J Exp Biol Advance Online Articles. First posted online on 2 October 2014 as doi:10.1242/jeb.107441
Access the most recent version at http://jeb.biologists.org/lookup/doi/10.1242/jeb.107441

Title:

An integrated transcriptomic and comparative genomic analysis of differential gene expression in Arctic charr (*Salvelinus alpinus*) following seawater exposure

Authors:
Joseph D. Norman[1*], Moira M. Ferguson[1], Roy G. Danzmann[1]

Affiliation:
[1]Department of Integrative Biology, University of Guelph, Guelph, Ontario, Canada, N1G 2W1

[*]**Corresponding author:**
Joseph D. Norman
Integrative Biology
Science Complex
University of Guelph
50 Stone Road East
Guelph, Ontario, Canada, N1G 2W1

Email: jnorman@uoguelph.ca
Telephone: 001 519 824-4120 ext. 58596
Fax: 001 519 767-1656

Running head:
Hypo-osmoregulation in Arctic charr

SUMMARY

High-throughput RNA sequencing was employed to compare expression profiles in two Arctic charr (*Salvelinus alpinus*) families post seawater exposure to identify genes and biological processes involved in hypo-osmoregulation and regulation of salinity tolerance. To further understand the genetic architecture of hypo-osmoregulation, the genomic organization of differentially expressed (DE) genes was also analysed. Using a *de novo* gill transcriptome assembly we found over 2300 contigs to be DE. Major transporters from the seawater mitochondrion-rich cell (MRC) complex were up-regulated in seawater. Expression ratios for 257 differentially expressed contigs were highly correlated between families, suggesting they are strictly regulated. Based on expression profiles and known molecular pathways we inferred that seawater exposure induced changes in methylation states and elevated peroxynitrite formation in gill. We hypothesized that concomitance between DE immune genes and the transition to a hypo-osmoregulatory state could be related to Cl^- sequestration by antimicrobial defence mechanisms. Gene Ontology analysis revealed that cell division genes were up-regulated, which

could reflect the proliferation of ATP1α1b-type seawater MRCs. Comparative genomics analyses suggest that hypo-osmoregulation is influenced by the relative proximities among a contingent of genes on Arctic charr linkage groups AC-4 and AC-12 that exhibit homologous affinities with a region on stickleback chromosome Ga-I. This supports the hypothesis that relative gene location along a chromosome is a property of the genetic architecture of hypo-osmoregulation. Evidence of non-random structure between hypo-osmoregulation candidate genes was found on AC-1/11 and AC-28, suggesting that interchromosomal rearrangements played a role in the evolution of hypo-osmoregulation in Arctic charr.

Keywords:

RNA-Seq, gene expression, osmoregulation, salinity tolerance, Salmonidae, *Salvelinus alpinus*

INTRODUCTION

Migration between freshwater and seawater habitats is an important stage in the ontogeny of anadromous salmonids (Hoar, 1988). Freshwater and seawater impose adverse and contrasting types of osmoregulatory stress, such that maintenance of internal ion homeostasis is paramount for survival. The transition from freshwater to seawater requires osmoregulatory mechanisms to switch from a state of ion absorption (i.e., hyper-osmoregulation) to one of ion excretion (i.e., hypo-osmoregulation). Gill tissue epithelial cells play an integral role in this process (Evans et al., 2005; Marshall and Grosell, 2006).

Mitochondrion-rich cells (MRCs) form a complex with accessory cells and pavement cells in the epithelial layer of gill tissue (Evans et al., 2005; Marshall and Grosell, 2006). In seawater this complex facilitates excretion of excess Cl^- and Na^+ from blood to seawater. Cl^- excretion is achieved via the collective function of three interdependent membrane-bound ion transporters found in MRCs: $Na^+/K^+/2Cl^-$, Na^+/K^+-ATPase, cystic fibrosis transmembrane conductance regulator. Na^+ excretion occurs through leaky paracellular pores located between MRCs and accessory cells (Silva et al., 1977; Marshall and Grosell, 2006), where permeability seems to be dependent upon the claudin isoform located at the tight junction (Furuse et al., 2001; Van Itallie et al., 2006; Tipsmark et al., 2008). Elevated expression of these transporters occurs concomitantly with an increase in the size and quantity of MRCs, which is controlled by cortisol

and growth hormone (McCormick, 2001; Marshall and Grosell, 2006). Despite the prominent role that MRCs have in hypo-osmoregulation, they comprise less than 10% of gill tissue epithelium, while pavement cells cover over 90% (Wilson and Laurent, 2002; Evans et al., 2005).

Variable functions for the genes regulating ion transport in teleost gill tissues can be achieved as a result of the whole genome duplications that teleost fishes underwent in their evolutionary origins (Spring, 1997; Jaillon et al., 2004). Furthermore, as a result of tandem duplications and an additional whole-genome duplication in salmonids (Allendorf and Thorgaard, 1984), these species possess a repertoire of functionally distinct isoforms of genes that are components of the seawater MRC complex. Na^+/K^+-ATPase isoforms *ATP1α1a* and *ATP1α1b* exhibit reciprocal expression in freshwater and seawater. In seawater, *ATP1α1b* is up-regulated and *ATP1α1a* is down-regulated, while the opposite occurs in freshwater (Richards et al., 2003; Mackie et al., 2005; Bystriansky et al., 2006; Nilsen et al., 2007; Larsen et al., 2008; Madsen et al., 2009; McCormick et al., 2009). *ATP1α1a* and *ATP1α1b* are also expressed in separate MRCs (McCormick et al., 2009). There are two *NKCC1* isoforms (i.e., *NKCC1a* and *NKCC1b*), but evidence suggests that only *NKCC1a* is significantly expressed in the gill of fishes in seawater (Cutler and Cramb, 2002; Tipsmark et al., 2002; Scott et al., 2004; Lorin-Nebel et al., 2006; Tse et al., 2006; Hiroi et al., 2008; Kang et al., 2010). In Atlantic salmon (*Salmo salar*), two *CFTR* isoforms are expressed in gill (i.e., *CFTRI* and *CFTRII*) (Chen et al., 2001; Mackie et al., 2005). Although both are up-regulated in seawater, temporal expression patterns are variable (Singer et al., 2002; Mackie et al., 2007; Aykanat et al., 2011). Five claudin isoforms have been detected in Atlantic salmon gill tissue; c*CLDN10E*, *CLDN27A*, *CLDN28A*, *CLDN28B*, and *CLDN30*. In seawater, *CLDN10E* mRNA is significantly up-regulated while *CLDN27A* and *CLDN30* mRNA is down-regulated (Tipsmark et al., 2008; Engelund et al., 2012). Overall, the pattern emerging seems to be an evolutionary divergence of hypo-osmoregulatory function towards distinct freshwater and seawater isoforms.

Large-scale transcriptomics analyses in a variety of teleosts have broadened our understanding of hypo-osmoregulation in fishes. Studies that assess differential gene expression in gill between fish in freshwater and seawater (Boutet et al., 2006; Kalujnaia et al., 2007; Evans

and Somero, 2008; Tine et al., 2008; Evans et al., 2011) demonstrate that hypo-osmoregulation is influenced by diverse biological processes that extend well beyond the mechanisms described by the seawater MRC complex. As the number of studies has increased, patterns have emerged suggesting hypo-osmoregulation in different taxa is influenced by some of the same biological processes. These include cell-cycle regulation, cell metabolism, protein synthesis and regulation, cytoskeleton and structural proteins, and immune system response (Boutet et al., 2006; Kalujnaia et al., 2008; Evans and Somero, 2008; Tine et al., 2008). Notable in their absence are controlled laboratory experiments on salmonids, species for which salinity tolerance has important implications from both life-history and economic perspectives. Salmonids have been studied in their natural state during different life-history stages in their anadromous life-cycle, although such studies are far from complete. For example Evans et al. (2011) examined the hyper-osmoregulatory aspects of gill function by sampling sockeye salmon (*Oncorhynchus nerka*) on their return to freshwater natal spawning grounds. Because their work was based on fish migrating from seawater to freshwater (instead of freshwater to seawater), it may only reflect part of the story about differential expression (DE) in genes involved in seawater acclimation.

Large-scale gene expression analysis can also be used to gain insight about the genetic basis of salinity tolerance. Salmonids exhibit diverse salinity tolerance capacities, both within and among species (Hoar, 1988; Delabbio et al., 1990; Schmitz et al., 1995; Singer et al., 2002; Mackie et al., 2005; Shrimpton et al., 2005; Bystriansky et al., 2006; Hiroi and MCormick, 2007; Nilsen et al., 2007). Within species, quantitative trait locus (i.e., QTL) studies indicate that variation has a genetic basis (Le Bras et al., 2011; Norman et al., 2011; Norman et al., 2012). Genes differentially regulated in Arctic charr (*Salvelinus alpinus*) with contrasting salinity tolerance capacities have been identified using large-scale gene expression analysis on gill tissue. Evidence suggests the genes that affect variation in salinity tolerance capacity might be different from the genes that are required for ionoregulation in hyper-osmotic conditions (Norman et al., 2014). Transcriptome-wide experiments assessing DE in Arctic charr between freshwater and seawater environments would permit further investigation of these differences. As the number of large-scale expression studies on salinity tolerance increases, comparisons between species may provide insight about the nature of the diverse salinity tolerance capacities that characterize

salmonids. Such work would also facilitate further research on the propensity of new candidate genes to confer genetically-based phenotypic variation.

Differences in the genomic organization of salinity tolerance candidate genes may also influence inter- and intraspecific variation in salinity tolerance capacity. Clustered genes in vertebrates are more likely to be co-expressed (Hurst et al., 2004; Ng et al., 2009; Woo et al., 2010) and co-methylated (McGowan et al., 2011) and thus are more likely to be involved in the same or closely-related biological pathways. For instance, candidate genes in salmonids show evidence of clustering along linkage groups (Norman et al., 2012) and similar patterns are evident on model teleost chromosomes (e.g., medaka, stickleback, zebrafish) (Norman et al., 2012) and on reconstructed proto-actinopterygian ancestral chromosomes (Kasahara et al., 2007). Current data indicate that inter-chromosomal rearrangements in the Atlantic salmon genome have produced unique combinations of salinity tolerance candidate genes, which may be related to the high salinity tolerance capacity that is characteristic of this species (Norman et al., 2012). However, these observations are based on relatively few candidate genes. The myriad candidate genes that are typically produced by large-scale gene expression analysis would permit a more in-depth characterization of these patterns. The production of candidate gene lists produced by large-scale gene expression analysis coupled with *in silico* comparative genomics approaches will facilitate a more in-depth investigation of these patterns, providing additional insight about the genetic architecture of salinity tolerance in salmonids.

In this study we collected large-scale gene expression data from Arctic charr to gain insight about the genes and biological processes that affect salinity tolerance in this species. Specifically, we used high-throughput mRNA sequencing technology to compare expression profiles between fish exposed to freshwater and seawater. We then investigated the genome organization of differentially expressed genes (DEGs) to gain insight about putative non-random patterns in the genetic architecture of salinity tolerance. Our specific objectives were to identify genes differentially expressed in Arctic charr gill tissue after transfer to seawater from freshwater, and to characterize which biological processes affect hypo-osmoregulation in this species. Based upon comparisons with other teleost species, inferences on the locations of the DEGs were made (i.e., putative retention of synteny blocks) and used to test if DEGs are

clustered, and whether or not genes or gene clusters are randomly distributed among Arctic charr linkage groups.

RESULTSDifferential expression analysis

Analyses conducted using EBSeq (Leng et al., 2013) revealed that 1075 and 1544 contigs were DE between seawater and freshwater treatments for family 10 (F10) and family 12 (F12), respectively (Fig. 1). For F10, 568 (52.8%) contigs were up-regulated and 507 were down-regulated, while for F12, 812 (53.9%) contigs were up-regulated and 732 were down-regulated. A comparison between families revealed that 257 contigs were DE in both families (Table S1). Interestingly, a striking congruence in both the direction and magnitude of expression was observed for all 257 contigs (Fig. 2; $F = 3118.10$; regression d.f. = 1; residual d.f. = 255; $R^2 = 0.924$; $P < 0.000001$). The majority (84.4%) of these exhibited an inter-familial difference in expression ratio that was less than 0.5 (i.e., a 1.4-fold increase) (Fig. 3), and slightly more contigs were up-regulated (51.7%; Table 1) than down-regulated (Table 2).

Contig annotation

Alignments against the non-redundant protein database at NCBI were successful for 60,565 of 108,645 assembled contigs (55.7%). To test if they were accurate, we aligned all unannotated contigs (i.e., 40,080) against EST databases for rainbow trout (*Oncorhynchus mykiss*) (release 8) and Atlantic salmon (release 6) from the Gene Index Project at the Dana-Farber Cancer Institute (Quakenbush et al., 2001), using criteria described in Materials and Methods. Significant alignments were observed for 36,879 contigs (76.7%). Thus, in total, 97,444 of 108,645 assembled contigs (89.6%) were verified by significant alignments to external databases. The number of annotated DE contigs for F10 and F12 was 832 (Table S2) and 1177 (Table S3), respectively.

Gene Ontology analysis

F10 and F12 shared 25 Gene Ontology (GO) categories significantly enriched for up-regulated genes (Table 3, Table S4-S9). Collectively, these categories represented biological processes related to cell division, cell proliferation, and responses to stress. In contrast, F10 and F12 shared only six categories enriched for down-regulated genes, which were related to

developmental processes, such as organ development. Tests for disproportionate representation of genes in biological process categories revealed that seven categories for up-regulated genes were different between families (Table 4). Together they represented two general processes, mRNA processing and splicing, and cellular metabolic processes involving nucleic acids. No differences were observed for down-regulated gene categories.

Proximity of differentially expressed genes

Comparisons of mean intergenic distance (IGD) between stickleback genes and genes DE in seawater gill tissue revealed that DEGs homologous with certain stickleback chromosomes were significantly closer to one another than the respective chromosomal-wide means (Fig. 4; Table S10). In F10 these patterns were found on Ga-II, -V, -XV, and -XVII, all of which exhibited $P < 0.001$. DEGs that aligned to Ga-II and -V were over 1 Mbp closer to one another on average, while those aligned to Ga-XV and -XVII were 660 kbp and 704 kbp closer to one another, respectively. For eight stickleback chromosomes the chromosomal-wide mean IGD was significantly lower than the mean IGD of the corresponding set of homologous DEGs from Arctic charr F10, which were 460 kbp to 1.13 Mbp further apart. In F12, DEGs that aligned to Ga-I, -IV, -V, and -X were significantly closer to one another than the corresponding chromosomal-wide means by at least 480 kbp. With the exception of Ga-X ($P = 0.0281$), these differences were also highly significant ($P \leq 0.0013$). Only four stickleback chromosomes (Ga-III, -VI, -VIII, and -XIV) had a mean IGD that was significantly lower than the corresponding set of homologous DEGs from F12. Consensus between families was evident for Ga-III, -V, and -VIII; however, only for Ga-V were the mean IGDs from DEGs significantly lower than the chromosomal-wide mean.

Characterization of differentially expressed gene clusters

Translated blast alignments (i.e., BLASTX) of DE contigs against the stickleback cDNA database yielded 507 hits for F10, of which 54.8% (i.e., 278 contigs) were up-regulated. For F12, 695 significant alignments were observed and of these 51.7% (i.e., 359 contigs) were up-regulated. In terms of DEGs located within 40 kbp of one another, 107 DEGs formed 50 clusters of two or more in F10 (Table S11), which contained five three-gene clusters and one four-gene cluster. In F12, 252 DEGs formed 105 clusters of two or more (Table S12). Most genes

occurred in clusters of two (72.3%); however, clusters of three (19.0%), four (5.7%), five (1.9%), and six (1.0%) were also evident. In F10, 36 clusters (50% were up-regulated) showed congruency in the direction of expression (i.e., genes in these clusters were either all up-regulated or all down-regulated), while in F12, 48 clusters were congruent (56% were up-regulated). Notably, the proportion of IGDs that were ≤ 40 kbp was highly similar between all protein-coding genes from the stickleback genome (63918/9180573 = 0.0069) and the DEGs from F10 (53/8457 = 0.0063) and F12 (126/18095 = 0.0069), which suggests that the proportion of DEGs within 40 kbp of one another was the same as would be expected by chance.

Enrichment of differentially expressed genes in synteny blocks

Three synteny blocks (i.e., chromosomal segments where a contingent of genes is conserved between two species) contained a greater number of DEGs than expected by chance (Table 5). All were observed in F10, while only the AC-1/Ga-XVII synteny block passed the FDR corrected threshold. No synteny blocks deviated from expectations for F12.

DISCUSSION

This is the first study to use high-throughput RNA sequencing in a controlled laboratory experiment to compare gene expression profiles in the gills of a salmonid species reared in freshwater and seawater. Using a *de novo* transcriptome assembly we conducted a large-scale analysis to gain insight about the genes and biological processes important for hypo-osmoregulation in Arctic charr. To make inferences about the conservation of candidate genes and biological processes in different taxa, we studied two families and compared our findings with those from similar studies on other salmonids. We examined gene cluster patterns and the distribution of DEGs to test hypotheses about the genetic architecture of salinity tolerance. Our large-scale gene expression analysis has provided a broad perspective of hypo-osmoregulation in Arctic charr, which will be a foundation for future studies to pursue answers to new questions about the physiological and genetic processes that influence hypo-osmoregulation.

Multiple salinity tolerance candidate genes from the seawater MRC complex (Evans et al., 2005; Marshall and Grosell, 2006) were DE in the gill of Arctic charr after transfer to seawater. Among the up-regulated genes were Na^+/K^+-ATPase subunit alpha 1b (*ATP1α1b*),

$Na^+/K^+/2Cl^-$ co-transporter 1a (*NKCC1a*), cystic fibrosis transmembrane conductance regulator II (*CFTRII*) and *CLDN10E* (Table S3). These findings confirm previous research in other salmonid species (Singer et al., 2002; Tipsmark et al., 2002; Richards et al., 2003; Mackie et al., 2005; Bystriansky et al., 2006; Nilsen et al., 2007; Larsen et al., 2008; Tipsmark et al., 2008; Madsen et al., 2009; McCormick et al., 2009). Furthermore, *ATP1α1a* was down-regulated in seawater, as expected given that *ATP1α1a* and *ATP1α1b* are reciprocally expressed (Richards et al., 2003; Mackie et al., 2005; Bystriansky et al., 2006; Nilsen et al., 2007; Larsen et al., 2008; Madsen et al., 2009; McCormick et al., 2009) and regulated (McCormick et al., 2009) between freshwater and seawater environments. This consistency with the general literature on hypo-osmoregulation indicates that our experimental design and analysis produced reliable data.

The two families exhibited different gene expression patterns for major transporters from the seawater MRC complex. *ATP1α1b*, *NKCC1a*, *CFTRI/II*, and *CLDN10E* were DE in F12 but not in F10, while *ATP1α1a* exhibited DE in both families. This difference is consistent with previous research showing that only F12 QTL colocalize with the locations of seawater MRC complex candidate genes (Norman et al., 2014). Genome scans for salinity tolerance QTL using the same fish revealed that only 17.6% (i.e., 3/17) were detected in both families. Moreover, QTL colocalize with *ATP1α1b* and *NKCC1* on Arctic charr linkage groups in F12 but not F10 (Norman et al., 2012). Thus, differences in salinity tolerance between F10 and F12 are evident at both nucleotide and transcriptional levels.

Our findings suggest that hypo-osmoregulation in Arctic charr may be regulated by a "core" gene subset that has limited capacity for plasticity in expression. In total, 257 DEGs from both families had expression ratios showing a remarkable consistency in magnitude and direction (Fig. 1). Predicting whether this pattern is conserved in other populations or in other salmonids is difficult, as current evidence is equivocal. For instance, the consistency in expression of this core subset might be related to the shared genetic background of the families, as both were derived from the same strain. Nevertheless, both families were generated by outbred crosses from unrelated parents and results from QTL experiments do support the concept of genetic heterogeneity between the families (Norman et al., 2011).

Comparing expression profiles among genes from the core subset provides clues about the molecular pathways involved, permitting inferences about biological significance to be made. For instance, changes in the direction of transcription of genes involved in methylation suggest that methylation states were altered in seawater-exposed fish. Adenosylhomocysteinase was up-regulated, and as part of the methionine cycle (Fig. 5), this enzyme catalyzes a reversible reaction that is thermodynamically favoured towards the production of S-adenosylhomocysteine from homocysteine and adenosine. S-adenosylhomocysteine is a potent inhibitor of methyltransferases (Hoffman et al., 1980), leading to increased hypomethylation levels of proteins, lipids, polyamines, and chromatin, which in turn have been linked to altered gene expression and cellular differentiation states (reviewed in James et al., 2002). Adenosine removal by adenosine kinase (up-regulated in F10) may reduce the availability of adenosine to adenosylhomocysteinase, thereby promoting conditions favouring the formation of homocysteine and adenosine. Gene transcription profiles for other components of the methionine cycle provide further support for this proposition: methyltransferases were up-regulated (both families), 5'-nucleotidase, which converts adenosine monophosphate to adenosine, was down-regulated (F12), and methionine adenosyltransferases, which catalyze the formation of S-adenosylmethionine from methionine, were up-regulated (F10). Since the gene sets that affect hyper- and hypo-osmoregulation are different, changes in methylation states should be anticipated when transcription is compared between freshwater and seawater exposed fish. Observations from other species support these findings: seawater-induced changes in methylation states have been observed in brown trout (Moran et al., 2013), and transcription of the betain-homocysteine S-methyltransferase gene in ayu (*Plecoglossus altivelis*) gill is reduced after transfer from freshwater to brackish water (Lu et al., 2010). Thus, changes in methylation states are likely part of the physiological transition to seawater in anadromous fishes.

Comparisons among transcriptional profiles for genes that are part of the same molecular pathways as inducible nitric oxide synthase (iNOS) provide clues about why immune response gene regulation coincides with elevated salinity and salinity tolerance capacity (Norman et al., 2014). Inducible nitric oxide synthase, highly up-regulated in core subset, produces nitric oxide (NO), a bio-signalling molecule that elicits many physiological responses (Pacher et al., 2007). NO produced by iNOS in particular, triggers an immune system response to pathogenic bacteria

and viruses (Lowenstein and Snyder, 1992). NO also increases the expression of cytochrome P450 (Zamora et al., 2001), which in turn, produces hydrogen peroxide that is catalyzed with chloride by myeloperoxidase (MPO) to produce hypochlorous acid (HOCl), a potent antimicrobial agent (Klebanoff, 2005). Interestingly, chloride availability may limit MPO activity (Pacher et al., 2007). If true, elevated plasma chloride levels may provide conditions that are conducive towards strengthening antimicrobial defence, which could have implications for ionoregulation, as hypochlorous acid production would necessitate chloride removal from blood plasma.

Elevated iNOS transcription likely fostered increased peroxynitrite formation, suggesting that further research about the putative impact and role of peroxynitrite in hypo-osmoregulation may be warranted. Peroxynitrite is a highly potent oxidizing compound formed from the spontaneous reaction of NO with superoxide (Huie and Padmaja, 1993). Although superoxide can be cytotoxic, it is detoxified by scavenging enzymes known as superoxide dismutases (SOD) (Pacher et al., 2007). In F12, SOD was down-regulated in seawater, suggesting that superoxide detoxification was reduced during hypo-osmoregulation. Moreover, superoxide detoxification by SOD is three times slower than peroxynitrite formation (Pohanka, 2013). Therefore increased superoxide levels combined with elevated NO production would likely result in increased peroxynitrite levels in seawater gill. Reduced transcription of SOD in high salinity tolerance capacity Arctic charr (Norman et al., 2014) also suggests that variation in salinity tolerance capacity may correlate with variation in peroxynitrite levels. Although peroxynitrite can protect against invading pathogens, it is also cytotoxic and can induce apoptosis and necrotic cell death (Pacher et al., 2007). Protection from pathogens would likely ease the transition to a hypo-osmoregulatory state, which can be metabolically demanding for salmonids (Rao, 1968; McCormick et al., 1989; Morgan and Iwama, 1999). Additionally, increased rates of cell death may accelerate cellular turnover in gill epithelia, where freshwater-type MRCs are replaced with seawater-type MRCs (McCormick et al., 2009). Hypomethylated DNA is also more susceptible to oxidative stress leading to increased rates of apoptosis and cellular turnover (James et al., 2002). Elevated cellular turnover may, in turn, more rapidly increase hypo-osmoregulatory capacity, conserving energy and mitigating the physiological stress conferred by a sub-optimal

osmoregulatory condition. Notably, peroxynitrite also modulates Na^+/K^+-ATPase activity via cysteine oxidation (Muriel and Sandoval, 2000; Varela et al., 2004).

While certain genes from the core subset have been implicated in hypo-osmoregulation by other studies, the majority are DE only in Arctic charr, providing limited support for the hypothesis that it is functionally conserved in other salmonids. We compared the results of this study with similar research on Atlantic salmon (Seear et al., 2010; Robertson and McCormick, 2012) and sockeye salmon (Evans et al., 2011). Only nine genes in sockeye salmon (Evans et al., 2011) and seven genes in Atlantic salmon (Seear et al., 2010; Robertson and McCormick, 2012) showed patterns of DE that were consistent with our observations (Table 6). It must be emphasized that differences in experimental design and in the technical platforms employed by others (Seear et al., 2010; Evans et al., 2011; Robertson and McCormick, 2012) may account for these differences.

The overrepresentation of up-regulated cell division genes suggests that MRC proliferation was occurring in the gill of seawater acclimating fish. Though short term (e.g., 5 hours) acclimation to a hyper-osmotic environment is characterized by the transformation of freshwater- to seawater-type MRCs (Hiroi et al., 1999), long term (i.e., days to weeks) acclimation involves an increase in the number of MRCs (McCormick et al., 2001; Evans et al., 2005; Hwang and Lee, 2007). In the Atlantic salmon gill, the seawater isoform *ATP1α1b* occurs in separate MRCs than its freshwater counterpart, *ATP1α1a* (McCormick et al., 2009). Considering that seawater acclimation in salmonids entails elevated *ATP1α1b* expression (Richards et al., 2003; Mackie et al., 2005; Bystriansky et al., 2006; Nilsen et al., 2007; Larsen et al., 2008; Madsen et al., 2009; McCormick et al., 2009), and that tissue samples were collected 10 days after transfer to seawater, it is plausible that the enrichment of up-regulated cell division genes could reflect the proliferation of *ATP1α1b*-type seawater MRCs. However, gill epithelium is composed of many different cell types, and pavement cells, which have a limited role in ionoregulation, comprise most of the epithelial surface area (> 90%), followed by MRCs (< 10%) (Wilson and Laurent, 2002; Evans et al., 2005). Therefore cell division gene enrichment is also likely evidential of the proliferation of other cell types (see Wilson and Laurent, 2002). For instance, iNOS, which may have a role in hypo-osmoregulation, localizes to small cells buried

deep within gill filaments (Ebbesson et al., 2005). Enhanced expression of the methionine cycle, specifically, methionine adenosyltransferase, is also regarded as essential in regulating cell cycle turnover rates (Lin et al., 2014), which complements the results from this study (Fig. 5). Enrichment of up-regulated genes related to cell division GO categories has also been described for other teleosts: cell-cycle regulation in sea bass (Boutet et al., 2006), cellular processes in tilapia (Tine et al., 2008), and cell-cycle in goby (Evans and Somero, 2008).

GO analyses suggest that the repression of developmental gene expression may be related to increased osmoregulatory stress. Overrepresentation of down-regulated genes was confined to a GO branch related to development (i.e., multicellular organismal development, system development, developmental processes, anatomical structure development, multicellular organismal process, and organ development). This appears paradoxical since cell division is integral to organ development and thus these processes would be expected to exhibit some congruency in expression. However, cellular turnover driven by senescence and apoptosis can occur independently of development (Pellettieri and Alvarado, 2007), while development necessitates cell division. Given that stress response genes were also up-regulated and overrepresented, we hypothesize that the stress induced by hyper-osmotic conditions may cause a repression of developmental gene expression. This would have to be temporary, however, perhaps until gill tissue and other ionoregulatory organs have transitioned to an optimal ion-excreting state, because the seawater phase of salmonid ontogeny is typically characterized by heightened growth rates. Repression of developmental gene expression may liberate energy for the transition of osmoregulatory tissues toward an ion-excreting state, as the metabolic demand of hypo-osmoregulation can be high, ranging from < 4% in cuthroat trout (*Oncorhynchus clarkii clarkii*) (Morgan and Iwama, 1999) to 30% in rainbow trout (Rao, 1968), and 20% of gill metabolism in Atlantic salmon (McCormick et al., 1989). To our knowledge, there are no data on the metabolic demands of hypo-osmoregulation in Arctic charr, although our hypothesis suggests that the cost would be high. The current study may have captured the transition period in gill physiology when hyper-osmoregulatory cells are being ablated prior to the metabolic machinery of hypo-osmoregulatory cells being fully activated.

Comparative genomics analyses provide support for the hypothesis that the relative location of salinity tolerance candidate genes along a chromosome is a feature of the genetic architecture of hypoosmoregulation. The physical clustering within linkage groups of DEGs in seawater gill tissue is consistent with previous observations in teleosts (Norman et al., 2012). The majority of genes that met our clustering criteria (i.e., ≤ 40 kbp) were also identified as paired by previous research conducted on the same fish, but on a smaller scale (Norman et al., 2012). Furthermore, DEGs that align to certain stickleback chromosomes tend to have lower mean IGDs than the corresponding chromosomal-wide means, and several of these chromosomes are homologous with salinity tolerance QTL. For instance, Ga-I, which contains DEGs from F12 that are on average 890 kbp closer together, is homologous with AC-4 and -12 (Norman et al., 2012), where salinity tolerance QTL have been localized in the same family (Norman et al., 2011). In addition, Ga-I not only contains two key genes from the seawater MRC model (i.e., *ATP1α1b* and *CLDN10E*), but an inversion on this chromosome is correlated with disparity between marine and freshwater stickleback ecotypes (Jones et al., 2012). Interestingly, *ATP1α1b* is positioned within this inversion while *CLDN10E* is positioned outside of it. Consequently, *ATP1α1b* and *CLDN10E* are 1.007 Mb apart on Ga-I in the freshwater ecotype and 1.012 Mb apart in the marine ecotype, which suggests that ecotype development could be influenced by changes in the relative proximity of these two genes along the chromosome. Salinity tolerance QTL localized to Atlantic salmon linkage groups AS-5 and -22 are also homologous with Ga-I (Norman et al., 2012). Norman et al. (2012), hypothesized that the superior hypo-osmoregulatory capacity that is characteristic of Atlantic salmon (Hoar, 1988; Hiroi and McCormick, 2007) could be related to the formation of novel gene clusters by interchromosomal rearrangements. Thus, evidence suggests that a contingent of genes on Ga-I and their homologous counterparts in other species (e.g., Arctic charr and Atlantic salmon) may have implications for hypo-osmoregulation. Other stickleback chromosomes where DEGs are significantly closer than expected and that share homologous affinities with Arctic charr salinity tolerance QTL are AC-18 & Ga-V (F12), AC-4 & Ga-I (F10) and AC-31 & Ga-X (F10) (Norman et al., 2011).

The genetic architecture of hypo-osmoregulation in Arctic charr shows some evidence of large-scale structure. We found that stickleback synteny blocks on Arctic charr linkage groups

AC-1, AC-11 (henceforth referred to as AC-1/11, due to the strong homeologous affinities of these linkage groups (Danzmann et al., 2005; Timusk et al., 2011; Norman et al., 2012)), and AC-28 contain a greater number of DEGs than expected by chance. These linkage groups also contain QTL for the salinity tolerance performance traits Na^+/K^+-ATPase activity and growth in seawater (Norman et al., 2011), suggesting that these QTL could be the product of multiple genes containing *cis*-regulatory polymorphisms. This also suggests that some of the phenotypic variation attributed to QTL could stem from disparity in expression rate and transcript availability. In addition, stickleback chromosomes Ga-XVII and Ga-XIV share syntenic blocks with AC-1/11 and AC-28, respectively. Together with their respective homeologues, Ga-XVII&XII and Ga-XIII&XIV primarily originated from the ancestral L and I chromosome lineages of actinopterygian fishes, respectively (Kasahara et al., 2007; Nakatani et al., 2007; Norman et al., 2012), and were duplicated with the whole-genome duplication event in the teleost ancestor ~ 350 MYA (Christoffels et al., 2004). Comparisons with the teleost ancestor reveal that Ga-XVII&XII and Ga-XIII&XIV have been affected by very few interchromosomal rearrangements since the stickleback and teleost ancestral lineages diverged. Furthermore, the interchromosomal rearrangements that have occurred reflect translocations mostly between these homeologous pairs. Ga-XVII (orthologous with ancestral chromosome L), contains a 575 kbp segment that is homologous with Ga-XIII (orthologous with ancestral chromosome I), which localizes to the AC-1/11 and Ga-XVII synteny block (Norman et al., 2012). Close inspection of this region reveals that a single gene, tubulin polyglutamylase complex subunit 2 (*TPGS2*), was DE in seawater. These findings suggest that interchromosomal rearrangements between orthologues of L and I in the most recent common ancestor of stickleback and Arctic charr could have contributed to the apparent large-scale structure of AC-1/11. Furthermore, this suggests that interchromosomal rearrangements may have played a role in the evolution of hypo-osmoregulation in Arctic charr, and is consistent with the findings of Norman et al. (2012), who found that interchromosomal rearrangements produced a coalescence of salinity tolerance candidate genes on certain chromosomes in Atlantic salmon, which they hypothesized could contribute to the high salinity tolerance capacity of that species. Further research is necessary to test these hypotheses.

This study characterized changes in gene expression profiles and biological processes that occurred in Arctic charr gill tissue during seawater acclimation. Gene expression profiles of major transporters from the seawater MRC complex were consistent with previous research for one family, and inconsistent for the other family. Despite these differences a subset of DEGs in both families showed a remarkable consistency in both magnitude and direction of expression. We hypothesized that this subset may be comprised of genes that have a limited capacity for plasticity in expression which may be integral for hypo-osmoregulation in Arctic charr. Examination of these genes suggested that in seawater exposed fish methylation states were changing and that peroxynitrite formation and antimicrobial defence may have implications for hypo-osmoregulation. Based on patterns from GO, we postulated that the enrichment of cell division genes could reflect the proliferation of *ATP1α1b*-type seawater MRCs, and that the stress induced by hyper-osmotic conditions may have caused a repression of developmental gene expression. Our findings provide additional support for previous research that suggests the relative proximity among a contingent of genes on Ga-I has important implications for hypo-osmoregulation. We also found that clustering patterns of DEGs were consistent with previous observations in salmonids and other teleosts, and that AC-1/11 and AC-28 show evidence of large-scale structure for hypo-osmoregulation candidate genes. Finally, we provided evidence that interchromosomal rearrangements have played a role in the evolution of hypo-osmoregulation in Arctic charr.

MATERIALS AND METHODS

Strain background and rearing

Two full-sib Arctic charr, families (F10 and F12) were created from fish descended from the anadromous population resident in the Fraser River in Labrador, Canada. Crosses were made in November of 2006 at the Coastal Zones Research Institute, Shippagan, New Brunswick (Canada). Eight months after hatching, 150 progeny per family were implanted with passive integrated transponder tags and transferred to St. Andrews Biological Station, St. Andrews, New Brunswick (Canada). There they were held in 1 m^3 freshwater tanks and reared under controlled simulated-natural conditions for photoperiod and water temperature. Tanks contained filtered and aerated freshwater (9.9-10.7°C, flow rate 18 $L \cdot min^{-1}$, dissolved O_2 10.0-10.6 $mg \cdot L^{-1}$). For the duration of sampling (i.e., June 9, 2008 to July 6, 2008) a 16 h:8 h light:dark photoperiod

regime was employed to minimize the confounding effects associated with a naturally changing photoperiod (for details see Norman et al., 2011). All experiments were carried out in accordance with the Canadian Council for Animal Care Guidelines under the University of Guelph Animal Care Committee approved protocol number 08R033.

Experimental protocol and sample selection

The salinity tolerance trials were conducted during June, 2008. Freshwater was replaced with 100% filtered seawater (salinity 31,000-33,000 $mg \cdot L^{-1}$, 10.5-11.9°C, flow rate 18 $L \cdot min^{-1}$, dissolved O_2 8.1-11.4 $mg \cdot L^{-1}$) over 24 hours. After ten days exposure to full strength seawater non-lethal gill tissue biopsies (McCormick et al., 1993) were collected for 50 fish per family. Samples were also collected from freshwater fish at this time. Each sample consisted of approximately 3-6 primary filaments, which were stored in 1 mL of RNAlater (Life Technologies) at 4°C for 24-hours, then transferred to -20°C for long-term storage. To compare expression profiles in gill tissue between fish exposed to seawater and freshwater, we selected six fish from seawater and three fish from freshwater from each family for mRNA sequencing (N=18). Notably, the same seawater-exposed fish used for this study were also used for a companion study, and thus were comprised of individuals that exhibited the greatest and least number of salinity tolerance QTL (see Norman et al. 2014).

RNA extraction and sequencing

Immediately prior to homogenization, gill tissue samples were removed from RNAlater and rinsed. Trizol reagent (Life Technologies) was used to extract total RNA according to the manufacturer's instructions. A disposable pestle grinder system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to homogenize tissue immersed in 1 mL Trizol. Purity of extracted total RNA was assessed with a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). For further details regarding sample preparation, we refer the reader to Norman et al. (2014). The Bio-Rad Experion system (Bio-Rad, Hercules, California, USA) was employed to assess RNA integrity. Only samples with an RNA quality index (RQI) of 8.0 or greater were submitted for mRNA sequencing. mRNA libraries were constructed separately for all 18 fish. Illumina sequencing was performed by the Clinical Genomics Centre (Mount Sinai Hospital, Toronto, Ontario, Canada). Sequencing libraries were

created according to the manufacturer's instructions (Illumina). Briefly, poly-A mRNA was isolated from total RNA and purified using poly-T oligo-attached magnetic beads. mRNA was fragmented into pieces using divalent cations under elevated temperature, and copied into cDNA using reverse transcriptase and random primers. Following the ligation of adaptors, cDNA templates were purified and amplified with PCR. Sequencing was performed with a HiSeq 2000 instrument (Illumina, San Diego, California, USA) and subjected to 100 cycles of paired-end sequencing. Image analysis, base-calling and quality value calculation were performed using Illumina's sequence analysis software, Casava (v1.8.2). The raw reads are available from the Sequence Read Archive (SRA) at the National Centre for Biotechnology Information (NCBI), under BioProject accession #SRP026259 and BioSample accession #SRS452215.

Quality control and de novo transcriptome construction

The procedures used for read pre-processing, the measures taken for quality control, and the steps for *de novo* construction of the reference transcriptome are described in detail by Norman et al., (2014). Briefly, the reads used to quantify expression were filtered and trimmed according to quality scores using FastQC and Trimmomatic software (Andrews 2010; Lohse et al., 2012). To facilitate de novo assembly, reads with rare k-mers, low quality reads, and identical reads were removed using Rnnotator (Martin et al., 2010). The Velvet-Oases software pipeline was employed to generate a single merged assembly from multiple assemblies spanning a range of k-mers. Redundancy in the merged assembly was reduced using CD-HIT-EST (Li and Godzik, 2006), producing a final consensus assembly of 108,645 contigs.

Differential expression analysis and sequence annotation

Read alignment to the reference transcriptome was conducted with RNA-Seq by Expectation Maximization (RSEM) (Li and Dewey, 2011). RSEM performs accurate transcript quantification when a reference genome is unavailable. It also allows for the inclusion of non-uniquely mapping reads in abundance estimates through the use of a statistical model that accounts for read mapping uncertainty. Since scaffolding was not used during assembly with Velvet-Oases, paired-end reads could not be used for accurate expression quantification. Accordingly, only forward reads were mapped since they typically yielded higher quality scores than reverse reads. Differential expression between fish exposed to seawater and freshwater was

analyzed separately for each family, using EBSeq (Leng et al., 2013). This software employs an empirical Bayes hierarchical model that accommodates read mapping uncertainty from RSEM. To account for differences in library size, the number of reads in each sample was adjusted by median normalization (Anders and Huber, 2010; Leng et al., 2013). Differential expression was established at a threshold of $P \leq 0.05$ and a B-H False Discovery Rate of $\alpha = 0.05$ (Sokal and Rohlf, 1995). Linear regression was employed using SPSS 16 (IBM) to compare the magnitude and direction of expression ratios for genes that were DE in both families.

Sequences were annotated using Blast2GO (Gotz et al., 2008). The single best alignment for each contig was obtained using BLASTX against the non-redundant protein sequence database at NCBI at an E-value threshold of 1E-5.

Gene Ontology analysis

The Biological Networks GO tool (BiNGO) (Maere et al., 2005) was used to perform hypergeometric and B-H False Discovery Rate tests ($\alpha = 0.05$) to identify overrepresented GO categories under the Biological Processes umbrella. Independent tests were conducted for each family for up-regulated and down-regulated genes. Data were entered in the form of official gene symbols and tests were run against the annotated reference *de novo* constructed transcriptome. All annotations were obtained from *Homo sapiens*. Fisher's exact tests followed by B-H False Discovery Rate tests ($\alpha = 0.05$) were performed to determine whether any GO categories contained different quantities of genes between families. These tests were done for up-regulated and down-regulated gene groups independently.

Characterization of differentially expressed gene clusters

BLAST+ (v2.2.26) (Camacho et al., 2009) was used to align all contigs showing DE between seawater and freshwater groups to a database of cDNA sequences for protein-coding genes in stickleback using the same alignment criteria noted previously (Ensembl v68) (Flicek et al., 2013; Jones et al., 2012). Based on a synteny map of Arctic charr and stickleback (Norman et al., 2012), patterns between gene location and DE were assessed to ascertain the extent that DEGs cluster along Arctic charr chromosomes. Genes were considered clustered if their coding regions were within 40 kbp of one another on a stickleback chromosome (Ng et al., 2009), as Ng

et al. (2009) detected a significant negative correlation ($R^2 = 0.50$) between gene coexpression and IGD in zebrafish, which was strongest ($R^2 = 0.79$) for genes 10 to 40 kbp apart.

Comparing the relative proximity of differentially expressed genes

To test whether DEGs were located in closer proximity to one another along a chromosome, permutation analysis was performed using the R statistical language (R Core Team 2013). The analysis included all of the DEGs that aligned to the stickleback genome. Tests were conducted independently for each chromosome. The IGDs among all genes on a chromosome were pooled with those from the DEGs that aligned to that chromosome. The difference between means was estimated between a random sample of the pooled data and the remaining non-sampled data over 10,000 iterations. The size of each random sample was equal to the number of DEGs that aligned to the chromosome in question. The observed mean difference was then compared to the distribution of mean differences from the 10,000 random samples. Significance was denoted for $P \leq 0.05$ following a B-H False Discovery Rate test to correct for multiple tests (Sokal and Rohlf, 1995). This analysis assumes that the relative proximity and distribution of genes located on stickleback chromosomes are conserved on Arctic charr chromosomes. We acknowledge that this may not be realistic across entire chromosomes, however, conserved synteny blocks between stickleback and Arctic charr chromosomes have been described (Norman et al., 2012).

Testing for enrichment of differentially expressed genes in synteny blocks

To determine if the quantity of DEGs in each of the Arctic charr -stickleback synteny blocks was greater than expected, two-tailed chi-squared tests of association were performed using 2X2 contingency tables. This test is an expansion of the analysis originally performed and described in detail by Norman et al. (2013). Briefly, for each synteny block and family, tests compared the quantity of genes showing significant DE in a synteny block and the total quantity of genes contained within that synteny block, with the quantity of genes in these two classes that were not contained within the synteny block. These tests assumed that the composition of genes within synteny blocks was not different between Arctic charr and stickleback. An adjustment to correct for multiple tests was done using a B-H False Discovery Rate test ($\alpha = 0.05$) (Sokal and Rohlf, 1995).

Physiological responses to gene expression data

The sample size was not large enough to warrant statistical analyses of covariation between gene expression and salinity tolerance phenotypes. However, in a previous study with the same fish (Norman et al. 2011), significant associations were found between variation in gill filament Na^+/K^+-ATPase activity and blood plasma osmolality with genetic variation on several Arctic charr linkage groups (i.e., chromosomes). Given that the fish chosen for this study were those individuals with the greatest and least number of salinity tolerance QTL, and considering that a QTL represents a statistical association between phenotypic variation and genetic variation, changes in gene expression are related to physiological responses.

ACKNOWLEDGEMENTS

The authors acknowledge Claude Pelletier of the Coastal Zones Research Institute for generating the family crosses and Brian Glebe, Steven Leadbeater, and Wilfred Young-Lai for overseeing the rearing and maintenance of fish at St. Andrews Biological Station. We also thank Xuejiang (Roger) Shi of the Clinical Genomics Centre at Mount Sinai Hospital for overseeing the mRNA sequencing. Special thanks to Xia Yue, Andrea Kocmarek, and Jing Zhang for help and advice in the laboratory. We also thank two anonymous reviewers for their comments, which improved the quality of the manuscript.

COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of research. J.D.N. performed experiments, analyzed data, interpreted results of experiments, prepared figures and drafted the manuscript. All authors edited, revised, and approved the final version of the manuscript.

FUNDING

This work was supported by a Natural Science and Engineering Research Council of Canada (NSERC) grant.

REFERENCES

Allendorf, F. and Thorgaard, G. (1984). Tetraploidy and the evolution of salmonid fishes. In *Evolutionary Genetics of Fishes*, (ed. B.J. Turner), pp. 1-46. New York: Plenum Press.

Anders, S. and Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biol.* **11**, R106.

Andrews S. (2010). FASTQC: A quality control tool for high throughput sequence data [Online] http://www. bioinformatics.babraham.ac.uk/projects/fastqc.

Aykanat, T., Thrower, F. P. and Heath, D. D. (2011). Rapid evolution of osmoregulatory function by modification of gene transcription in steelhead trout. *Genetica* **139**, 233-242.

Boutet, I., Ky, C. L. L. and Bonhomme, F. (2006). A transcriptomic approach of salinity response in the euryhaline teleost, *Dicentrarchus labrax*. *Gene* **379**, 40-50.

Bystriansky, J. S., Richards, J. G., Schulte, P. M. and Ballantyne, J. S. (2006). Reciprocal expression of gill Na+/K+-ATPase alpha-subunit isoforms alpha 1a and alpha 1b during seawater acclimation of three salmonid fishes that vary in their salinity tolerance. *J. Exp. Biol.* **209**, 1848-1858.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K. and Madden, T. L. (2009). BLAST plus : architecture and applications. *BMC Bioinformatics* **10**, 421.

Chen, J. M., Cutler, C., Jacques, C., Boeuf, G., Denamur, E., Lecointre, G., Mercier, B., Cramb, G. and Ferec, C. (2001). A combined analysis of the cystic fibrosis transmembrane conductance regulator: Implications for structure and disease models. *Mol. Biol. Evol.* **18**, 1771-1788.

Christoffels, A., Koh, E. G. L., Chia, J. M., Brenner, S., Aparicio, S. and Venkatesh, B. (2004). Fugu genome analysis provides evidence for a whole-genome duplication early during the evolution of ray-finned fishes. *Mol. Biol. Evol.* 21, 1146-1151.

Cutler, C. P. and Cramb, G. (2002). Two isoforms of the Na+/K+/2CI(-) cotransporter are expressed in the European eel (*Anguilla anguilla*). *BBA Biomembranes* **1566**, 92-103.

Danzmann, R. G., Cairney, M., Davidson, W. S., Ferguson, M. M., Gharbi, K., Guyomard, R., Holm, L. E., Leder, E., Okamoto, N., Ozaki, A. et al., (2005). A comparative analysis of the rainbow trout genome with 2 other species of fish (Arctic charr and Atlantic salmon) within the tetraploid derivative Salmonidae family (subfamily : Salmoninae). *Genome* **48**, 1037-1051.

Delabbio, J. L., Glebe, B. D. and Sreedharan, A. (1990). Variation in growth and survival between 2 anadromous strains of Canadian Arctic charr (*Salvelinus alpinus*) during long-term saltwater rearing. *Aquaculture* **85**, 259-270.

Ebbesson, L. O. E., Tipsmark, C. K., Holmqvist, B., Nilsen, T., Andersson, E., Stefansson, S. O., and Madsen, S. S. (2005). Nitric oxide in the gill of Atlantic salmon: colocalization with and inhibition of Na+,K+-ATPase. *J. Exp. Biol.* **280**, 1011-1017.

Engelund, M. B., Yu, A. S. L., Li, J., Madsen, S. S., Faergeman, N. J. and Tipsmark, C. K. (2012). Functional characterization and localization of a gill-specific claudin isoform in Atlantic salmon. *Am. J. Physiol.-Reg. I.* **302**, R300-R311.

Evans, D. H., Piermarini, P. M. and Choe, K. P. (2005). The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* 85, 97-177.

Evans, T. G. and Somero, G. N. (2008). A microarray-based transcriptomic time-course of hyper- and hypo-osmotic stress signaling events in the euryhaline fish *Gillichthys mirabilis*: osmosensors to effectors. *J. Exp. Biol.* **211**, 3636-3649.

Evans, T. G., Hammill, E., Kaukinen, K., Schulze, A. D., Patterson, D. A., English, K. K., Curtis, J. M. R. and Miller, K. M. (2011). Transcriptomics of environmental acclimatization and survival in wild adult Pacific sockeye salmon (*Oncorhynchus nerka*) during spawning migration. *Mol. Ecol.* **20**, 4472-4489.

Flicek, P., Ahmed, I., Amode, M. R., Barrell, D., Beal, K., Brent, S., Carvalho-Silva, D., Clapham, P., Coates, G., Fairley, S. et al., (2013). Ensembl 2013. *Nucleic Acids Res.* **41**, D48-D55.

Furuse, M., Furuse, K., Sasaki, H. and Tsukita, S. (2001). Conversion of Zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. *J. Cell Biol.* 153, 263-272.

Gotz, S., Garcia-Gomez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., Robles, M., Talon, M., Dopazo, J. and Conesa, A. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res.* **36**, 3420-3435.

Hiroi, J. and McCormick, S. D. (2007). Variation in salinity tolerance, gill Na+/K+-ATPase, Na+/K+/2Cl(-) cotransporter and mitochondria-rich cell distribution in three salmonids *Salvelinus namaycush*, *Salvelinus fontinalis* and *Salmo salar*. *J. Exp. Biol.* **210**, 1015-1024.

Hiroi, J., Yasumasu, S., McCormick, S. D., Hwang, P.-P. and Kaneko, T. (2008). Evidence for an apical Na-Cl cotransporter involved in ion uptake in a teleost fish. *J. Exp. Biol.* **211**, 2584-2599.

Hiroi, J., Kaneko, T. and Tanaka, M. (1999). In vivo sequential changes in chloride cell morphology in the yolk-sac membrane of Mozambique tilapia (*Oreochromis mossambicus*) embryos and larvae during seawater adaptation. *J. Exp. Biol.* **202**, 3485-3495.

Hoar, W. (1988). The physiology of smolting salmonids. In *Fish Physiology, vol. XIV* (eds. W. Hoar and D. Randall), pp. 275-343. New York: Academic Press.

Hoffman, D. R., Marion, D. W., Cornatzer, W. E., and Duerre, J. A. (1980). S-adenosylmethionine and S-adenosylhomocysteine metabolism in isolated rat liver. *J. Biol. Chem.* **255**, 10822 10827.

Huie, R. E., and Padjama, S. (1993). The reaction of NO with superoxide. *Free Radical Res.* **18**, 195-199.

Hurst, L. D., Pal, C. and Lercher, M. J. (2004). The evolutionary dynamics of eukaryotic gene order. *Nat. Rev. Genet.* **5**, 299-310.

Hwang, P.-P. and Lee, T.-H. (2007). New insights into fish ion regulation and mitochondrion-rich cells. *Comp. Biochem. Phys. A* **148**, 479-497.

Jaillon, O., Aury, J. M., Brunet, F., Petit, J. L., Stange-Thomann, N., Mauceli, E., Bouneau, L., Fischer, C., Ozouf-Costaz, C., Bernot, A. et al., (2004). Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature* **431**, 946-957.

James, S. J., Melnyk, S., Pogribna, M., Pogribny, I. P., and Caudill, M. A. (2002). Elevation in S-adenosylhomocysteine and DNA hypomethylation: potential epigenetic mechanisms for homocysteine-related pathology. *J. Nutr.* **132**, 2361S-2366S.

Jones, F. C., Grabherr, M. G., Chan, Y. F., Russell, P., Mauceli, E., Johnson, J., Swofford, R., Pirun, M., Zody, M. C., White, S. et al., (2012). The genomic basis of adaptive evolution in threespine sticklebacks. *Nature* **484**, 55-61.

Kalujnaia, S., McWilliam, I. S., Zaguinaiko, V. A., Feilen, A. L., Nicholson, J., Hazon, N., Cutler, C. P. and Cramb, G. (2007). Transcriptomic approach to the study of osmoregulation in the European eel *Anguilla anguilla*. *Physiol. Genomics* **31**, 385-401.

Kasahara, M., Naruse, K., Sasaki, S., Nakatani, Y., Qu, W., Ahsan, B., Yamada, T., Nagayasu, Y., Doi, K., Kasai, Y. et al., (2007). The medaka draft genome and insights into vertebrate genome evolution. *Nature* **447**, 714-719.

Kang, C.-K., Tsai, H.-J., Liu, C.-C., Lee, T.-H. and Hwang, P.-P. (2010). Salinity-dependent expression of a Na+, K+, 2Cl(-) cotransporter in gills of the brackish medaka *Oryzias dancena*: A molecular correlate for hyposmoregulatory endurance. *Comp. Biochem. Phys. A* **157**, 7-18.

Klebanoff, S. J. (2005). Myeloperoxidase: friend or foe. *J. Leukocyte Biol*. **77**, 598-625.

Larsen, P. F., Nielsen, E. E., Koed, A., Thomsen, D. S., Olsvik, P. A. and Loeschcke, V. (2008). Interpopulation differences in expression of candidate genes for salinity tolerance in winter migrating anadromous brown trout (*Salmo trutta* L.). *BMC Genet.* **9**, 12.

Le Bras, Y., Dechamp, N., Krieg, F., Filangi, O., Guyomard, R., Boussaha, M., Bovenhuis, H., Pottinger, T. G., Prunet, P., Le Roy, P. et al., (2011). Detection of QTL with effects on osmoregulation capacities in the rainbow trout (*Oncorhynchus mykiss*). *BMC Genet*. **12**, 46.

Leng, N., Dawson, J. A., Thomson, J. A., Ruotti, V., Rissman, A. I., Smits, B. M. G., Haag, J. D., Gould, M. N., Stewart, R. M. and Kendziorski, C. (2013). EBSeq: an empirical Bayes hierarchical model for inference in RNA-seq experiments. *Bioinformatics* **29**, 1035-1043.

Li, B. and Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**, 323.

Li, W. and Godzik, A. (2006). Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* **22**, 1658-1659.

Lin, D. W., Chung, B. P., and Kaiser, P. (2014). S-adenosylmethionine limitation induces p38 mitogen-activated protein kinase and triggers cell cycle arrest in G1. *J. Cell Sci.* **127**, 50-59.

Lohse, M., Bolger, A. M., Nagel, A., Fernie, A. R., Lunn, J. E., Stitt, M. and Usadel, B. (2012). RobiNA: a user-friendly, integrated software solution for RNA-Seq-based transcriptomics. *Nucleic Acids Res.* **40**, W622-W627.

Lorin-Nebel, C. and Charmantier, G. (2007). The ontogeny of osmoregulation in teleosts. *Oceanis S. D.* **30**, 349-384.

Lowenstein, C. J., and Snyder, S. H. (1992). Nitric oxide, a novel biologic messenger. *Cell* **70,** 705-707.

Lu, J. X., Chen, J., Huang, Z. A., Shi, Y. H., Wang, F. (2010). Proteomic analysis on the alteration of protein expression in gills of ayu (*Plecoglossus altivelis*) associated with salinity change. *Comp. Biochem. Phys. D* **5**, 185-189.

Mackie, P., Wright, P. A., Glebe, B. D. and Ballantyne, J. S. (2005). Osmoregulation and gene expression of Na+/K+ ATPase in families of Atlantic salmon (*Salmo salar*) smolts. *Can. J. Fish. Aquat. Sci.* **62**, 2661-2672.

Mackie, P. M., Gharbi, K., Ballantyne, J. S., McCormick, S. D. and Wright, P. A. (2007). Na+/K+/2Cl(-) cotransporter and *CFTR* gill expression after seawater transfer in smolts (0(+)) of different Atlantic salmon (*Salmo salar*) families. *Aquaculture* **272**, 625-635.

Madsen, S. S., Kiilerich, P. and Tipsmark, C. K. (2009). Multiplicity of expression of Na(+),K(+)-ATPase alpha-subunit isoforms in the gill of Atlantic salmon (*Salmo salar*): cellular localisation and absolute quantification in response to salinity change. *J. Exp. Biol.* **212**, 78-88.

Maere, S., Heymans, K. and Kuiper, M. (2005). BiNGO: a Cytoscape plugin to assess overrepresentation of Gene Ontology categories in biological networks. *Bioinformatics* **21**, 3448-3449.

Marshall, W. and Grosell, M. (2006). Ion transport, osmoregulation, and acid-base balance. In *The Physiology of Fishes*, (eds. D. Evans and J. Claiborne), pp. 177-230. Boca Raton, FL: CRC Press.

Martin, J., Bruno, V. M., Fang, Z., Meng, X., Blow, M., Zhang, T., Sherlock, G., Snyder, M. and Wang, Z. (2010). Rnnotator: an automated de novo transcriptome assembly pipeline from stranded RNA-Seq reads. *BMC Genomics* **11**, 663.

McCormick, S. D. (1993). Methods for nonlethal gill biopsy and measurement of Na+,K+-ATPase activity. *Can. J. Aquat. Sci.* **50**, 656-658.

McCormick, S. D. (2001). Endocrine control of osmoregulation in teleost fish. Am. Zool. **41**, 781-794.

McCormick, S. D., Moyes, C. D. and Ballantyne, J. S. (1989). Influence of salinity on the energetics of gill and kidney of Atlantic salmon (*Salmo salar*). *Fish Physiol. Biochem.* **6**, 243-254.

McCormick, S. D., Regish, A. M. and Christensen, A. K. (2009). Distinct freshwater and seawater isoforms of Na(+)/K(+)-ATPase in gill chloride cells of Atlantic salmon. *J. Exp. Biol.* **212**, 3994-4001.

McGowan, P. O., Suderman, M., Sasaki, A., Huang, T. C. T., Hallett, M., Meaney, M. J. and Szyf, M. (2011). Broad epigenetic signature of maternal care in the brain of adult rats. *PLOS One* **6**, 2.

Moran, P., Francisco, M. R., Megias, M., Covelo-Soto, L., and Perez-Figueroa, A. (2013). Environmental induced methylation changes associated with seawater adaptation in brown trout. *Aquaculture* **392-395**, 77-83.

Morgan, J. D. and Iwama, G. K. (1999). Energy cost of NaCl transport in isolated gills of cutthroat trout. *Am. J. Physiol.-Reg. I* **277**, R631-R639.

Muriel, P. and Sandoval, G. (2000). Nitric oxide and peroxynitrite anion modulate liver plasma membrane fluidity and Na+/K+-ATPase activity. *Nitric Oxide-Biol. Ch.* **4**, 333-342.

Nakatani, Y., Takeda, H., Kohara, Y. and Morishita, S. (2007). Reconstruction of the vertebrate ancestral genome reveals dynamic genome reorganization in early vertebrates. *Genome Res.* **17**, 1254-1265.

Ng, Y. K., Wu, W. and Zhang, L. (2009). Positive correlation between gene coexpression and positional clustering in the zebrafish genome. *BMC Genomics* **10**, 42.

Nilsen, T. O., Ebbesson, L. O. E., Madsen, S. S., McCormick, S. D., Andersson, E., Bjornsson, B. T., Prunet, P. and Stefansson, S. O. (2007). Differential expression of gill Na+,K+-ATPase alpha- and beta-subunits, Na+,K+,2Cl(-) cotransporter and *CFTR* anion channel in juvenile anadromous and landlocked Atlantic salmon Salmo salar. *J. Exp. Biol.* **210**, 2885-2896.

Norman, J. D., Danzmann, R. G., Glebe, B. and Ferguson, M. M. (2011). The genetic basis of salinity tolerance in Arctic charr (*Salvelinus alpinus*). *BMC Genet.* **12**, 81.

Norman, J. D., Robinson, M., Glebe, B., Ferguson, M. M. and Danzmann, R. G. (2012). Genomic arrangement of salinity tolerance QTLs in salmonids: A comparative analysis of Atlantic salmon (*Salmo salar*) with Arctic charr (*Salvelinus alpinus*) and rainbow trout (*Oncorhynchus mykiss*). *BMC Genomics* **13**, 420.

Norman, J. D., Ferguson, M. M., and Danzmann, R. G. (2014). Transcriptomics of salinity tolerance capacity in Arctic charr (*Salvelinus alpinus*): A comparison of gene expression profiles between divergent QTL genotypes. *Physiol. Genomics* **46**, 123-137.

Pacher, P., Beckman, J. S., and Liaudet, L. (2007). Nitric oxide and peroxynitrite in health and disease. *Physiol. Rev.* **87**, 315-424.

Pellettieri, J. and Alvarado, A. S. (2007). Cell turnover and adult tissue homeostasis: From humans to planarians. *Annu. Rev. Genet.* **41**, 83-105.

Pohanka, M. (2013). Role of oxidative stress in infectious diseases. *Folia Microbiol.* **58**, 503-513.

Quackenbush, J., Cho, J., Lee, D., Liang, F., Holt, I., Karamycheva, S., Parvizi, B., Pertea, G., Sultana, R. and White, J. (2001). The TIGR Gene Indices: analysis of gene transcript sequences in highly sampled eukaryotic species. *Nucleic Acids Res.* **29**, 159-164.

R Core Team. (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Rao, G. M. M. (1968). Oxygen consumption of rainbow trout (*Salmo gairdneri*) in relation to activity and salinity. *Can. J. Zool.* **46**, 781.

Richards, J. G., Semple, J. W., Bystriansky, J. S. and Schulte, P. M. (2003). Na+/K+-ATPase (alpha-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. *J. Exp. Biol.* **206**, 4475-4486.

Robertson, L. S. and McCormick, S. D. (2012). Transcriptional profiling of the parr-smolt transformation in Atlantic salmon. *Comp. Biochem. Phys. D* **7**, 351-360.

Schmitz, M. (1995). Seasonal changes in hypoosmoregulatory ability in landlocked and anadromous populations of Arctic charr, *Salvelinus alpinus*, and Atlantic salmon, *Salmo salar*. *Environ. Biol. Fish.* **42**, 401-412.

Scott, G. R., Richards, J. G., Forbush, B., Isenring, P. and Schulte, P. M. (2004). Changes in gene expression in gills of the euryhaline killifish *Fundulus heteroclitus* after abrupt salinity transfer. *Am. J. Physiol-Cell Ph.* **287**, C300-C309.

Seear, P. J., Carmichael, S. N., Talbot, R., Taggart, J. B., Bron, J. E. and Sweeney, G. E. (2010). Differential gene expression during smoltification of Atlantic salmon (*Salmo salar* L.): a first large-scale microarray study. *Mar. Biotechnol.* **12**, 126-140.

Shrimpton, J. M., Patterson, D. A., Richards, J. G., Cooke, S. J., Schulte, P. M., Hinch, S. G. and Farrell, A. P. (2005). Ionoregulatory changes in different populations of maturing sockeye salmon *Oncorhynchus nerka* during ocean and river migration. *J. Exp. Biol.* **208**, 4069-4078.

Silva, P., Solomon, R., Spokes, K. and Epstein, F. (1977). Ouabain inhibition of gill Na-K-ATPase: relationship to active chloride transport. *J. Exp. Zool.* **199**, 419-426.

Singer, T. D., Clements, K. M., Semple, J. W., Schulte, P. M., Bystriansky, J. S., Finstad, B., Fleming, I. A. and McKinley, R. S. (2002). Seawater tolerance and gene expression in two strains of Atlantic salmon smolts. *Can. J. Fish Aquat. Sci.* **59**, 125-135.

Sokal, R. and Rohlf, F. (1995). Biometry. New York: W.H. Freeman and Company.

Spring, J. (1997). Hypothesis: vertebrate evolution by interspecific hybridisation - Are we polyploid*? FEBS Lett.* **400**, 2–8.

Timusk E. R., Ferguson M. M., Moghadam H. K., Norman J. D., Wilson C. C., Danzmann R. G. (2011). Genome evolution in the fish family Salmonidae: generation of a brook charr genetic map and comparisons among charrs (Arctic charr and brook charr) with rainbow trout. *BMC Genet.* **12,** 68, 2011.

Tine, M., de Lorgeril, J., D'Cotta, H., Pepey, E., Bonhomme, F., Baroiller, J. F. and Durand, J.-D. (2008). Transcriptional responses of the black-chinned tilapia *Sarotherodon melanotheron* to salinity extremes. *Mar. Genomics* **1**, 37-46.

Tipsmark, C. K., Madsen, S. S., Seidelin, M., Christensen, A. S., Cutler, C. P. and Cramb, G. (2002). Dynamics of Na+,K+,2Cl(-) cotransporter and Na+,K+-ATPase expression in the branchial epithelium of brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar). J. Exp. Zool.* **293**, 106-118.

Tipsmark, C. K., Kiilerich, P., Nilsen, T. O., Ebbesson, L. O. E., Stefansson, S. O. and Madsen, S. S. (2008). Branchial expression patterns of claudin isoforms in Atlantic salmon during seawater acclimation and smoltification. *Am. J. Physiol-Reg. I.* **294**, R1563-R1574.

Tse, W. K. F., Au, D. W. T. and Wong, C. K. C. (2006). Characterization of ion channel and transporter mRNA expressions in isolated gill chloride and pavement cells of seawater acclimating eels. *Biochem. Bioph. Res. Co*. **346**, 1181-1190.

Van Itallie, C. M., Rogan, S., Yu, A., Vidal, L. S., Holmes, J. and Anderson, J. M. (2006). Two splice variants of claudin-10 in the kidney create paracellular pores with different ion selectivities. *Am. J. Physiol-Renal* **291**, F1288-F1299.

Varela, M., Herrera, M., and Gavin, J. L. (2004). Inhibition of Na-K-ATPase in thick ascending limbs by NO depends on O2- and is diminished by a high salt diet. *Am. J. Physiol-Renal* **287**, F224-F230.

Wilson, J. M., Laurent, P. (2002). Fish gill morphology: Inside out. *J. Exp. Zool*. **293**, 192-213.

Woo, Y. H., Walker, M. and Churchill, G. A. (2010). Coordinated expression domains in mammalian genomes. *PLOS One* **5**, 8.

Zamora, R., Vodovotz, Y., Alarcon, L., Betten, B., Loughran, P. A., Aulak, K. S., Steuhr, D. J., Gibson, K. F., and Billar, T. R. (2001). Nitric oxide from the inducible nitric oxide synthase (iNOS) increase the expression of cytochrome P450 2E1 in iNOS-null hepatocytes in the absence of inflammatory stimuli. *Arch. Biochem. Biophys*. **390**, 287-294.

906 **Table 1.** Genes up-regulated in Arctic charr (*Salvelinus alpinus*) seawater gill libraries for
907 families 10 and 12 combined that exhibit mean expression ratios ≥ 1.
908

Hit accession	Hit description	Mean ER	Mean P-Value
XP_003961679	splicing factor 3b subunit 1-like	6.7	0.000196
XP_788663	histamine H3 receptor-like	5.2	0.000311
CAC82808	inducible nitric oxide synthase	5.2	0.000878
XP_003612234	atp synthase subunit a chloroplastic	4.9	0.001639
ABC72122	Myeloperoxidase	4.7	0.000581
NP_001098159	transcription factor c-myb	4.3	0.024577
XP_003228519	adenosylhomocysteinase 3-like	4.1	< 0.000001
XP_003440931	2-oxoglutarate receptor 1-like	3.8	0.034657
XP_003455055	uncharacterized protein LOC100705794	3.5	0.020761
ACI69118	transmembrane and tpr repeat-containing protein 4	3.3	< 0.000001
XP_001642069	predicted protein	3.2	0.025506
NP_001167620	cytochrome P450, family 1, subfamily B, polypeptide 1	3.2	< 0.000001
EHH17669	hypothetical protein EGK_14124, partia	3.2	< 0.000001
ACI69602	c-c motif chemokine 19 precursor	2.9	0.008692
AAX24831	sjchgc03009 protein	2.7	< 0.000001
AAX25032	sjchgc04714 protein	2.7	< 0.000001
AAQ10900	cytochrome p450 1a	2.6	0.000143
NP_001092900	asteroid homolog 1	2.4	0.030242
XP_003459735	novel protein vertebrate tumor necrosis factor receptor member 21	2.4	0.023096
AAQ10899	cytochrome p450 1a	2.4	0.001698
ACM09026	gtpase imap family member 7	2.3	0.027571
ACH85356	hypothetical protein	2.1	< 0.000001
ACJ02099	FGF2	2.0	0.002211
NP_001134448	e3 ubiquitin-protein ligase rnf138	1.9	0.000081
NP_998587	udp glucuronosyltransferase 1 family polypeptide a3 precursor	1.9	0.008261
CBN81775	aryl hydrocarbon receptor repressor	1.4	0.026637
ACM08262	c-c motif chemokine 20 precursor	1.4	0.002684
XP_003444970	probable g-protein coupled receptor 61-like	1.3	0.017361
ADG29182	prostate stem cell antigen precursor-like	1.3	0.002470
NP_001116443	myxovirus (influenza virus) resistance g	1.2	0.015531
XP_003451252	egl nine homolog 3-like isoform X1	1.2	0.000165
XP_001333205	hypothetical protein LOC796178	1.1	0.022922

Hit accession	Hit description	Mean ER	Mean P-Value
CBN81435	centrosomal protein of 55 kda	1.1	0.018376
XP_003457336	forkhead box protein q1-like	1.1	< 0.000001
XP_003456889	interleukin-1 receptor accessory protein-like	1.1	0.000341
XP_002733521	RETRotransposon-like family member (retr-1)-like	1.1	0.000024
ACI69358	cd83 antigen precursor	1.1	0.013459
CAG03861	unnamed protein product	1.0	0.003472
NP_001134708	tumor necrosis factor receptor superfamily member 5 precursor	1.0	0.022405
XP_003453070	ribonuclease zc3h12a	1.0	0.007395
NP_001133906	g1 s-specific cyclin-e2	1.0	0.018579

Hit accessions are from the NCBI non-redundant protein database; Mean ER is the mean expression from families 10 and 12; expression ratios represent the $\log_2$ fold-change in gene expression in seawater gill tissue relative to freshwater gill tissue; Mean P-Value represents the mean FDR-corrected P-value from families 10 and 12.

916 **Table 2.** Genes down-regulated in Arctic charr (*Salvelinus alpinus*) seawater gill libraries for
917 families 10 and 12 combined that exhibit mean expression ratios ≥ 1.
918

Hit accession	Hit description	Mean ER	Mean P-Value
NP_001073337	orexin receptor type 2	-3.6	0.006369
XP_003746712	PREDICTED: uncharacterized protein LOC100906026	-3.3	0.021946
NP_001117748	glucose transporter 1A	-2.9	0.001800
CBN81991	Villin-1	-2.8	0.005164
XP_001304408	hypothetical protein	-2.8	0.000179
XP_003965142	phosphatidylinositol 4-phosphate 5-kinase-like protein 1-like	-2.7	0.000001
XP_003440665	ferm domain-containing protein 5-like	-2.7	0.002285
XP_003961509	vesicular glutamate transporter 1-like	-2.5	0.019769
XP_003447126	cat eye syndrome critical region protein 5 homolog	-2.5	0.004807
NP_001134781	vesicle-associated membrane protein 5-like	-2.4	0.000040
XP_003453591	regulator of g-protein signaling 4-like	-2.4	0.000020
BAF37936	tc1-like transporase	-2.3	0.020455
XP_001921093	estrogen-related receptor gamma	-2.2	0.000044
CAG06489	unnamed protein product	-2.1	0.000001
XP_003969602	iroquois-class homeodomain protein irx-3-like	-2.0	< 0.000001
XP_001631851	predicted protein	-2.0	0.000389
ACN60195	retrotransposable element tf2 155 kda protein type 3	-1.9	0.003592
NP_956302	pancreatic progenitor cell differentiation and proliferation factor b	-1.9	< 0.000001
XP_002958534	hypothetical protein VOLCADRAFT_69561	-1.9	0.000082
XP_003452618	zinc transporter ZIP3-like isoform X1	-1.8	< 0.000001
XP_002733851	retrotransposon-like family member (retr-1)-like	-1.8	0.000832
NP_001140147	cdp-diacylglycerol--serine o-phosphatidyltransferase	-1.7	0.006255
ACM09149	sulfotransferase 6b1	-1.7	0.000633
ZP_09047056	hypothetical protein HMPREF1020_01135	-1.7	0.000005
AFR53956	Transposase	-1.6	0.009962
XP_002933173	hypothetical protein LOC100488659	-1.6	0.000098
ACO09698	sulfotransferase 6b1	-1.6	0.000062

Hit accession	Hit description	Mean ER	Mean P-Value
ADX97065	amine sulfotransferase	-1.5	0.001560
XP_003497853	prominin-2-like isoform 2	-1.5	0.006462
XP_003449393	hypothetical protein LOC100690300	-1.5	0.003664
XP_001339010	rho gtpase-activating protein 24	-1.5	< 0.000001
XP_003458639	metastasis suppressor protein 1	-1.4	0.000046
XP_003439033	synapsin-2-like isoform X1	-1.4	0.001324
XP_001632820	predicted protein	-1.4	0.001776
BAC10574	nrf2-associated protein keap1	-1.4	< 0.000001
ACO09696	claudin 8	-1.3	< 0.000001
XP_003963031	ring finger protein 223-like	-1.3	0.000049
XP_003969444	hepacam family member 2-like	-1.3	0.000358
XP_003978005	protein fam69c-like	-1.2	0.003272
XP_003448007	mucolipin-1-like	-1.2	0.023517
XP_003199551	uncharacterized protein LOC321155	-1.2	0.000027
XP_003969864	membrane progestin receptor gamma-b-like	-1.2	0.000016
XP_003965239	g-protein-signaling modulator 1-like	-1.2	0.000460
XP_003459874	seizure protein 6 homolog	-1.2	0.002602
XP_003440685	delta-like protein 4-like	-1.2	< 0.000001
ABQ01988	reverse transcriptase-like protein	-1.1	0.024643
CAC81754	corticotropin-releasing factor receptor type 2	-1.1	0.000010

See Table 1 for legend.

923 **Table 3.** Overrepresented Gene Ontology categories for up- and down-regulated genes combined
924 from Arctic charr (*Salvelinus alpinus*) seawater gill libraries for families 10 and 12 combined.
925

GO ID	Description	P-Value	Mean PD	Mean FE
Up-regulated				
51301	cell division	3.06E-12	0.112	4.5
22403	cell cycle phase	3.85E-12	0.120	3.9
280	nuclear division	4.63E-12	0.098	5.1
7067	Mitosis	4.63E-12	0.098	5.1
87	M phase of mitotic cell cycle	1.04E-11	0.099	5.0
48285	organelle fission	1.05E-11	0.097	4.9
279	M phase	1.79E-11	0.109	4.3
278	mitotic cell cycle	7.78E-10	0.107	3.6
7049	cell cycle	4.43E-09	0.137	2.8
22402	cell cycle process	8.40E-09	0.114	3.0
7059	chromosome segregation	4.49E-06	0.047	6.6
51726	regulation of cell cycle	3.08E-04	0.065	2.5
6996	organelle organization	5.48E-04	0.098	1.8
75	cell cycle checkpoint	7.53E-04	0.037	4.6
6323	DNA packaging	3.29E-03	0.030	6.3
30261	chromosome condensation	4.54E-03	0.022	9.9
6259	DNA metabolic process	7.27E-03	0.089	2.8
71103	DNA conformation change	7.62E-03	0.036	5.5
6260	DNA replication	1.47E-02	0.066	4.4
51983	regulation of chromosome segregation	1.56E-02	0.013	14.6
7093	mitotic cell cycle checkpoint	1.86E-02	0.023	5.3
8283	cell proliferation	1.88E-02	0.044	2.2
6261	DNA-dependent DNA replication	2.37E-02	0.036	6.8
9404	toxin metabolic process	2.40E-02	0.011	18.1
6950	response to stress	2.52E-02	0.072	1.5
Down-regulated				
7275	multicellular organismal development	8.68E-06	0.172	1.8
48731	system development	3.68E-05	0.157	1.8
32502	developmental process	4.58E-05	0.172	1.7
48856	anatomical structure development	6.92E-05	0.158	1.8
32501	multicellular organismal process	1.84E-04	0.181	1.6

GO ID	Description	P-Value	Mean PD	Mean FE
48513	organ development	3.10E-03	0.127	1.9

GO ID = Gene Ontology category identification number; P-value = corrected FDR P-value statistic; Mean PD = the mean from families 10 and 12 of the proportion difference of genes comprising experimental and reference Gene Ontology categories (i.e., PD = [proportion genes in experimental - proportion genes in reference]); Mean FE = mean fold-enrichment, or the mean ratio from families 10 and 12 of the proportion of genes comprising experimental and reference Gene Ontology categories (i.e., FE = [proportion genes in experimental / proportion genes in reference]).

936 **Table 4**. Interfamilial differences in Gene Ontology category (GO) overrepresentation for up-
937 regulated genes from Arctic charr (*Salvelinus alpinus*) seawater gill libraries.
938

GO accession	GO description	P-F10	P-F12	P-Value
8380	RNA splicing	0.081	0.022	0.0018
6397	mRNA processing	0.089	0.028	0.0022
6396	RNA processing	0.119	0.041	0.0008
90304	nucleic acid metabolic process	0.315	0.184	0.0004
6139	nucleobase-containing compound metabolic process	0.349	0.222	0.0011
34641	cellular nitrogen compound metabolic process	0.357	0.234	0.0017
6807	nitrogen compound metabolic process	0.370	0.250	0.0027

939
940 P-F10 and P-F12 represent the proportion of up-regulated genes that occur within the respective
941 GO category for families 10 and 12, respectively; P-Value statistics are presented for only those
942 that pass the FDR corrected threshold of 0.003 ($\alpha = 0.05$); no significant interfamilial differences
943 were found for down-regulated genes.
944
945

Table 5. The number of genes differentially expressed in Arctic charr (*Salvelinus alpinus*) gill after transfer from freshwater to seawater that localize to conserved synteny blocks on three-spined stickleback (*Gasterosteus aculeatus*) chromosomes.

AC	GA Synteny			F10		F12	
LG	Chr.	# Genes	Block (Mbp)	# DEGs	P	# DEGs	P
1	**XVII**	**282**	**5.921**	**18 (8.3)**	**0.001***	12	0.787
4	I	838	21.343	26	0.819	31	0.211
4	XIX	13	0.146	1	0.341	2	0.091
4	I&XIX	851	21.489	27	0.725	33	0.312
5	VII	98	1.336			1	0.098
6	IV	342	7.485	12	0.563	21	0.191
7	XII	335	5.649	10	0.988	16	0.883
8	V	637	10.407	17	0.653	29	0.952
11	**XVII**	**479**	**10.007**	**24 (14.5)**	**0.010**	19	0.514
12	I	47	0.477			1	0.433
13	IX	201	5.585	9	0.220	15	0.066
15	II	223	6.841	6	0.809	15	0.148
17	XII	362	7.480	9	0.591	16	0.871
18	V	39	0.479			2	0.881
19	III	451	8.255	10	0.348	19	0.699
20	XI	924	14.063	26	0.775	34	0.178
20	XVI	264	4.260	9	0.680	14	0.601
20	XI&XVI	1188	18.324	35	0.959	48	0.346
21	IX	184	2.859	8	0.284	6	0.399
23	VII	243	3.525	8	0.772	5	0.064
23	XX	471	8.168	12	0.591	27	0.255
23	VII&XX	714	11.693	20	0.133	32	0.878
24	IV	22	0.241	1	0.675	2	0.347
25	I	526	13.043	13	0.501	23	0.804
28	**XIV**	**431**	**9.642**	**22 (13)**	**0.010**	24	0.353
31	X	80	1.584	4	0.302	5	0.504
32	III	471	9.273	10	0.280	19	0.563
34	IX	160	4.005	7	0.309	11	0.191
35	XX	457	7.898	12	0.666	27	0.196
36	XVI	273	7.232	12	0.175	12	0.874
37	VII	385	10.836	10	0.669	18	0.948
39	XIII	173	4.173	6	0.706	9	0.718

Arctic charr linkage groups (AC LG) obtained from Timusk et al., 2011; stickleback chromosomes (chr) obtained from Ensembl (v68) (Flicek et al., 2013; Jones et al., 2012); synteny block sizes obtained from Norman et al., 2012; numbers in parentheses for family 10 (F10) and family 12 (F12) differentially expressed genes (DEG) represent expected values from 2X2 chi-square test for independence; bold values are significant at $P < 0.05$; * values pass FDR significance threshold ($\alpha = 0.05$).

957 **Table 6.** Comparison of genes differentially expressed in Arctic charr (*Salvelinus alpinus*) and
958 sockeye salmon (*Oncorhynchus nerka*) seawater gill, and Atlantic salmon (*Salmo salar*)
959 freshwater smolt gill tissue.
960

Gene annotation				
Description	**Gene ID**	**AC**	**SS**	**AS**
NADH dehydrogenase 1 alpha subcomplex subunit 9, mitochondrial precursor	209732928	+	+	
Mitochondrial ribosomal protein 63	209736992	+	+	
Argininosuccinate synthase	213514834	+	+	
Tumor-associated calcium signal transducer 2 precursor	213514296	+	-	
Zinc finger protein Gfi-1b	213514862	+	-	
Zymogen granule membrane protein 16 precursor	221222226	+	-	
bloodthirsty-2	401664016	+	-	
THAP domain-containing protein 11	213512444	+		+
transcription factor Spi-C	213514860	+		+
rab GDP dissociation inhibitor beta	213515058	+		+
Non-histone chromosomal protein H6	221219742	+		+
Importin subunit alpha-2	223647006	+		+
FK506-binding protein 4	225707240	+		+
PREDICTED: frizzled-8-like	348520010	+		+
FGF2	209962463	-/+	-	
TC1-like transposase	357575246	-/+	-	
reverse transcriptase-like protein	146147385	-	-	
apolipoprotein CII precursor	185133660	-	-	
secreted acidic cysteine rich glycoprotein	197632219	-	-	+
SPARC precursor	209155872	-	-	
unnamed protein product	47224919	-		+
lumican precursor	213514094	-		+
Pirin	213514874	-		+
EPS8L1a-like protein	374722810	-		+
beta-globin	1431593		+	+
beta-globin	1431608		+	+
hemoglobin subunit alpha	185134045		+	+
Hemoglobin subunit alpha-4	209731594		+	+
Hemoglobin subunit beta	209735786		+	+
Type-1 angiotensin II receptor-associated protein-like	213512516		+	+
tumor protein D52	213514032		+	+
Hemoglobin subunit alpha	223646832		+	+

Gene annotation				
Description	**Gene ID**	**AC**	**SS**	**AS**
Hemoglobin subunit beta-1	223646870		+	+
FAM139A	224613362		+	+
40 kDa peptidyl-prolyl cis-trans isomerase	225703320		+	+
conserved hypothetical protein	294886057		+	+
PREDICTED: ETS-related transcription factor Elf-2-like	410917177		+	+
C-C motif chemokine 19 precursor	209733266		-	+
fish virus induced TRIM protein	255983023		-	+
Hemoglobin subunit beta	209732622			+

Up-regulated genes denoted by "+"; down-regulated genes denoted by "-"; data for Arctic charr (AC) is from this study; data for sockeye salmon (SS) was obtained from Evans et al., (2011), and for Atlantic salmon (AS) from Seear et al., (2010) and Robertson and McCormick (2012); genes assigned +/- consist of independent contigs that align to the same reference sequence but exhibit divergent expression profiles.

Figure Captions

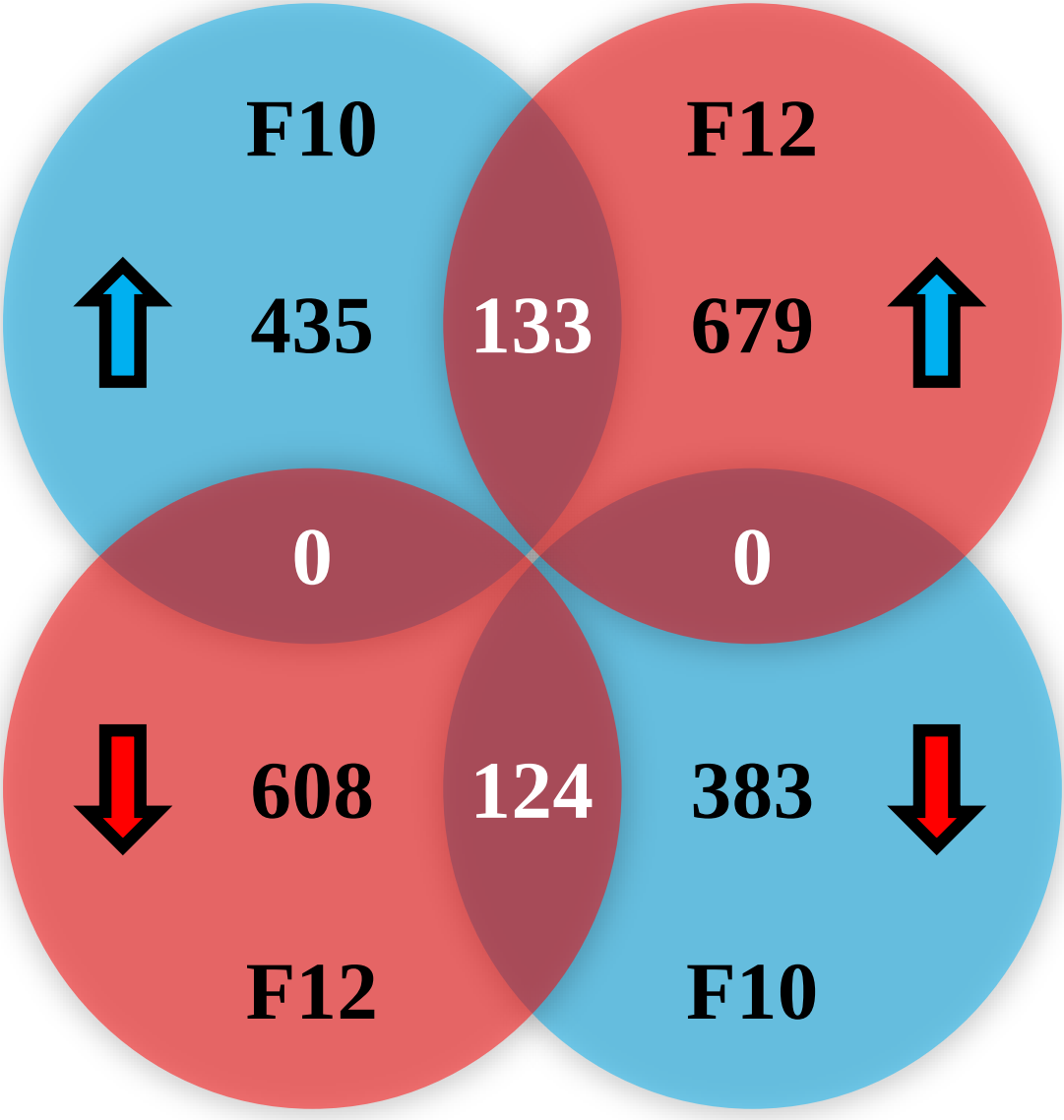
Fig. 1. Comparison of genes differentially expressed in seawater gill tissue from Arctic charr (*Salvelinus alpinus*) families 10 and 12. A description of these genes is provided in Table S1.

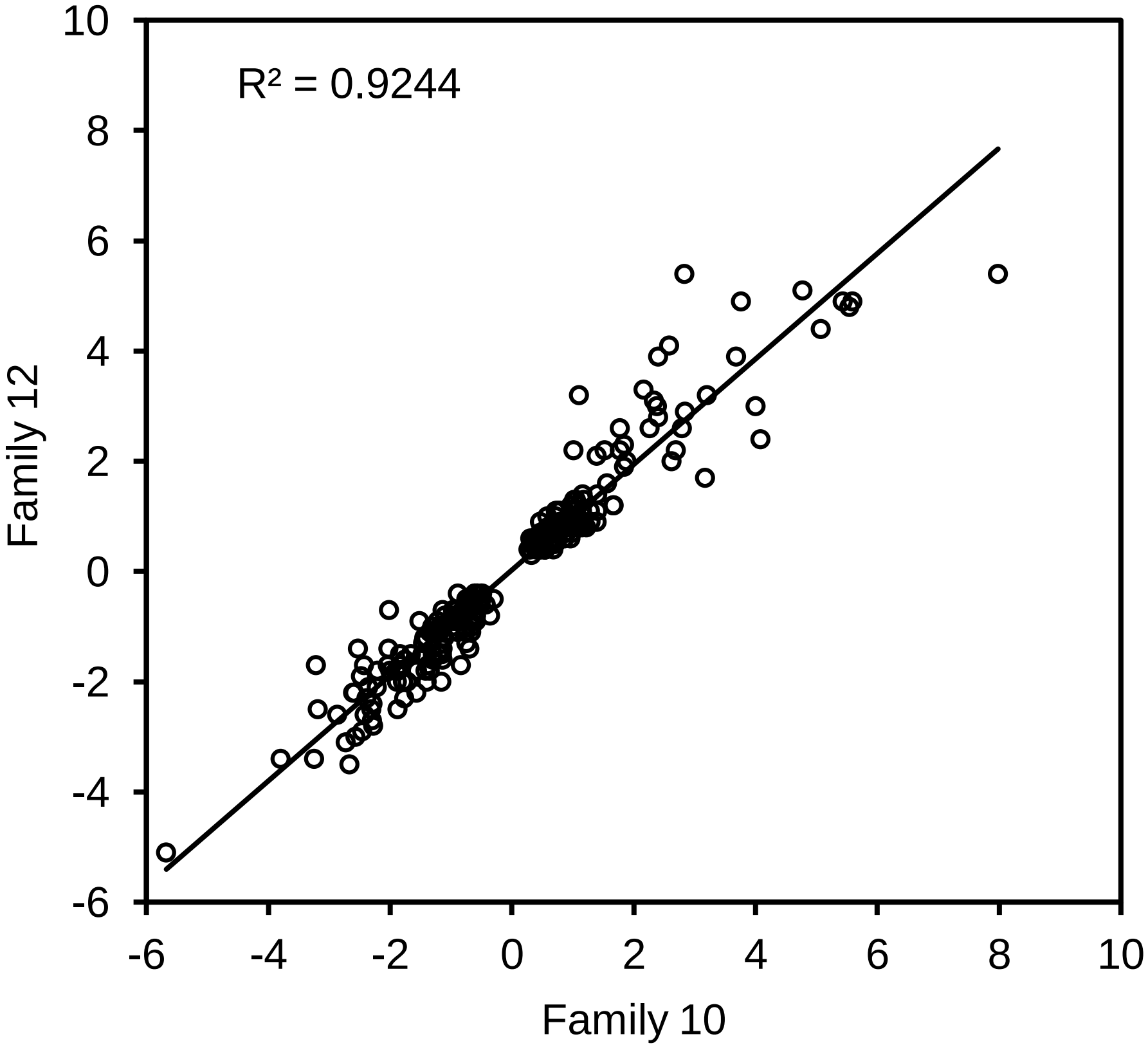
Fig. 2. Relationship between $\log_2$ expression ratios for genes differentially expressed in seawater gill tissue from two Arctic charr (*Salvelinus alpinus*) families.

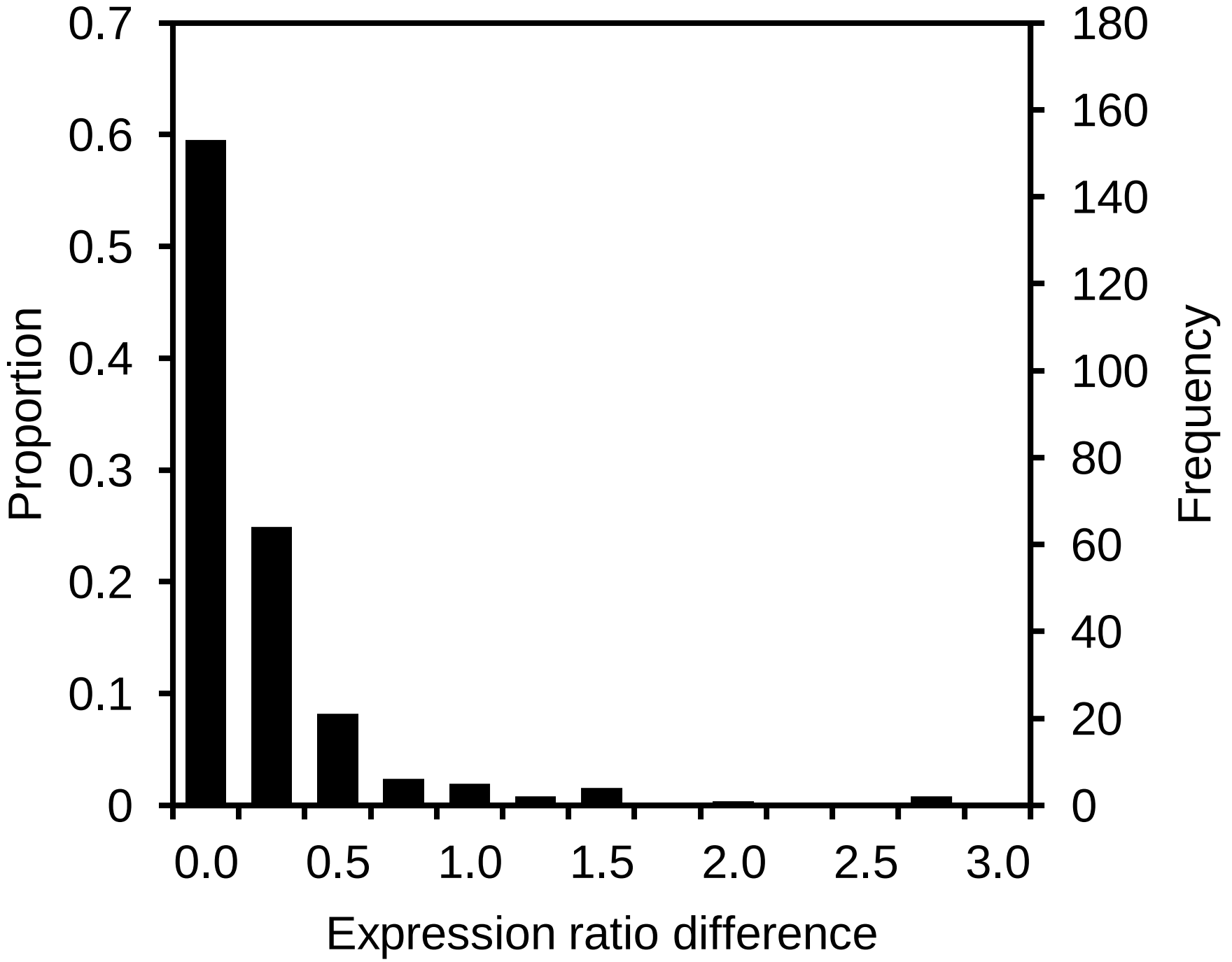
Fig. 3. Proportion and frequency distribution of the difference in $\log_2$ expression ratios between genes differentially expressed in seawater gill tissue from two Arctic charr (*Salvelinus alpinus*) families.

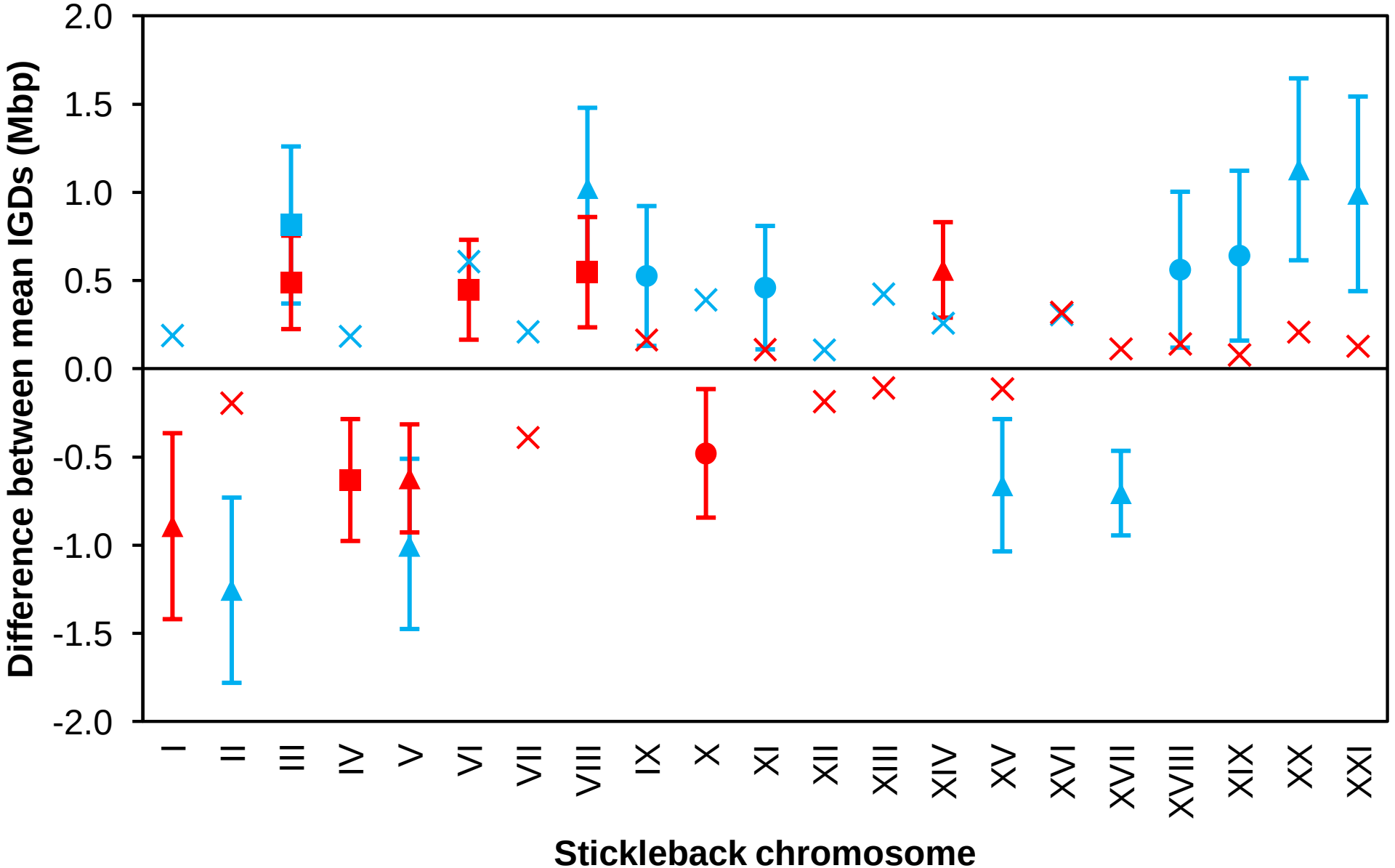
Fig. 4. Chromosomal comparisons of mean intergenic distances (IGDs) of stickleback (*Gasterosteus aculeatus*) genes and genes differentially expressed in seawater gill tissue from two Arctic charr (*Salvelinus alpinus*) families. False discovery rate-corrected significance is denoted by triangles ($P \leq 0.001$), squares ($P \leq 0.01$) and circles ($P \leq 0.05$), for families 10 (blue) and 12 (red), which are bound by 95% confidence intervals. The mean difference in megabase pairs (Mbp) was calculated by subtracting the mean IGD from differentially expressed genes that align to a particular stickleback chromosome from the mean IGD of the entire stickleback chromosome.

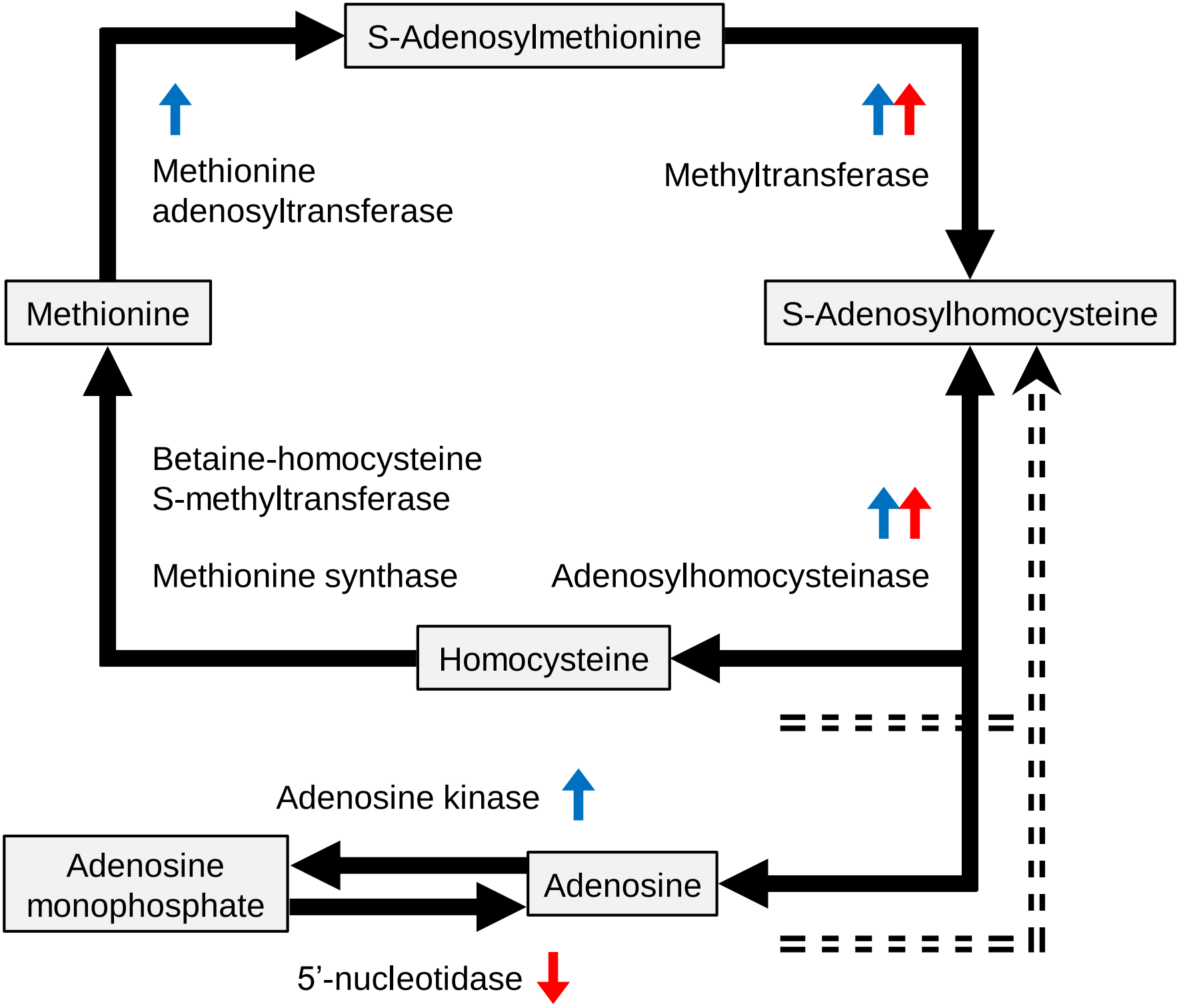
Fig. 5. The methionine cycle. Blue (family 10) and red (family 12) arrows denote the directional change in transcription of the respective enyzme genes observed in Arctic charr (*Salvelinus alpinus*) gill tissue after 10-days of exposure to full strength seawater. Genes for all of the enzymes in the methionine cycle were expressed, including cystathionine beta-synthase (EC 4.2.1.22); enzymes absent arrows were not differentially expressed. The dashed line indicates the thermodynamically favoured direction of the reversible reaction catalyzed by adenosylhomocysteinase. Methyltransferase (EC 2.1.1); adenosylhomocysteinase (EC 3.3.1.1); adenosine kinase (EC 2.7.1.20); 5'-nucleotidase (EC 3.1.3.5); betaine-homocysteine S-methyltransferase (EC 2.1.1.5); methionine synthase (EC 2.1.1.13); methionine adenosyltransferase (EC 2.5.1.6). Figure modified from James et al., (2002).


F10
435
133
F12
679
0
0
608
124
383
F12
F10


R² = 0.9244
Family 12
Family 10
10
8
6
4
2
0
-2
-4
-6
-6
-4
-2
0
2
4
6
8
10


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Proportion
180
160
140
120
100
80
60
40
20
0
Frequency
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Expression ratio difference


Difference between mean IGDs (Mbp)
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
XIX
XX
XXI
Stickleback chromosome


S-Adenosylmethionine
Methionine adenosyltransferase
Methyltransferase
Methionine
S-Adenosylhomocysteine
Betaine-homocysteine S-methyltransferase
Methionine synthase
Adenosylhomocysteinase
Homocysteine
Adenosine kinase
Adenosine monophosphate
Adenosine
5'-nucleotidase