent (Fluidigm), and amplified cDNA. Assay mix (5 μL) was prepared with 10 μmol·L^{-1} primers and 3 μmol·L^{-1} probes for each assay. Sample mix and assay mix were then added into the inlets on the Fluidigm 96.96 dynamic array. PCR was performed on the BioMark with the following conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Statistical analyses

Biomark Real-Time analysis software (Fluidigm; www.fluidigm.com) was used to determine cycle threshold (Ct) for each assay. Amplification curves of all assays were visually evaluated for unusual curve shapes. Using R statistical software, we calculated efficiencies for each assay using the slope of a regression between Ct values and serial dilutions. Points falling significantly outside of the linear relationship between Ct and known RNA concentration (typically found on the extreme ends; i.e., lowest RNA concentration) were removed to improve the accuracy of assay efficiency estimates. Only assays with efficiencies between 1.80 and 2.20, and with proper amplifications on both dynamic arrays, were considered for subsequent analyses. In total, 24 assays did not meet these criteria and were removed, leaving a total of 33 host gene assays and 15 infectious agents. One housekeeping gene (*EF1a*) was removed due to poor efficiency (<1.80). Host gene expression was normalized with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), with the first delta as the mean of the two reference genes and the second delta as the pooled sample made at the cDNA step. Normalizing to the pooled sample centers the data by showing expression relative to the population mean. Individual samples were assessed for an indication of poor quality (low expression of reference genes), and five samples were removed that were higher than 2 × SD from the mean Ct of either reference gene. Thus, all subsequent analyses were completed with 114 samples (Table 2).

To assess survival of gill-biopsied smolts, acoustic data were compiled into detection histories for each individual with available paired genomic data (n = 114). Survival in the river was calculated by dividing the number of river-released individuals detected in the estuary by the number released. Survival to Northern Strait of Georgia (NSOG) was calculated in a similar manner; however, separate estimates were calculated for each release group (i.e., for marine-release smolts from release to NSOG and for river-released individuals travelling from the estuary to NSOG). Two smolts migrated south through the Juan De Fuca Strait; however, gill biopsies were not taken from these individuals. To identify migration fate, individual smolts were then categorized into one of three groups based on regions where survival was known to be poorest (Healy et al. 2017): (*i*) smolts that were released in the river and never detected (i.e., assumed river mortalities or smolts that failed to reach the estuary; RM), (*ii*) smolts from either release group that did not survive the initial portion of the marine migration to the NSOG subarray (UN), and (*iii*) individuals that were successful migrants to at least the NSOG subarray (SU).

Because a microfluidics approach can show a large difference in Ct values compared with single assay systems, infectious agent assay Ct values were converted to copy number (amount of RNA copies in sample; i.e., load). To do this, we used the serial dilution of the artificial positive construct clones to create a standard curve between Ct and known concentration of RNA copies (Miller

Table 2. Summary table of Seymour River hatchery steelhead (*Oncorhynchus mykiss*) smolt tagging and survival data by release group.

				Assumed river mortalities (RM)	Survived river		Unsuccessful to NSOG (UN)	Successful past NSOG (SU)	
Release location	Sample size	Length (mm; SD)	Mass (g; SD)	Count	Count	Segment survival (%)	Count	Count	Cumulative survival (%)
River	46	203.9 (15.9)	81.3 (23.6)	9	37	80	24	13	28
Marine	68	202.7 (12.6)	80.0 (17.8)	NA	NA	NA	22	46	68

Note: NSOG = Northern Strait of Georgia.

et al. 2016). Infectious agent loads were then log-transformed to improve normality for subsequent analyses.

To describe any interrelationships among infectious agents, smolt body condition, and migration fate on gene expression, we used constrained ordination in the form of redundancy analyses (RDA). In preparation for RDA, a new variable, "relative infectious agent burden" (RIB; Bass 2018) was first calculated by

$$\sum_{i=1}^{m}\left(\frac{L_i}{\mathrm{Lmax}_i}\right)$$

where L_i is the RNA load of the ith infectious agent, and Lmax_i is the maximum load of the ith agent summed across all infectious agents (m) present in the individual. Thus, this metric considers infectious agent(s) present, as well as their relative load. Smolt mass (g) was modelled as a function of FL (mm), and we used the residuals of this relationship as a metric of body condition (i.e., larger length–weight residuals = larger mass for a given fork length). An overall RDA model was run using the package vegan (Borcard et al. 2011; Oksanen et al. 2008) in R.

RDA combines regression with PCA (Zuur et al. 2007) to test the relationship of multiple explanatory factors on a response matrix of data (in the present case, the gene expression matrix). Separate Monte Carlo permutations tests can be used to assess the significance of the entire model (i.e., whether the response matrix is associated with any explanatory variables), investigate which individual RDA axes represent variation that is more structured than random (i.e., test if gene clustering on individual canonical axes is not just randomly distributed), as well as test which individual explanatory factors are significant predictors of the response matrix (Legendre et al. 2011). Monte Carlo permutation tests calculate a p value based on the proportion of permuted test statistic values larger than the true unpermuted value of the statistic for a one-tailed ANOVA test (Borcard et al. 2011). For our analyses, the gene expression matrix of all individuals was the response variable, and scaled explanatory variables included infectious agents, RIB, length–weight residuals, and migration fate (model: gene expression matrix ~ "*Ca.* B. cysticola" + *F. psychrophilum* + RIB + length–weight residuals + migration fate). The model fit, as well as axes and terms, were assessed using Monte Carlo permutation tests. To reduce the probability of type I errors in our analyses, only genes that were tightly linked in RDA ordination space to migratory fate groups were further assessed by one-way ANOVAs with post hoc Tukey's honest significance tests. Five genes in closest ordination space to the RM group (*Il-17D*, *RPL6*, *MMP13*, *IFNα*, and *C5aR*), as well as five closest to SU smolts (*NKA α1b*, *NKA b1*, *hep*, *SAA*, and *C7*) were chosen for these analyses (see Results).

Because our RDA didn't allow us to directly test the relationship between migration fate and infectious agents, we ran a separate ANOVA comparing the loads of each agent that was detected among migration fate groups. For each infectious agent detected, we also compared the presence of each detected infectious agent among fate groups using a separate Pearson's χ^2 goodness-of-fit test.

To test whether length–weight residuals (i.e., body condition) varied by migration fate groups, we used a one-way ANOVA with post hoc Tukey's honest significance tests. Next, to test if length–weight residuals predicted residence time (as in Hanson et al. 2011), a generalized linear model was run with log-transformed (to improve normality) residence time as the response and length–weight residuals as the explanatory variable. Additionally, because most residence times were less than 1 day (Healy et al. 2017), we categorized smolts by either "long" residency (≥24 h) or "short" residency (<24 h) and used a one-way ANOVA to see if length–weight residuals differed between these two groups.

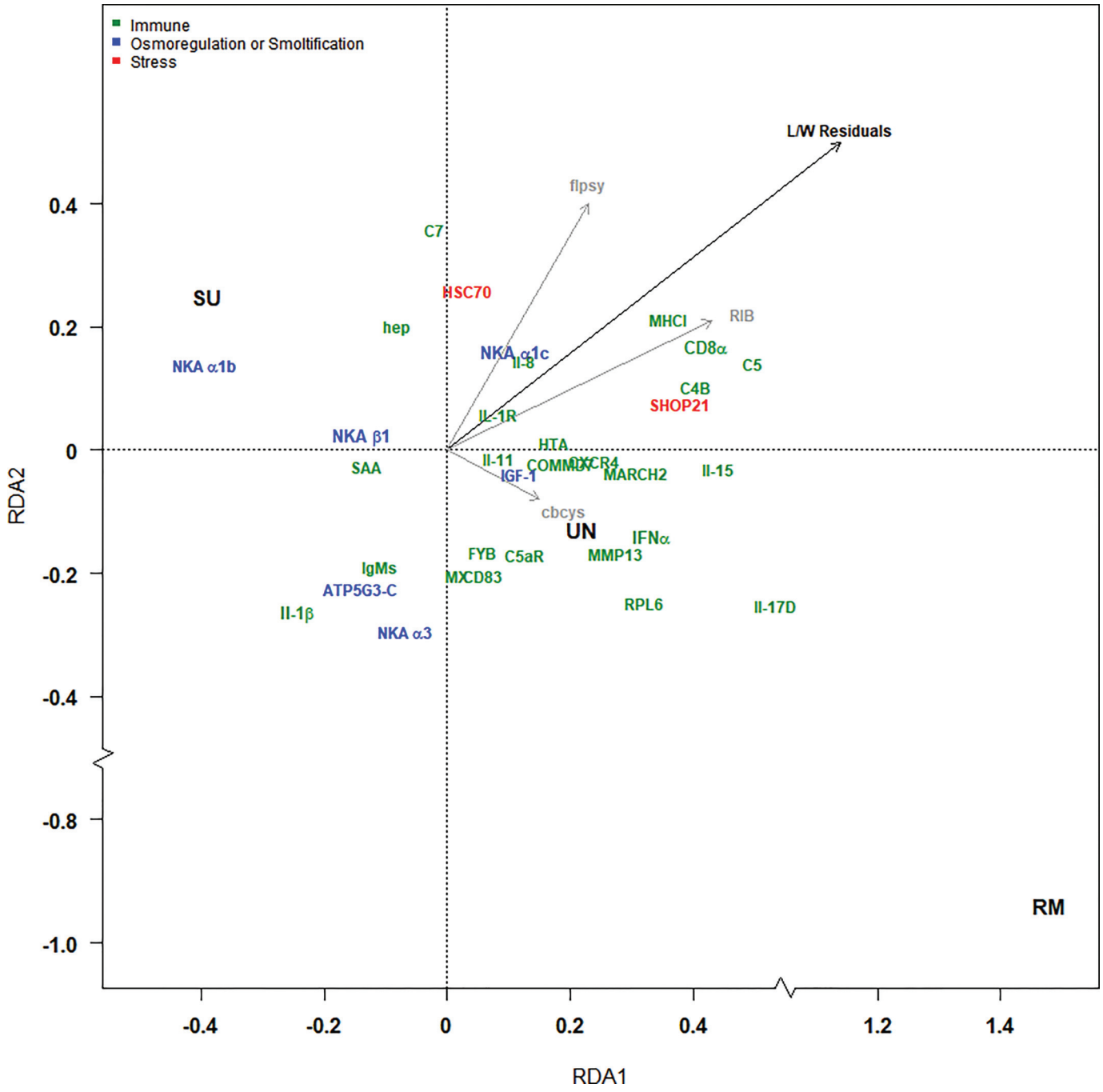
Because any potential differences between the two release groups (river- and marine-release) could bias our results, we carried out several tests assessing the physiological condition of these groups. First, we ran a separate RDA model with a similar structure to the previously described RDA model, with the exception of replacing migration fate with release group (model: gene expression matrix ~ "*Ca.* B. cysticola" + *F. psychrophilum* + RIB + length–weight residuals + release group). Release group was appropriate to assess in a separate model because migration fate and release group are confounding variables (e.g., only river-release fish could be classified "assumed river mortalities"). Thus, the inclusion of both of these factors in one RDA may have resulted in overfitting the model. The model fit and the significance of axes and terms were tested using Monte Carlo permutations tests. We also compared the presence of each infectious agent between release groups by using Pearson's χ^2 goodness-of-fit tests. To assess if loads of infectious agents differed between release groups, we ran separate ANOVAs comparing release groups for each infectious agent detected. All statistical analyses were completed in R (RStudio, v1.0.136; R Core Team 2015; www.rstudio.com).

Results

Smolt survival was poorest in two regions of the migration. In particular, for river-released smolts with accompanying gene expression data, 37 of 46 were detected in the estuary (80% survival) just ~2.5 km downstream of the release site (Table 2). In the marine environment, survival varied between groups to the NSOG array. For river-released smolts that had to travel through Burrard Inlet, only 13 were detected at NSOG (35% survival), compared with 46 of 68 marine-released smolts (68% survival; Table 2), which were released just beyond Burrard Inlet. These survival calculations agree with survival estimates from a parallel study using both biopsied and nonbiopsied steelhead smolts, which also found the river and Burrard Inlet to be regions of poor survival (Healy et al. 2017).

Two of 18 infectious agents monitored (Table 1) were detected, both of which were bacteria. *Flavobacterium psychrophilum* was present in 71 samples (~62%) and "*Candidatus* Branchiomonas cysticola" in 15 samples (~13%). There was no indication that migration fate was associated with either loads (ANOVAs: *F. psychrophilum*, $F_{[2,68]} = 1.069$, $p = 0.35$; "*Ca.* B. cysticola", $F_{[1,13]} = 0.013$, $p = 0.91$) or presence (*F. psychrophilum*, $\chi^2 = 0.382$, df = 2, $p = 0.826$; "*Ca.* B. cysticola", $\chi^2 = 1.543$, df = 2, $p = 0.462$) of these infectious agents. No difference in loads were detected between release groups (ANOVAs: "*Ca.* B. cysticola", $F_{[1,13]} = 0.220$, $p = 0.647$; *F. psychrophilum*, $F_{[1,69]} = 0.704$, $p = 0.404$). "*Candidatus* B. cysticola" presence did not vary between groups (marine-released: ~15%; river-released: ~11%; $\chi^2 = 0.097$, df = 1, $p = 0.755$); however, presence of *F. psychrophilum* was

Fig. 2. Redundancy analyses (RDA) ordination plot of Seymour River Hatchery steelhead (*Oncorhynchus mykiss*) gene expression data from nonlethal gill biopsies. RDA1 and RDA2 were determined to be significant, and all significant ($p < 0.05$) covariates are in black, while nonsignificant variables ($p \geq 0.05$) are in grey. Migration fate centroids are indicated as follows: RM = river mortalities (river-released smolts never detected on the estuary receivers); SU = successful migrants, to at least the NSOG subarray (both release groups); UN = unsuccessful migrants (both release groups not detected at or beyond NSOG). Genes are coloured according to their primary known function from the available literature; however, many genes are known to have multiple physiological associations. Note that both RDA1 and RDA2 have axes breaks. [Colour version available online.]



determined to be higher in marine-released (~72%) relative to river-released smolts (~48%; $\chi^2 = 5.866$, df = 1, $p = 0.015$).

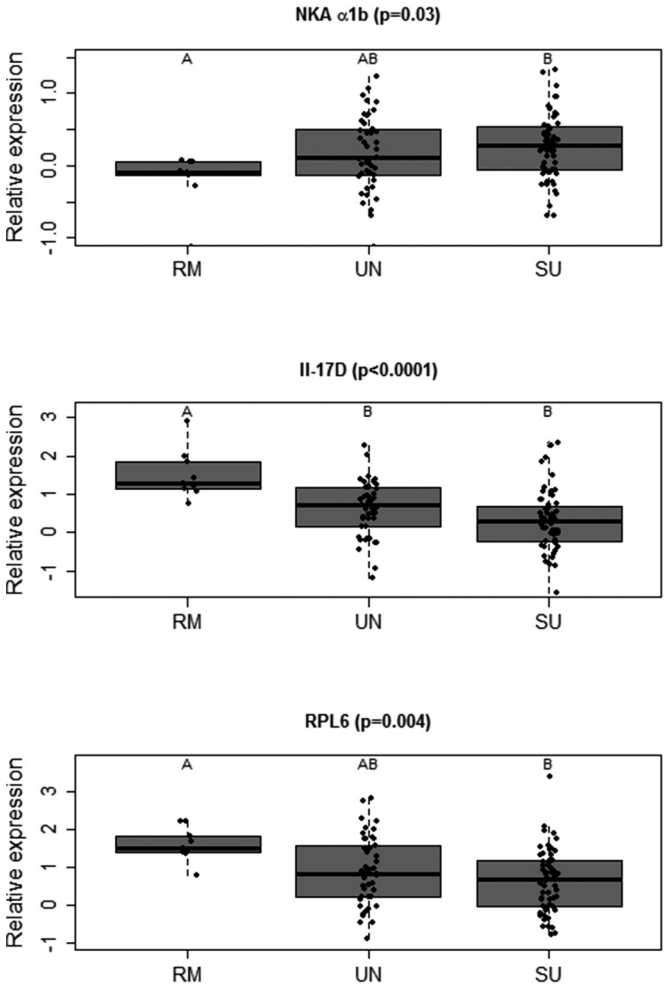
The RDA model including migration fate (model: gene expression matrix ~ "*Ca.* B. cysticola" + *F. psychrophilum* + RIB + length–weight residuals + migration fate) was significant (Monte Carlo permutations test, $F_{[6,107]} = 1.495$, $p = 0.005$; Fig. 2). All five factors ("*Ca.* B. cysticola", *F. psychrophilum*, RIB, length–weight residuals, migration fate) combined accounted for ~8% of the variance in the gene expression data. The first two RDA axes were determined to be significant (Monte Carlo permutation tests, RDA1, $F_{[1,107]} = 4.229$, $p = 0.001$; RDA2, $F_{[1,107]} = 2.024$, $p = 0.039$) and explained 3.5%, and 1.6% of the variance in the gene expression data, respectively (i.e., cumulatively ~5.2%). Two explanatory factors were found to be significantly related to the gene expression matrix (at $p < 0.05$): migration fate (Monte Carlo permutations test, $F_{[2,107]} = 1.5013$, $p = 0.045$), and length–weight residuals (Monte Carlo permuta-

Table 3. Summary statistics for the redundancy analysis (RDA) of the migration fate model (model: gene expression matrix ~ cbcys + flavo + RIB + length–weight residuals + migration fate) of Seymour River steelhead.

Variable	df	Variance	*F*	*p*
Relative infectious agent burden	1	0.4496	1.580	0.099
"*Candidatus* Branchiomonas cysticola"	1	0.2594	0.9115	0.515
Flavobacterium psychrophilum	1	0.2794	0.9820	0.451
Length–weight residuals	1	0.7097	2.4940	**0.003**
Migration fate	2	0.8544	1.5013	**0.045**
Residual	107	30.4475		

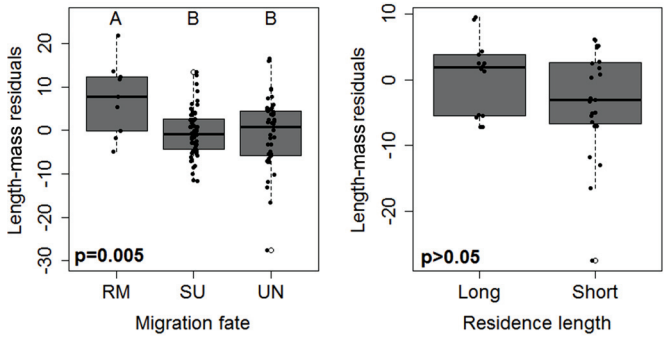
Note: Significant *p* values are shown in bold ($p < 0.05$).

Fig. 3. Boxplots of relative gene expression of three genes significant ($p < 0.05$) among migration fate groups. Individual hatchery steelhead (*Oncorhynchus mykiss*) smolts are shown by the black dots, while migration fate groupings are shown by the individual boxes. RM = river mortalities (or smolts never detected on the estuary receivers); SU = successful migrants, to at least the NSOG subarray; UN = unsuccessful migrants (did not make it to NSOG). Different letters denote Tukey's honest significance test statistical significance among fate groups for each gene.



tions test, $F_{[1,107]} = 2.494$, $p = 0.003$; Table 3). RDA1 was most associated with several genes loading positively (i.e., *Il-17D*, *C5*, *Il-15*, *CD8α*, *C4B*, *SHOP21*, and *MHCI*) and negatively (i.e., *NKA α1b*, *Il-1β*, *ATP5G3-C*, *SAA*, *NKA b1*, *IgMs*, *hep*, and *NKA α3*) along this axis (Fig. 2). RDA2 was positively associated with *C7*, *HSC70*, *MHCI*, *CD8α*, *NKA α1c*, and *hep* and most negatively with *NKA α3*, *Il-1β*, *Il-17D*, *RPL6*, *ATP5G3-C*, and *MX*.

Fig. 4. Relationship between length–weight residuals of hatchery steelhead (*Oncorhynchus mykiss*) smolts and migration fate (left panel). Estuary residence period (for just river-released smolts; right panel) is shown by duration (long: ≥24 h; short: <24 h). Letters above each migration fate group (left panel) shows Tukey's honest significance test statistical significance among groups. Each black point represents an individual smolt.



The overall RDA ordination revealed survival fate groups clustered separately in ordination space along the first two axes (Fig. 2). Successful (SU) smolts clustered negatively on RDA1 and positively on RDA2, while RM individuals (river-released smolts that failed to reach the estuary) clustered far away from all other fate groups (positively on RDA1, negatively on RDA2). RM smolts clustered closest with five immune genes (*Il-17D*, *RPL6*, *MMP13*, *IFNα*, and *C5aR*). In contrast, successful migrants through the system were associated with the osmoregulatory genes *NKA α1b* and *NKA b1*, but were also in close ordination space with the immune genes *C7*, *SAA*, and *hep*. These genes closest to SU smolts were also furthest in ordination space from RM smolts (Fig. 2). Closer analyses of these ten candidate genes revealed that *Il-17D* (ANOVA, $F_{[2,111]} = 11.065$, $p < 0.0001$), *NKA α1b* (ANOVA, $F_{[2,111]} = 3.607$, $p = 0.03$), and *RPL6* (ANOVA, $F_{[2,111]} = 5.687$, $p = 0.004$) were most associated with migration fate (i.e., $p < 0.05$; Fig. 3). On the RDA ordination, UN smolts (i.e., assumed to have not survived in the marine environment pre-NSOG) clustered closest to the center of the RDA ordination.

Measures of river residency were tested as continuous (time between first and last estuary detection) and categorical (duration <24 h or ≥24 h) variables. Smolt length–weight residuals on their own did not influence estuary residency by time (generalized linear model, $F_{[1,35]} = 1.852$, $p = 0.182$) or duration (ANOVA, $F_{[1,35]} = 2.361$, $p = 0.133$) (Fig. 4). However, length–weight residuals were higher for RM smolts compared with the other fate groups (ANOVA, $F_{[2,111]} = 5.589$, $p = 0.005$) (Fig. 4).

The RDA investigating the relationship between release group on gene expression indicated the model (gene expression matrix ~ "*Ca.* B. cysticola" + *F. psychrophilum* + RIB + length–weight residuals + release group) was significant (Monte Carlo permutations test, $F_{[5,108]} = 1.911$, $p = 0.001$). The five explanatory factors accounted for ~8% of the variance in gene expression data. The first two axes were significant (RDA1: $F_{[1,108]} = 4.464$, $p = 0.001$; RDA2: $F_{[1,108]} = 2.834$, $p = 0.003$), and three terms were significantly associated with gene expression: release group ($F_{[1,108]} = 3.191$, $p = 0.002$), length–weight residuals ($F_{[1,108]} = 2.45$, $p = 0.004$), and RIB ($F_{[1,108]} = 2.137$, $p = 0.033$; Table 4). River-released smolts were most associated with *CD83*, but also several other immune (e.g., *Il-1β*, *C5aR*, *RPL6*, *MX*) and osmoregulatory genes (e.g., *NKA α3*, *NKA b1*), while marine-released smolts were primarily clustered with *C7* and *HSC70* (Fig. 5).

Discussion

The present study shows that the Seymour River and the first marine embayment (Burrard Inlet) were associated with poor

Fig. 5. Redundancy analyses (RDA) ordination plot of Seymour River Hatchery steelhead (*Oncorhynchus mykiss*) showing differences between release groups. RDA1 and RDA2 were determined to be significant, and all significant ($p < 0.05$) covariates are in black, while nonsignificant variables are in grey. Migration-release group centroids are indicated as follows: MR = marine-release and RR = river-release. Genes are coloured according to their primary known function from the available literature; however, many genes are known to have multiple physiological associations. [Colour version available online.]

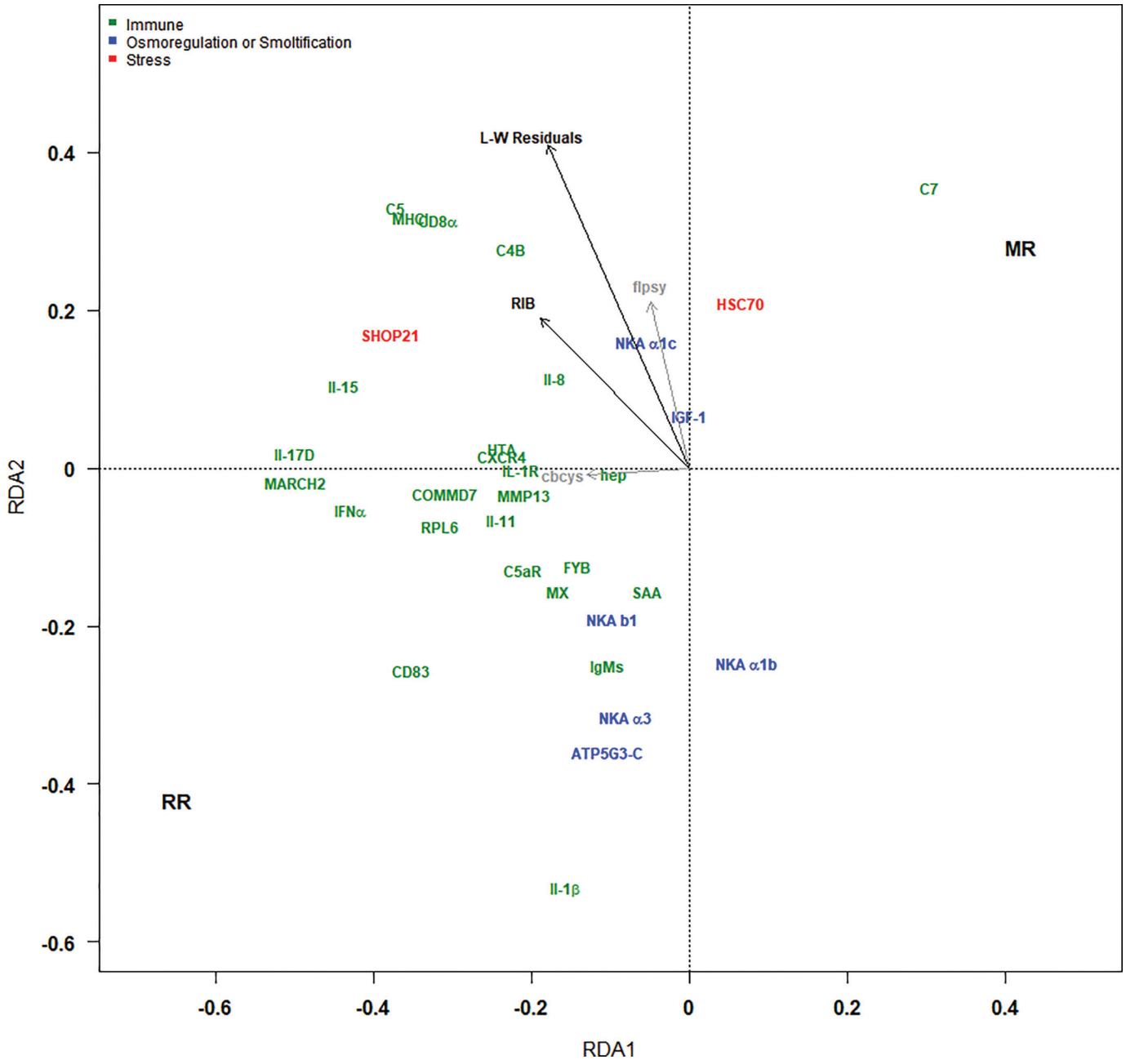


Table 4. Summary statistics for the redundancy analysis (RDA) of the model testing for an effect of release group on gene expression (model: gene expression matrix ~ "*Ca.* B. cysticola" + *F. psychrophilum* + RIB + residuals + release group).

Variable	df	Variance	*F*	*p*
Release group	1	0.8958	3.1911	**0.002**
Relative infectious agent burden	1	0.5999	2.1370	**0.033**
Length–weight residuals	1	0.7013	2.4984	**0.004**
"*Candidatus* Branchiomonas cysticola"	1	0.2629	0.9366	0.463
Flavobacterium psychrophilum	1	0.2227	0.7933	0.637
Residual	108	31.2913		

Note: Significant *p* values are shown in bold ($p < 0.05$).

survival for biopsied smolts, particularly given their short distances (~2.5 and ~18 km, respectively). As expected, our survival calculations are consistent with a parallel telemetry study reporting survival estimates of biopsied and nonbiopsied steelhead (Healy et al. 2017), as well as prior work using the same marine and release locations (Balfry et al. 2011). Differences in survival between the two release groups to the NSOG array have previously been reported and were determined to not be a result of differences in travel time alone (Healy et al. 2017). The present study enhances our knowledge of how the physiological condition of hatchery steelhead smolts can influence migration fate through these high-risk landscapes. By combining acoustic telemetry with

HT-qRT-PCR of nonlethal gill biopsies, we identified several important genes that were related to fate, as well as identified several infectious agents present in the population of hatchery steelhead.

Two infectious agents, "*Ca.* B. cysticola" and *F. psychrophilum*, were detected in gill samples from acoustically tagged smolts, but neither had any apparent influence on smolt gene expression profiles or migration fate. "*Candidatus* B. cysticola" is a recently discovered bacterium that may be associated with proliferative gill disease (Toenshoff et al. 2012); however, "*Ca.* B. cysticola" is not necessarily always associated with mortality (Bass et al. 2017; Gunnarsson et al. 2017; Teffer et al. 2017). Presence of this infectious agent in a fish may be the result of a secondary infection (Tengs and Rimstad 2017); however, some studies have hypothesized that "*Ca.* B. cysticola" could be part of the normal microflora present on the gills (Steinum et al. 2009; Toenshoff et al. 2012). Thus, the association between the presence of this infectious agent and disease in salmonids warrants further investigation. *Flavobacterium psychrophilum* is a common bacterium associated with mortality of salmon in aquaculture facilities worldwide (Nematollahi et al. 2003), but its presence does not always equate to disease (Decostere et al. 2000; Nematollahi et al. 2003). Susceptibility to *F. psychrophilum* infection for juvenile rainbow trout (nonanadromous *O. mykiss*) may be age-dependent, as older individuals (>5 months) are most successful at avoiding disease states (Decostere et al. 2001), which may help explain why this infectious agent did not influence smolt survival in the present study. An important limitation is that PCR can detect RNA of an infectious agent in fish, but cannot distinguish between individuals in a carrier or disease state. Recent work has paired histopathology with HT-qRT-PCR techniques and identified a suite of host genes that may help in distinguishing between disease states for viral infections (Miller et al. 2017). Applying this methodology to other types of infectious agents (e.g., bacteria) will vastly improve our ability to identify important genes indicative of fish in disease states and identify more clear links among infectious agents, disease, and migration fate.

The use of nonlethal gill biopsies in the present study likely limited our ability to detect infectious agents. Many bacteria and viruses are thought to enter fish via the gills, gut, or skin (Khimmakthong et al. 2013; Schönherz et al. 2012; Tobback et al. 2010), but can then move to infect other internal tissues in later stages of infection (Bradford et al. 2010). Therefore, because only steelhead gills were biopsied, we may have missed infectious agents present in other tissues. Furthermore, because of the small sizes of gill tissue taken from smolts, we had to normalize RNA to a concentration to ~25% of levels typically used with larger samples taken from adult fish (Miller et al. 2016). These low concentrations likely contributed to an increase in false negatives for infectious agents. Therefore, we consider it likely that there was a higher presence of the two detected agents in the population than we estimated here (62% for *F. psychrophilum* and ~13% for "*Ca.* B. cysticola"), as well as other infectious agents we may not have detected (or did not assay for) that could have influenced migration fate. A previous telemetry study with this population of steelhead found that vaccination of smolts against several infectious agents (*Aeromonas salmonicida*, *Listonella anguillarum*, and *Vibrio salmonicida*) appeared to enhance survival, indicating that infectious agents may play a role in smolt migratory success (Balfry et al. 2011).

Positioning of smolts across the top two RDA ordination axes provided insight into the variances in physiological condition relating to survivors to at least NSOG (SU) and river-released smolts that failed to reach the estuary (RM). Genes related to the smoltification process were in close ordination space to survivors, including important osmoregulatory isoforms associated with the saltwater transition (e.g., *NKA α1b* and *NKA b1*; Richards 2003; Stefansson et al. 2007). Additionally, hepcidin (*hep*), which has been linked to inflammation and iron metabolism (Ganz 2003; Raida and Buchmann 2009), as well as *C7*, which is hypothesized to link the acute and adaptive immune systems (Gonzalez et al. 2007), were positively associated with successful smolts. In contrast, RM smolts showed association primarily with genes indicative of an inflammatory response, such as *Il-17D* (Zou and Secombes 2016), *Il-15* (Komatsu et al. 2009; Wang et al. 2007), *RPL6* (Kumar et al. 2014; Miller et al. 2014), and *MMP13*, which may signify chronic inflammation at the gills (Castro et al. 2013; Krasnov et al. 2012; Tadiso et al. 2011). Of the immune genes we investigated, *Il-17D* and *RPL6* were most associated with fate for migrating smolts, with RM individuals showing significantly higher relative expression of these genes than other individuals. Transcriptome signals related to inflammatory genes at the gills have previously been linked to survival in salmonids in multiple studies, regardless of the cause (Drenner 2006; Jeffries et al. 2012, 2014; Miller et al. 2011, 2014; Teffer et al. 2017). Multiple mechanisms can induce inflammatory responses, including aquatic contaminants (Eder et al. 2009; Schmidt-Posthaus et al. 2001), stress (Castro et al. 2011; Verleih et al. 2015), and infectious agents (Kvellestad et al. 2005; Raida et al. 2011; Raida and Buchmann 2009; Tadiso et al. 2011). Thus, an indication of an inflammatory response in RM fish suggests these individuals were in poor condition relative to other smolts, which could have reduced swimming performance (Castro et al. 2013) and (or) increase susceptibility to predation (Hostetter et al. 2012; Tucker et al. 2016) in fresh water. Alternately, gill inflammation along with reduced indicators of smoltification may result in failure of fish to migrate out of the river system (Sutherland et al. 2014).

The ability to adapt to changes in salinity is integral for smolts migrating from freshwater natal streams to the marine environment (Robertson and McCormick 2012; Schreck et al. 2006; Stich et al. 2015). In the present study, the expression of Na^+, K^+-ATPase isoform α1b (*NKA α1b*) was associated with migration fate, with RM smolts showing lower relative expression of *NKA α1b* compared with other migration fate groups. *NKA α1b* is thought to be particularly important for saltwater entry (Bystriansky 2006), and higher expression of this isoform at the gills can be associated with the parr–smolt transition in the spring (Robertson and McCormick 2012; Stefansson et al. 2007), suggesting that steelhead that were never detected in the river estuary may not have been fully developed as smolts to enter the marine environment. Similarly, a positive association with the stress gene heat shock protein 70 (*HSC70*; Boone and Vijayan 2002; Lewis et al. 2010) for successful smolts could indicate an increased tolerance for transfer to seawater (Niu et al. 2008). The expression patterns of other osmoregulatory genes (*NKA α1c*, *NKA b1*, and *NKA α3*) were not particularly indicative of survival; however, *NKA α1c* levels tend to not be associated with transfer to salt water (Richards 2003). No adjustments were made for carrying out multiple gene-by-gene analyses, so these results should be considered cautiously; however, we only employed post hoc tests based on genes identified to be important from our RDA, therefore reducing our probability of type I error.

The freshwater survival results should be interpreted cautiously because we cannot directly conclude that all RM smolts represented mortalities. Juvenile steelhead are known to exhibit migratory plasticity, with some individuals in the population showing anadromy, while others remain in fresh water as residents (i.e., "residualize"; reviewed in Kendall et al. 2015). As parr transform into smolts, they develop a more fusiform body morphology (i.e., smaller length–weight residuals in this study) that prepares them for marine migration (Nichols et al. 2008; Stefansson et al. 2007). In species with both migratory and nonmigratory forms (such as steelhead), larger length–weight residuals (sometimes referred to by a similar metric: "condition factor") can be an indication of freshwater residualization (Hausch and Melnychuk 2012; Tipping et al. 2003). Our results indicate that RM

smolts had larger length–weight residuals than other groups. Similarly, lower levels of *NKA α1b* (such as was seen for RM individuals) could be an indication of a smolt residualizing in fresh water (Hanson et al. 2011). Even though the river estuary receivers had a detection probability of 100% (Healy et al. 2017), there were no detections here for the last 11 days prior to recovery; therefore, some of the RM smolts may have remained in the river as residents or delayed for longer in the river prior to emigration. If smolts remained in the river, this likely resulted in an underestimation of river survival. While stream home ranges can be small for juvenile resident trout (<1–2 km; Hartman et al. 2012; Harvey et al. 2005), residency rates are typically only ~5% in hatchery steelhead (Hausch and Melnychuk 2012), and smolts released close to the river estuary (such as in the present study) are significantly less likely to residualize (Hausch and Melnychuk 2012). Therefore, it is unlikely that all the RM smolts represent residualized fish. Regardless, incorporating HT-qRT-PCR with acoustic telemetry allowed us to detect physiological differences at the molecular level, which demarcates RM smolts from other fate groups.

An important consideration is that our RDA results are on ordinations that explain ~8% of the variance in the gene expression data. While this is low, previous work with adult sockeye salmon using principal component analyses related migration survival to axes that explained ~12% of the variance in the data, but with substantially more genes (i.e., thousands of genes; Miller et al. 2011). Other external factors can influence smolt gene expression (Evans et al. 2011), including temperature (Beckman et al. 1998; Verleih et al. 2015), light levels (Stefansson et al. 2007), and other infectious agents that were not included in our panel of assays. Additionally, survival can be influenced by many factors such as currents and (or) flow (Perry et al. 2013), food availability (Beamish and Mahnken 2001), and predators (Berejikian et al. 2016; Hostetter et al. 2012). Therefore, for the present study, the low variance explained can likely be attributed to the limited explanatory variables in the RDA models, as well as our constrained inclusion of genes on the qPCR dynamic arrays (i.e., potentially important assays that were not run and (or) that did not meet our quality standards for efficiency). Targeting a larger subset of genes known to be important to the smolt life stage could provide a clearer understanding of intrinsic factors influencing outmigration fate.

Infectious agent and gene expression profiles were found to be slightly different between the two release groups, which could confound interpretations of our results. Marine-released smolts had higher detected presence of *F. psychrophilum* and clustered with different genes in RDA ordination space than river-released smolts. These variable immune gene expression profiles could be a result of the disparate presence of *F. psychrophilum* among groups. Our acoustic tagging procedure was kept consistent across all surgeries, with smolts being randomly selected from tanks and tagging alternating between release groups (i.e., both release groups were tagged simultaneously, with successive smolts tagged and placed in alternate pens for release throughout). Thus, the gene expression differences detected between groups were most likely due to an unknown factor we could not identify. Regardless, this slight difference in gene expression by group may have biased our results. Although these differences in physiological state may have contributed to the poor survival observed at NSOG for river-released relative to marine-released smolts, survival was similar to previous estimates (Balfry et al. 2011). Other external factors, such as predation, likely play a more prominent role in survival through these early portions of marine migration (Berejikian et al. 2016; Healy et al. 2017) and may explain why differences in smolt condition did not appear to significantly influence survival here.

Linking telemetry with transcriptome profiles and infectious agents, such as in the present study, allows the rare opportunity to identify factors operating at the molecular level that influence migratory fate for smolts in the wild. Disease likely plays an important role in salmonid migration survival (Jeffries et al. 2014; Miller et al. 2011, 2014), but can be particularly challenging to study, as mortality is seldom observed in migrating fish (Miller et al. 2014). While the present study found no indication that infectious agents influenced migration fate, we found immune gene profiles that were associated with fate for migrating steelhead. We highlighted the early riverine portion of outmigration to be a region where the expression of several genes were particularly important determinants of fate, consistent with similar work with sockeye smolts in British Columbia (Jeffries et al. 2014). Because factors operating during this critical life stage can be linked to population productivity (Irvine and Akenhead 2013; Kendall et al. 2017; Moore et al. 2012), identifying relationships between smolt physiology and migration fate will be crucial for future conservation and increased population predictive capabilities.

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