

A proteomics approach reveals divergent molecular responses to salinity in populations of European whitefish (*Coregonus lavaretus*)

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Abstract

Osmoregulation is a vital physiological function for fish, as it helps maintain a stable intracellular concentration of ions in environments of variable salinities. We focused on a primarily freshwater species, the European whitefish (*Coregonus lavaretus*), to investigate the molecular mechanisms underlying salinity tolerance and examine whether these mechanisms differ between genetically similar populations that spawn in freshwater vs. brackishwater environments. A common garden experiment involving 27 families in two populations and five salinity treatments together with a large-scale, high-resolution mass spectrometry experiment that quantified 1500 proteins was conducted to assess phenotypic and proteomic responses during early development, from fertilization until hatching, in the studied populations. The populations displayed drastically different phenotypic and proteomic responses to salinity. Freshwater-spawning whitefish showed a significantly higher mortality rate in higher salinity treatments. Calcium, an ion involved in osmotic stress sensing, had a central role in the observed proteomic responses. Brackishwater-spawning fish were capable of viable osmoregulation, which was modulated by cortisol, an important seawater-adaptation hormone in teleost fish. Several proteins were identified to play key roles in osmoregulation, most importantly a highly conserved cytokine, tumour necrosis factor, whereas calcium receptor activities were associated with salinity adaptation. These results imply that individuals from these populations are most likely adapted to their local environments, even though the baseline level of genetic divergence between them is low ($F_{ST} = 0.049$). They also provide clues for choosing candidate loci for studying the molecular basis of salinity adaptation in other species. Further, our approach provides an example of how proteomic methods can be successfully used to obtain novel insights into the molecular mechanisms behind adaptation in non-model organism.

Keywords: Baltic Sea, brackish water adaptation, calcium ions, glucocorticoid signalling pathway, label-free protein quantification, shotgun proteomics

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Introduction

Fish, like all vertebrates, need to maintain a stable intracellular concentration of salts. As a result, in freshwater (FW) conditions, fish need to remain hyperosmotic,

struggling against constant salt loss and overhydration, whereas in seawater, they are challenged with salt overload and dehydration. Efficient osmoregulation is therefore a vital physiological function in aquatic organisms, enabling short- and/or long-term survival in environments of different salinities. Considerable effort has been invested to understand the processes by which fish acclimate and/or adapt to different salinities. At the

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functional genetics level, it is only recently that a comprehensive view on the molecular architecture behind osmoregulation has begun to emerge (Evans *et al.* 2005; Evans 2008, 2010). This recent progress has been fuelled by the use of various transcription profiling methods (Evans 2010; Larsen *et al.* 2011). Changes in transcription under osmotic stress can provide important information about the genes involved in osmoregulation (e.g. Chapman *et al.* 2011; Lockwood & Somero 2011). However, there are several limitations to this approach. First, proteins and not mRNA are the typical effectors of biological function, and protein abundance does not always correlate with the abundance of the corresponding transcripts (Cox & Mann 2007; Lu *et al.* 2007; Gstai-ger & Aebersold 2009; Schwanhausser *et al.* 2011). Second, important regulatory signalling events take place only downstream transcription and thus cannot be captured by transcription profiling (Evans 2010).

Large-scale protein quantification can help fill this gap and improve our understanding of the dynamics of signalling networks involved in osmoregulation in fish (Forné *et al.* 2010). Recent technological and methodological advances in mass spectrometry (MS)-based proteomics now enable the global analysis of cellular and organismal proteomes (Aebersold & Mann 2003; Mallick & Kuster 2010). In quantitative proteomics, several MS-based strategies can be employed for targeted or global protein expression profiling (Domon & Aebersold 2010). Shotgun quantitative proteomics, in particular, is a promising approach for large-scale proteome analysis. Compared with other MS-based methods, it requires no prior knowledge about the peptides or proteins of the study system, and therefore, it provides an unbiased way to describe proteome responses to environmental stress, even when genome sequence information is lacking (Domon & Aebersold 2010; Tomanek 2011). In shotgun proteomics, absolute or relative protein abundances are measured by metabolic protein, chemical peptide, or protein labelling or by label-free methods. Each approach has its own particular strengths and weaknesses, but in general, labelling offers better accuracy, while label-free enables higher proteome coverage and greater dynamic range of quantification (Bantscheff *et al.* 2007; Neilson *et al.* 2011). However, recent methodological and bioinformatic advances have greatly improved the accuracy of label-free strategies, making them attractive for high-throughput and cost-effective proteomic profiling (Cox *et al.* 2011; Hubner & Mann 2011).

The European whitefish species complex belongs to subfamily *Coregoninae* (family *Salmonidae*) and is well known for its great diversity in morphology, life history and ecological preferences (Østbye *et al.* 2006 and references therein). Having a broad circumpolar distribution,

Coregonus lavaretus sensu lato can be found in different kinds of fresh-, brackish- and saltwater habitats (e.g. Østbye *et al.* 2005). In Scandinavia, all native whitefish belong to same species, *C. lavaretus* sensu lato (Himberg & Lehtonen 1995). Nevertheless, distinct brackish-water (BW)-spawning and FW-spawning ecological forms, also termed ecotypes, have been recognized in the Baltic Sea region (Himberg & Lehtonen 1995). According to a microsatellite analysis, genetic distances between Baltic Sea-spawning and FW whitefish forms are rather small, with pairwise F_{ST} estimates varying from 0.01 to 0.06 (Säisä *et al.* 2008). However, it is not known whether the BW- and FW-spawning whitefish populations exhibit significant differences in salinity tolerance that would indicate adaptation to their local environments. Moreover, it is not known which molecular processes are potentially involved in adaptation to different salinity conditions in whitefish, or whether such adaptations even exist.

The primary objectives of this study were threefold. First, we aimed to estimate fertilization success, embryo salinity tolerance and early growth of BW- and FW-spawning whitefish from the Baltic Sea at different salinities. We focused on salinity tolerance in developing embryos, from fertilization until hatching, as early life stages in teleosts are more sensitive to salinity stress than are adult fish (Holliday 1969; De March 1989; Madsen *et al.* 1996). Second, we used a half-sib breeding design to evaluate the maternal and paternal influences on fertilization and embryonic survival. Third, we applied a label-free shotgun quantitative proteomics method and measured the expression of hundreds of proteins in response to salinity. By employing protein-protein interaction (PPI) modelling and network analysis, we identified key signalling pathways and proteins involved in osmoregulation.

Materials and methods

Sample collection, common garden experiment and survival measurements

Ripe sea-spawning whitefish individuals, also known as lesser sparsely rakered whitefish (*Coregonus lavaretus widegreni*), were collected on 9 November 2007 from the spawning grounds off the coast of the Åland Islands in the Baltic Sea (lat. 60°18'40.84", long. 20°12'4.46"). Eggs and milt of Lake Päijänne-spawning whitefish (Rautalampi stock, also known as northern densely rakered whitefish, *C. lavaretus pallasi*) were collected on 21 November 2007 from individuals kept in the Laukaa hatchery in central Finland. These two populations are subsequently referred to as BW and FW whitefish, respectively. Eggs and milt of nine females and nine

males were collected in dry plastic bags, stored on ice, and immediately transferred to the laboratory for artificial fertilization. The mating design within each of the two populations consisted of three full-factorial mating matrices; within each matrix, eggs of three females were fertilized by three different males. Fertilization was carried out in five different salinities (0, 2, 4, 6, and 10 ppt) to cover the conditions naturally occupied by the FW whitefish (~0 ppt) and BW whitefish populations (~4 to ~8 ppt) used in this study. Fertilized eggs from each cross were subsequently split into three replicate clutches, then placed and reared at constant temperature (6 °C) on Petri dish well plates until hatching (six wells per plate, diameter 36 mm; BW whitefish: mean number of eggs per well 19.9, median 20, range 10–31, SD 3.83; FW whitefish: mean number of eggs per well 20.3, median 20, range 12–35, SD 4.07), similar to Wedekind *et al.* (2001). Well positions, 81 in total, were randomized within each salinity treatment (14 plates in total per treatment). Water was changed weekly throughout the experiment and dead eggs removed twice per week. Petri dishes were photographed four times during the experiment, and the number of live eggs counted to obtain estimates of fertilization success and survival during the early developmental stage (*Stage 1*; until 28 and 39 days post-fertilization for FW and BW whitefish, respectively), during the eyed-egg stage (*Stage 2*; 70 and 84 days post-fertilization for FW and BW whitefish, respectively) and between the eyed-egg stage and hatching (*Stage 3*). Similar to Willoughby & Roberts (1992), we used malachite green (0.25 mg/L) to treat developing embryos against fungal infection. Hatched fry were anaesthetized by an MS-222 overdose, photographed against a millimetre grid for length measurements, snap frozen in liquid nitrogen and stored at –80 °C for the proteomic experiment.

Statistical analysis of the survival and length data

The effect of salinity on survival was investigated by applying generalized linear models (McCullagh & Nelder 1989) using the PROC GENMOD module of SAS v.9.2 (SAS Institute Inc., Cary, NC, USA). The ratio of the number of eggs at the end of each stage to the number of eggs at the end of the preceding stage was connected with the logit link function to the binomial distribution of the predicted values of the linear model. Differences in survival between FW and BW whitefish populations under the same salinity treatment were compared with the predicted differences (=log odds) (see, e.g., Saloniemi *et al.* 2004). The effect of salinity on the length of fry at hatching was investigated by applying a mixed model using the PROC MIXED module of SAS, wherein population and salinity were treated as

fixed factors, and female, male and female*male were treated as random factors.

Protein extraction, fractionation, MS acquisition

Eight hatch-stage samples per salinity per population were randomly selected from two of the common garden conditions (0 and 10 ppt) in both populations (32 individuals in total, 12 and 13 different families from FW and BW whitefish, respectively). Proteins were isolated using standard sodium dodecyl sulphate (SDS)-based extraction method. Detailed protocols of protein extraction, in-solution digestion, and Nano-LC-MS/MS are given in Appendix S1 (Supporting information).

Protein identification and quantification

Proteins were identified and quantified using MaxQuant v.1.1.1.36 software (Cox & Mann 2008). For the Andromeda search (Cox *et al.* 2011), cysteine carbamidomethylation was used as fixed modification, with oxidation of methionine and N-terminal acetylation as variable modifications. MS/MS tolerance was set to 0.5 Da for the collision-induced dissociation (CID) fragmentation, and the eight top peaks per 100 Da were analysed. For protein identification, a minimum of two peptides, one of which was unique, was required. The minimum peptide length was set to six amino acids and the maximum false discovery rate (FDR) to 1% for both peptides and proteins. The FDR was estimated from the number of reverse hits from a target-decoy search. The Atlantic salmon (*Salmo salar*) protein sequences submitted to UniProt (<http://www.uniprot.org>, release 2010_11, 9497 entries) were used as a search database. Salmonid UniProt sequences are an efficient resource for this purpose in salmonid species (Papakostas *et al.* 2010). To minimize the identification of multiple entries of the same protein, the database was curated for redundancy. Using Cd-hit v.4.3 (Li & Godzik 2006), identical sequences were removed and only the longest entry was retained, leaving 8984 sequences in the end. The remaining redundancy was handled by MaxQuant. Whenever the set of identified peptides for one protein was identical to or completely contained in that of another protein, both proteins were joined in the same protein group by MaxQuant (Cox & Mann 2008). Last, a list of common contaminants provided with MaxQuant v.1.1.1.36 was included in the search.

Protein quantification was based on both unique and 'razor' peptides. 'Razor' peptides are the shared peptides that are most parsimoniously associated with the group that has the highest number of identified peptides (Nesvizhskii & Aebersold 2005; Cox & Mann

2008). The use of both unique and 'razor' peptides for protein quantification has been suggested to be a good compromise between unequivocal peptide assignment and more accurate quantification (Cox & Mann 2008; Hubner & Mann 2011). For each fraction, peptides were matched across different LC-MS/MS runs on the basis of mass and retention time ('match between runs' option in MaxQuant) using the default time window of 2 min. Manual inspection of the chromatographic shifts between samples verified the suitability of this option. To increase the quantification accuracy, co-fragmented peptides were distinguished by enabling the second peptide option in MaxQuant (Cox *et al.* 2011). Last, the option for label-free quantification (LFQ) was also enabled.

Normalization, ANOVA test of significance and q-value calculation

The LFQ algorithm of MaxQuant performs normalization across samples after retention time alignment, matching between runs and assembling protein identifications. However, normalization of the reported LFQ values across biological replicates can be employed to minimize technical variation in label-free experiments (J. Cox, personal communication). Therefore, the LFQ intensities in each whitefish population were log₂-transformed, loess-normalized using the median values across biological replicates as a reference set and then evaluated for normal distribution using the Kolmogorov–Smirnov non-parametric test. In the end, an ANOVA test of significance between salinities (0 and 10 ppt) was performed using a robust linear model. These analyses were carried out with DanteR v.1.0.1.1, an R package for the analysis of proteomic data (updated edition of DANTE, Polpitiya *et al.* 2008). Kolmogorov–Smirnov tests were performed in SPSS (Statistical Package for the Social Sciences) v16. ANOVA is typically used for normally distributed ion-intensity data and is even suggested to be superior than other methods, such as those assigning significance to features like fold changes (Waanders *et al.* 2009; Neilson *et al.* 2011). Last, to obtain an FDR-based estimate for the lists of significant proteins, *q*-values were calculated with the program QVALUE (Storey & Tibshirani 2003). *Q*-value is an extension of FDR describing the proportion of false positives incurred within a set of significant features (Storey & Tibshirani 2003).

Gene ontology enrichment tests

Hypergeometric tests for gene ontology (GO) term enrichment in the significant protein lists were performed with BiNGO 2.44 (Maere *et al.* 2005) in Cytoscape 2.8.0 (Shannon *et al.* 2003). One of the advantages

of BiNGO is the visualization of significantly over-represented GO categories in the context of the GO hierarchy, enabling easier discrimination between the significant terms furthest down the hierarchy (more detailed, terminal GO terms) and the more general ones that may be significant as a result of term interdependency (Maere *et al.* 2005; Rhee *et al.* 2008). The proportion of the quantified whitefish proteome (i.e. all quantified proteins) was used as a reference set. To overcome the poor annotation of the Atlantic salmon proteins, the GO terms from the human (*Homo sapiens*) orthologues were employed. Human UniProtKB/Swiss-Prot identifiers were retrieved using local blastp (ncbi_blast_2.2.25 with default blastp parameters). For every Atlantic salmon protein, the top blastp hit was selected. The *E*-value threshold was set to 1.00E-06 after manually inspecting the blastp results for correct orthologue identifications. In the case of protein groups (multiple entries that could not be differentiated on the basis of peptide evidence), a single orthologue was decided on the basis of the reported 'majority proteins'. A majority protein is that entry in the group with the most peptide evidence. Multiple majority proteins for a group are typically involved the same top blastp hit. In a few cases, this was not true, and the hit with the lowest *E*-value was selected. These cases typically involved different isoforms of the same protein, thus sharing the same GO annotations. Ontology and human annotations were downloaded from the Gene Ontology web site (<http://www.geneontology.org/>) and were current as of 07.06.2011 and 03.06.2011, respectively. *Q*-values for the lists of significant GO terms were calculated with QVALUE.

Protein–protein interaction modelling and network analysis

Protein–protein interaction network analysis was based on models inferred with PPI spider (Antonov *et al.* 2009) using the human UniProtKB/Swiss-Prot orthologue identifiers for the significant proteins of the BW and the FW whitefish. For a given set of genes, PPI spider (available as a web tool via <http://www.bioprofiling.de/>) identifies sub-networks in a global reference PPI network from the IntAct database (<http://www.ebi.ac.uk/intact/>) that minimize the interaction distances among the genes. Three models are considered, namely D1, D2 and D3, which correspond to sub-networks that allow zero, one or two intermediate ('missing') nodes between list members, respectively. The significance of each model is then assessed according to a Monte Carlo simulation procedure (Antonov *et al.* 2009, 2010). The most significant PPI spider model was subsequently analysed with Ingenuity Pathways

Analysis v.9.0 (IPA), a system that transforms gene lists into sets of relevant networks based on records maintained in the Ingenuity Pathways Knowledge Base (IPKB). The aim was to use the statistically treated PPI model as scaffold for the IPA network generation algorithm to identify highly interconnected pathways that were probably to represent fundamental biological functions. To increase network confidence and specificity, only experimentally observed direct relationships between genes were considered in the IPA analysis. Because IPKB also contains findings from medical research, data sources involved in cancer, diseases, toxicity, pathogen-influenced signals and xenobiotic signals were not considered in the analysis. Last, the networks generated by IPA were incorporated into Cytoscape 2.8 and explored with Cyto-Hubba v1.2 (<http://hub.iis.sinica.edu.tw/cytoHubba/>), a Cytoscape plug-in version of Hubba (Lin *et al.* 2008) that computes several node indices describing network topology. In particular, the molecules in each network were described according to their *degree*, a node topological index that corresponds to the number of nodes adjacent to a given node (Lin *et al.* 2008), and *radiality*, a node centrality index that takes into account the diameter of the network by means of shortest paths (Scardoni *et al.* 2009). These measures of connectedness and reachability can reveal nodes holding central positions in a network, which, in the case of PPI networks, have often been associated with biologically important proteins (Jeong *et al.* 2001; He & Zhang 2006). *Degree* and *radiality* were also employed to classify the canonical pathways that the IPA software identified in the two networks. This was a topology-based pathway classification and was derived from the arithmetic means of the *degree* and *radiality* scores of the pathway molecules present in the networks. This approach can highlight signalling pathways of functional significance in PPI networks (Przulj *et al.* 2004).

Results

Effect of salinity on fertilization, survival and length of fry

Salinity had a highly significant effect on the fertilization success and the survival of the FW whitefish, in contrast to BW whitefish, which performed nearly equally in all salinity treatments. At *Stage 1*, the fertilization success and/or immediate post-fertilization survival of the FW whitefish decreased significantly with increasing salinity ($\chi^2 = 20.4$, $P < 0.0001$), whereas BW whitefish showed only small decreases in fertilization success and survival ($\chi^2 = 4.5$, $P < 0.03$) in the highest salinity treatment (6 ppt vs. 10 ppt: $\chi^2 = 6.9$, $P = 0.01$; Fig. 1A). At *Stage 2*,

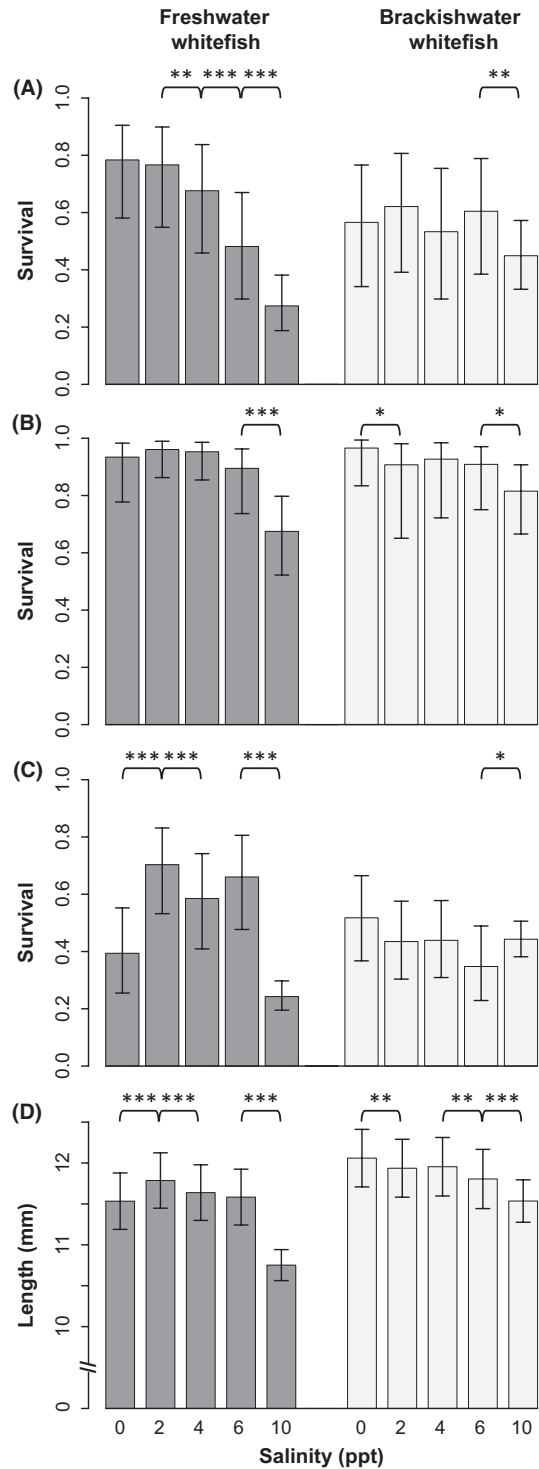


Fig. 1 Effects of different salinities on (A) fertilization success and survival during early development, (B) survival from early development until the eyed-egg stage, (C) survival from the eyed-egg stage until hatching, and (D) length at hatching. Asterisks indicate significance between salinities (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

the effect of salinity on survival was again significant for the FW whitefish ($\chi^2 = 8.1$, $P = 0.005$), but salinity had only a small effect on BW whitefish survival ($\chi^2 = 3.85$, $P = 0.05$) in the highest salinity (6 ppt vs. 10 ppt: $\chi^2 = 6.1$, $P = 0.01$; 0 ppt vs. 2 ppt: $\chi^2 = 4.0$, $P = 0.05$; Fig. 1B). Survival at *Stage 3*, from the eyed-egg stage until hatching, was similar at all salinities for the BW whitefish ($\chi^2 = 0.17$, $P = 0.680$; 6 ppt vs. 10 ppt: $\chi^2 = 4.2$, $P = 0.04$). Freshwater whitefish survival rates from the eyed-egg stage until hatching were potentially affected by a fungal infection causing lowered overall survival in 0 ppt salinity (0 ppt vs. 2 ppt: $\chi^2 = 16.7$, $P < 0.0001$), but despite this, the lowest survival was again observed in the 10 ppt salinity treatment (Fig. 1C).

The employed mating design allowed us to test for the contribution of females and males to the survival of the eggs in different salinities. Interestingly, males had no significant effect on survival at any of the three stages, whereas females had a significant effect, implying a strong maternal effect in the trait variation (males: *Stage 1*: d.f. = 12, $\chi^2 = 17.7$, $P = 0.12$; *Stage 2*: d.f. = 12, $\chi^2 = 13.8$, $P = 0.30$; *Stage 3*: d.f. = 12, $\chi^2 = 14.8$, $P = 0.25$; females: *Stage 1*: d.f. = 12; $\chi^2 = 25.0$, $P = 0.015$; *Stage 2*: d.f. = 12, $\chi^2 = 27.1$, $P = 0.008$; *Stage 3*: d.f. = 10, $\chi^2 = 26.4$, $P = 0.003$).

Salinity treatment had a highly significant effect on the length of the hatching fry in both populations (FW whitefish: $F_{1,1948} = 141.34$, $P < 0.0001$; BW whitefish: $F_{1,1356} = 127.1$, $P < 0.0001$). However, FW whitefish reared in the highest salinity environment appeared to have been affected most, with hatching fry $\sim 10\%$ smaller than their counterparts in lower salinities (Fig. 1). Length and survival data can be found in the Tables S5 and S6 (Supporting information) (see Data accessibility section).

Identified and quantified proteins

A total of 1500 proteins and protein groups were quantified, after removing contaminant and reverse hits (Table S1, Supporting information). These proteins were identified on the basis of 8160 highly confident peptides, of which 6696 were unique (Table S1, Supporting information). Mean sequence coverage was 22.32% (all peptides), 15.74% (unique peptides) or 18.67% ('razor' and unique peptides that were used for quantification). On average, each protein was quantified by 5.44 peptides. Overall, 73 (q -value = 0.18) and 42 (q -value = 0.31) proteins were differentially expressed between salinity treatments in FW and BW whitefish, respectively (Fig. 2, Table S1, Supporting information). Of these proteins, only six were common to both populations. In the FW whitefish, most of the significant proteins (61) were overexpressed in 10 ppt salinity, and only 12 were underexpressed. Similarly, in BW whitefish, most of the

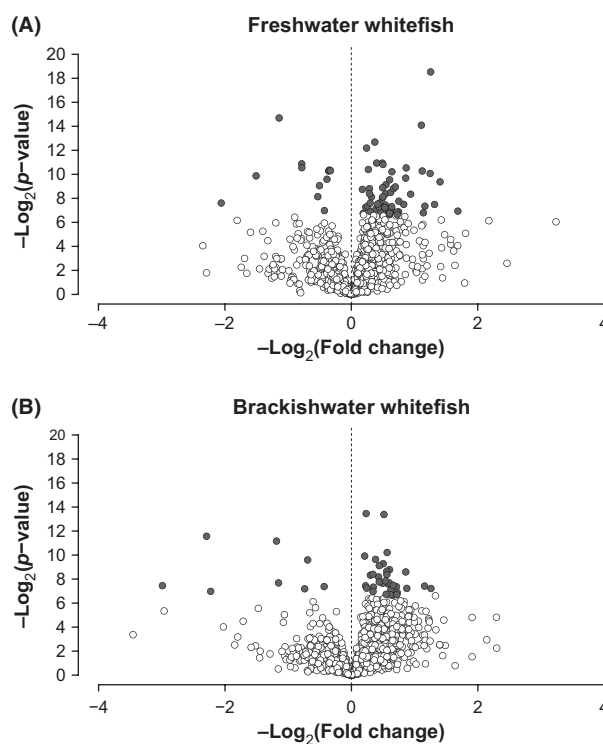


Fig. 2 Scatter plots showing the distribution of the protein expression measurements for the 1500 quantified proteins in the two whitefish populations, according to the $-\log_2 P$ value of the ANOVA test and the $-\log_2$ fold change in expression between the 0 ppt and 10 ppt salinities. Dark circles indicate proteins with $P < 0.01$, and positive fold changes represent upregulation in higher salinity. (A) In the brackishwater whitefish, 34 proteins were significantly upregulated and eight significantly downregulated in 10 ppt salinity. (B) Likewise, freshwater whitefish had 61 and 12 proteins significantly up- and downregulated in 10 ppt salinity, respectively.

significant proteins (34) were overexpressed in 10 ppt, and only eight were underexpressed (Fig. 2).

Gene ontology term enrichment analysis

The significant proteins were enriched for 76 and 142 GO terms in the FW and BW whitefish, respectively ($P < 0.05$). Using the GO hierarchy, these two lists were narrowed to 32 and 61 terminal GO terms, respectively (Table S2, Supporting information). Table 1 shows the most significant biological process, cellular component, and molecular function terminal GO terms for each whitefish population. For the FW whitefish, different terminal GO terms involved in *calcium ion transport* and *calcium channel regulator activity* were detected in the biological process and molecular function categories, respectively. In BW whitefish, terms involved in *sodium ion transport* were detected among the biological processes (Table 1).

Table 1 The three most significantly enriched terminal GO terms in the lists of significant proteins of the whitefish populations

GO category	Freshwater whitefish		Brackishwater whitefish	
	GO term (number of genes)	<i>P</i> -value	GO term (number of genes)	<i>P</i> -value
BP	Intracellular sequestering of iron ion (2)	3.50E-03	Protein K63-linked ubiquitination (2)	6.93E-03
	Retinol metabolic process (2)	3.50E-03	Sodium ion transport (2)	1.13E-02
	Calcium ion transport (3)	9.12E-03	Glycogen catabolic process (2)	1.13E-02
CC	Sarcoplasmic reticulum (3)	9.21E-03	Stress granule (2)	1.65E-02
	Eukaryotic translation initiation factor 2 complex (2)	1.02E-02	Muscle myosin complex (2)	1.65E-02
	Postsynaptic density (2)	1.02E-02	Nuclear matrix (2)	2.95E-02
MF	Translation factor activity, nucleic acid binding (6)	2.88E-03	Hsp70 protein binding (2)	1.22E-03
	Calcium channel regulator activity (2)	3.36E-03	Structural constituent of muscle (3)	1.14E-02
	Nuclease activity (4)	6.82E-03	Protein domain specific binding (4)	3.19E-02

BP, biological process; CC, cellular component; MF, molecular function; GO, gene ontology.

For the full list of significant GO terms, *q*-values were estimated at 0.14, 0.56 and 0.07 in freshwater whitefish and 0.26, 0.11 and 0.06 in brackishwater whitefish for biological process, cellular component and molecular function, respectively. The complete list of significant GO terms and the gene identifiers included in each case can be found in Table S2 (Supporting information).

Protein–protein interaction models and network analyses

A single ($P < 0.01$) PPI model with one intermediate ('missing') interaction molecule between the significant proteins (D2 model) was identified in each whitefish population. The model for the FW whitefish involved 39 proteins in total, 22 of which were significant. The BW-whitefish model concerned 43 proteins in total, 24 of which were significant (Fig. S1, Supporting information). There was very little overlap between the two models in terms of shared proteins. Five proteins were shared in common, three of which were significant (Fig. S1, Supporting information). In FW whitefish, all significant proteins of the PPI model were overexpressed in higher salinity. Likewise, the majority of the significant proteins in the model for the BW whitefish, 21 in total, were overexpressed in higher salinity.

In every case, the network analysis with the IPA software resulted in a single high-scoring network (Fig. S2, Supporting information). Figure 3 shows the distribution of the proteins according to their *degree* and *radiality* in each of the networks. The cytoskeletal proteins actin and plectin and the heat shock protein HSP90 were distinguished as top interacting proteins in FW whitefish. HSP70, HSP90 and tumour necrosis factor (TNF) were among the proteins positioned as top interacting nodes in the network of the BW whitefish (Fig. 3). Table 2 shows the results of the topology-based pathway classification for every case. Tight junction signalling was the highest interacting pathway in the network for the FW whitefish, and glucocorticoid receptor signalling was highest in BW whitefish (Table 2). Figure 4 highlights the glucocorticoid signal-

ling protein interactions produced in the context of the PPI model for the BW whitefish.

Discussion

Phenotypic response to salinity: survival and embryonic growth

Distinct differences between FW and BW whitefish in response to varying salinity conditions during the fertilization and embryonic phases were evident from the common garden experiment. In particular, for the FW whitefish, the overall survival was about 40% lower in 6 ppt and 10 ppt salinities compared with the BW whitefish, and this observation was consistent across different developmental stages (Fig. 1). In contrast to the phenotypic differentiation, the overall neutral genetic divergence between the two whitefish populations was rather low (overall $F_{ST} = 0.049$, 95% CI = 0.03–0.07, based on 15 microsatellite loci; unpublished results). These results suggest that lesser sparsely raked whitefish from the Baltic Sea are most likely adapted to the brackish water environment. Our results are also in concordance with the earlier observations of upper salinity tolerance limits of 10.2 ppt (Jäger *et al.* 1981) and 4.8 ppt (Albert *et al.* 2004) for the whitefish embryos originating from southern Baltic Sea and Lake Peipsi populations, respectively. The significant effect of the dams on survival in both populations indicates a major role of maternal components in salinity tolerance. This effect is not surprising because the maternal investment in the amount and/or quality of resources devoted to offspring is as an important source of phenotypic variation in many taxa, including fish (Mous-

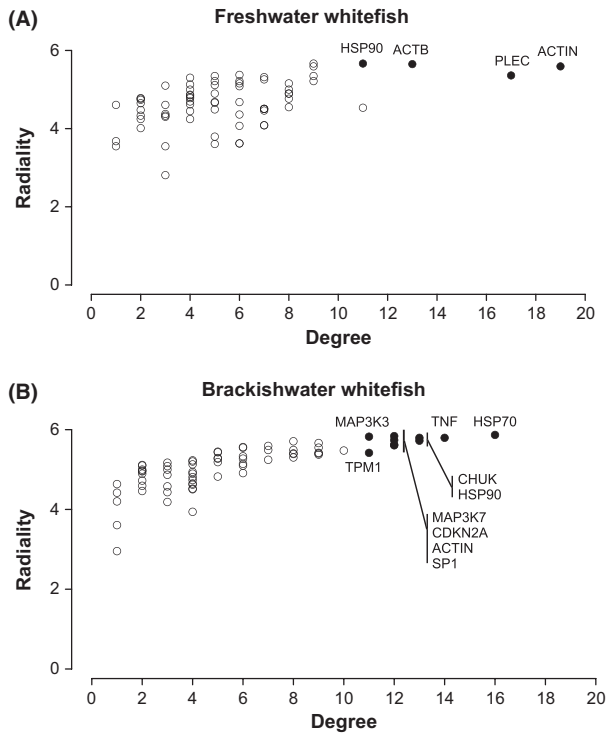


Fig. 3 Scatter plot showing the distribution of the proteins in (A) freshwater and (B) brackishwater whitefish, according to the *degree* (topological index) and *radiality* (centrality index) calculated for the networks that were generated with the Ingenuity Pathways Analysis (IPA) software. Gene names are given for those proteins that corresponded to nodes with a *degree* >10.5 and a *radiality* >5 (black-filled circles). The *degree* and *radiality* values for all proteins, as well as the networks generated with IPA, can be found in the Supporting information (Fig. S2 and Table S3, Supporting information). Gene names (further details in Table S4, Supporting information): ACTB, actin beta; ACTIN, actin; CDKN2A, cyclin-dependent kinase inhibitor 2A; CHUK, conserved helix-loop-helix ubiquitous kinase; HSP70 and HSP90, heat shock protein 70 and 90; MAP3K3 and MAP3K7, mitogen-activated protein kinase kinase kinase 3 and 7; PLEC, plectin; SP1, sp1 transcription factor; TNF, tumour necrosis factor; TPM1, tropomyosin 1.

seau & Fox 1998; Perry *et al.* 2005). However, it remains unclear to what degree differences in the salinity tolerance of the two whitefish populations are because of the environment experienced by the dams prior to the development of gametes.

In addition to drastic differences in survival, we found a significant effect of salinity on early embryonic growth, as FW whitefish fry exhibited accelerated growth in moderate salinities (2–6 ppt) compared with 0 ppt. This phenomenon has been described in several FW species, such as ruffe (*Gymnocephalus cernuus*) and common carp (*Cyprinus carpio*), and is suggested to be related to reductions in the ionic and osmotic gradients between the external and internal medium, which enable osmoregulation with a smaller energetic cost (Lam & Sharma 1985; Albert *et al.* 2006). Interestingly, such a response was not observed in BW whitefish. For both populations, the increased cost of osmoregulation was evident at the 10 ppt water, as fry were distinctively smaller than their counterparts in lower salinities.

Proteomic response to salinity in freshwater whitefish: osmotic stress related to calcium ion imbalance

In concordance with the lower survival and decreased body size of FW whitefish in 10 ppt salinity, the observed proteomic response suggests osmotic stress and specifically, problems in cell volume regulation associated with calcium ion imbalance. The biological process *calcium ion transport* and molecular function *calcium channel regulator activity* were two of the most significantly enriched terminal GO terms in the list of significant FW-whitefish proteins (Table 1). The proteins under these GO terms were all overexpressed in higher salinity (Table S1, Supporting information), suggesting a positive relationship between calcium ion transport activity and salinity in FW whitefish. In addition, annexin A6 (*Homo sapiens* orthologue: ANXA6), one of the proteins included in *calcium ion transport*,

Table 2 The pathways with the highest *degree* (topological index) and *radiality* (centrality index) according to the network analysis of this study. The proteins involved in each pathway for each network and their individual *degree* and *radiality* scores are provided in Table S3 (Supporting information)

Freshwater whitefish		Brackishwater whitefish	
Pathway name	Degree/radiality	Pathway name	Degree/radiality
Tight junction signalling	10.4/5.30	Glucocorticoid receptor signalling	9.27/5.52
NRF2-mediated oxidative stress response	9.14/5.19	TNRF2 signalling	9.00/5.44
VEGF signalling	8.33/4.51	NF-kB signalling	8.78/5.48
Regulation of eIF4 and p70S6K signalling	6.56/4.17	ILK signalling	7.83/5.30
EIF2 signalling	5.23/4.14	Actin cytoskeleton signalling	6.83/5.25

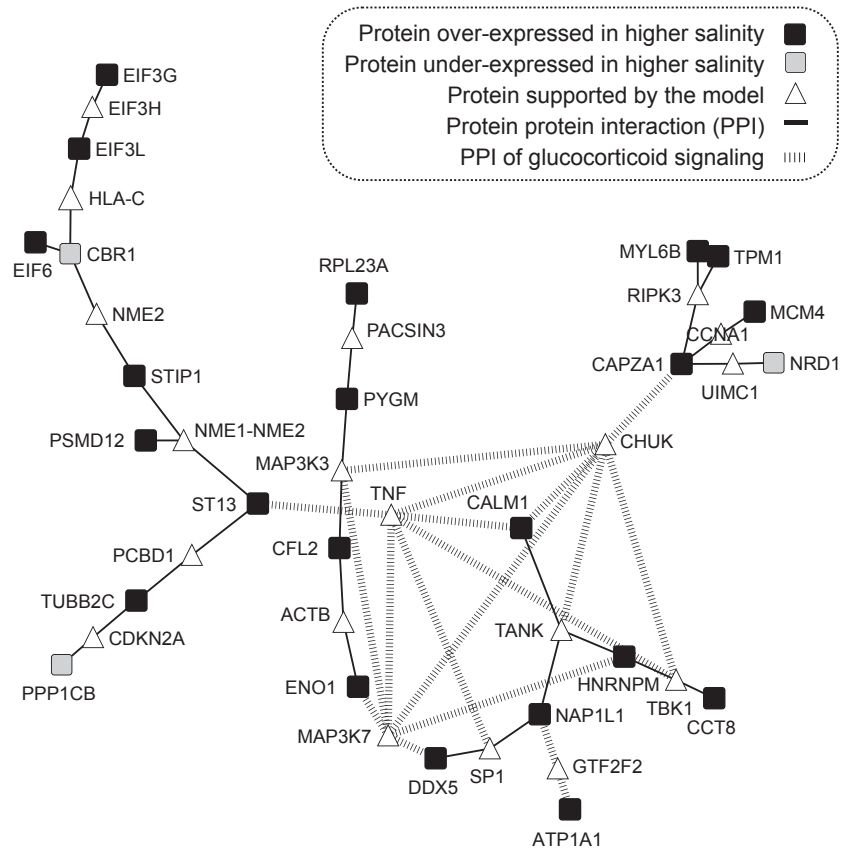


Fig. 4 The most significant protein–protein interaction (PPI) model for the brackishwater whitefish on the basis of the observed proteomic responses to salinity. Highlighted are the interactions involved in glucocorticoid signalling, a pathway associated with seawater adaptation in teleosts. The PPI model was visualized in Cytoscape using a spring-embedded algorithm to obtain a force-directed view of the model and gain perspective about the central and peripheral architecture of the model. Proteins of the glucocorticoid signalling pathway (TNF, CHUK, MAP3K7, and GTF2F2) occupied a central position in the model and were linked to proteins important for osmoregulation, such as the alpha subunit of sodium/potassium ATPase (ATP1A1). Glucocorticoid signalling interactions were inferred from the network analysis that was performed with the Ingenuity Pathways Analysis (IPA) software. Details about the PPI model, the IPA-generated network and the involved proteins can be found in the Supporting information (Figs S1B and S2B, Table S4, Supporting information). Gene names (in alphabetic order): ACTB, actin beta; ATP1A, ATPase Na⁺/K⁺ transporting alpha 1; CALM1, calmodulin 1; CAPZA1, capping protein muscle Z-line alpha 1; CBR1, carbonyl reductase 1; CCNA1, cyclin A1; CCT8, chaperonin containing TCP1 subunit 8; CDKN2A, cyclin-dependent kinase inhibitor 2A; CFL2, cofilin 2; CHUK, conserved helix-loop-helix ubiquitous kinase; DDX5, DEAD box polypeptide 5; EIF3G, EIF3H, and EIF3L, eukaryotic translation initiation factor 3 subunit G, H, and L; EIF6, eukaryotic translation initiation factor 6; ENO1, enolase 1; GTF2F2, general transcription factor IIF polypeptide 2; HLA-C, major histocompatibility complex class I, C; HNRNPM, heterogeneous nuclear ribonucleoprotein M; MAP3K3 and MAP3K7, mitogen-activated protein kinase kinase kinase 3 and 7; MCM4, minichromosome maintenance complex component 4; MYL6B, myosin light chain 6B; NAP1L1, nucleosome assembly protein 1-like 1; NME1 and NME2, nucleoside diphosphate kinase A and B; NRD1, nardilysin; PACSIN3, protein kinase C and casein kinase substrate in neurons 3; PCBD1, pterin 4 alpha carbinolamine dehydratase; PPP1CB, protein phosphatase 1 catalytic subunit beta isozyme; PSMD12, 26S proteasome non-ATPase regulatory subunit 12; PYGM, phosphorylase glycogen muscle; RIPK3, receptor-interacting serine-threonine kinase 3; RPL23A, ribosomal protein L23a; SP1, sp1 transcription factor; ST13, suppression of tumorigenicity 13; STIP1, stress-induced phosphoprotein 1; TANK, TRAF family member-associated NFKB activator; TBK1, TANK-binding kinase 1; TNF, tumour necrosis factor; TPM1, tropomyosin 1; TUBB2C, tubulin beta 2C; UIMC1, ubiquitin interaction motif containing 1.

was involved in the PPI model of the FW whitefish (Fig. S1A, Supporting information). Another annexin, ANXA4, was also involved in this PPI model and was also overexpressed in higher salinity (Fig. S1A, Supporting information). We focused on proteins of the PPI models because there is additional statistical and func-

tional evidence that these proteins are true positives. In addition, protein interaction network analyses are highly relevant for investigating signal transduction pathways and identifying proteins that are important in modulating the flow of information in signalling networks (Vinayagam *et al.* 2011). Annexins are an evolu-

tionarily conserved family of calcium- and phospholipid-binding proteins that interact with membranes upon increases in intracellular calcium ion concentrations or during cytoplasmic acidification (Gerke & Moss 2002). The ANXA6 protein, in particular, has a role in attenuating transmembrane calcium ion entry upon intracellular calcium increase (Monastyrskaya *et al.* 2009). It is therefore reasonable to assume that the studied FW whitefish are challenged by disturbances related to calcium ion homeostasis when grown in 10 ppt salinity. Exposure of FW fish or FW-acclimated euryhaline fish to saline conditions is often accompanied by a large influx of calcium ions (Suresh & Jayaraman 1983; Prodocimo *et al.* 2007).

Calcium ions are important second messengers for several cellular signalling events. In fish, among other taxa, calcium ions are involved in osmosensing by modulating the activity of calcium receptors, which are salinity sensors (Nearing *et al.* 2002; Chang & Shoback 2004; Fiol & Kültz 2007). However, an intracellular calcium increase does not indicate an osmoregulatory response *per se*, but rather, an *a posteriori* disruption of permeability control, typically associated with anisotonic cell volume regulation and the opening of tight junctions (Prodocimo *et al.* 2007); osmotic stress seems to be the case for FW whitefish. Tight junction signalling proteins and proteins involved in actin filament structure and organization (ACTIN, ACTB, PLEC) were discovered as pivotal components of the proteomic response to increased salinity in FW whitefish (Fig. 3A, Fig. S2A, Supporting information). Notably, cellular osmotic changes are mediated by the cytoskeleton, particularly by the actin filament system, and intracellular calcium is an important second messenger related to this process (Bennett & Weeds 1986; Chowdhury *et al.* 1992). Plectin (PLEC), in particular, was significantly overexpressed in higher salinity and was also involved in the PPI model of the FW whitefish. Plectin plays a cytoprotective role against osmotic stress (Osmanagic-Myers *et al.* 2006) and participates in the formation of a variety of plasma membrane-cytoskeleton junctional complexes (Wiche 1998). Tight junction signalling, on the other hand, has many roles, including maintaining ionic and osmotic balance between cells, and intracellular calcium ions may serve as signals influencing the permeability of tight junctions (Shen *et al.* 2011). Altogether, our proteomic data support the phenotypic results that FW whitefish suffer chronic osmotic stress in 10 ppt salinity. Perhaps the most interesting observation is that calcium ions seem to be carriers of the main signals that are generated under these conditions. As calcium is also an important second messenger for salinity sensing (Fiol & Kültz 2007), we suspect that mechanisms involved in calcium sensing and/or homo-

eostasis (Loretz 2008) are associated with the higher mortality of the FW whitefish in the 10 ppt salinity.

Proteomic response to salinity in brackishwater whitefish: molecular mechanisms of salinity adaptation

We identified several proteins, particularly those involved in the PPI model, that have been previously associated with salinity acclimation and/or adaptation in teleost fish. The most striking case is seemingly the alpha subunit of the sodium/potassium ATPase (ATP1A1) protein. This protein was overexpressed in BW whitefish at 10 ppt and included in the PPI model (Fig. 4), and *sodium ion transport* was among the most significantly enriched terminal GO terms for biological processes in BW whitefish (Table 1). Sodium/potassium ATPases (NKAs) are ion transporters with key roles in tissues such as fish gills during osmoregulation (McCormick *et al.* 2009; Hwang *et al.* 2011). In salmonids, several ATP1A1 isoforms have tissue-specific distributions and salinity-specific expression profiles (Richards *et al.* 2003; McCormick *et al.* 2009). The *a* isoform has been implicated in FW acclimation and the *b* isoform in seawater acclimation (Richards *et al.* 2003; Bystriansky *et al.* 2006; Madsen *et al.* 2009; McCormick *et al.* 2009). In this study, the ATP1A1 protein that was identified and quantified was the *b* isoform (UniProt accession: C0H8U1, Table S1, Supporting information). Significant overexpression of the *b* isoform at higher salinity fits the seawater-adaptation hypothesis for the BW whitefish.

Heat shock cognate 70 (Hsc70)-interacting protein (*H. sapiens* orthologue: ST13) is another case of a salinity-induced protein in BW whitefish that has been associated with salinity acclimation/adaptation in teleosts. Hsc70 was overexpressed in higher salinity and was involved in the BW whitefish PPI model (Fig. 4). It is an adaptor protein that mediates the association of HSP70 with HSP90 protein families. Notably, HSP70 and HSP90 were both identified as top interacting nodes in the BW whitefish network (Fig. 3B), and *Hsp70 protein binding* was the most significant terminal GO term for molecular function in BW whitefish (Table 1). HSP70 expression can be induced by a variety of stressors in fish, including osmotic stress (Iwama *et al.* 1998). The transcription of Hsc70 is strongly induced by salinity in the gills of the silver sea bream, *Sparus sarba* (Deane & Woo 2004). Hemmer-Hansen *et al.* (2007) found evidence for ongoing selection in the Hsc70 gene of European flounder (*Platichthys flesus* L.) populations throughout the northeastern Atlantic, particularly between the western and central Baltic Sea. They highlighted temperature and salinity as the probably selective agents because of the strong transition of

both of these environmental parameters in the zone connecting the marine Atlantic and brackish Baltic Sea.

The observation that glucocorticoid signalling was the predominant pathway behind the proteomic response of BW whitefish (Table 2, Fig. 4, Fig. S2B, Supporting information) is perhaps the finding that best demonstrates that the observed proteomic responses in BW whitefish are involved in osmoregulation. Glucocorticoid receptor is a transcription factor that regulates several genes in a positive or negative way (McKay & Cidlowski 1999; De Bosscher *et al.* 2003). It is the receptor to which cortisol and other glucocorticoids bind. Notably, cortisol is the major corticosteroid in teleosts and is an osmotic stress hormone that enables fish to acclimate to environmental salinity changes (McCormick 1995, 2001). A highly conserved pleiotropic cytokine, TNF, was identified as a carrier of the main extracellular signal in the BW whitefish network (Fig. 3B). The central position of TNF was evident not only in the IPA-generated network of the BW whitefish (Fig. S2B, Supporting information) but also in the PPI model (Fig. 4). Dowd *et al.* (2010) have recognized TNF as a key modulator of the protein-level osmoregulatory responses in elasmobranch fishes. They suggested that the activity of TNF is associated with the activity of polyvalent cation receptors (CaRs), which are salinity sensors in both elasmobranchs and teleosts (Nearing *et al.* 2002), as well as with the activity of the angiotensin II system, which has been implicated in blood volume regulation under different salinities both in elasmobranchs (Dowd *et al.* 2010 and references) and in teleosts (e.g. Wong *et al.* 2006). CaRs are G protein-coupled receptors (GPCRs), a large superfamily of membrane receptors that are involved in the activation of glucocorticoid signalling (Tasker *et al.* 2006). CaR-induced TNF transcription has been repeatedly recognized as a critical step to regulate ion and water balance in cells (Abdullah *et al.* 2008a,b), whereas GPCRs can mediate TNF-induced NF- κ B activation (Patial *et al.* 2010), which is required to qualify angiotensin II-induced signalling cascades (Zahradka *et al.* 2002). Notably, NF- κ B signalling was also among the top interacting pathways in the BW whitefish network (Table 2). Finally, CaRs have evolutionarily conserved intracellular functional domains important for MAPK cascade activation (Loretz 2008). Interestingly, MAP3K3 and MAP3K7 were both identified as top interacting molecules in the BW whitefish network (Fig. 3B). These findings indicate that glucocorticoid signalling plays an important role in salinity tolerance in BW whitefish. Altogether, our proteomic results suggest that TNF and CaR proteins could serve as important mediators of the signalling events that take place during osmoregulation in both teleosts and elasmobranchs.

Identification of novel key proteins related to osmoregulation in teleosts

For the PPI model of the BW whitefish, there is extensive statistical, functional and phenotypic evidence, in addition to previously published results, to suggest that the model in Fig. 4 adequately describes the proteins and interactions involved in osmoregulation. Interestingly, many of the significant proteins in this model have never been assigned such a role in teleosts. Describing the function of each of the significant proteins in the model and their potential roles in osmoregulation is beyond the scope of this work. Below, we illustrate two examples of such proteins that, in addition, were found to interact directly with pivotal components of the BW whitefish network (Fig. 3B).

The first case concerns the heterogeneous nuclear ribonucleoprotein M (*H. sapiens* orthologue: HNRNPM), a protein that was significantly upregulated by salinity in BW whitefish (Table S1, Supporting information) and a member of the PPI model (Fig. 4) that interacted directly with TNF, a top interacting protein of the BW whitefish network (Fig. 3B, Fig. S2B, Supporting information). HNRPs represent a group of evolutionary conserved proteins associated with splicing regulation (Kiesler *et al.* 2005; Hovhannisyan & Carstens 2007). HNRPs are ubiquitous in the nucleus, but in some cases, they can also be expressed as cell surface proteins (e.g. Bajenova *et al.* 2001). HNRPs can modulate gene expression by affecting pre-mRNA splicing or mRNA stability, particularly in response to physical or chemical cellular stress (Biamonti & Caceres 2009). Interestingly, HNRNPMs are targets for sumoylation (Vassileva & Matunis 2004; Schimmel *et al.* 2008). Small ubiquitin-related modifiers (SUMOs) are post-translationally conjugated to other proteins and regulate a wide range of biological processes (Gareau & Lima 2010). Increasing evidence suggests important roles for sumoylation in the responses to stressors, such as osmotic, hypoxic, heat, oxidative and genotoxic stresses (Tempé *et al.* 2008).

The second case is the stress-induced phosphoprotein 1 (*H. sapiens* orthologue: STIP1), which was also significantly upregulated by salinity in BW whitefish (Table S1, Supporting information) and involved in the PPI model (Fig. 4). As a member of the BW whitefish network (Fig. S2B, Supporting information), STIP1 interacted closely with HSP70 and HSP90, both of which were among the top interacting molecules of the network (Fig. 3B), which is not surprising because STIP1 is an adaptor protein that coordinates the functions of HSP70 and HSP90 (Song & Masison 2005; Onuoha *et al.* 2008). Most importantly, STIP1 has a critical role in the assembly of the glucocorticoid receptor by specifically binding Hsp90 and mediating its association to Hsp70

before these are incorporated into the glucocorticoid receptor heterocomplex (Pratt *et al.* 2006). Interestingly, single-nucleotide polymorphisms in STIP1 have been associated with differences in the strength of the corticoid response in asthmatic individuals treated with corticosteroids, highlighting the role of STIP1 in the glucocorticoid signalling pathway (Hawkins *et al.* 2009).

Evolutionary and ecological implications

Our results reveal aspects of the molecular architecture behind osmotic stress adaptation in a salmonid fish species at the protein level, but how universal can these findings expected to be? This is a difficult question to answer as currently there is a lack of similar studies in other teleost species. That said, current knowledge concurs with the view that much of the structure of the PPI models and networks should be evolutionary conserved. This is based on the fact that many of the genes and pathways implicated in these models and networks have been found to have an osmoregulatory role in other fish species, whereas central genes and pathways like glucocorticoid signalling or TNF have been suggested to be important components of the osmotic stress response in teleosts or both teleosts and elasmobranchs, respectively (McCormick 2001; Dowd *et al.* 2010).

The potential role of calcium ions and their respective receptors, CaRs, appears to be of particular evolutionary and ecological significance in osmotic stress adaptation in fish. We have demonstrated that apparent osmotic stress is linked to calcium ion imbalance in FW whitefish and viable osmoregulatory response with CaR-mediated TNF activation in BW whitefish. Calcium ions have earlier been recognized to contribute significantly to osmosensory signal transduction in fish with CaRs activated by changes in calcium ion concentration in the environment (Nearing *et al.* 2002; Chang & Shoback 2004; Fiol & Kültz 2007). CaR sensitivity in particular is tuned to detect physiologically meaningful changes of calcium concentration in the presence of other ions in the water that act as agonists (Nearing *et al.* 2002; Chang & Shoback 2004). Therefore, according to the results of this study, CaRs are good candidates to be involved in salinity adaptation in fish. Further study is warranted, however, to shed more light on the molecular mechanisms by which aquatic organisms survive and reproduce in environments of different salinities.

In conclusion, our study takes a systems-wide proteomics view of osmotic response in teleost fishes and further compares the responses to salinity of two different populations of the same species. The drastically different phenotypic responses were accompanied by different proteomic responses. Combining protein expression data with protein interaction network analysis enabled

us to examine the signal transduction pathways that occupied central positions in the generated networks. For the viable osmoregulation observed in the BW whitefish, we discovered the importance of glucocorticoid signalling downstream of the cortisol-activated signalling cascade, with TNF having a central role in this pathway. On the other hand, osmotic stress and high mortality in FW whitefish were associated with a disruption of calcium ion homeostasis. The latter, along with the facts that TNF function interacts closely with calcium receptors and that calcium receptors are salinity sensors in fish, suggests a role for these receptors in the observed responses. Altogether, our results indicate that lesser sparsely raked whitefish from the Baltic Sea is most likely adapted to the BW environment. This finding has broad conservation and environmental interest because whitefish is a commercially important species and is stocked routinely in many countries. Last, our molecular approach provides an excellent example of how potentially adaptive traits can be studied using modern proteomic methods in non-model organisms.

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muscle cells. *Journal of Molecular and Cellular Cardiology*, **34**, 1609–1621.

A.V., C.R.P., J.-P.V. and S.P. are interested in understanding patterns of genetic and phenotypic variation in natural fish populations using ecological genomic tools. M.H. is focused in the study of whitefishes from the Baltic Sea region using population genetic approaches. L.P. is interested in mass spectrometry based quantitative proteomics.

Data accessibility

The mass spectrometry files, search database, version of the MaxQuant software together with the results of the Andromeda search and label-free quantification have been deposited in Proteome Commons Tranche repository, [https://proteomecommons.org/tranche/\(search for 'Papakostas' under Data>Search Data\)](https://proteomecommons.org/tranche/(search%20for%20%27Papakostas%27%20under%20Data%26Search%20Data)). Length and survival data can be found in the Tables S5 and S6 (Supporting information).

Supporting information

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

Appendix S1 Materials and methods.

Fig. S1 The significant ($P < 0.01$) protein-protein interaction models found in the (A) freshwater and (B) brackishwater whitefish, based on the observed proteomic responses to salinity.

Fig. S2 The highest scoring IPA networks for the (A) freshwater (IPA score = 70) and (B) brackishwater (IPA score = 81) whitefish.

Table S1 The protein IDs, human orthologs, peptide counts, sequence coverage and label-free quantification (LFQ) intensities before and after normalization for the 1500 proteins and protein groups that have been identified and quantified in the studied freshwater (FW) and brackishwater (BW) whitefish.

Table S2 The IDs, descriptions and P -values of the GO terms that were found enriched ($P < 0.05$) for the lists of significant proteins in freshwater and brackishwater whitefish.

Table S3 The calculated *degree* and *radiality* indices for the proteins of the freshwater- and brackishwater-whitefish networks that were generated with the IPA software.

Table S4 Molecule and interaction information of the freshwater and brackishwater whitefish networks that were generated with the IPA software.

Table S5 Length of hatched whitefish of brackishwater and freshwater origin.

Table S6 Stage-specific survival of brackishwater and freshwater whitefish.

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