



Changes in muscle and blood plasma proteomes of Atlantic salmon (*Salmo salar*) induced by crowding

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ABSTRACT

Crowding is a well-known stressor for fish and may, depending on its severity and duration, induce several physiological changes to the organisms. The present work aimed to identify changes in muscle and blood plasma proteomes of Atlantic salmon induced by crowding (>200 kg fish/m³, 40 min). A total of 27 and 17 proteins showed altered abundance in muscle and blood plasma, respectively, from control and crowded salmon. The main changes in muscle were related to structural proteins (actin, myosin heavy and light chains, tropomyosin) and enzymes involved in energy production (creatine kinase, enolase, phosphoglycerate kinase), while apolipoprotein A-I, angiotensinogen and complement component C3 were found to change in the blood plasma. All these proteins are involved in different secondary and tertiary stress responses, including altered energy metabolism, osmotic regulation and immune function. The changes observed in energy metabolism indicated a shift towards anaerobic energy production. Moreover, the changes observed in proteins of the immune system indicate that short-term exposure to crowding may induce changes in the immune system of salmon. In conclusion, the changes observed in muscle and blood plasma proteomes not only correspond with the physiological stress response in fish, but also highlights the mechanisms causing an accelerated muscle pH decline and *rigor mortis* contraction in crowded salmon.

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1. Introduction

When an animal is under the influence of a stressor, hormones such as cortisol and catecholamines are released into its blood stream, leading to a series of secondary effects that involve energy metabolism, respiratory function, immune function, and osmotic regulation, and tertiary effects on performance factors such as growth and disease resistance (Iwama et al., 1997). The most common indicator of stress in fish is an increase in plasma cortisol concentration, and the major physiological roles of cortisol are regulation of the hydromineral balance and energy metabolism (Wendelaar Bonga, 1997).

A large number of studies have been performed regarding the stress response in fish. Most of these have studied the effects on levels of hormones, enzymes and metabolites, while less is known regarding the molecular mechanisms underlying these physiological responses. However, several reports on gene expression in different tissues related to stress response in fish are now available. Gene expression in liver has been studied in response to handling stress in rainbow trout (Wiseman et al., 2007) and confinement exposure of gilthead sea bream (Calduch-Giner et al., 2010), and several genes related to

different metabolic processes, proteolytic pathways and immune function were altered due to these treatments. Similarly, proteins related to immune function were also found to change in rainbow trout liver due to low-water stress (Momoda et al., 2007). Handling stress of rainbow trout has also been found to affect expression of genes related to mitochondria, extracellular matrix and endopeptidases in kidney, while glycolytic enzymes and motor protein were affected in the brain (Krasnov et al., 2005). The stress response in fish has also been studied by proteomics, and changes in heat shock protein 70 and a calcium-binding protein have been reported in response to acute cadmium exposure in gill tissue of Japanese flounder (Ling et al., 2009), while proteins related to energy metabolism and cytoskeleton and actin assembly were found to change in response to anoxia in developing zebrafish embryos (Mendelsohn et al., 2009). Recently, comparative proteomics has revealed changes in liver proteins involved in lipid transport and oxidation, chaperoning, ammonia metabolism and cytoskeleton as a result of chronic stress in gilthead seabream (Alves et al., 2010). Proteomics has also been applied to study changes in muscle proteins of rainbow trout in response to pre-slaughter stress (Morzel et al., 2006), and the majority of these proteins were related to energy metabolism and structural assembly of the muscle.

Handling prior to slaughter is known to induce stress and thereby negatively affects meat quality of several animal species, including fish (Lowe et al., 1993), pigs (Støier et al., 2001), and cattle (Beltran

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et al., 1997). In fish, increased metabolic rates, reduced energy reserves in the muscle at slaughter, and accumulation of lactic acid from anaerobic metabolism have great impact on *postmortem* fillet quality (Pottinger, 2001). More specifically, the fillet quality of Atlantic salmon (*Salmo salar*) is affected by several stress-inducing *antemortem* factors such as handling and crowding, and these are reported to alter *rigor mortis* contraction, muscle pH decline, and fillet texture (Skjervold et al., 1999, 2001). However, more knowledge is needed regarding the underlying mechanisms for these effects.

In a previous study, we reported that salmon subjected to a crowding stressor (>200 kg fish/m³) for 40 min resulted in an increased level of plasma cortisol, lactate, and osmolality, and a decreased level of plasma glucose (Veiseth et al., 2006). Moreover, the crowded salmon were also found to have reduced muscle pH values immediately after slaughter, and they exhibited an accelerated and extended *rigor mortis* contraction. The objective of this study was to examine the stress-related changes in muscle and blood plasma proteomes of the Atlantic salmon from the previous study, and identify possible links to physiological stress responses and fillet quality attributes.

2. Material and methods

2.1. Animals and tissue sampling

The Atlantic salmon (*S. salar*, AquaGen stock, 1+ smolts stocked in seawater two years prior to sampling) used in this experiment were selected at random from a group of 10 metric tons kept in a 700-m³ resting net-pen in the sea. A total of 20 fish with a mean ($\pm$ SD) body weight of 4.8 ± 1.2 kg were sampled in this experiment. Half of the fish were carefully removed from the net-pen (~ 14 kg fish/m³) one at a time (Control), while the other half were crowded (>200 kg fish/m³) for 40 min prior to slaughter (Crowded) in order to mimic the crowding conditions often seen during commercial slaughter of salmon. All fish were killed by a blow to the head, the gill arches were cut, and the fish were subsequently eviscerated, washed, and filleted by hand. Immediately after killing the fish, but before gill cutting, blood samples were taken from the caudal vein using a heparinised hypodermic needle. The blood samples were centrifuged for 10 min at 1500 $\times$ g, after which blood plasma was collected, frozen in liquid nitrogen, and stored at -80°C . Muscle samples were taken, from the region below the dorsal fin and above the midline, within 5 min of slaughter, snap frozen in liquid nitrogen and stored at -80°C until further processing.

2.2. Sample preparations

The muscle samples from individual fish were extracted using two different approaches; SDS and TES extraction. The SDS extracts were prepared by homogenising 120 mg muscle tissue in 2 mL SDS-solubilisation buffer (2% SDS, 60 mM Tris, pH 8.3, 100 mM DTT) using a Polytron (PT 1200 E, Kinematic AG, Lucerne, Switzerland), and the resulting homogenate was boiled for 2 min, and left to cool at room temperature for 30 min. Homogenates were re-homogenised prior to addition of 200 μL RNase/DNase-buffer (0.5 mg/mL RNase A and 1 mg/mL DNase II (Cat. 109 169 and 104 159, respectively; L. Hoffman-La Roche Ltd., Basel, Switzerland) in 0.05 M MgCl₂, 0.5 M Tris, pH 7.0), incubated at room temperature for 30 min, and finally boiled again for 2 min. Immediately after boiling, the samples were homogenised one final time, before 1 mL of the homogenate was transferred into an Eppendorf tube for centrifugation at 16,100 $\times$ g for 15 min at 20°C . In order to remove most of the fat in the samples, 800 μL was carefully removed and transferred into a new tube for a second centrifugation.

The TES extracts were prepared by homogenising 200 mg muscle tissue in 1 mL TES-buffer (10 mM Tris, pH 7.6, 1 mM EDTA, 0.25 M

sucrose) using a Polytron. The homogenates were transferred into microcentrifuge tubes and centrifuged at 9500 $\times$ g, 4°C , for 30 min, and the TES-soluble proteins were collected. The blood plasma samples were thawed on ice and centrifuged at 1500 $\times$ g for 5 min at 4°C before protein determination and preparation for two-dimensional gel electrophoresis (2-DE). Protein concentrations were measured with a commercial kit (RC DC Protein Assay, Bio-Rad Laboratories Inc., Hercules, CA) using BSA as standard. All extracts were stored at -80°C until further preparation for two-dimensional gel electrophoresis (2-DE).

2.3. Two-dimensional gel electrophoresis

The 2 muscle extracts (SDS and TES) and the blood plasma samples from individual fish were run as three separate experiments, resulting in a total of 60 gels. The samples in each treatment group were randomised prior to 2-DE, and an equal number of samples from each treatment were run in each batch (10 gels per batch). Immobilised pH gradient (IPG) strips (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) spanning the pH region 4–7 were used for the isoelectric focusing (IEF). The samples were diluted in IEF sample buffer (8 M Urea, 2 M Thiourea, 1.5% CHAPS, 50 mM DTT, 0.1% IPG-buffer pH 4–7, 10 mM Tris, pH 8.3; for the SDS extracts 0.1% SDS was also included), and allowed to stand for a minimum of 1 h prior to use. For analytical 2-DE, 75, 100, and 500 μg proteins from Plasma, TES, and SDS samples, respectively, were loaded onto each IPG strip by active (50 V) in-gel rehydration using a PROTEAN[®] IEF Cell (Bio-Rad Laboratories Inc.), while 500 and 1000 μg proteins were loaded of all extracts for preparative 2-DE. Low voltage (250 V) was applied in the initial step of the IEF, followed by a rapid increase to 10,000 V, where the voltage was kept until a total of 60,000, 45,000, and 80,000 Vh was reached for Plasma, TES, and SDS samples, respectively.

Following IEF, the IPG strips were incubated in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl, pH 8.8) twice for 15 min. To the first and second equilibration steps 2% (w/v) DTT and 5% (w/v) iodoacetamide was added, respectively. The second dimension polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% separating gels (29:1 ratio of acrylamide to bisacrylamide, 0.1% SDS, 375 mM Tris-HCl, pH 8.8), using the Ettan DALTtwelve system (GE Healthcare Bio-Sciences AB). The visualisation of proteins was performed by silver staining according to (Blum et al., 1987) for the TES and Plasma samples, while a colloidal Coomassie Brilliant Blue staining (Rabilloud and Charmont, 2000) was used for the SDS samples. Proteins on the preparative gels were visualised by standard Coomassie blue (R-250) stain for the SDS samples and an MS-compatible silver stain (Shevchenko et al., 1996) for the TES and plasma samples.

2.4. Image analysis and statistical analysis

The gels were scanned using an office scanner (Epson Perfection 4990 Photo, Epson America Inc., Long Beach, CA) with a resolution of 300 dpi, and analysed using the PDQuest software, version 7.2.0 (Bio-Rad Laboratories Inc.). The three sets of samples, Plasma, TES, and SDS, were analysed separately. Spots were automatically detected and matched with spots from the other gels, and all artefacts and mismatched spots were manually removed. The spot volumes were normalised by dividing the raw volume of each spot in a gel by the total volume of valid spots in that gel. The resulting spot tables were imported into Unscrambler v 9.2 (CAMO A/S, Norway) for statistical analysis. Missing values were imputed using the PCA method present in the Unscrambler software. The aim of this method is to impute values in such a way as to keep the PCA model on the original data unchanged.

The first part of the statistical analysis was to remove the batch effect from the measured protein volumes. Partial least squares

regression (PLSR) was used to model the batch information based on the protein volumes and the residuals from this model were subsequently used for further analyses. Differences in protein expression with regard to treatment were found by an ANOVA model using the treatment categories as regressor and corrected protein volumes as response. Individual significance levels of each protein were adjusted with the rotation test to correct for false positives due to multiple hypothesis testing (Langsrud, 2005). Significance level after adjusting was set to 5%, meaning that 5% of the reported proteins could be expected to be false positives. The ANOVA was performed with the 50–50 MANOVA software (<http://www.langsrud.com/stat>). An additional analysis was performed in Matlab (The Mathworks inc., MA, USA), using the cross model validation (CMV) method (Anderssen et al., 2006). CMV is based on regular PLS but uses an additional cross validation level as a strategy to adjust reported significance levels for individual proteins. The significance for each protein is based on what percentage of the validation loops that protein was marked as significant at the 5% level. Each of these extra validation loops reports a list of significant proteins at the 5% level and the total adjusted significance for each protein after the CMV procedure measures in how many of these lists that protein appears. For this experiment, the percentage level was set at 70%. As found by Grove et al. (2008), variable selection from 2-DE data will differ when different statistical methods are used, and in this study we chose a conservative method for selection of significant variables, by only reporting the protein spots that were selected both with univariate ANOVA and multivariate CMV. Combining these methods should increase the certainty of those proteins marked as significant in this study.

2.5. Protein identification

The protein spots of interest were cut out from preparative gels and processed according to Jensen et al. (1998) with slight modifications. Briefly, the gel plugs were washed twice with 100 μ L 50% acetonitrile (ACN) for 15 min with gentle shaking at room temperature, and subsequently dried in a speed-vac centrifuge (ISS 110 SpeedVac System, Thermo Savant) for 20 min. The gel plugs were then incubated with 100 μ L of 10 mM DTT in 100 mM NH_4HCO_3 with gentle shaking for 45 min at 56 °C, followed by incubation in 100 μ L 55 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min at room temperature in the dark. The gel plugs were washed twice with 100 μ L of a 1:1 solution of ACN and 50 mM NH_4HCO_3 at room temperature for 15 min, followed by 20 min drying in a speed-vac centrifuge. Five μ L of modified porcine trypsin (30 ng/ μ L, sequencing grade; Promega, Madison, WI) was added to the dried gel plugs and incubated on ice for 30 min, before 25 μ L 50 mM NH_4HCO_3 was added followed by an overnight incubation at 37 °C.

Small disks of C18 Empore Disks (3 M, Minneapolis, MN) were placed into GelLoader Tips (Eppendorf AG, Hamburg, Germany) according to Rappsilber et al. (2003), and these microcolumns were used to desalt and further concentrate the peptide mixtures prior to MS analysis. A 5-mL syringe was used to force liquid through the columns, and 10 μ L of the peptide mixtures were loaded onto the columns, and washed with 20 μ L 0.1% TFA. Peptides were eluted with 0.8 μ L matrix solution (α -cyano-4-hydroxy-cinnamic acid (Bruker Daltonics, Bremen, Germany) saturated in a 1:1 solution of ACN and 0.1% TFA) and spotted directly onto the MALDI-TOF target plate.

An Ultraflex MALDI-TOF/TOF mass spectrometer with a LIFT module (Bruker Daltonics) was used for mass analyses of the peptide mixtures. External calibration of the PMF spectra was performed with a standard peptide mixture (Peptide calibration standard I, Bruker Daltonics), while the internal calibration was performed with the trypsin autolytic products. FlexAnalysis 2.4 (Version 1.1.3, Bruker Daltonics) was used to create the peak list, using median baseline subtraction with 0.8 in flatness and smoothing with Savitzky-Golay

with 0.2 m/z in width. BioTools 3.0 (Version 1.0, Bruker Daltonics) was used for interpretation of MS and MS/MS spectra, and proteins were identified by peptide mass fingerprinting (PMF) using the database search program MASCOT (<http://www.matrixscience.com>). An accuracy of 100 ppm was used in the search criteria, with carbamidomethyl (C) and oxidation (M) as fixed and variable modifications, respectively. MS/MS analysis and repeated MASCOT database searches of a minimum of two precursor ions recognised in the PMF search were performed to confirm the PMF-based protein identification. Following the database searches, the number of peptide matches, sequence coverage, molecular weight, and pI were used to evaluate the results.

3. Results

The muscle samples in this study were analysed using two extraction methods; SDS and TES extraction (Figs. 1 and 2, respectively). While the SDS extraction can be considered a whole muscle homogenate, including the less soluble structural proteins of muscle, the TES extraction mainly includes the more soluble proteins, and can be referred to as a soluble muscle extract. A total of 376 and 459 spots were observed in the 2-DEs from the SDS and TES extractions, respectively. In the SDS-extracted muscle samples, 20 protein spots were significantly changed in abundance between the control and crowded salmon, and 8 of these were increased while the others were decreased in the crowded salmon. The differences in abundance between the two groups were relatively small and ranged from -1.5 to $+1.5$ -fold. In the TES-extracted muscle samples, 1 protein was increased while 6 were decreased in abundance in the crowded salmon, and the differences ranged from -1.7 to $+1.1$ -fold.

In the 2-DE from the blood plasma samples 474 protein spots were observed, and 17 of these were significantly changed in abundance between the control and crowded salmon (Fig. 3). Eleven of these had an increased abundance in the crowded salmon, while the remaining 6 spots showed a decreased abundance. The differences in abundance between these protein spots in control and crowded salmon ranged from -1.9 to $+9.9$ -fold, and thus showed a larger difference in protein expression than what was observed in the muscle sample extracts.

Based on the results from the statistical analyses, all spots that were found to differ significantly in abundance between control and crowded salmon were selected for identification. Out of the 44 protein spots showing altered abundance, 25 spots were successfully identified. Among the 19 protein spots that were not identified, 8 protein spots were not identified due to low protein concentration and 11 protein spots were not identified even with good quality PMF and MS/MS spectra. Among the 25 identified spots, 20 were identified by matching peptide data to salmon protein sequences in the database, while the remaining spots were identified due to interspecies homology to rainbow trout and seabass protein sequences (Tables 1 and 2).

Among the identified protein spots from the SDS muscle extracts (Table 1) two proteins were found in several spots on the 2-DE; myosin heavy chain (spots 3712 and 2410) and actin (spots 1206, 1301, 3202, 3203, 7301, and 8217). Some of the actin spots were fragments and others the full-length versions of this protein. Two additional structural proteins were also found to be decreased and increased in abundance, respectively; tropomyosin (spot 0201) and myosin regulatory light chain 2 (spot 0102). In addition to structural proteins, significant increases in abundance in the crowded salmon were also found for proteins involved in energy metabolism; creatine kinase (spot 8207), enolase (spot 8404 and 8411), and phosphoglycerate kinase 1 (spot 6206). From the TES muscle extracts, only two of the significantly altered proteins were successfully identified (Table 1), and both of these decreased in abundance in the crowded

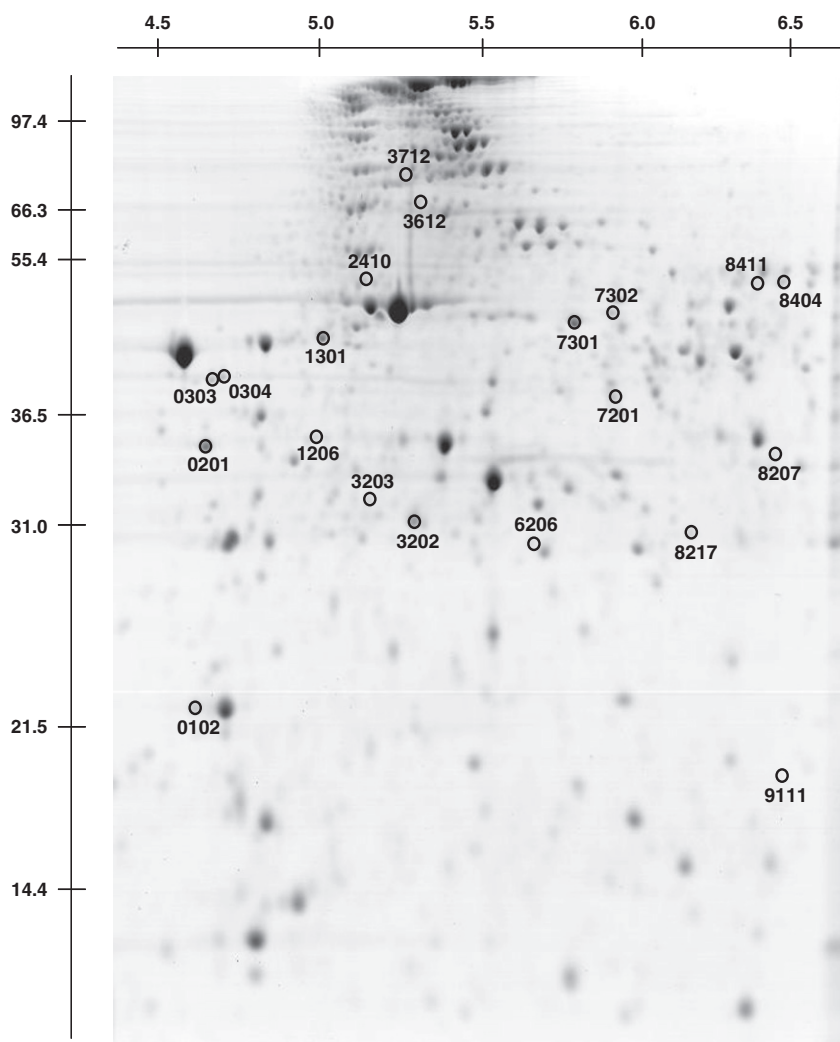


Fig. 1. A representative 2-DE gel of an SDS extract from salmon muscle. Protein (500 µg) was loaded, and 2-DE was performed using a pH range of 4–7 in the first dimension and SDS-PAGE (12%) in the second dimension. Proteins were visualized by colloidal Coomassie brilliant blue staining, and protein spots that were found to change due to crowding stress are numbered.

salmon; apolipoprotein A-I (spot 0108) and methylmalonate-semialdehyde dehydrogenase (spot 3601).

Nine of the proteins found to significantly change in abundance in the blood plasma samples were successfully identified (Table 2). Of the spots that were shown to decrease with crowding, three were identified as the transport protein apolipoprotein A-I (spots 1205, 2207 and 1308), while the other was an immunoglobulin light chain precursor (spot 7207). Among the spots found to increase with crowding were two different isoforms of actin (spots 2402 and 4304), angiotensinogen (spot 7522), and fragments of complement component 3, a protein of the immune system (spots 5902 and 7611).

4. Discussion

In order to get a comprehensive overview over the protein alterations induced by crowding stress, both muscle and blood plasma proteomes were analysed in this experiment. While alterations in both proteomes would give information regarding the general stress response in the fish, changes in the muscle proteome could in addition provide a link to the changes observed in several muscle quality traits as a result of the crowding procedure.

The crowded salmon investigated in this experiment showed classic signs of both primary and secondary stress response, such as increased levels of plasma cortisol, lactate, and osmolality, and a decreased level of plasma glucose (Veiseth et al., 2006). In addition to

these classic signs of stress response, the crowded salmon in this experiment were also found to have reduced muscle pH values immediately after slaughter, and they exhibited an accelerated and extended *rigor mortis* contraction (Veiseth et al., 2006).

By visual inspection of the salmon during the crowding treatment, we observed that the crowding lead to intense physical activity in the salmon, which would increase the energy demand in the muscle. Moreover, due to the duration of the crowding treatment, we observed a switch towards anaerobic energy production as seen by the increased plasma lactate concentration in this fish. This increase in plasma lactate concentration was similar to earlier reports of confinement stress in tilapia (Vijayan et al., 1997), exhaustive exercise in rainbow trout (Milligan, 1996) and crowding stress in Atlantic salmon (Skjervold et al., 1999, 2001). Moreover, a gene expression study in liver from rainbow trout subjected to an acute stressor showed an up-regulation of genes involved in energy metabolism (Wiseman et al., 2007). Indeed, several of the proteins with altered abundance in the muscle in our study were related to energy metabolism. One of the most immediate available sources of energy in muscle is through the transfer of phosphate from creatine phosphate (CP) to ADP yielding ATP and creatine. Creatine kinase is the enzyme that catalyses this reversible transfer of phosphate between CP and ATP, and a fragment of this enzyme was found to increase in abundance in the crowded salmon in this experiment. The degradation of creatine kinase observed in this study could be a result

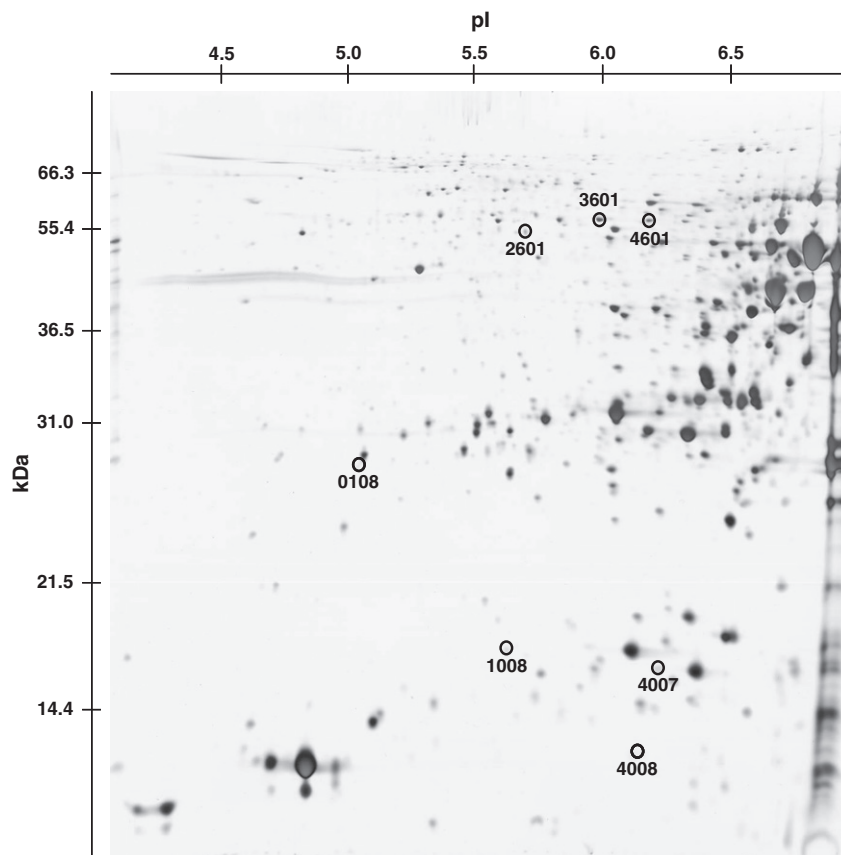


Fig. 2. A representative 2-DE gel of a TES extract from salmon muscle. Protein (100 µg) was loaded, and 2-DE was performed using a pH range of 4–7 in the first dimension and SDS-PAGE (12%) in the second dimension. Proteins were visualized by silver staining, and protein spots that were found to change due to crowding stress are numbered.

of reduced need for this enzyme once the CP reserve is emptied, or simply a result of more general protein degradation taking place under these stress conditions. Several groups using proteome analyses have reported changes in creatine kinase in relation to *postmortem* storage of mammalian muscle (Jia et al., 2007; Lametsch et al., 2002) and during anoxia in developing zebrafish embryos (Mendelsohn et al., 2009).

A second source of energy in the muscle is through the anaerobic energy production taking place in the glycolysis. In this experiment, 2 isoforms of enolase 3 were found to increase in the crowded salmon. Enolase 3 is a muscle specific enzyme that catalyses the ninth step of glycolysis. The increase in relative abundance of this enzyme in crowded salmon corresponds well with the observed changes in plasma lactate and glucose concentrations, since it may indicate an accelerated glycolysis. This would lead to decreased glucose and increased lactate levels, since they are the initial substrate and final product of the anaerobic glycolysis, respectively. A fragment of phosphoglycerate kinase 1, an enzyme that catalyses the seventh step of glycolysis, was also found to increase in the crowded salmon. Changes in these and several other enzymes involved in energy metabolism has previously been found for crowded trout (Morzel et al., 2006) and during *postmortem* storage of bovine muscles (Jia et al., 2006, 2007). On the contrary, high stocking density and handling have recently been found to reduce the level of several glycolytic enzymes in gilthead seabream liver, however this was thought to be a result of the activation of liver glycogenolysis and gluconeogenesis in order to maintain a sufficient supply of glucose to other organs, including muscle (Alves et al., 2010). In addition to the enzymes involved in energy metabolism, the abundance of methylmalonate-semialdehyde dehydrogenase was found to decrease in abundance in the crowded salmon in this experiment. This enzyme is involved in amino acid metabolism, specifically the valine and pyrimidine catabolic pathways. Gene

expression studies of the mummichog fish have shown a reduced expression profile of this gene as a result of chronic pollutant exposure (Oleksiak, 2008). Together these results indicate that stress induced by different stressors (i.e. crowding and pollution) can affect the amino acid metabolism in fish muscle.

Most of the identified proteins that changed in the crowded salmon muscle were related to structural proteins that are essential for muscle contraction, and six out of ten of these proteins were fragments. These fragments are not likely to be a result of proteolysis during the protein extraction procedure, but if so, it would affect the two treatment groups similarly since all samples were extracted simultaneously. Thus, the occurrence of these fragments indicates an increased level of proteolysis in the crowded salmon muscle. Previous reports have shown that cortisol may affect the synthesis and degradation of skeletal muscle proteins (McGrath and Goldspink, 1982; Odedra et al., 1983). Whether the changes observed in this experiment are directly related to cortisol concentration is not known, but crowding of rainbow trout prior to slaughter induced changes in several structural proteins in the muscle (Morzel et al., 2006). Moreover, stress prior to slaughter of Atlantic salmon has also been suggested to accelerate cathepsin activity resulting in faster muscle degradation (Bahuaud et al., 2010). Thus, our study provides additional support that stress may lead to an increased protein degradation process in the muscle.

One of the proteins found to decrease in abundance due to crowding in this study was apolipoprotein A-I (ApoA-I). This is the main protein constituent of high density lipoprotein (HDL), which is known to play a role in reverse cholesterol transport from tissues to the liver and in lipid metabolism. A similar decrease in ApoA-I abundance has also been found in plasma from pigs during road transport (Piñeiro et al., 2007). Moreover, ApoA-I has also been suggested to play a role in hyperosmotic regulation in amphidromous

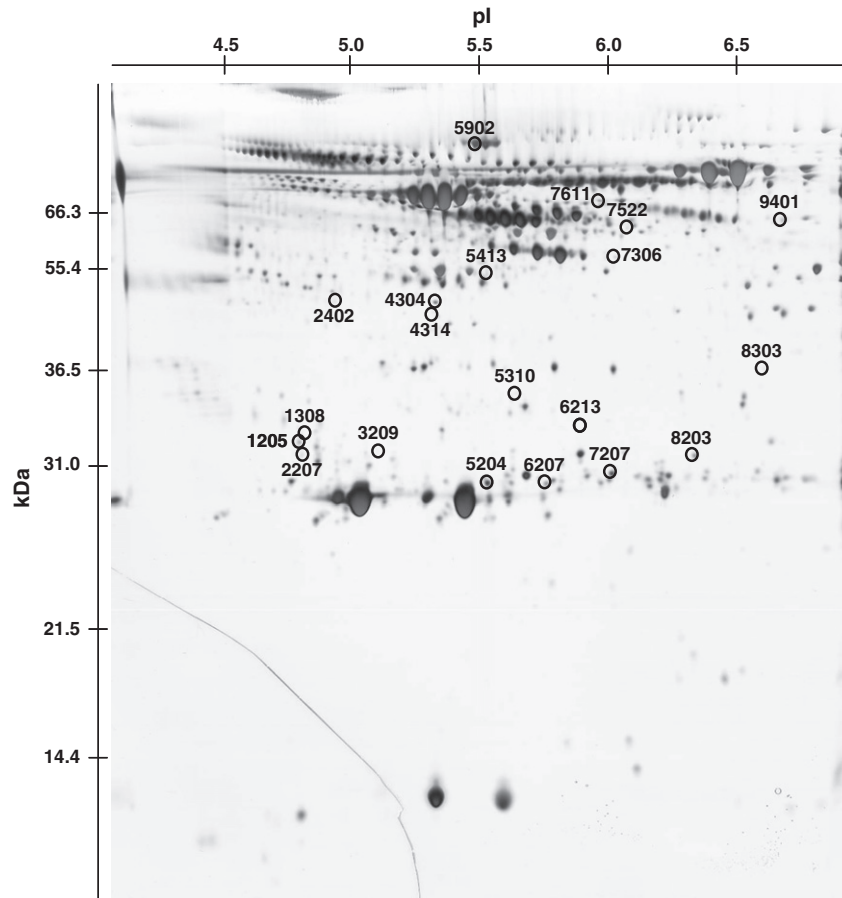


Fig. 3. A representative 2-DE gel of blood plasma from salmon. Protein (75 µg) was loaded, and 2-DE was performed using a pH range of 4–7 in the first dimension and SDS-PAGE (12%) in the second dimension. Proteins were visualized by silver staining, and protein spots that were found to change due to crowding stress are numbered.

fish, illustrated by its down-regulation when fish are transferred from freshwater to brackish water (Chen et al., 2009). Both of these functions of ApoA-I correspond well with the crowding-induced stress response and changes in osmolality seen in this study.

ApoA-I has also been reported to play a possible role in regulation of the complement system in fish (Magnadottir, 2006). The complement system is an important part of the innate mechanisms of immunity, and previous studies have shown that the complement

Table 1

Protein spots with significantly altered abundance between muscle from control and crowded salmon.

Spot number	Accession number ^a	Protein identification (species)	Fold change ^b	SC ^c	kDa/pI		PM ^d
					Observed	Theoretical	
<i>Proteins increased in abundance in the crowded salmon</i>							
SDS 8207	gi 197632231	Creatine kinase (<i>Salmo salar</i>), fragment	+ 1.5	29%	32.1/6.4	43.0/6.44	11
SDS 3712	gi 755771	Myosin heavy chain (<i>Oncorhynchus mykiss</i>), fragment	+ 1.4	18%	63.9/5.3	80.4/5.36	12
SDS 8217	gi 185133138	Fast myotomal muscle actin (<i>Salmo salar</i>), fragment	+ 1.4	38%	29.2/6.2	42.2/5.22	16
SDS 8411	gi 213513724	Enolase 3-2 (<i>Salmo salar</i>)	+ 1.4	45%	48.8/6.4	47.7/6.61	14
SDS 0102	gi 185132088	Myosin regulatory light chain 2 (<i>Salmo salar</i>)	+ 1.3	44%	20.5/4.6	19.1/4.72	7
SDS 6206	gi 197631857	Phosphoglycerate kinase 1 (<i>Salmo salar</i>), fragment	+ 1.3	8%	28.6/5.7	44.9/8.31	3
SDS 8404	gi 213511756	Enolase 3-1 (<i>Salmo salar</i>)	+ 1.2	47%	48.8/6.5	47.6/6.81	17
<i>Proteins decreased in abundance in the crowded salmon</i>							
TES 0108	gi 213512936	Apolipoprotein A-I (<i>Salmo salar</i>)	− 1.7	37%	29.0/5.0	29.7/5.17	19
SDS 2410	gi 755771	Myosin heavy chain (<i>Oncorhynchus mykiss</i>), fragment	− 1.4	19%	49.5/5.1	80.4/5.36	16
TES 3601	gi 218505653	Methylmalonate-semialdehyde dehydrogenase (<i>Salmo salar</i>)	− 1.3	29%	57.3/6.0	57.3/6.48	12
SDS 1206	gi 185133138	Fast myotomal muscle actin (<i>Salmo salar</i>), fragment	− 1.3	11%	32.8/5.0	42.2/5.22	3
SDS 0201	gi 185132405	Fast myotomal muscle tropomyosin (<i>Salmo salar</i>)	− 1.1	26%	32.4/4.6	32.5/4.63	10
SDS 1301	gi 10953948	Fast myotomal muscle actin (<i>Salmo salar</i>)	− 1.1	30%	41.3/5.0	42.2/5.22	9
SDS 3202	gi 185133138	Fast myotomal muscle actin (<i>Salmo salar</i>), fragment	− 1.1	16%	29.8/5.3	42.2/5.22	7
SDS 3203	gi 185133138	Fast myotomal muscle actin (<i>Salmo salar</i>), fragment	− 1.1	14%	30.6/5.2	42.2/5.22	4
SDS 7301	gi 185133138	Fast myotomal muscle actin (<i>Salmo salar</i>)	− 1.1	34%	42.8/5.8	42.2/5.22	14

^a NCBI nr ID accession number.

^b Change in abundance in crowded relative to control salmon.

^c Percentage of sequence coverage.

^d Number of peptides matched.

Table 2

Protein spots with significantly altered abundance between blood plasma from control and crowded salmon.

Spot number	Accession number ^a	Protein identification (Species)	Fold change ^b	SC ^c	kDa/pI		PM ^d
					Observed	Theoretical	
<i>Proteins increased in abundance in the crowded salmon</i>							
4304	gi 27805142	Beta actin (<i>Dicentrarchus labrax</i>)	+ 1.9	33%	48.1/5.3	42.1/5.29	13
7522	gi 213511078	Angiotensinogen (<i>Salmo salar</i>)	+ 1.9	38%	63.6/6.1	52.0/7.64	13
5902	gi 431608	Complement component C3 (<i>Oncorhynchus mykiss</i>), fragment	+ 1.7	12%	79.3/5.5	181.5/6.11	19
7611	gi 431608	Complement component C3 (<i>Oncorhynchus mykiss</i>), fragment	+ 1.5	6%	68.6/6.0	181.5/6.11	10
2402	gi 185133138	Fast myotomal muscle actin (<i>Salmo salar</i>)	+ 1.4	25%	48.4/4.9	42.2/5.22	10
<i>Proteins decreased in abundance in the crowded salmon</i>							
1205	gi 213512936	Apolipoprotein A-I (<i>Salmo salar</i>)	− 1.9	47%	31.9/4.8	29.7/5.17	22
2207	gi 213512936	Apolipoprotein A-I (<i>Salmo salar</i>)	− 1.9	18%	31.5/4.8	29.7/5.17	8
1308	gi 213512936	Apolipoprotein A-I (<i>Salmo salar</i>)	− 1.7	18%	32.2/4.8	29.7/5.17	10
7207	gi 10505167	Immunoglobulin light chain precursor (<i>Salmo salar</i>)	− 1.7	28%	30.7/6.0	26.5/6.29	6

^a NCBI nr ID accession number.^b Change in abundance in crowded relative to control salmon.^c Percentage of sequence coverage.^d Number of peptides matched.

system in fish is affected by both short-term and long-term crowding stress (Montero et al., 1999; Ortuño et al., 2001; Yin et al., 1995). In this study, two fragments of the complement component C3 were found to increase in the crowded salmon. This protein is known to play a central role in the activation of the three pathways of the complement system (Sahu and Lambris, 2001), however from this study it is not possible to conclude if the occurrence of these two fragments have any physiological relevance. Finally, there was a decrease in the abundance of an immunoglobulin light chain precursor. Thus, the results in this study indicate that a short-term (i.e. 40 min) exposure of salmon to crowding stress may induce changes in their immune system.

Angiotensinogen is a precursor to angiotensin, and plays a critical role in blood pressure regulation. Previous reports have shown that the gene coding for angiotensinogen is differentially expressed dependent on salinity acclimation in European flounder (Larsen et al., 2007, 2008). To our knowledge, this is the first report of changes in angiotensinogen level as a result of crowding of fish. However, this alteration corresponds well with the observed changes in the blood circulatory system during stress in fish.

Identification of protein spots extracted from the 2-DE gels has been a challenge in this experiment, as only 25 out of 44 protein spots were successfully identified. The reason for this lack of identification was for some of these protein spots related to the low amounts of protein present in the spot. This often resulted in a PMF spectrum where the autolytic peptides of trypsin completely overshadowed the peptides belonging to the protein of interest. However, MS analysis of about half of the unidentified protein spots resulted in good quality PMF and MS/MS spectra, and still did not yield a positive identification of the proteins. This could be caused by the lack of a complete genome sequence for the Atlantic salmon, and consequently, deviation in amino acid sequences in salmon proteins compared to the corresponding proteins in other annotated species may result in unsuccessful identification by MS.

5. Conclusions

The results from the proteome analyses in this study are in accordance with physiological measurements of stress response in these salmon (Veiseth et al., 2006). The proteins that were found to change in abundance are involved in several well-known secondary and tertiary stress responses, including altered energy metabolism and osmotic regulation. Changes were also observed in proteins related to the immune system, indicating that short-term exposure to crowding may induce changes in the immune system of salmon. Moreover, the changes observed in different metabolic proteins in the

muscle, indicating a shift towards anaerobic energy production, can explain the accelerated *postmortem* pH decline and *rigor mortis* contraction of the crowded salmon.

Author contribution

Eva Veiseth-Kent:
Experimental design; collection and analysis of data; preparation of manuscript.
Harald Grove:
Data analysis; reviewed and commented on the manuscript.
Ellen M. Færgestad:
Data analysis; reviewed and commented on the manuscript.
Svein O. Fjæra:
Experimental design; collection of data; reviewed and commented on the manuscript.

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