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Divergent immunity and energetic programs in the gills of migratory and resident *Oncorhynchus mykiss*

BEN J. G. SUTHERLAND,[1] KYLE C. HANSON,[2] JOHANNA R. JANTZEN,[1] BEN F. KOOP[1] and CHRISTIAN T. SMITH[2]

[1]*Department of Biology, Centre for Biomedical Research, University of Victoria, 3800 Finnerty Road, Victoria, British Columbia V8W 3N5, Canada,* [2]*Abernathy Fish Technology Center, U.S. Fish and Wildlife Service, 1440 Abernathy Creek Road, Longview, Washington 98632, USA*

Abstract

Divergent life history strategies occur in steelhead or rainbow trout *Oncorhynchus mykiss*, and many populations produce both migrant (anadromous fish that move to the ocean after rearing) and resident (do not migrate and remain in fresh water) individuals. Mechanisms leading to each type are only partially understood; while the general tendency of a population is heritable, individual tendency may be plastic, influenced by local environment. Steelhead hatchery programmes aim to mitigate losses in wild stocks by producing trout that will migrate to the ocean and not compete with wild trout for limited freshwater resources. To increase our understanding of gill function in these migratory or resident phenotypes, here we compare gill transcriptome profiles of hatchery-released fish either at the release site (residents) or five river kilometres downstream while still in full fresh water (migrants). To test whether any of these genes can be used as predictive markers for smoltification, we compared these genes between migrant-like and undifferentiated trout while still in the hatchery in a common environment (prerelease). Results confirmed the gradual process of smoltification, and the importance of energetics, gill remodelling and ion transport capacity for migrants. Additionally, residents overexpressed transcripts involved in antiviral defences, potentially for immune surveillance via dendritic cells in the gills. The best smoltification marker candidate was *protein s100a4*, expression of which was highly correlated with Na^+, K^+ ATPase (NKA) activity and smolt-like morphology in pre- and postrelease trout gills.

Keywords: ecological genomics, immunity, migration, smoltification, steelhead, transcriptomics

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Introduction

Migration from the natal site to a location of more abundant resources is a nearly ubiquitous feature of the life histories of salmonid fishes (Family Salmonidae). *Oncorhynchus mykiss* is a species that exhibits tremendous variability in the timing, extent and propensity for migration. Most populations with access to the ocean include both anadromous (steelhead) and resident (rainbow trout) individuals. While there is typically little, if any, population structure between the two types (Olsen *et al*. 2006), the proportion of individuals which migrate can vary among adjacent populations (Hayes *et al*. 2012). Individuals that migrate to the marine environment face increased risks associated with predation and other factors, but benefit from increased feeding opportunities, growth and fecundity, especially for females (reviewed by Quinn 2005). Thorpe (1994) characterized smoltification as a 'developmental conflict', in which juvenile *O. mykiss* can adopt different life history strategies during each year of fresh water residence. First year resident fish may begin sexual maturation and attempt to spawn (precocial residents), may remain in

Correspondence: Christian T Smith, Fax: (360) 636-1855;
E-mail: christian_smith@fws.gov

fresh water until migration in a future year (parr) or may migrate from natal streams to the ocean (smolts).

Juvenile steelhead migration is preceded by the initiation of the parr-smolt transformation, a suite of physical, behavioural and physiological changes collectively termed 'smoltification' (Folmar & Dickhoff 1980; McCormick & Saunders 1987; Hoar 1988). Morphological changes result in a more streamlined body shape and silver coloration (Hoar 1988). Freshwater parr are territorial bottom dwellers that exhibit positive rheotaxis and stationary positioning in the river, whereas smolts are less territorial, surface-oriented and more prone to change river positioning (Thorpe & Morgan 1978; Gibson 1983). Gills undergo extensive changes during smoltification in preparation for a change from a hypoosmotic to hyperosmotic environment (reviewed by Evans 1984) such as increasing gill Na^+, K^+ ATPase (NKA) activity (Hoar 1988).

Quantitative genetics revealed an overall high heritability for anadromous behaviour in *O. mykiss*, but also a remarkably high level of variability among families and lineages (Thrower *et al.* 2004). In this work, it was proposed that balancing selection is a possible mechanism to explain retention of a substantial amount of variation, despite high heritability and high selective pressure on that trait (e.g. retention of the ability to produce smolts by a population above an impassable barrier to migration). Divergent life history strategies adopted by individual *O. mykiss* may thus be seen as an example of developmental plasticity whereby genotype interacts with environmental and biological cues (Björnsson 1997; Stefansson *et al.* 2007; Berejikian *et al.* 2013) rather than the result of a fixed genotype (Thorpe 1994; Thorpe & Metcalfe 1998; Thorpe *et al.* 1998). Several physiological indicators have been linked to migratory or resident tendency among salmon including sex steroid levels (Larsen *et al.* 2004), growth hormone levels (Sweeting *et al.* 1985), gill NKA activity (Gale *et al.* 2009), plasma sodium concentration or osmolality (Kennedy *et al.* 2007), and levels of thyroid hormone and plasma insulin-like growth factor I (McCormick *et al.* 2002). Studies of male Chinook salmon *O. tshawytscha* have indicated that physiological factors may be predictive of the developmental paths towards anadromy or residency several months prior to the initiation of migratory behaviour (Shearer & Swanson 2000; Shearer *et al.* 2006). The plasticity of this life history trait, the potential for identifying predictive markers and the economic and cultural importance of *O. mykiss* make this a good model for studying the molecular basis of anadromy.

Identification of genomic regions associated with anadromy and residency has been accomplished by a series of association mapping studies. Initial work focused on clonal lines of *O. mykiss* exhibiting predominantly resident or anadromous life histories and revealed several quantitative trait loci (QTL), including one associated with multiple traits (Nichols *et al.* 2008). Martinez *et al.* (2011) performed a genome scan on wild *O. mykiss* from two adjacent populations (one anadromous and the other landlocked by a barrier waterfall) and observed several outlier loci (i.e. markers exhibiting greater or less divergence than expected based on a simulated neutral distribution) potentially associated with genomic regions under disruptive selection. Next-generation sequencing of restriction-site-associated DNA (RAD) tags has facilitated an order-of-magnitude increase in the numbers of markers which may be included in association studies, and has thus increased the resolution provided by recent comparisons of anadromous and resident *O. mykiss* populations (Hale *et al.* 2013; Hecht *et al.* 2013). Additionally, wild anadromous and resident P1 crosses enabled the analysis of F2 individuals to identify 19 QTL regions, including two chromosomal positions with 7 associated traits each (Hecht *et al.* 2012). Challenges to using genetic association to identify genomic regions influencing anadromy include the confounding of traits with population demography and the effect of the environment on an individual's phenotype. Such challenges have been addressed by comparing results between studies of different populations and marker sets and identifying regions consistently associated with anadromy. Genes associated with development rate, osmoregulatory function and reproductive maturity are among those that have been observed in regions associated with anadromy to date (e.g. Hale *et al.* 2013; Hecht *et al.* 2013).

Complementary insight into the mechanisms behind the phenotypic plasticity of anadromy has been gained by transcriptome analyses of gill tissue in migratory and nonmigratory individuals. Nilsen *et al.* (2007) used a candidate gene approach with quantitative PCR and identified up-regulation of NKA subunits, a Na^+, K^+, $2Cl^-$ cotransporter and a cystic fibrosis transmembrane conductance regulator (*cftr*) in the gills of Atlantic salmon *Salmo salar* during smoltification. Transcriptomic studies have additionally revealed increased expression of genes associated with metabolism (e.g. α- and β-*globin* and *cytochrome c*) and osmoregulation (e.g., *NKA-α1*) in Atlantic salmon undergoing smoltification (Seear *et al.* 2010; Robertson & McCormick 2012; Lemmetyinen *et al.* 2013). Furthermore, transcriptomic analysis of the gills of brook charr *Salvelinus fontinalis* identified migrant overexpression of genes involved with metabolism and development relative to residents (Boulet *et al.* 2012). In this study, several innate immunity genes were also overexpressed in smolts, including *c-type lectin 2*. Interestingly, this gene was also overexpressed in migrant Atlantic salmon (Seear *et al.* 2010). These

transcriptomic investigations have provided methods to identify genes and functions not typically considered in candidate gene approaches. Enrichment of functional categories using databases such as Gene Ontology or KEGG pathways in combination with accessible tools for applying enrichment analysis (e.g. The Database for Annotation, Visualization and Integrated Discovery (DAVID); Huang *et al.* 2009) provides further insight on activated pathways or biological functions in nonmodel organisms (see review by Primmer *et al.* 2013). Forthcoming advancements for ecologists also include the potential expansion into ecological associations for genes (Pavey *et al.* 2012).

To identify molecular differences between resident and anadromous forms of *O. mykiss*, we used a transcriptomic approach (Koop *et al.* 2008; Jantzen *et al.* 2011a) to compare gene expression in the gills of migrating and nonmigrating hatchery-reared individuals in Abernathy Creek prior to salt water entry. Eight days after release from the hatchery, gill transcriptomes of fish remaining in the creek (residents) or on route to the ocean but still in fresh water (migrants) were compared. Differentially expressed genes from this comparison were subsequently tested for expression differences in an earlier prerelease sampling from the common environment of the hatchery, contrasting individuals with a migratory type with an undifferentiated morphology. This analysis provided insight on genes associated with migration with reduced influence of variable environments, identifying predictive candidates for smoltification. A later sampling of residents 48 days after hatchery release allowed for a comparison of short- and long-term resident profiles to investigate changes over time in residents. This study provides insight on mechanisms associated with a migratory phenotype as well as predictive markers to aid efforts to increase the success of hatchery releases.

Materials and methods

Experimental design and sampling

Abernathy Creek flows into the Columbia River approximately 87 river kilometres (rkm) from the Pacific Ocean (46°22′N, 123°14′W; Fig. 1a). Abernathy Creek is full fresh water; monitoring stations halfway between the Pacific Ocean and the Abernathy Creek tributary (e.g., Marsh Island) rarely register an increase in salinity (probe depth 5.4 m; Center for Coastal Margin Observation & Prediction; www.stccmop.org/datamart/observation_network). Abernathy Fish Technology Center (AFTC) is a research hatchery on Abernathy Creek 5.4 rkm from the confluence with the Columbia River. Adult hatchery-origin winter steelhead returning to the

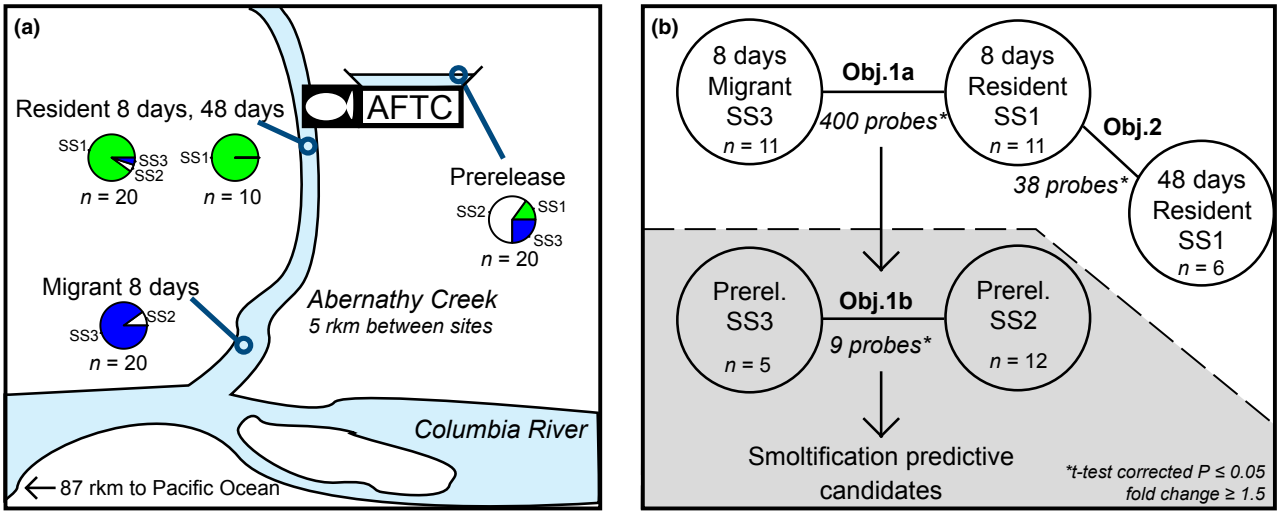

Fig. 1 Study site and experimental design. (a) Fish were reared in a hatchery at the Abernathy Fish Technology Center (AFTC) and released into Abernathy Creek. Prior to the release, samples were taken from within the hatchery (prerelease). Sampling of migrants (8 days) occurred downstream at the weir, and of residents (8 and 48 days) at the release site. As displayed in the pie charts, the majority of resident fish had a smoltscale (SS) 1 classification, migrants were mostly SS3, and prerelease were a mix of all three classifications. The numbers of samples collected at each site (*n*) are shown beside pie charts. (b) Objective (Obj.) 1 identifies differentially expressed probes between the postrelease migrants and residents at day 8 and then uses this list to test for differences between SS3 and the undifferentiated SS2 while fish were still in the hatchery (prerelease). Obj.2 compared residents from 8 days postrelease to residents 48 days postrelease to identify the effect of time in the creek. The numbers of samples used in gene expression work for each condition (*n*) are shown within the circles. rkm = river kilometres; d = day; prerel = prerelease.

hatchery were captured and spawned in February through March 2011. Juveniles were raised on well water in circular tanks in a hatchery building until September 2011 when they were transferred to an outdoor raceway fed by unfiltered water from Abernathy Creek. In May 2012, all individuals (approximately 20 000) were released into Abernathy Creek adjacent to the hatchery. The majority of migratory fish from this location leave the system within 13 days after release from the facility (Hanson *et al.* 2011).

Fish used in the present study originated from two hatchery release groups, the first on May 1 and the second on May 15. The sampling design is shown in Fig. 1a. Prior to both of these releases (13 days prerelease), samples were taken from the hatchery raceways. After both releases (8 days postrelease), fish remaining in the creek near the hatchery were captured via electrofishing (residents). After the May 15 release (8 days postrelease), fish were captured via a rotary screwtrap in a weir located at the mouth of the creek (migrants). Finally, residents were also sampled 48 days after the second release near the hatchery. For the remainder of the study, we refer to fish captured near the hatchery as 'residents' and fish captured at the screwtrap as 'migrants', assuming that they are generally representative of smolts. The flexible nature of *O. mykiss* life history makes it possible that some of the resident individuals would have eventually become smolts and migrated; however, their capture near the hatchery several days after release (and the observation that most had a low smoltscale score; see below) suggests that most would have remained in fresh water for some time. All fish raised at AFTC were marked using coded-wire tags and adipose fin clips, and these marks were used to distinguish recaptured study fish from their wild conspecifics. Rearing and handling followed the Guidelines for the Use of Fishes in Research (Nickum *et al.* 2004).

At each sampling event, ten individual hatchery-raised fish were sampled, with the exception of the weir screwtrap, at which 20 individuals were sampled to make up for the lack of the migrant sampling of the first hatchery release. Fish were euthanized by a sharp blow to the head, weighed, measured and photographed. Each fish was assigned a 'smoltscale' rating of 1–3 based on body shape (stocky or fusiform) and coloration (parr marks, reddish or silver hue to the body), with 1 being a typical parr (darker coloration with highly visible parr marks and stocky body shape), 3 being a typical smolt (silvery coloration lacking parr marks and highly fusiform body shape) and 2 being intermediate or having characteristics of both (or if not all members of the sampling crew agreed on the rating). Gills were rapidly removed for each fish, with the left gill being preserved in RNAlater (QIAGEN) for gene expression studies, and the right gill being preserved in ice-cold SEI buffer (250 mM sucrose, 10 mM Na_2-EDTA, 50 mM imidazole, pH = 7.3) for NKA activity assays. Additionally, a fin clip was preserved in 95% ethanol and stored at ambient temperature for genetic sex determination. Samples in RNAlater were stored overnight at 4 °C and then transferred to −20 °C. Samples in SEI were transferred to −80 °C within thirty minutes of sampling.

Gill Na^+, K^+ ATPase (NKA) activity, weights and sex identification

Gill Na^+,K^+-ATPase (NKA) activity was measured as μmol inorganic phosphate [P_i]/mg protein/h using the method described by McCormick (1993). NKA activity differences were investigated between pre- and postrelease samples and among smoltscale classifications by two-way ANOVA with post hoc Tukey's HSD tests in the statistical environment R (R Development Core Team 2012). Weight and length measurements were contrasted between day 8 residents and migrants by a two-tailed *t*-test in R.

DNA was extracted from each fin clip using a DNeasy Blood and Tissue Kit (QIAGEN). The genetic sex marker OmyY1, ~94% accurate for steelhead from Abernathy Creek (Brunelli *et al.* 2008) was used to identify the sex of each individual. Individuals exhibiting both the control (autosomal or x-linked) band and the Y-chromosome-specific band were identified as males, those exhibiting only the common band were identified as females, and those exhibiting no bands were classified as 'unknown' and were not used for the transcriptomic analysis.

RNA extraction, cDNA synthesis and reference pool generation

A subset of the samples was used for gene expression analysis, using only smoltscale 3 migrants (n = 11) and smoltscale 1 residents (n = 11 (8 days); n = 6 (48 days); Fig. 1b) for postrelease samples. Individuals with all three smoltscale ratings were used from the prerelease condition (n = 5, 12, 3 for smoltscale 3, 2, 1, respectively). Gill samples were randomized and total RNA was extracted using TRIzol (Invitrogen) by physical disruption with a MixerMill (Retsch). Total RNA was then purified through RNeasy columns with on-column DNase digestion to remove any genomic DNA (QIAGEN). Total RNA was quantified and quality-checked by spectrophotometry (NanoDrop-1000) and 1% agarose gel electrophoresis and then stored at −80 °C. cDNA synthesis and cRNA amplification were performed on 200 ng input total RNA using the Low Input Quick Amp system (Agilent; v6.5) incorporating

Cy5- or Cy3-CTP (PerkinElmer). A common reference pool to be hybridized alongside experimental samples on all arrays (Churchill 2002) was generated by synthesizing Cy3-labelled cRNA from two individuals from each of the six collections (four individuals for the migrant collection to improve the balance of the pool; Table S1, Supporting information) and then pooling equimolar amounts of each Cy3-labelled sample. All cRNA samples were quality-checked for appropriate specific activity as per manufacturers' protocol (Agilent) and kept at −80 °C in the dark until hybridization.

Microarray hybridization, quantification, normalization and filtering

Samples and reference material were combined, fragmented and hybridized to randomized-order 44K cGRASP salmonid microarrays (Jantzen *et al.* 2011a) as per manufacturers' instructions with stabilization solution to prevent ozone-related problems (Agilent; v6.5). All slides were scanned on a PerkinElmer ScanArray® Express at 5 μm using photomultiplier settings optimized for ~1–2% of all probes on the array to be saturated (Cy5: 70; Cy3: 65). Probe intensities were quantified in Imagene 8.0 (BioDiscovery) using an eArray GAL file (Agilent; Design ID: 025055). Poor or control probes were flagged for downstream filtering, and the median background intensity was subtracted from the median probe intensity for each probe. Samples were loaded into GeneSpring 11.5.1 (Agilent). All sample files have been uploaded to GEO under the accession GSE48844. Negative raw values were set at 1.0, each array was normalized by an intensity-dependent *Lowess* (Agilent; Yang *et al.* 2002), and a baseline to median transformation of normalized expression values was performed per gene (Agilent). Control probes and any probes not passing the following quality control thresholds were removed: raw values ≥500 in both channels and no poor quality flags in at least 65% of the samples in any one experimental condition.

Differential expression and functional analysis

To test the validity of pooling first and second release day 8 residents, these groups were contrasted by a two-sample *t*-test (Benjamini–Hochberg multiple test corrected $P \leq 0.05$, fold change ≥1.5). As no differential expression was identified, samples from these groups were pooled (day eight residents). This cut-off was used for all subsequent transcriptome comparisons. Differential expression was tested by two-sample *t*-test between the day eight residents ($n = 11$) and migrants ($n = 11$; objective (Obj.) 1a; Fig. 1b). Any genes found differentially expressed in this comparison were then tested for differential expression by two-sample *t*-test between the prerelease samples with smoltscale 3 ($n = 5$) and 2 ($n = 12$) classifications to produce candidates for smoltification predictor genes (Obj.1b). Smoltscale 1 was not used in this prerelease comparison due to the low numbers of smoltscale 1 samples collected (only two with a successful genetic sex identification). Differential expression was also investigated between day eight ($n = 11$) and day 48 residents ($n = 6$) by two-sample *t*-test to identify genes changing over time in the creek (Obj.2). For each gene list, functional category enrichment was determined by comparing Entrez-ID identifiers of the differentially expressed gene list to the background list (all probes passing quality control filters; Obj.1 $n = 13\,974$; Obj.2 $n = 14\,422$) in DAVID Bioinformatics (v6.7; Huang *et al.* 2009). Enriched Gene Ontology categories ($P \leq 0.05$; number of genes in gene list in category ≥4) were filtered for redundancy using GO trimming with a soft trim threshold of 0.80 (Jantzen *et al.* 2011b).

Reverse transcription–quantitative polymerase chain reaction (qPCR)

The same samples used in the microarray experiment were also analysed by qPCR to validate several functions and predictive genes identified in the transcriptomic analysis. Genes of interest were selected based on presence in enriched functional categories, predictive potential or for particular interest in relevance to the study system. Candidate reference genes included *eukaryotic translation initiation factor 4 h* (*if4h*), *U6 snRNA-associated Sm-like protein* (*lsm8*) and *mRNA turnover 4 homolog* (*mrto4*). Reference gene primer design and normalizer suitability were previously reported (Sutherland *et al.* 2014) and were confirmed here for *O. mykiss* by standard curve efficiency testing and analysis of stable expression across conditions in the microarray analysis. Gene of interest primers were designed in Primer3 (Rozen & Skaletsky 2000). Primer sequences and efficiency values can be found in Table S2 (Supporting information).

Total RNA was reverse-transcribed to cDNA using the SuperScript III Synthesis System (Invitrogen), and cDNA samples were diluted 20-fold. To test primer efficiency, equimolar amounts of undiluted cDNA from each condition were pooled, diluted sevenfold and then used to create a five-point, fivefold serial dilution. All primers had 90–110% efficiency (Table S2, Supporting information). Single amplicons were identified by melt curve analysis, and amplicon identity was confirmed by sequencing on a 3730 DNA Analyzer (Applied Biosystems) as previously reported (Sutherland *et al.* 2011). Samples were quantified in duplicate using SsoFast EvaGreen reagents (Bio-Rad) on an MX3000P (Agilent).

Duplicate wells were within 0.5 cycle values for 712 of 735 sample-target combinations.

Data were imported into qBASE (BioGazelle) for geNORM analysis to confirm the stability of selected normalizers (Vandesompele *et al.* 2002). Sample–gene combinations were normalized using the geometric mean of the three normalizer genes and primer-specific efficiencies. Correlation was tested between log2 values from the microarray and qPCR for each gene tested to confirm trends identified in the microarray analysis. Analysis of qPCR results was performed on log2 normalized relative quantities in R using linear models contrasting resident and migrants at day eight postrelease.

Results

Morphology and physiology

At eight days postrelease, the collections at either the release site (residents; mean smoltscale = 1.1) or the weir (migrants; mean smoltscale = 2.9) yielded individuals with very distinct phenotypes (Fig. 1a). The hatchery (prerelease; mean smoltscale = 2.1) yielded an intermediate collection with individuals of all three smoltscale classifications. At eight days, migrants were greater in length than residents ($P < 0.001$), but not in weight ($P = 0.076$). Of the 70 samples collected, 30 were female, 37 were male, and three were unknown (Table S1, Supporting information). Morphological data are available in Table S1 (Supporting information), and photographs of each individual are available on Dryad (doi:10.5061/dryad.3p545).

Na^+, K^+ ATPase (NKA) activity among smoltscale classes

Gill NKA activity (measured as μmol P_i/mg protein/h) was highest in individuals with a smoltscale classification of 3 (median = 3.2), followed by smoltscale 2 (median = 1.7) and lowest in smoltscale 1 (median = 1.02; Fig. 2). There was no effect of whether samples were taken before or after hatchery release (2-way ANOVA main or interaction effect $P > 0.3$). Smoltscale classification groups were all different from each other in NKA activity (Tukey's HSD $P < 0.01$). Observed values for each fish are listed in Table S1 (Supporting information).

Comparative transcriptomics of migrant and resident gills

Differential gene expression in the gills of migrants and residents indicated functional differences occurring during the smoltification process prior to seawater exposure (Obj.1a; Fig. 1b). In the 8-day postrelease comparison of migrants and residents, migrants overexpressed 218 probes (133 uniquely annotated) and residents overexpressed 182 probes (104 uniquely annotated; Data S1, Supporting information).

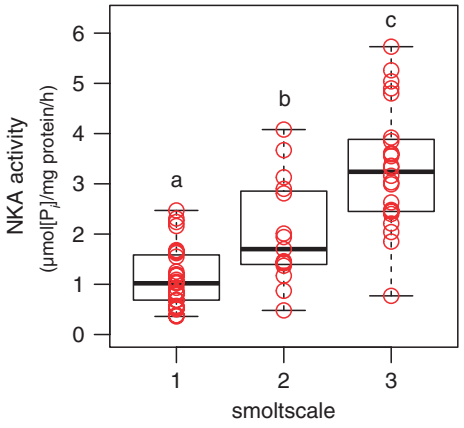

Fig. 2 Na^+, K^+ ATPase (NKA) activity and smoltscale. NKA activity increased between each smoltscale classification (Tukey's HSD $P < 0.01$), independent of whether the sample was pre- or postrelease. The boxplot displays median and interquartile range, and circles are all individual datapoints. Conditions that do not share a letter above the boxplot are significantly different from each other.

Functional enrichment in the migrant overexpression list indicated the importance of energy production (GO: mitochondrial part; 12 genes $P < 0.02$; Table S3, Supporting information). Genes involved in hormone response were also enriched in the migrant gills (GO: cellular response to hormone stimulus; 4 genes $P < 0.05$). Many transporter genes were overexpressed compared with residents, including *cystic fibrosis transmembrane conductance regulator* (*cftr*) and *sodium/potassium-transporting ATPase subunit alpha-1* (*atp1a1*; Table 1). Genes involved in energy production overexpressed in migrants included *cytochrome c* and *glyceraldehyde-3-phosphate dehydrogenase*. Apoptosis-related transcripts were also highly overexpressed in the migrants, including *apoptosis inducing factor 1* and *dna-damage inducible transcript 4*. Additionally, genes involved in calcium homeostasis and FK506-binding were also overexpressed (e.g., *FK506-binding protein 5* > 4.5-fold; Table 1).

Functional enrichment in the resident overexpression list indicated immunity and inflammation (GO: inflammatory response; 7 genes; GO: immune response; 9 genes; $P < 0.001$; Table S3, Supporting information) and included antiviral immunity-related genes *interferon regulatory factor 1* and *8*, *interferon-induced protein 44*, *antigen peptide transporter 1*, *pyrin* and *tumour necrosis factor ligand superfamily member 10* (Table 1). *ATP-binding cassette subfamily B member 9* was also overexpressed in residents; this gene is strongly expressed during monocyte

Table 1 Selected differentially expressed genes related to calcium, transport, energy and immunity. Genes associated with these functions are shown with the microarray probe identifier, corrected *P*-value and linear fold change for both 8-day migrant compared with 8-day resident, and prerelease smoltscale 3 compared with smoltscale 2. Genes not significant in the prerelease condition are denoted with a dash. This table contains approximately 10% of total differentially expressed genes found in the migrant/resident day eight comparison

			Postrelease		Prerelease	
Function	Gene	Probe ID	Corr. *P*-value	Mig/Res F.C.	Corr. *P*-value	SS3/SS2 F.C.
Calcium	Protein S100-A4	C153R120	1.4E-05	40.18	3.1E-02	7.01
	Protein-glutamine gamma-glutamyltransferase 5	C076R113	7.3E-05	6.62	—	—
	Cytochrome P450 24A1, mitochondrial	C195R083	5.9E-03	2.72	6.4E-03	2.12
	Calcitonin-1	C244R120	6.4E-04	2.90	—	—
	Calcitonin gene-related peptide	C266R110	6.5E-04	2.87	—	—
	Arylsulfatase D	C110R071	1.7E-03	1.90	—	—
Transport	Cystic fibrosis transmembrane conductance regulator	C161R157	1.5E-03	10.74	4.0E-02	2.33
	Gap junction gamma-1 protein	C045R113	1.7E-03	2.25	—	—
	Sodium/potassium-transporting ATPase subunit alpha-1	C230R144	1.8E-03	2.04	6.4E-03	1.69
Energy and Growth	Glyceraldehyde-3-phosphate dehydrogenase	C146R081	3.2E-04	2.50	9.5E-03	1.79
	ATPase family AAA domain-containing protein 3	C162R023	8.3E-04	2.25	—	—
	Cytochrome c	C133R020	3.2E-02	1.75	—	—
	Growth hormone-regulated TBC protein 1-A	C225R154	8.3E-04	2.00	—	—
Immunity	FK506-binding protein 5	C148R059	6.0E-04	4.98	—	—
	C-X-C chemokine receptor type 4	C204R072	8.5E-03	2.77	—	—
	Pyrin	C103R085	2.3E-03	−1.85	—	—
	Transcription factor RelB	C104R157	2.0E-04	−1.57	—	—
	Tumour necrosis factor ligand superfamily member 10	C257R074	1.2E-03	−1.94	—	—
	ATP-binding cassette subfamily B member 9	C142R063	4.7E-03	−1.86	—	—
	Antigen peptide transporter 1	C100R014	8.6E-03	−1.76	—	—
	Tapasin-related protein	C083R147	5.9E-03	−1.62	—	—
	Mitogen-activated protein kinase kinase kinase 14	C060R057	2.5E-04	−1.88	—	—
	Interferon regulatory factor 8	C084R105	6.5E-03	−1.99	—	—
	Interferon regulatory factor 1	C189R008	4.7E-04	−2.80	—	—
	Interferon-induced protein 44	C105R064	7.8E-03	−4.63	—	—

SS, smoltscale; F.C., fold change.

differentiation to dendritic cells and macrophages (Demirel *et al.* 2007). A secreted protease, *serine protease 27*, was also overexpressed. This antiviral defence-related suite of genes was specific to the residents and is a main difference from migrants. The resident list was also enriched in genes involved in transcription factor activity (8 genes $P < 0.05$).

Predictive smoltification candidates

Genes identified as overexpressed in migrants were also tested for expression differences in prerelease smoltscale 3 and smoltscale 2 individuals (Obj. 1b in Fig. 1b). Of the 218 overexpressed probes in the 8-day migrants, eight were also overexpressed in the prerelease smoltscale 3 relative to the smoltscale 2 condition. Also, of the 182 underexpressed in 8-day migrants (i.e. overexpressed in 8-day residents), one was underexpressed in prerelease smoltscale 3 condition. These genes are the predictive candidates for smoltification. Several of these have biological functions relevant to smoltification, including *protein s100a4*, *cytochrome p450 24a1*, *glyceraldehyde-3-phosphate dehydrogenase*, *cystic fibrosis transmembrane conductance regulator* and *sodium/potassium-transporting ATPase*

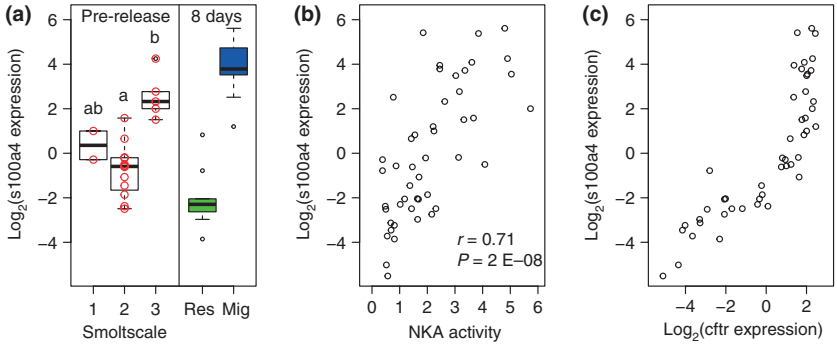

Fig. 3 Predictive candidates for smoltification. (a) *s100a4* expression was higher in prerelease smoltscale (SS) 3 than SS2 conditions, and in day eight postrelease migrants relative to residents. (b) *s100a4* expression was highly correlated with Na^+, K^+ ATPase (NKA) activity in individuals from all collections and smoltscale classifications. (c) *s100a4* was correlated with *cystic fibrosis transmembrane receptor* (*cftr*), although *cftr* hit a ceiling of expression, whereas *s100a4* continued increasing. Boxplot displays median and interquartile range, and large circles in prerelease conditions are individual datapoints. Small circles outside of whiskers are outliers, greater than 1.5 times outside the range. Expression data shown are from qPCR analysis.

subunit alpha-1 (Table 1). The gene with the most significant differential expression in the postrelease samples (*protein s100a4*) also had the greatest difference in the prerelease comparison (>6 fold; Fig. 3a). *s100a4* was highly correlated with NKA values in all collections ($r = 0.71$; $P < 0.001$; Fig. 3b). *s100a4* and *cftr* were highly correlated in all samples ($r = 0.81$; $P < 0.001$); however, *cftr* expression reached a maximum of expression, while *s100a4* continued increasing (Fig. 3c).

Changes over time in resident gill

A small number of genes were differentially expressed between the 8-day and 48-day residents (36 probes with 17 uniquely annotated), indicating the estimates of the 8-day residents reflects that of the 48-day residents. Of the 10 uniquely annotated genes up-regulated over time, several were involved in immune-related processes, such as *mucin-4*, *Toll-like receptor 13 precursor*, *T-cell receptor alpha chain V region CTL-F3 precursor* and *mitogen-activated protein kinase kinase kinase kinase 5*. Additionally, both *carbonic anhydrase 4* and *12* were up-regulated over time. Down-regulated genes over time in the residents included *c-type lectin domain family 4 member m*, and several mitochondrial-related transcripts, including *cytochrome c oxidase subunit VIIa-related protein* and *mitochondrial 28S ribosomal precursor*. The full list of differential probes is provided in Data S1 (Supporting information).

qPCR validation and exploration

The selected normalizers had high reference target stability (collective M value 0.335; CV 0.132; Hellemans *et al.* 2007), and microarray and qPCR data correlated well (Fig. S1, Supporting information). qPCR confirmed the expression of genes from several functional trends, including the overexpression of antiviral immunity transcripts in residents and overexpression of cation transport, energetics and apoptosis genes in the migrants. Due to the interest in C-type lectins in migrating salmon (Seear *et al.* 2010; Evans *et al.* 2011; Boulet *et al.* 2012), we also compared the day eight migrants and residents using qPCR results for *c-type lectin family 4 member m*. Without the multiple test correction that was applied to the microarray data, this gene was found to be overexpressed in the migrants (qPCR $P = 0.016$) and inversely regulated relative to the overexpressed antiviral immunity transcripts in the residents (Fig. 4). *irf1* increased over time in the residents, and *clec4 m* decreased over time in the residents. However, without a 48-day migrant condition, we were unable to determine whether this pattern was due to development and would have also occurred in the 48-day migrants, or if it was a continuation of the trajectory of each phenotype.

Discussion

Divergent morphology and gill transcriptome profiles between migrant-like and resident-like *O. mykiss* began while the fish were still in the hatchery, exemplifying the gradual process of smoltification. After hatchery release, functions overexpressed in the migrants included general preparatory mechanisms for smoltification, such as energy generation (e.g. oxygen transport proteins and mitochondrion components) and ion transport (e.g. *cftr* and *NKA subunit alpha 1*β) as previously identified in Atlantic salmon (Seear *et al.* 2010) and brook charr *Salvelinus fontinalis* (Boulet *et al.* 2012). Gill

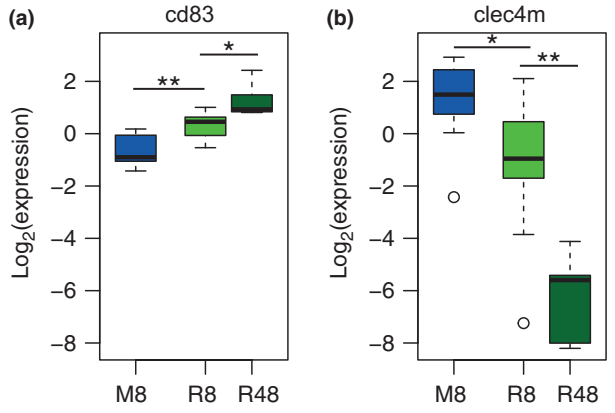

Fig. 4 Divergent immunity programs in residents and migrants. (a) At day eight, residents overexpressed transcripts associated with antiviral immunity, for example *cd83*. The expression of this gene was found to increase over time in the creek in residents (qPCR $P = 0.012$). (b) In contrast, at day eight, migrants overexpressed a potential pathogen recognition receptor *c-type lectin family 4 member m* (qPCR $P = 0.016$). In the creek, this gene continued to decrease over time. Boxplot displays median and interquartile range. Circles outside of whiskers are outliers, greater than 1.5 times outside the range. M8 = migrant day 8; R8 = resident day 8; R48 = resident day 48. Expression data shown are from qPCR analysis. $*P \leq 0.05$, $**P \leq 0.001$.

remodelling may occur during smoltification, similar to that which occurs due to changing osmotic demands (Evans 1984; Hoar 1988); this may explain apoptosis-related transcripts identified here as well as in brook charr (Boulet *et al.* 2012). The later collection of residents 48 days postrelease allowed for the confirmation of resident molecular phenotypes identified in the 8-day postrelease collection. It is possible that small differences in the environment between the sites (5 rkm apart) such as temperature or current, as well as differences in capture methods (e.g., electrofishing or rotary screwtrap), could affect the profiled transcriptome. However, the prerelease comparison provided a common environment and capture method for comparing phenotypes, and consistencies with previous work indicate the reliability of profiles identified here.

Innate immunity pattern recognition receptors (PRRs) may be important for migratory fish due to the likelihood of encountering novel pathogens in seawater transfer (Boulet *et al.* 2012). Migrant overexpression of various C-type lectins has been identified in other salmonid species (Seear *et al.* 2010; Evans *et al.* 2011; Boulet *et al.* 2012). Here, we found migrant overexpression of *c-type lectin domain family 4 member m* (*clec4m*) by qPCR. Innate immunity is important for defence against salmon lice (Johnson & Albright 1992; Jones 2001), an example of a parasite first encountered soon after seawater entry. Although the pathogen-associated molecular patterns (PAMPs) recognized by these lectins are not known, evaluating future samples for known pathogen presence and lectin expression may provide more insight.

Residents overexpressed antiviral genes compared with migrants, and this expression remained high over time in the creek. This may indicate an infection in residents and not in migrants, although both were exposed to similar environments in the same stream for the same amount of time only a few kilometres apart. Fish were exposed to unfiltered creek water in the hatchery, but additional pathogen exposure and additional stressors may occur upon release. For example, infectious hematopoietic necrosis virus (IHNV) has been detected in Abernathy Creek in recent years (Susan Gutenberger, Lower Columbia River Fish Health Center, personal communication), and exposure to IHNV is known to induce the expression of type I interferon-related genes in the gills of *O. mykiss* (Purcell *et al.* 2011). Alternatively, the antiviral signature may not be an indication of an infection, but rather a constitutive surveillance defence in the residents (e.g. intrinsic immunity; Bieniasz 2004) that is suppressed in the migrants. Due to the relative nature of the migrant and resident expression profiles, it is difficult to say which of these two possibilities is true (suppression in migrants or induction in residents). Pipefish *Syngnathus typhle* exposed to salinity stress have reduced capacity to respond to a *Vibrio* infection (Birrer *et al.* 2012); energetic constraints can reduce immune competence.

The resident antiviral signature may originate from dendritic cells, important antigen presentation cells bridging innate and adaptive immunity (Banchereau & Steinman 1998). Recently, dendritic cell functional analogues were identified in rainbow trout that had elevated *cd83* expression (Bassity & Clark 2012), a marker of dendritic cell maturation (Lechmann *et al.* 2002). Here, residents overexpressed *cd83* in the gills, an important tissue for immune surveillance. Similar overexpression was identified for *interferon response factor 1* (*irf1*), a transcription factor directly involved in inducing *cd83* expression in maturing dendritic cells (Stein *et al.* 2013). The antiviral signature and C-type lectin expression point to very different immune responses of resident and migrant juvenile *O. mykiss*, while both are still in fresh water.

Although divergence in antiviral immunity transcription was not identified in prerelease samples, there were only a few samples in this collection with a smolt-scale 1 (resident) phenotype (the majority of fish released from AFTC are thought to migrate). It is possible with a larger sample size, we may have captured more fish with a resident phenotype, especially if the initiation of a migratory trajectory occurs months prior

to release (as in Chinook salmon; Shearer & Swanson 2000). Another possibility is that the expression of antiviral genes underlies unhealthy or infected individuals that will not migrate (and become residents). A combined approach using gene expression and pathogen titres in large numbers of individuals, combined with movement data based on passive integrated transponder (PIT) tags, might provide more insight as to why the resident samples were overexpressing these genes.

Genes exhibiting early divergence between resident and migrant phenotypes may provide insight on upstream inducers of smoltification in the gill and function as predictive gene expression markers for likelihood of smoltification. Both *cftr* and *s100a4* correlated with NKA activity. However, *cftr* reached an expression maximum, while *s100a4* expression continued to increase alongside NKA activity. The *cftr* maximum was observed in both the microarray and qPCR data, and the qPCR primers were shown to function over a range well beyond that in the samples. It is likely that *cftr*, being a saltwater inducible gene (Singer *et al.* 2002), only increases to a certain extent until salt water is present. The strongest predictive candidate is *s100a4*.

S100 proteins are a family of EF-hand Ca^{2+} -binding proteins that regulate a broad range of biological processes in vertebrates (Hermann *et al.* 2012). Fourteen S100 subfamilies have been described in teleosts, and in zebrafish *Danio rerio*, expression patterns are often tissue- and life stage-specific (Kraemer *et al.* 2008). S100 proteins are fine-tuned to free intracellular Ca^{2+} and refine cell-specific activities (Hermann *et al.* 2012). Upon binding Ca^{2+}, S100 proteins change conformation and interact with specific targets. S100 proteins also may activate cellular ion channels (Hermann *et al.* 2012) and have been shown to have proliferative functions through epidermal growth factor ligand binding (Klingelhöfer *et al.* 2009). Interestingly, in mammalian liver tissue, *s100a4* was up-regulated by hypertonic stress (Rivard *et al.* 2007). An S100 protein was already identified as involved in the differences between resident and anadromous populations of brook charr; crosses of the two populations resulted in suppressed expression of genes in hybrids, including *protein s100a1*, potentially due to the disruption of finely tuned regulatory regions (Mavarez *et al.* 2009). We identified divergence in gill *s100a4* expression between phenotypes while all fish were in a common environment and still in fresh water. However, to determine whether the expression change is inducing gill changes for smoltification or whether it is a downstream effect of smoltification requires further work.

Calcium has important signalling and nutritional roles during smoltification, and several calcium-related genes were overexpressed in migrants (Table 1). Growth hormone levels increase during smoltification, inducing rapid growth and additional nutrient requirements (e.g. increased calcium and phosphate). Activated vitamin D3 (calcitriol) induces uptake of these nutrients (Lock *et al.* 2007) and increases transcription of calcium channels in *Danio rerio* (Lin *et al.* 2012). Lin *et al.* (2012) found that while fish were in low ambient calcium, calcium influx correlated with *vitamin D3 receptor* (*vdr*) expression. These authors also identified a negative feedback loop, similar to that in mammals, in which calcitriol induces the expression of its inactivator, *1,25-dihydroxyvitamin D3 24-hydroxylase, mitochondrial* (*cyp24a1*), and suppresses its activator *25-hydroxyvitamin D-1 alpha hydroxylase, mitochondrial* (*cyp27b1*). Here, we identified overexpression of *vitamin D3 receptor* and the calcitriol inactivator *cyp24a1* in migrants, suggesting calcitriol activation may be occurring in the migrants, as has been identified in Atlantic salmon in fresh water during smoltification preparation (Graff *et al.* 2004) and while in transitional concentrations (50:50 salt water/fresh water; Lock *et al.* 2007). Here, migrants also overexpressed *calcitonin-1*, which promotes calcium uptake into bones from blood, calcium-binding *arylsulfatase d* and others (Table 1), indicating the importance of calcium homeostasis in migrants.

The ubiquitous yet variable nature of migratory components of salmonid life history leads to the question of the broader relevance of differential expression patterns revealed here. Observations of populations above migratory barriers which continue to produce smolts (e.g. Thrower *et al.* 2004; Martinez *et al.* 2011) and qualitative and quantitative similarities in gene expression in the gills of anadromous and land-locked salmon (Lemmetyinen *et al.* 2013) suggest that it might be interesting to examine differentially expressed genes observed here more broadly within *O. mykiss* and also in species with greater divergence between anadromous and land-locked populations (e.g. bull trout *Salvelinus confluentus*). Presently, we are sampling steelhead populations from other Distinct Population Segments (Federal Register Vol. 71, No. 3, 5 January 2006, pp. 834-862) to examine whether patterns observed here are exhibited more broadly by the species. We are also performing more extensive temporal sampling from the juvenile life history phase to observe the timing of changes in expression of genes, including the earlier induction of *s100a4*, and plan to manipulate diets, photoperiods and other hatchery rearing conditions with the aim of improving our understanding of the roles of these genes in smoltification. The investigation of the potential trade-offs in energetics and antiviral immunity will also be important to continue to improve our understanding of the ecological immunology of migrating salmon.

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BJGS contributed to study design, performed RNA extractions, microarray work and analysis, qPCR analysis and wrote the article. JRJ performed qPCR work. BFK contributed to study design and article preparation. KCH performed NKA activity assays. KCH and CTS designed the study, collected samples and wrote the article.

Data accessibility

Microarray files: GEO submission GSE48844. Photographs and additional files: Dryad submission doi: 10.5061/dryad.3p545

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Correlation between qPCR and microarray.

Table S1 Sample information.

Table S2 Primer table.

Table S3 Gene Ontology enrichment in migrants or residents.

Data S1 Total differential gene lists.