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A proteomic approach to assess the host response in gills of farmed Atlantic salmon *Salmo salar* L. affected by amoebic gill disease

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ABSTRACT

Amoebic gill disease (AGD), caused by the marine ectoparasite *Neoparamoeba perurans*, is one of the most significant infectious diseases for Atlantic salmon (*Salmo salar*) aquaculture. Upon colonisation of the host gills, the parasite induces a marked hyperplasia and fusion of the lamellar epithelium, which can severely compromise the host if left untreated. In order to investigate the host response during the disease at a protein level, sequential gill samples were collected and analysed using two-dimensional electrophoresis (2DE), with peptides of interest subjected to LC-MS/MS. Samples were obtained from an experimental challenge using naïve Atlantic salmon smolts and cultured *N. perurans*. Sampling points were set at 1, 2, 3, 7, 14 and 21 days post-infection, and included both sub-clinical and clinical stages of the disease. A total of 23 proteins differentially expressed between non-infected and infected individuals were successfully identified by LC-MS/MS. Findings included upregulation of prohibitin, cyclophilin A, apolipoprotein *A*1, ictacalcin, RhoGDP dissociation inhibitor α, components of the heat shock proteins 70 family and histones H3a and H4, and downregulation of peroxiredoxin-5 and cofilin. Among the most significant protein functions identified were cell cycle regulation, cytoskeletal regulation, oxidative metabolism and immunity. This is the first sequential proteomic analysis of gills during *N. perurans* infection. It is believed that the use of non-target screening techniques can contribute to the knowledge of gill responses to injury and pathogenic insults, including AGD.

1. Introduction

Amoebic gill disease (AGD) is a parasitic disease caused by the marine ectoparasite *Neoparamoeba perurans*. AGD was first described in farmed Atlantic salmon *Salmo salar* in Tasmania in 1986 (Munday, 1986), but since then it has been reported from numerous countries worldwide and in different marine finfish species. In recent years, AGD has become one of the most significant infectious diseases for Atlantic salmon aquaculture in Europe, causing severe economic losses to the industry (Rodger, 2014). Colonisation of the host gills by the parasite induces a marked hyperplasia and fusion of the lamellar epithelium, with associated increase in mucous cell numbers and increased mucus production. Macroscopically, lesions are noted as white raised mucoid patches (Adams et al., 2004; Adams and Nowak, 2003; Taylor et al., 2009; Zilberg and Munday, 2000).

Several studies have investigated the host response of Atlantic salmon to AGD, but most of the research to date has focused on the gene expression of classical immune molecules (Benedicenti et al., 2015; Morrison et al., 2007; Morrison et al., 2012; Pennacchi et al., 2014). Other studies have used non-target screening techniques, such as DNA microarrays (Morrison et al., 2006; Wynne et al., 2008a; Young et al., 2008), but significant knowledge gaps still remain. Proteomics is the large-scale study of proteins, including their structure and functions, and the proteome is the protein profile of a cell or tissue at a specific time point and under specific environmental conditions. There is a paucity of the application of proteomic techniques in fish and/or aquaculture studies, but interest has increased in recent years (Rodrigues et al., 2012). The use of proteomics to assess the host response of Atlantic salmon to AGD has been previously reported by Valdenegro-Vega et al. (2014), and proteins involved in cell to cell signalling, inflammation, and cell cycle regulation were identified in gill mucus of affected fish. Other proteomic studies on Atlantic salmon are Braceland et al. (2013) and Easy and Ross (2009), which assessed the immunity and

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pathogenesis of pancreas disease (salmonid alphavirus) and sea lice (*Lepeophtheirus salmonis*) respectively. In addition to disease, pathogen or immune based studies, proteomics research in aquaculture has also examined the effect of environmental stressors and toxins on different fish and shellfish species, and it is in this area where much of the gill proteomic studies are found (Ling et al., 2009; Lopes Rocha et al., 2015; Lu et al., 2010).

The characterization of the protein profile of a given disease can help to understand the disease pathogenesis and immune response, and to identify potential biomarkers (Ceciliani et al., 2014; Sanchez-Ovejero et al., 2016). The study presented here aims to investigate the gill response at the protein level of farmed Atlantic salmon during experimentally induced AGD using two-dimension electrophoresis (2DE) and subsequent protein identification via mass spectrometry (MS). In order to assess the changes involved during disease progression, several sampling points at sub-clinical and clinical stages of the disease were included. On the approach to assess the overall system, gill samples taken comprised whole gill arches after removal of the arch cartilage. Therefore, individual samples included mucus, gill filaments and gill circulating blood. Infected samples from clinical stages included both lesion and non-lesion areas.

2. Materials and methods

2.1. Amoeba culture

N. perurans were harvested from naturally infected Atlantic salmon from a commercial farm based on the west coast of Ireland. Gill mucus containing the parasite was collected and transferred into 0.2 μm-filtered sterile sea water. This solution was then plated into marine malt yeast agar plates (MMYA; 0.01% malt, 0.01% yeast, 2% bacteriology agar, sterile sea water at 35 practical salinity units (PSU)) and incubated at 18 °C. To limit bacterial growth, plates were washed every two days by changing the overlay water with new filtered sterile sea water. Amoebae were sub-cultured by inoculating free-floating amoebae onto fresh MMYA plates. The identity of the amoebae was confirmed by real-time polymerase chain reaction (RT-PCR) as described by Downes et al. (2015). Amoebae were kept in culture for 42 days from collection and seeding until the in-vivo challenge.

2.2. Fish husbandry and in-vivo challenge

Naive Atlantic salmon smolts (~85 g) were obtained from a commercial facility and kept at the Daithi O'Murchu Marine Research Station (Bantry, Co. Cork, Ireland) for experimental purposes. Fish ($n = 300$) were distributed into four circular 1000 L tanks ($n = 75$) and left to acclimatise for 10 days. Sea water temperature ranged from 10.5 °C to 11.5 °C and salinity was 35 PSU for the whole experimental period. Fish were fed a commercial salmon diet at 1% body weight per day. Prior to the inoculation of the infected tanks with *N. perurans*, the water level of all tanks was reduced to 300 L. Two of the four tanks were challenged with 1800 amoebae/L (infected tanks), whereas the other two tanks were used as negative controls (non-infected tanks). Following inoculation, both the infected and negative control tanks were maintained for 4 h at 300 L and subsequently refilled to 1000 L. This work was authorised by the Health Products Regulatory Authority (HPRA) in Ireland under project authorisation number AE19114/P001, following the Animals Scientific Procedures Act 1986 (Directive 2010/63/EU transposed into Irish law by S.I. No 543 of 2012).

2.3. Sampling procedures

Progression of the disease was assessed by gross examination of the gills at 1, 2, 3, 7, 14 and 21 days post-infection. At each sampling point, 10 fish per tank were anaesthetised (tricaine methanesulfonate) and gills scored as per the commercial standard protocol. In Atlantic salmon aquaculture a gross scoring system is applied to assess the severity and stage of AGD. The currently used system (adapted from Taylor et al., 2009) examines all gills arches from each individual for presence of AGD lesions, and ranges the severity of the macroscopic lesions from 0 (non-affected) to 5 (severely affected). Three of the ten fish examined from each tank were euthanized with an overdose of anaesthetic (tricaine methanesulfonate) and gill samples taken for RT-PCR, histopathology and proteomics analysis. The remaining seven fish per tank were allowed to recover in clean oxygenated seawater and returned to the trial tanks. Prior to the sampling of the gills, fish were bled to minimize blood contamination during the excision of the gill samples. Gill swabs from the 1st, 3rd and 4th right gill arches of each fish were obtained, and analysed using a RT-PCR designed for detecting *N. perurans* as described by Downes et al. (2015). For histopathology, the four left gill arches of each fish were sampled and fixed in 10% neutral buffered formalin. Samples were processed and embedded in paraffin wax blocks, sectioned (3–5 μm), and stained with haematoxylin and eosin (H&E). Histopathology was undertaken to confirm AGD and to assess presence of microscopic AGD lesions at the early stages of the infection. For proteomic analysis, the 2nd right gill arch from each fish was excised, the arch cartilage removed, and the remaining gill filaments snap frozen in dry ice and kept at −80 °C until used.

2.4. Sample preparation and two-dimensional electrophoresis (2DE)

Each gill sample was weighed and homogenized (T10 basic Ultra-Turrax®, IKA) in 1:10 lysis buffer (50 mM Tris-HCl pH 8.0, 1% Triton X-100). Homogenates were vortexed for 15 min, centrifuged for 10 min (10,000 g at 4 °C), and the resulting supernatant filtrated using 0.45 μm cellulose acetate syringe filters (Minisart®). Total protein concentration of each individual sample was determined by the bicinchoninic acid (BCA) assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific) using a bovine serum albumin (BSA) standard curve.

Based on the RT-PCR results, four out of the six time points were selected for proteomic analysis (2, 7, 14 and 21 days post-infection). For each of the selected time points, the protein samples of all positive and all negative individuals were pooled, resulting in six fish per pool, and a total of four negative (non-infected) and four positive (infected) pools. Pooling was done to minimize the biological variation between individuals and tanks, and each fish in the pool contributed with the same amount of protein (not volume). Each pooled sample was run and analysed in triplicate, thus a total of 24 gels were produced. Pooled samples containing a total of 252 μg of protein (42 μg of protein from each fish) were solubilized in rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte®) (BioRad) and separated by pI using 11 cm pH 3–10 Immobilized pH Gradient (IPG) strips (BioRad). Loaded IPG strips were rehydrated for 12 h and focused in a PROTEAN® i12™ IEF system (BioRad) for 35,000 Vh. Focused strips were equilibrated in two consecutive steps: 20 min in reducing buffer (6 M urea, 0.375 M Tris–HCl pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol, 2% (w/v) DTT) and 20 min in alkylating buffer (6 M urea, 0.375 M Tris–HCl pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol, 2.5% (w/v) iodoacetamide) (Sigma-Aldrich). IPG strips were then placed into 4–15% Criterion™ Tris-HCl Gels (BioRad) and subjected to electrophoresis at 200 V. Subsequently, gels were stained for 1 h in a 0.3% (w/v) Coomassie Brilliant Blue R-250 solution containing 10% acetic acid, 45% methanol and 45% dH_2O, and then de-stained using a solution of 20% acetic acid, 7.5% methanol and 72.5% dH_2O.

2.5. Gel image analysis

Gels were scanned using a PharosFX™ Plus molecular imager (BioRad), and uploaded into the SameSpots software (Totallab, Nonlinear Dynamics, Newcastle UK), which automatically aligned the different gels. The normalised volumes of each detected spot were used to identify the protein spots differently expressed between groups using the

between-subject experimental design. Results were filtered using the programme's statistical analysis function, with only those with an ANOVA significance score of <0.05 being considered for further analysis. Twenty-three spots were chosen for protein identification.

2.6. Spot preparation and mass spectrometry

Selected spots were manually excised using a sterile scalpel and placed in individual vials for in-gel digestion. Gel pieces were washed three times in 100 μL of 50 mM ammonium bicarbonate, 50% v/v methanol and then twice in 100 μL 75% v/v acetonitrile, before drying. Gel pieces were rehydrated with trypsin solution (20 μg trypsin/mL 20 mM ammonium bicarbonate), and incubated for 4 h at 37 °C. Peptides were extracted from the gel pieces by washing twice in 100 μL of 50% v/v acetonitrile/0.1% v/v trifluoroacetic acid, before being transferred in solution to a fresh 96 well plate and dried before mass spectrometric (MS) analysis.

Peptides were solubilized in 2% acetonitrile with 0.1% trifluoroacetic acid and fractionated on a nanoflow uHPLC system (Thermo RSLCnano) before online analysis by electrospray ionisation (ESI) mass spectrometry on an Amazon ion trap MS/MS (Bruker Daltonics). Peptide separation was performed on a Pepmap C18 reversed phase column (LC Packings). Peptides were desalted and concentrated for 4 min on a C18 trap column followed by an acetonitrile gradient (in 0.1% v/v formic acid) (3.2–32% v/v 4–27 min, 32% to 80% v/v 27–36 min, held at 80% v/v 36–41 min and re-equilibration at 3.2%) for a total time of 45 min. A fixed solvent flow rate of 0.3 μL/min was used for the analytical column. The trap column solvent flow rate was fixed at 25 μL/min using 2% acetonitrile with 0.1% v/v trifluoroacetic acid. Mass spectrometric (MS) analysis was performed using a continuous duty cycle of survey MS scan followed by up to ten MS/MS analyses of the most abundant peptides, choosing the most intense multiply charged ions with dynamic exclusion for 120 s.

MS data were processed using Data Analysis software (Bruker) and the automated Matrix Science Mascot Daemon server (v2.4.1). Protein identifications were assigned using the Mascot search engine to interrogate protein sequences in the Actinopterygii taxonomy of the NCBI GenBank database, allowing a mass tolerance of 0.4 Da for both MS and MS/MS analyses.

3. Results and discussion

3.1. In-vivo challenge, RT-PCR and histopathology

The first gross AGD lesions were noted at 14 days post-infection (p.i), when the average gill scores for both infected tanks were 1.9. At the last sampling point (21 days p.i), the average gill score for the two infected tanks was 3.3 ± 0.1. Gill lesions were distributed homogeneously between individuals, with all fish sampled at each time point showing similar gill scores. Although no consistent macroscopic AGD lesions were noted at the earlier stages, half of the fish examined at 7 days p.i showed minor pale areas on the gills that may have indicated early lesions. Histopathology assessment revealed mild multifocal hyperplasia and fusion of the lamellar epithelium at 2 days p.i, but the first consistent microscopic AGD lesions (proliferation and fusion of the lamellar epithelium with presence of amoebae) were observed at 7 days p.i. All the challenged fish sampled at 1 day p.i. tested negative by RT-PCR, but analysis of gill swabs confirmed the presence of *N. perurans* from 2 days p.i onwards in the challenged fish. Three out of the six, and one out of the six challenged fish sampled at 2 and 3 days p.i, respectively were positive by RT-PCR. Based on these PCR results, sampling points 1 and 3 days p.i were not included in the proteomic analysis. All challenged fish sampled at 7, 14 and 21 days p.i tested positive for *N. perurans* by RT-PCR. A rapid increase in the gill score was observed from day 7 to day 14 p.i. Such rapid disease progression can also occur in field cases with naive smolts (*pers. obs.*), but a lower infection dose may prove beneficial in future in-vivo studies to slow down the disease development. The first gross lesions were noted at approximately two weeks after the first RT-PCR detection. This coincides with what has been observed in field cases (*pers. obs.*). Non-infected samples subjected to gill scores, RT-PCR and histopathology analysis, remained negative for the duration of the experiment.

3.2. 2DE and protein identification

The average protein concentration of all individual samples was 7.1 μg/μL ($n = 48$, SD $\pm$ 0.89), and no significant differences were noted between protein concentrations of non-infected and infected samples. In total, 549 spots were identified by the SameSpots software, of which 39 spots showed statistically significant differences over the course of infection. Twenty-three of these 39 spots (Fig. 1) were successfully characterised by LC-MS/MS analysis and identified using the MASCOT database restricted to Actinopterygii. The mean fold changes for each spot identified (at specified time points) are detailed in Table 1.

Several proteins with functions in immunity, cellular proliferation and/or oxidative metabolism were differentially expressed between infected and non-infected fish at different stages of the trial. Cellular hyperplasia and excess mucus production are key features of AGD. Oxidative stress (imbalance between oxidants and antioxidant defences) has been previously suggested to occur during the clinical stages of AGD (Loo et al., 2012; Wynne et al., 2008a). The functions and potential roles on the pathogenesis of AGD of the most relevant proteins identified are discussed here; together with current knowledge gaps and future research needs.

Prohibitin (PHB) (spot 1463) was over-expressed during the clinical stages of the disease, with fold changes of $+1.2$ and $+1.6$ at 14 and 21 days p.i respectively. PHB is a highly conserved protein mainly located in the cell mitochondria, nucleus and plasma membrane (Peng et al., 2015), although it has also been recently detected in human airway gland mucus (Joo et al., 2015). PHB is a multifunctional protein with roles in cell proliferation, mitochondrial function, cell signalling, oxidative stress, and inflammation (Merkwirth and Langer, 2009; Mishra et al., 2010). Upon oxidative stress, PHB is up-regulated to prevent oxidative-induced apoptosis and inflammation (Theiss et al., 2007), which could explain the increase seen in this study. PHB is up-regulated in certain types of cancer and cancer cell lines (Cao et al., 2016; Cheng et al., 2014; Guo et al., 2012; Sievers et al., 2010), where it is positively associated with cell survival, proliferation and migration. Along these lines, Rajalingam et al. (2005) showed the direct association of PHB with epithelial cell migration, through its interaction with the Ras-induced Raf-MEK-ERK activation pathway, which is one the best characterised MAPK signalling pathways and controls important functions such as cell proliferation, apoptosis and differentiation (McCubrey et al., 2007). The interaction between c-Raf and prohibitin was also proven by Polier et al. (2012). Interestingly, another pathogenic amoeba, *Entamoeba histolytica*, has been shown to activate multiple MAPK pathways (although primarily the ERK pathway) through the interaction of the parasitic Gal/GalNAc adherence lectin with the host intestinal epithelial cells (Rawal et al., 2005). Interactions between parasitic lectins and host glycoconjugates are known to play a significant role in the adhesion and interaction between parasites and hosts, and a lectin-mediated attachment has been suggested for *N. perurans* (Nowak et al., 2014). In humans, the ERK-MAPK pathway has also been associated with mucin production (Dilly et al., 2015; Perrais et al., 2002; Song et al., 2005) and Th2-type response (Lee et al., 2006; Yamashita et al., 2005), both factors linked to AGD. The ERK-MAPK pathway in breast cancer has also been implicated in the positive regulation under physiological stress conditions of the anterior gradient protein 2 (Zweitzig et al., 2007), a protein associated with AGD pathogenesis (Morrison et al., 2006). Although extrapolation between species and pathological conditions may not be possible at the current state of knowledge, the potential involvement of the ERK-MAPK pathway in AGD may be worthy of

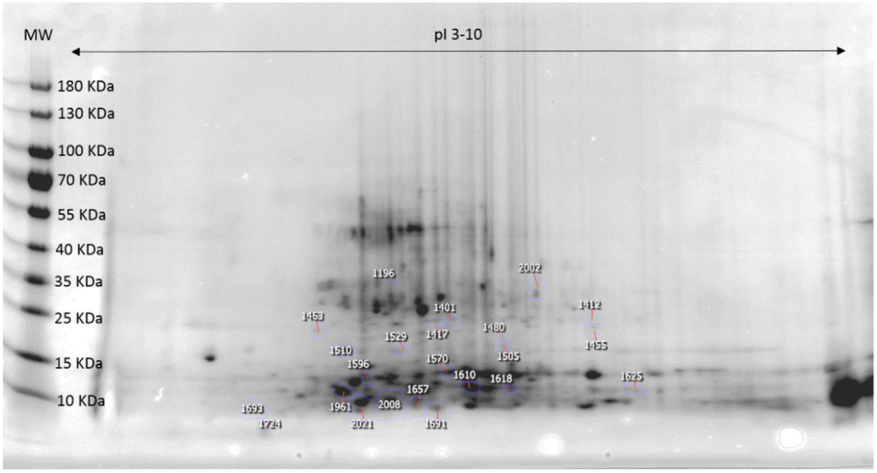

Fig. 1. Atlantic salmon gill protein spots on 2DE selected for mass spectrometry analysis (gel image corresponds to pool of positive samples at 14 days p.i.).

further investigation. Finally, PHB can also bind to C3 and enhance complement activation (Mishra et al., 2007). The complement C3 factor was upregulated using gel free proteomics in gill mucus of AGD affected fish (Valdenegro-Vega et al., 2014).

Rho GDP dissociation inhibitor α (RhoGDIα) (spot 1510) was overexpressed at 14 (+1.2) and 21 (+1.4) days p.i. RhoGDIs are key regulators of RhoGTPases, a sub-family of the Ras proteins superfamily which regulate actin cytoskeletal dynamics. RhoGTPases are therefore involved in many aspects of cell regulation, including morphology and migration, gene transcription, and cell cycle progression (Etienne-Manneville and Hall, 2002). Although initially described as negative regulators, recent evidence suggests that RhoGDIs also act as positive regulators of RhoGTPases (Wang et al., 2014; Zhao et al., 2008); which in this case may suggest that the increase seen at the late stages of the infection challenge is associated with gill morphological and structural changes (cell hyperplasia and fusion) occurring during AGD. In relation to the ERK-MAPK pathway discussed in the above paragraph, RhoGTPases have been described to be necessary for Ras-stimulated Raf activation (Li et al., 2001). In Valdenegro-Vega et al. (2014), RhoGDIα was also detected but appeared downregulated. In that study, however, the challenged fish were not naïve to *N. perurans* and only the gill mucus was analysed, factors that complicate a comparison between results. In Morrison et al. (2006), the related Rho GTPase activating protein IIA was up-regulated in AGD affected fish.

2-Peptidylprolyl isomerase A (PPIA) (spot 1625), more well known as cyclophilin A (CypA), was overexpressed at 2 (+1.3), 7 (+1.3) and 14 (+1.5) days p.i. CypA, a member of the immunophilin superfamily, is involved in several physiological processes such as inflammation, infection, cell proliferation and oxidative stress. CypA secretion is promoted during inflammation, acting as a potent chemoattractant for neutrophils, monocytes and T cells (Huang et al., 2015; Xu et al., 1992). CypA up-regulation occurs in different types of inflammation including sepsis, autoimmune disease and allergy (Arora et al., 2005). CypA is also up-regulated upon oxidative stress to prevent oxidative stress-induced apoptosis (Jin et al., 2000). CypA has also been associated with cell growth, tumorigenesis and drug resistance in a range of cancers (Lavin and Mc Gee, 2015; Lee and Kim, 2010). The exact role of CypA on the pathogenesis of AGD is unclear with the existing data and requires further investigation. However, the fact that it was up-regulated from 2 to 14 days p.i may indicate its involvement in more than one physiological process. In relation to the previously suggested cell proliferation pathway, CypA has also been reported to activate the ERK-MAPK cascade (Philp and Wang, 2003; Yang et al., 2007).

Cofilin-2-like protein was detected in spot 1412, showing negative fold changes of −1.4 and −1.2 at 14 and 21 days p.i respectively. Cofilin (CFL) is an actin-binding protein with important roles in regulating actin cytoskeletal dynamics, as well as in mitochondrial induced apoptosis (Lennicke et al., 2015). CFL is a key target of oxidation, which causes it to lose its affinity for actin and to translocate from the cytoplasm to the mitochondria where it induces apoptosis (Klamt et al., 2009; Zdanov et al., 2010). Reduction in CFL protein levels results in inhibition of apoptosis, while CFL over-expression increases apoptosis (Chua et al., 2003). The CFL decrease seen here may be inhibiting oxidative-induced apoptosis in the proliferated gill epithelium, but the mechanism of this is unknown. Due to its role in regulating actin dynamics, CFL can also affect cell motility and migration. Over-expression of CFL-1 has been associated with suppressed mobility of lung cancer cells (Wang et al., 2016). CFL is also involved in T-cell activation and migration, and oxidation of CFL induces T-cell hyporesponsiveness (Klemke et al., 2008; Samstag et al., 2013).

Peroxiredoxin-5 (PRDX5) (spot 1455) appeared down-regulated at 14 and 21 days p.i, showing fold changes of −1.6 and −1.7 respectively. Peroxiredoxins are cytoprotective antioxidant enzymes, but important immune functions of peroxiredoxins are also recognised for both mammals (Park et al., 2016) and fish (Valero et al., 2015). In a transcriptomic study, Wynne et al. (2008a) described down-regulation of at least four genes with antioxidant properties in AGD affected fish, suggesting that fish at the terminal stages of AGD may be suffering from oxidative stress. In a subsequent study, Loo et al. (2012) showed downregulation of peroxiredoxin 1 in Atlantic salmon gills at 14 days p.i with *N. perurans*. Peroxiredoxin downregulation was also noted in AGD affected fish in the transcriptomic study carried out by Morrison et al. (2006). Information on the response of host peroxiredoxins to parasitosis is scarce in comparison to data available for bacterial and viral infections. Despite this, depletion of host antioxidant enzymes (including peroxiredoxin) has been reported for a range of parasitic diseases and hosts (Al-Wahab et al., 2009; Castellanos-Martínez et al., 2014; Koinarski et al., 2005), although the mechanisms underlying these observations are not well understood. In one of the few studies found in fish, Pérez-Sánchez et al. (2011) assessed the mRNA expression of PRDX1 to PRDX6 in head kidney of gilthead sea bream (*Sparus aurata*) challenged with the intestinal parasite *Enteromyxum leei*. They compared un-infected fish with (a) exposed but not infected fish, and (b) exposed and infected fish. Exposed but not infected fish showed upregulation of PRDX 1, 2 and 3, while exposed and infected fish showed downregulation of PRDX 1, 3, 5 and 6. In the study presented here, PRDX 5 was significantly down-regulated at the late stages of the infection. At the early infection stages, PRDX5 appeared up-regulated but the levels were not statistically significant and therefore are not shown in the results (Table 1). However, this hypothetical first up- and subsequent down-

Table 1
2DE protein spots demonstrating significant ($p < 0.05$) fold changes in salmon gills post inoculation with *N. perurans* and identified by LC-MS/MS in combination with searches in the MASCOT database restricted to Actinopterygii.

Spot number	Accession number	Protein identification [Species]	Mean fold change (n = 3)	Estimated MW (Da)	Protein MASCOT Score	Matches[a]	Sequences[b]	Main biological functions
1693	gi| 208479658	Histone H3a, partial [*Tahuantinsuyoa macantzatza*]	+2.1 (7 d p.i)	12,394	51	6(1)	4(1)	DNA metabolism
1505	gi| 831294759	Histone H3-like [*Clupea harengus*]	+1.6 (7 d p.i) +1.5 (14d p.i)	24,384	57	3(1)	3(1)	DNA metabolism
1961	gi| 584032662	Histone H4-like, partial [*Neolamprologus brichardi*]	+1.2 (2 d p.i) +1.7 (7 d p.i)	10,915	119	16(4)	6(2)	DNA metabolism
1196	gi| 223646978	α-Enolase [*Salmo salar*]	+1.6 (14 d p.i)	47,484	213	13(6	6(4)	Carbohydrate metabolism
1529	gi| 929293264	Pyruvate dehydrogenase E1 component subunit β, mitochondrial [*Salmo salar*]	−1.5 (2 d p.i)	43,434	133	5(3)	4(2)	Carbohydrate metabolism
1401	gi| 209,737,406	Apolipoprotein A-I-1 precursor [*Salmo salar*]	+1.2 (14 d p.i) +1.3 (21 d p.i)	27,657	251	40(10)	15(7)	Lipid transport and metabolism
1596	gi| 213512628	ATP synthase subunit α, mitochondrial [*Salmo salar*]	+1.3 (14 d p.i)	57,212	451	25(12)	8(6)	Energy metabolism
	gi| 929097585	Haemoglobin subunit β-like [*Salmo salar*]		16,199	303	24(10)	9(6)	Oxygen transport
2021	gi| 209732604	Ictacalcin [*Salmo salar*]	+1.2 (14 d p.i) +1.3 (21 d p.i)	10,336	312	11(8)	1(1)	Calcium binding
2008	gi| 209732604	Ictacalcin [*Salmo salar*]	+1.1 (21 d p.i)	10,336	181	5(4)	1(1)	Calcium binding
1691	gi| 929097585	Haemoglobin subunit β-like [*Salmo salar*]	−1.2 (21 d p.i)	16,199	119	18(4)	7(2)	Oxygen transport
1570	gi| 929097585	Haemoglobin subunit β-like [*Salmo salar*]	+1.4 (14 d p.i) −1.5 (21 d p.i)	16,199	337	34(9)	12(5)	Oxygen transport
	gi|1431593	β-globin [*Salmo salar*]		16,215	261	32(9)	12(6)	
1657	gi|1431598	β-globin [*Salmo salar*]	+1.4 (14 d p.i) +1.1 (21 d p.i)	12,821	106	10(3)	4(2)	Oxygen transport
	gi| 185132519	Haemoglobin subunit β [*Salmo salar*]		16,229	94	9(3)	4(2)	
	gi| 209732604	Ictacalcin [*Salmo salar*]		10,336	73	1(1)	1(1)	Calcium binding
1480	gi| 185132519	Haemoglobin subunit β [*Salmo salar*]	+1.6 (7 d p.i) +1.5 (14 d p.i)	16,229	159	5(3)	3(2)	Oxygen transport
	gi| 213512524	Transgelin-2 [*Salmo salar*]		21,303	63	2(1)	2(1)	Structural (actin binding)
1417	gi|1431598	β-globin [*Salmo salar*]	+1.4 (21 d p.i)	12,821	142	7(4)	6(3)	Oxygen transport
	gi| 929221500	Keratin, type I cytoskeletal 13-like [*Salmo salar*]		49,325	82	12(2)	9(2)	Structural
1724	gi|1431593	β-globin [*Salmo salar*]	+2.0 (7 d p.i)	16,215	72	4(1)	2(1)	Oxygen transport
	gi| 884957753	Keratin, type I cytoskeletal 13-like [*Esox lucius*]		32,877	55	3(1)	2(1)	Structural
1412	gi| 929088871	Cofilin-2-like [*Salmo salar*]	−1.4 (14 d p.i) −1.2 (21 d p.i)	18,943	123	17(3)	4(1)	Structural (actin binding)
2002	gi| 213514058	Heat shock cognate 71 kDa protein [*Salmo salar*]	+1.6 (21 d p.i)	71,581	669	62(8)	18(9)	Stress response
	gi| 209155490	Heat shock cognate 70 kDa protein [*Salmo salar*]		72,339	367	29(10)	12(7)	
	gi| 213513866	Heat shock protein 70 [*Salmo salar*]		71,214	262	15(7)	7(5)	
1455	gi| 209730614	Peroxiredoxin-5, mitochondrial precursor [*Salmo salar*]	−1.6 (14 d p.i) −1.7 (21 d p.i)	20,369	216	29(10)	6(6)	Antioxidant response
1625	gi| 213514672	2-peptidylprolyl isomerase A (Cyclophilin A) [*Salmo salar*]	+1.3 (2 d p.i) +1.3 (7 d p.i) +1.5 (14 d p.i)	17,834	163	8(6)	3(3)	Immune response
	gi| 185132519	Haemoglobin subunit β [*Salmo salar*]		16,229	152	10(4)	6(3)	Oxygen transport
1463	gi| 213515458	Prohibitin [*Salmo salar*]	+1.2 (14 d p.i) +1.6 (21 d p.i)	29,810	149	13(5)	7(5)	Cell survival and mitochondrial function
1510	gi| 39645438	Rho GDP dissociation inhibitor (GDI) α [*Danio rerio*]	+1.2 (14 d p.i) +1.4 (21 d p.i)	23,147	81	7(2)	3(2)	Signalling
1610	gi| 688562948	Uncharacterized protein LOC100006146 [*Danio rerio*]	−1.4 (2 d p.i)	103,577	263	30(9)	11(4)	Uncharacterized
	gi| 961996792	Uncharacterized protein LOC106906734 [*Poecilia mexicana*]		46,459	259	25(8)	9(3)	
	gi|1431592	α-globin [*Salmo salar*]		13,176	147	15(5)	4(2)	
1618	gi| 688563016	Uncharacterized protein LOC100002181 [*Danio rerio*]	−1.4 (7 d p.i)	59,409	297	28(8)	10(3)	Uncharacterized
	gi|	Uncharacterized protein LOC105935932		43,965	297	27(8)	9(3)	

(continued on next page)

Table 1 (*continued*)

Spot number	Accession number	Protein identification [Species]	Mean fold change (n = 3)	Estimated MW (Da)	Protein MASCOT Score	Matches[a]	Sequences[b]	Main biological functions
	831482706 gi| 688562948	[*Fundulus heteroclitus*] Uncharacterized protein LOC100006146 [*Danio rerio*]		103,577	280	30(8)	11(3)	

[a] Total number of peptide matches. The number in parenthesis indicates the number of matches above the significance threshold ($p < 0.05$).
[b] Total number of distinct peptide sequences. The number in parenthesis indicates the number of matches above the significance threshold ($p < 0.05$).

regulation is in line with that described by Pérez-Sánchez et al. (2011), and may reflect the role of peroxiredoxins in the initial innate immune response.

Apolipoprotein A-I-1 precursor (pro-ApoA-I-1) was identified from one spot (spot 1401), and appeared upregulated at 14 and 21 days p.i (+1.2 and +1.3 fold increase respectively). Apolipoprotein A1 (ApoA-1) and apolipoprotein A2 (ApoA-2) are the most abundant apolipoproteins in fish (Dietrich et al., 2014), and both are protein fractions of high-density lipoproteins (HDL). HDL are the most abundant plasma lipoproteins in the majority of vertebrates, but they are present at even higher levels in fish (Dietrich et al., 2014), since most fish species use lipids as their primary energy source (Watanabe, 1982), in contrast to mammals which use carbohydrates. Apolipoproteins are involved in the metabolism and transport of lipids, but important immune functions are also recognised. Mammals have a wide range of apolipoproteins, some of which are considered to be negative (including ApoA-1) and others positive acute phase proteins (Carpintero et al., 2005; Hyka et al., 2001; Khovidhunkit et al., 2004). In mammals, ApoA-1 has been shown to have antioxidant, anti-tumorigenic, and anti-inflammatory activity (including anti-inflammatory role in airway hyperresponsiveness) (Park et al., 2013; Wang et al., 2010; Zamanian-Daryoush et al., 2013). In fish, up-regulation of ApoA-1 has been reported following bacterial (Caipang et al., 2008; Pridgeon and Klesius, 2013; Wei et al., 2015), viral (Braceland et al., 2013; Wei et al., 2015) and parasitic infections (Easy and Ross, 2009; Mohapatra et al., 2016; Pardo et al., 2012; Valdenegro-Vega et al., 2014), and antibacterial activity of ApoA-1 has been demonstrated in-vitro (Concha et al., 2003; Dietrich et al., 2014; Villarroel et al., 2007). ApoA-1 was also observed upregulated (fold change of +2.5) in Valdenegro-Vega et al. (2014). Although the mechanisms underlying these reported observations are not known, it is agreed that ApoA-1 plays a role in the innate immune response of fish. In Atlantic cod (*Gadus morhua*), ApoA-1 appeared to be closely associated with the C3 component of the complement system (Magnadóttir and Lange, 2004). ApoA-1 is present in liver, plasma, mucus and mucosal surfaces (gills, skin and intestine) of fish (Easy and Ross, 2009; Valdenegro-Vega et al., 2014; Villarroel et al., 2007). Besides its synthesis in the hepatocytes, Smith et al. (2005) described the synthesis of the precursor of ApoA-1 in rainbow trout gills in-vitro, and suggested that ApoA-1 may also play a role in the barrier properties (i.e. permeability) of the gills of freshwater fish.

Components of the heat shock proteins 70 family (Hsp70s) were detected in spot 2002, showing a +1.6 fold increase at 21 days p.i. Heat shock proteins (HSPs) are a group of chaperone proteins that include several different molecular weight class families, of which the Hsp70 family represents the most highly conserved (Rohde et al., 2005). Expression of Hsp70 significantly increases in response to both abiotic and biotic factors including oxidative stress and infection (Basu et al., 2002; Iwama et al., 1998). Several fish studies have reported over-expression of Hsp70 during bacterial (Ackerman and Iwama, 2001; Forsyth et al., 1997), viral (Zhang et al., 2013) and parasitic infections (Sitjà-Bobadilla et al., 2008). Expression of Hsp70s in gill and mucus has been previously detected in proteomic studies (Jurado et al., 2015; Ling et al., 2009; Lu et al., 2010; Provan et al., 2013; Sanahuja and Ibarz, 2015). In a recent paper, Hsp70 genes were up-regulated in gills of channel catfish after infection with *Flavobacterium columnare* (Song et al., 2016), and Hsp70 has also been up-regulated in gills in response to toxic and salinity insults (Ling et al., 2009; Lu et al., 2010). HSPs are known to be involved in the activation of both innate and adaptive immune responses. HSPs (including Hsp70) can activate antigen presenting cells (dendritic cells and macrophages) and trigger the pro-inflammatory cytokine cascade by acting as independent immunogens or by binding to exogenous or endogenous antigenic peptides (Colaco et al., 2013; Milani et al., 2002; Osterloh and Breloer, 2008; Prohászka and Füst, 2004; Srivastava, 2002). HSP-antigen complexes also facilitate the loading and presentation of the antigenic peptides by MHC-I and MHC-II to CD8+ and CD4+ T cells, inducing the adaptive immune response (Fischer et al., 2010; Srivastava, 2002; Suzue et al., 1997). In mammals, HSPs have also been widely studied in relation to cancer. Hsp70 is overexpressed in cancer cells, and can inhibit apoptosis and interact with p53 tumour suppressor protein (Dudeja et al., 2009; Fourie et al., 1997; Rérole et al., 2011). Morrison et al. (2006) reported down-regulation of the p53 protein in AGD lesions, and suggested that the proliferative response of the gill epithelial cells may be mediated by the inhibition of p53. Human studies have also shown HSP70 to be involved in airway mucin secretion (Fang et al., 2013; Park et al., 2006) and asthma (Vignola et al., 1995), and both in mammals and zebrafish, the ERK-MAPK pathway has been reported to induce expression of Hsp70 (Keller et al., 2008).

Ictacalcin was identified in two spots (2021 and 2008). Both spots showed a mild volume increase at the last sampling points, with fold changes of +1.2 (14 days p.i) and +1.3 (21 days p.i) for spot 2021, and fold change of +1.1 (21 days p.i) for spot 2008. Ictacalcin is a novel member of the S100 family of calcium-binding proteins only present in teleosts, and first described in channel catfish *Ictalurus punctatus* (Bettini et al., 1994). Calcium-binding proteins play an important role in the regulation of many cellular functions, including enzymatic regulation, maintenance of cell shape and motility, modulation of signal transduction pathways and regulation of calcium homeostasis (Kraemer et al., 2008). S100 proteins in mammals have also been reported to be pro-inflammatory, acting as damage associated molecular patterns (DAMPs), chemoattractants and to function as antimicrobial peptides (Pouwels et al., 2014; Yang et al., 2001). In Atlantic salmon, ictacalcin levels were found to be increased in skin mucus of individuals infected with sea lice (Easy and Ross, 2009). The specific functions of ictacalcin are as yet unknown, but it has been found to be highly expressed in epithelial cells (including skin and gills) of channel catfish (Porta et al., 1996) and zebrafish *Danio rerio* (Hsiao et al., 2003; Kraemer et al., 2008).

Partial histones H3a and H4 were upregulated at the earlier infection stages in spots 1693, 1961 and 1505. Histone H3a-partial showed a fold change of +2.1 at 7 days p.i in spot 1693, and fold changes of +1.6 and +1.5 at 7 and 14 days p.i respectively in spot 1505. Partial histone H4 detected in spot 1961 showed fold increases of +1.2 and +1.7 at 2 and 7 days p.i respectively. Histones are primary components of chromatin in eukaryotic cells, but in addition to their structural role they are also known to be involved in cellular signalling and innate immunity. Histones are known antimicrobial peptides (AMPs) in a wide range of invertebrate and vertebrate species including fish (Dráb et al., 2014; Nikapitiya et al., 2013; Noga et al., 2011; Tagai et al., 2011). Besides of their antibacterial activity, histones are major components of the neutrophil extracellular traps (NETs), innate defense mechanism against

bacteria and parasites, and histone-like proteins from fish have proven lethal to the parasitic dinoflagellate *Amyloodinium ocellatum* (Noga et al., 2001). Several naturally occurring AMPs share sequence identity with portions of various histone subunits (Pavia et al., 2012). Uncharacterized protein sequences were down-regulated in spots 1610 (−1.4 at 2 days p.i) and 1618 (−1.4 at 7 days p.i). The uncharacterized sequences, available in GenBank, were blast searched against the Atlantic salmon genome database and showed significant similarity with fractions of histones sequences. Although it can only be speculated that these uncharacterized spots represent histone-derived peptides, their downregulation at the early stages of infection differs from the upregulation noted for the partial histones H3a and H4. While some studies have shown a relationship between decreased expression of AMPs and increased disease susceptibility (Noga et al., 2011), further studies are needed to establish the role of histones, histone-derived peptides, and other antimicrobial peptides on the innate immunity of Atlantic salmon gills to parasites, and specifically to *N. perurans*.

Haemoglobin subunit β (Hgbβ) or globin β was identified as the main protein present in six spots (1724, 1657, 1691, 1480, 1570 and 1417). Hgbβ was up-regulated at 7 and/or 14 days p.i in four of the spots (1724, 1657, 1480 and 1570) showing fold changes ranging from 1.4 to 2. At 21 days p.i, Hgbβ was downregulated in spots 1570 (−1.5) and 1691 (−1.2), but upregulated in spots 1657 (+1.1) and 1417 (+1.4). In the up-regulated spots at 21 days p.i, however, Hgbβ was detected together with ictacalcin in spot 1657, and with keratin type I cytoskeletal 13-like in spot 1417, both proteins that could be having an effect on the upregulation detected. Keratin 13 and keratin 4 are characteristic of non-keratinised mucosal stratified squamous epithelium (Moll et al., 2008), the type of epithelium in advanced/severe AGD lesions. Peptides derived from haemoglobin are also recognised AMPs in different animal species. In the channel catfish (*Ictalurus punctatus*), peptides derived from the β-chain of haemoglobin were upregulated in the gill epithelium in response to *Ichthyophthirius multifiliis* infection (Ullal et al., 2008). In-vitro, one peptide (HbbP-1) showed lethal activity against the trophont stages of *I. multifiliis* (Ullal et al., 2008) and *A. ocellatum* (Ullal and Noga, 2010). Haemoglobin depletion in advanced stages of AGD has been reported at gene and protein levels by Young et al. (2008) and Nowak (2012) respectively. The apparent decrease, however, could be due to respiratory (Nowak, 2012) or structural gill changes in AGD (increased volume ratio of epithelium versus blood vessels).

The proteomic analysis carried out in this study demonstrated significant changes in the gill proteome of previously naïve Atlantic salmon smolts infected with *N. perurans*. Differences observed between infected and non-infected individuals were, however, lower than expected considering the degree of pathology that occurred during the challenge trial. AGD lesions occur focally or multifocally in the gills, and therefore the inclusion of whole gill arches in the samples may be diluting some of the changes. Targeting lesion and non-lesion areas separately could be applied in future proteomic studies, but this would not be possible for the early stages of the infection when lesions are still not visible macroscopically. Pooling of individuals was the chosen approach for this study. Pooling of samples may mask minor intra-individual biological variations, but it is an accepted method to assess trends in homogeneous populations, and it has previously been used in other proteomic and biomedical studies directed to identify potential disease biomarkers (Braceland et al., 2013; Kuleš et al., 2014).

Most of the significant differences encountered occurred at the clinical stages of the disease (14 and 21 days p.i). The gill pathology (grossly and by histopathology) in early or sub-clinical AGD cases is mild and focal, which could explain the low changes noted at the first sampling points (2 and 7 days p.i). Previous transcriptomic and proteomic studies have reported no or minor expression of acute phase response (APR) molecules in AGD affected fish. Wynne et al. (2008a, 2008b) reported no APR in naïve fish infected for the first time (Wynne et al., 2008a), but showed presence of APR molecules in fish that had suffered three consecutive *N. perurans* infections (Wynne et al., 2008b). Nevertheless, preliminary molecular changes could have been neutralised by the dilution effect discussed above, or by inter-individual differences between fish of the same pool. Although the population was homogeneous and the infection developed evenly among individuals, some degree of variation between individuals is to be expected.

2DE is one of the most widely used proteomics techniques, and a suitable approach for descriptive and comparative proteome analysis. However, it also faces some challenges such as the underrepresentation of proteins expressed at lower levels and the limitations for high molecular weight proteins (>150 kDa). The majority of the spots revealed here showed a pI between 5 and 8. Future studies could be enhanced by using narrower pH range IPG strips for the separation of the different gill proteins, by the use of more sensitive staining methods (i.e. silver staining), and by using non-gel based proteomic approaches of liquid chromatography and mass spectrometry (Almeida et al., 2015; Lippolis and Reinhardt, 2011).

Although AGD is a parasitic disease, it also has many hallmarks of a proliferative condition. Accordingly, the use of non-target screening techniques can contribute to the knowledge of gill response to AGD through the identification of novel proteins and potential pathways. Oxidative stress has been previously suggested as one of the mechanisms underlying AGD pathogenesis, and in view of the results presented here, oxidative metabolism on AGD affected gills should be further investigated. Comparative pathology between similar diseases in different animal species (e.g. airway hyperplasia and hyperresponsiveness) and pathological agents (e.g. other pathogenic amoebae) can also be of great value on elucidating AGD pathogenesis, and should be used more in fish diseases studies. Pathogenic amoeba can resist host reactive oxygen defences (Moonah et al., 2013; Santi-Rocca et al., 2009), but oxidative stress can lead to host tissue damage. Oxidative stress has proven to induce cellular hyperplasia, Th2 response and mucus production (Frossi et al., 2003; King et al., 2006; Rada et al., 2011; Vital et al., 2016), all factors described for AGD. Besides physiological alterations, amoeba secretions and interactions between amoeba and host cells may be triggering certain molecules and pathways contributing to the AGD pathology. *Naegleria fowleri* trophozoites have been found to induce expression of mucin genes in a human airway epithelial cell line and in a mouse model via ROS production (Cervantes-Sandoval et al., 2009).

4. Conclusions

Gill diseases are currently one of the most important health challenges in Atlantic salmon farming. This study is the first proteomic analysis of gill changes during AGD, and the results obtained contribute to the ongoing understanding of the mechanisms and markers of the disease. The use of proteomic techniques can benefit our understanding of gill and mucus responses to injury and pathogenic insults, and such work may be valuable in identifying means of reducing their impacts.

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