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CHANGES IN ATLANTIC SALMON (*SALMO SALAR*) MUCUS COMPONENTS FOLLOWING SHORT AND LONG-TERM HANDLING STRESS

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Abstract

This study examines molecular-level changes in Atlantic salmon (*Salmo salar*) epidermal mucus proteins following short and long-term handling stress in an effort to better understand how the mucus responds to stressors. Short-term stress consisted of a single removal of fish from water for 15 sec followed by mucus and blood sampling at 0 (prior to stress), 3 and 24 hr post-stress. Long-term stress consisted of daily removal of fish from water for 15 sec over 4 weeks, with mucus and blood samples taken daily prior to the stress event and at 7, 14 and 21 days. Mucus enzyme activity (lysozyme, alkaline phosphatase, and cathepsin B) and actin fragmentation patterns in mucus were examined. Previous work had shown changes in cortisol levels following a short-term handling stress; however, in the current study, no change in cortisol levels was detected for long-term handling stress. In the short-term study, there were no observable changes in mucus lysozyme, alkaline phosphatase or cathepsin B activity. For the long-term stress study, a high level of individual variability was observed with respect to mucus enzyme activities. There was a significant difference in lysozyme activity for both control and stressed fish at day 21 compared to other sampling days. Immunoblotting showed actin breakdown products in the mucus of fish exposed to short and long-term handling stress with some distinctions based on sampling time. Protein fragmentation suggests the presence of proteolytic activity that may be undetectable by standard enzymatic assays. There also appeared to be a shift towards a more thorough breakdown of actin in the stressed group over the course of the long-term handling stress study. Taken together, these results suggest changes in mucus proteases in response to handling stress. The variability in Atlantic salmon mucus enzymes levels and actin fragmentation patterns suggests other triggers for inducing changes in mucus protein composition that need to be further investigated in order to better understand the role of mucus in the response of Atlantic salmon to external stressors.

Keywords: Atlantic salmon; mucus; long-term handling, acute stress; cortisol; immunoblotting; protease; actin

1. Introduction

The physiological response of fish to stressors has been well studied; however, little attention has been directed to the associated molecular responses of fish to stress. The stress response in fish involves a complex cascade of behavioral and physiological changes at three levels (Chrousos, 1998; Barton, 2002). The primary response is characterized by the release of catecholamine hormones including adrenaline and noradrenaline from chromaffin tissue, and corticosteroids, including cortisol, from interrenal tissue (Reid *et al.*, 1998; Donaldson, 1981. Secondary responses are defined by metabolic changes including increases in glucose, increased heat shock protein (HSP) production and a concomitant decrease in tissue glycogen (Pickering, 1989; Mommsen *et al.*, 1999). The final or tertiary response is identified by changes in animal performance such as the ability to mount an effective disease response (Wedemeyer & McLeay, 1981; Barton, 2002).

Elevation in plasma cortisol levels are frequently used as an indicator of stress in teleosts (Barton & Iwama, 1991; Wendelaar Bonga, 1997). Although acclimation to stressors occurs following repeated stress events, chronic stress can reduce growth performance and it can result in shorter post-stress responses compared to fish stressed only once (Barton, 1987; Jentoft *et al.*, 2005; Hosoya *et al.*, 2007). Fast *et al.*, (2008) reported an increase in plasma cortisol in Atlantic salmon exposed to a short-term handling stress. However, they did not detect

any change in response to long-term handling stress. It has been suggested that early cortisol-activated immune responses help maintain essential defense mechanisms over longer periods of time (Tort *et al.*, 2004); however, the effect on key components of fish immunity is not clearly understood (Espelid *et al.*, 1996).

Since fish are immersed in water they are susceptible to fluctuations in water conditions such as temperature, salinity and environmental contaminants (Cossins &Crawford, 2005). In teleost fish, epidermal mucus plays roles in respiration, ionic and osmotic regulation, reproduction, excretion, and in protection against microorganisms, toxins, pollutants and hydrolytic enzymes (Shephard, 1994; Bansil *et al.*, 1995; MacPherson *et al.*, 2005; Knowles & Boucher, 2002). Fish mucus also contains many biological compounds with a variety of roles in maintaining homeostasis.

The stress response in fish causes the release of a number of innate immune factors into the serum to offset the effects of the stressor (Ellis, 1981). If tissue damage is involved, there is an increase in the levels of proteins responsible for tissue repair, including C-reactive protein and elements of the complement system (Bayne & Gerwick, 2001). Thus the presence of these proteins in mucus and serum can be used as indicators of a physiological response to environmental stressors. Several examples of protein bioindicators exist in the literature including heat shock protein 70 (HSP70), which is one of the most commonly expressed proteins in rainbow trout following heat shock (Heredia-Middleton *et al.*, 2008). Nonetheless, the use of hsps as stress

indicators is in question due to variability in the cellular response according to tissue type, hsp family and stressor (Iwama *et al* 2004). Suppression of synthesis of histone-like proteins, which are broad-spectrum antimicrobial peptides, following chronic stress suggests their potential use as a stress indicator (Robinette & Noga, 2001).

Protein bioindicators which have been shown to change in response to environmental disturbances include several classes of enzymes. In order to combat bacteriological challenge there can be an increase in mucus lysozyme, a carbohydrate-digesting enzyme which lyses both Gram-negative and Gram-positive bacteria (Fletcher & White, 1973; Shephard, 1994). In addition to lysozyme, fish skin mucus contains a variety of other enzymes including proteolytic enzymes or proteases (Hartley, 1960; Aranishi & Nakane, 1997; Cho *et al.*, 2002; Salles *et al.*, 2007), which have not been examined in terms of the stress response. Proteases regulate many physiological functions such as blood coagulation, fibrinolysis, the complement system, food digestion, inflammation and immune response (Fujita *et al.*, 2004; Zhu *et al.*, 2006). Cysteine proteases in mucus such as cathepsin B and L, have shown bacteriolytic activity against fish pathogens (Aranishi, 1997). A complex series of serine protease cascades has been shown to be essential in the formation of lipid barriers and epidermal cell construction (Ovaere *et al*, 2009). Changes in the levels or activities of serine proteases have been shown to result in various skin pathologies. Alkaline phosphatase is another mucus enzyme found in fish and is believed to act as an antibacterial agent as well as aiding in wound healing (Iger & Abraham, 1990,

1997). Wounding in fish activates a series of proteases, including matrix metalloproteases (MMPs) which are controlled by tissue inhibitors of metalloproteases or TIMPs (Iger and Abraham, 1990; Martin *et al.*, 1997; Brew *et al.*, 2000). Breakdown of the extracellular matrix (ECM) involves the degradation of actin by proteases which bind to recognition sites within the actin cytoskeleton (Song *et al.*, 1997).

Actin is a ubiquitous protein in eukaryotic cells and is involved with cellular structure and movement (Weeds, 1982). Actin and transferrin degradation products have been observed in the mucus of sea lice infected salmon and were suggested to result from proteolytic breakdown (Easy & Ross, 2009). There was a weak positive correlation identified between cortisol levels and transferrin cleavage suggesting a link between stress and protease activity in the mucus of Atlantic salmon.

In this study we will examine the response of specific relevant targets in mucus to short term and long term stress. In particular, we aim to more fully describe the response of fish to stress and to uncover potential new targets for stress marker development. The study strove to further investigate the potential correlation between mucus actin fragmentation and plasma cortisol levels. We used techniques for fish mucus sampling that do not involve physical abrasion of the epidermal surface and therefore may provide a less invasive method for acquiring samples for stress analysis. Thus, fish mucus could provide a readily available tissue that can be sampled non-lethally for analysis of stress biomarkers. Parameters investigated included the relationship between actin

fragmentation, enzyme activity and cortisol levels following short and long-term handling stress.

2. Materials and Methods

2.1. Animal husbandry

Atlantic salmon smolts were reared at the NRC Institute for Marine Biosciences (NRC-IMB) Marine Research Station (Ketch Harbour, NS, Canada). Juveniles were initially held in two 500 l holding tanks supplied with ambient flow-through seawater at a temperature of 10 ± 1°C and greater than 90% oxygen saturation. Fish were fed twice daily (8:00 and 15:00 h) at a rate of 1.5% of average body mass with a commercial Atlantic salmon diet (EWOS, Vancouver, BC, Canada). Three weeks prior to the start of the experiment, fish (mass 144.4 ± 20 g, length 25.7 ± 2 cm) were transferred to sixteen 120 l tanks (20 fish/tank) and allowed to acclimate. During this period, water flow, temperature and oxygen saturation were 8.0 l min^{-1}, 10 ± 1°C and 90%, respectively. Daily photoperiod was 10 h light, 14 h dark.

2.2. Stress studies

Short and long-term stress studies were carried out as described by Fast *et al.* (2008). Each study consisted of two experimental groups: control and stressed (four replicate tanks in each). In the short term stress study, fish in the stress group were subjected to a single handling stress which consisted of 15 s out of the water. Blood and mucus samples were collected at 0, 3 and 24 h post-

stress. In the long-term stress study, fish in the stress group were subjected to a daily handling stress (15 s out of the water) for 4 weeks. This procedure was carried out either at 8:00 a.m. or 3:00 p.m. to prevent the fish from anticipating the stressor. Feedings were usually at 8:00 a.m. and 3:00 p.m., but the timing of one of the feedings was adjusted so that it occurred 1 h after handling. During this study, fish (2 per replicate tank) were sampled prior to initiation of the stress protocol (0 week) and 1, 2, 3 and 4 weeks after it began. Blood and mucus samples were taken just prior to the handling stress of the day, and were collected at least 18 h after the last stress was applied.

2.3. Mucus and blood collection

Fish were kept off-feed for 24 h prior to sampling. Fish were placed into 10 litre buckets containing a lethal dose of anaesthetic (200 mg $l^{-1)}$ tricaine methansulphonaste (Syndel Laboratories, Vancouver, British Columbia) and then transferred into plastic bags containing 10 ml of 100 mM NH_4HC0_3 pH 7.8 (Ross *et al.*, 2000). The bags were gently agitated for approximately 1 min to collect epidermal mucus. Fish were then removed and the bags placed on ice. In order to remove foreign matter from mucus, samples were centrifuged at 2730 X *g* for 15 minutes at 4° C, aliquoted into sterile tubes containing 1 mM PMSF and 50 mM EDTA (protease inhibitors) and stored at –80° C until use. After thawing on ice, mucus samples were transferred to dialysis bags (3500 MW cutoff) and dialyzed for 48 hours against 10 mM Tris-HCl pH 8.0 in order to remove salt. Following dialysis, samples were centrifuged at 4° C at 200 X *g* for 20 minutes at

Alkaline phosphatase (AP) activity was determined by incubating 5 µL of mucus reconstituted in 100mM ammonium bicarbonate, 1 mM $MgCl_2$, pH 7.8, at 30° C for 15 min. Fifty µL of 4 mM p-nitrophenol phosphate substrate was added and the absorbance was measured at 405 nm over a 30 min period at 30° C. One unit of activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol product in 1 min.

Cathepsin B activity was assayed using a modified method of Barrett and Kirschke (1981). Briefly, 5 µL of mucus reconstituted in 0.1 M sodium phosphate buffer (pH 6.0) was incubated with 20 µL of assay buffer (0.1 M sodium phosphate, 0.08% (w/v) Brij, 1 mM EDTA, pH 6.0), 20 µL of 1 mM dithiothreitol and 60 µL of milli-Q water in a 96-well microtitre plate for 5 min at 30° C. Then 50 µL of 25 µM carbobenzoxy-L-phenylalanyl- L-arginyl-4-methylcoumaryl-7-amide substrate was added. The fluorescence of 7-amino-4-methylcoumarin (AMC) was measured continuously for 30 min at 30° C at an excitation wavelength of 380 nm and an emission wavelength of 405 nm. The initial rate of reaction was used to calculate enzyme activity and 1 U of activity was defined as the amount of enzyme required to release 1 µmol of substrate in 1 min.

2.6 Testing of anti-actin antibody and enzymatic digestion of actin

In order to test the specificity of the anti-actin antibody and the effects of cysteine protease activity on actin, 5 µg of Atlantic salmon epidermal mucus protein and bovine actin were treated separately with 2 µl cathepsin B (2 U/ml) at neutral pH under reducing conditions in sample buffer (0.1 M sodium phosphate,

0.08% (w/v) Brij, 1 mM EDTA, pH 6.0). This was done in triplicate. Following an overnight incubation at 30° C digestion patterns of actin and undigested actin were visualized on SDS-PAGE and immunoblots.

2.7 SDS-Page and Immunodetection

Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Briefly, samples including bovine actin (Sigma), were dissolved in an equal volume of sample buffer (0.5 M Tris-HCl pH 6.8, 4% SDS, 25% glycerol, 0.01% bromophenol blue (SIGMA), heated at 95° C for 5 min and loaded onto a 12% SDS-PAGE gel and electrophoresed at 150 V for 90 min (Laemmli, 1970). Broad-range unstained molecular markers were run on each gel (Bio-Rad). Each lane contained 20 µg or protein. Polyacrylamide gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 15 min and then proteins were electroblotted onto 0.2 µm nitrocellulose membranes at 100 V for 1 h (Towbin *et al.*, 1979). Membranes were blocked in a blocking buffer composed of 5% skim milk in 20 mM Tris-HCl, 8% NaCl, 0.1% Tween 20, pH 7.6 (TBST). Mouse monoclonal anti-actin antibody (Sigma) was added at a 1:1000 dilution and incubated at 4° C overnight on a rotating shaker at 70 RPM. Blots were washed three times with TBS-T for 20 min at room temperature (RT). Membranes were incubated with goat anti-Mouse IgG–peroxidase conjugated antibody (Sigma) as the secondary antibody at a 1:2000 dilution in blocking buffer for one hour with shaking at RT. Following a further three washes for 20 min at RT with TBS-T and a final rinse with TBS (no Tween)

proteins were visualized using ECL detection chemistry (GE Health Sciences, Pittsburgh, PA). Bands were scanned using a HP Scanjet 4570C (Hewlett-Packard, Palo Alto, CA., USA). Quantification of actin and actin breakdown products were carried out using Scion imaging software (Maryland, USA). In order to correct for background density, mean densities of homogenous regions in the blots were subtracted from individual spot densities. Bovine actin was run as a positive control on each gel.

2.8 Statistics

Data were analysed using Prism 4 (GraphPad Software). Data are represented as mean standard deviation. A two way analysis of variance was used to compare between enzyme activities of mucus isolated from stressed and control fish at different time points. Non-normal data were analysed using the Kruskal-Wallace non-parametric statistical test.

3 Results

3.1. Enzyme assays

For the short-term stress study, alkaline phosphatase and cathepsin B activity were at or below assay detection limits (data not shown). There were no significant differences in lysozyme activity between individual samples isolated at different time points or between control and stressed groups (Table 1).

In the long-term stress study, there was a high level of variability in individual mucus enzyme activities (Table 2). For lysozyme, there was no

significant difference in activity between stressed and non-stressed fish within each sampling period; however, mucus lysozyme activity was significantly elevated in both stressed and control fish at day 21 when compared to the other sampling times. Similarly, there were no significant differences in mucus cathepsin B and alkaline phosphatase either between groups (stress and control) or within groups over the four time points.

A Pearson's product-moment test was run to identify correlations between cortisol levels and mucus enzyme activities. For the short-term stress study at time 0, there was no detectable association between cortisol levels and lysozyme activity (Table 1). At 3 hours post-handling stress, there was an overall weak positive association identified. By 24 hours, lysozyme activity in mucus showed a strong positive association with cortisol levels.

In the long-term stress study there was little to no association between lysozyme activity and cortisol at days 0, 7 and 21; however, there was a weak positive association at day 14 (Table 2). For alkaline phosphatase, there was no association recorded between cortisol levels and enzyme activity at all time points. Cathepsin activity showed a weak positive association at day 21. Cathepsin activity did not correlate with cortisol levels at the other time points.

Pearson's product-moment correlations for enzyme activity vs. cortisol levels examined over all time points showed no detectable association for lysozyme, cathepsin and alkaline phosphatase , $r = -0.10$, -0.16 and 0.045 respectively.

3.2. Immunoblotting

Actin

Immunoblotting was performed in order to examine actin and actin breakdown products in Atlantic salmon epidermal mucus in response to handing stress. For identification purposes, a pattern identification key was generated that illustrated the prevalent banding patterns (Fig. 1).

Banding pattern A is comprised of two bands, one at 42 kDa, a mass corresponding to intact actin, and one at approximately 28 kDa, which appears to be a breakdown. Patterns that lacked multiple bands below 28 kDa were designated as "A". Banding pattern B contains up to 7 bands; actin at 42 kDa and bands at approximately 40, 34, 29, 28, 25 and 24 kDa. Patterns with non-distinct bands within the 42 to 29 kDa range, but with distinctive bands between 20-24 kDa, were identified as "B". Pattern C represents the absence of bands. For simplicity, dimers, trimers of actin at approximately 80 and 120 kDa and bands of low intensity as determined visually are not included in the banding key.

For the short-term handling stress study (Fig. 2 and 3) there is a three band pattern at 3 hours post-handling in both control and stressed fish. There were no noticeable differences in actin fragmentation patterns between treatment groups.

Immunoblots of mucus isolated from the long-term stress study are illustrated in figures 4-7 representing day 0, day 7, 14 and day 21 post-handling stress, respectively. At time 0, there was a variety of banding patterns with no clear predominance of one banding type present (Fig. 4). At 7 days post-stress, 4

of 6 mucus samples showed no actin on the blots (pattern C) for the stressed fish, with 2 showing the A pattern (Fig. 5). For mucus from control fish, 4 of 5 illustrated the A pattern. By day 14 post-stress, 4 of 5 samples from stressed fish showed a B pattern with 1 illustrating the A pattern (Fig. 6). For the controls at day 14, 4 of 6 showed the A pattern, with 2 showing a B pattern. By day 21, 3 of 6 samples from stressed fish showed the A pattern and 3 showed the B pattern (Fig. 7). For control fish at day 21, 6 of 6 showed the A pattern.

3.3.1 Recognition of proteolytic fragments of actin by anti-actin antibodies

Specificity of the anti-bovine actin antibody was tested in an SDS-PAGE and immunoblot (Fig. 8). In A and B the results of cathepsin B digestion of actin show that the anti-bovine actin antibody appears to recognize proteolytically derived bovine actin fragments. To show that the anti-actin antibody recognized salmon actin fragments and that the fragments labeled in salmon mucus were proteolytically derived, mucus was incubated with cathepsin B. In mucus treated with cathepsin B, there were several more bands visible on the SDS-PAGE than for the untreated mucus sample (Fig. 8C, lanes 1 and 2 respectively). Immunoblots of the same gel, (Fig. 8D) demonstrated that the anti-actin antibody recognized the cathepsin-derived mucus actin breakdown products.

4. Discussion

Environmental stressors can lead to changes in an organism's proteome (Nesatyy & Suter, 2008). Transferrin and actin fragments were previously identified in mucus of Atlantic salmon and changes in actin fragmentation was

observed in sea lice-infected and non-infected fish (Easy & Ross, 2009). A weak correlation between transferrin cleavage and fish plasma cortisol levels was also identified (Easy & Ross, 2009). Thus, the current paper set out to examine changes in Atlantic salmon epidermal mucus proteins following a handling stress and to explore potential relationships between actin cleavage and handling stress.

The mucus of fish acts as a reservoir for a broad range of enzymes including proteases such as cathepsin B, carbohydrases such as lysozyme and other enzymes involved in the innate immune response including alkaline phosphatase and antimicrobial peptides (Alexander & Ingram, 1992; Shephard, 1994). Measuring changes in enzyme activity following a stress-related event may help in understanding an organism's response to stress. The current study illustrates the proteolytic breakdown of actin in mucus suggesting the action of mucus enzymes that may be triggered by a prior stimulus. To further examine this process, enzyme assays were conducted on mucus collected at each time point from the short and long-term stress studies. Comparison of lysozyme activities between mucus samples from stressed and control fish did not show any significant differences. For the short-term stress study, cathepsin B and alkaline phosphatase activity were below assay detection levels, which are in contrast to the higher levels observed in the long-term stress study. The low activities may be due to enzyme degradation or the presence of enzyme inhibitors found in fish serum which could "leak" into the mucus (Aranishi, 1997). In the long-term stress study there was a significant difference in lysozyme

activity for both stressed and control samples at day 21 compared to samples at other time points, suggesting an event such as a change in feeding schedule, light activity, water pH or temperature may have occurred that led to a change in the status of the fish. Variations in lysozyme activity have previously been attributed to sex, diet, maturity and genetic variation (Balfry & Iwama, 2004). Changes in mucus lysozyme activity have also been shown to vary seasonally and between fish species (Schrock *et al.*, 2001).

Alkaline phosphatase and cathepsin B activities varied greatly, however differences between treatment groups were not significant. Changes in alkaline phosphatase and cathepsin B activity have previously been noted in response to microbial, physical and chemical stress (Iger & Abraham, 1990, 1997; Aranishi & Mano, 2000; Ross *et al*, 2000).

In studies by Fast *et al* (2008), cortisol levels spiked within 3 hours following a short-term stress; however, there was no change in cortisol levels for long-term handling stress (data not shown). For the short-term stress study there was little to no association between cortisol levels and lysozyme activity for the first 7 days of the study. However at day 14 there was a weak positive association between lysozyme activity and cortisol levels. This correlation became a strong positive association by day 21. This suggests fish may upregulate lysozyme over time in response to environmental cues. This is not unprecedented, as Fagan *et al.*, (2003) detected lysozyme activity in epidermal mucus of Atlantic salmon with a significant increase noted during the smoltification process.

In the long-term stress study there were no significant correlations noted between cortisol levels and enzyme activity except for weak positive associations identified in lysozyme and cathepsin at days 14 and 21 respectively. This suggests that the link between cortisol levels and enzyme activity is not clear for long-term handling stress. The wide variation observed in individual enzyme activities and lack of or minimal correlation with cortisol suggests that there may be other triggers influencing enzyme activity. Such triggers could include physical disturbances such as abrasion due to overcrowding resulting in an inflammatory response.

Actin is a ubiquitous protein and it is used in molecular and proteomic studies as a base-line marker due to its highly conserved sequence across species. It was believed that actin is expressed and maintained in a consistent fashion by the animal however, there is increasing evidence that expression of actin varies depending on biochemical stimuli, therefore, its role as a stable and constitutive control is in doubt (Zupanc *et al.*, 2006, Ruan & Li, 2007). In the current study, actin was used as a signature protein and amount of expression was not a factor. Immunoblotting of Atlantic salmon epidermal mucus showed actin and actin fragments as a series of banding patterns. These fragmentation patterns suggest proteolytic degradation of actin. However, they may also be the result of non-specific binding of the anti-bovine actin antibody to other proteins present in the mucus. To ensure this antibody recognizes actin fragments and that the extra bands were not due to non-specific binding bovine actin and Atlantic salmon epidermal mucus were incubated with cathepsin B and run on

SDS-PAGE and immunoblotted. The data show that the actin antibody clearly recognizes both bovine actin and teleost actin breakdown products. This suggests that bands visible on the blots may not be due solely to non-specific binding.

For ease of analysis, a banding key was derived to characterize fragmentation patterns. The B pattern indicated a more thorough breakdown of actin than the A pattern suggesting an increase in protease activity. There was a shift to the B pattern over time that was more evident in mucus isolated from stressed fish than from the control fish. By the end of the study at day 21, all of the control fish exhibited the A pattern while only 50% of the fish from the stressed group had the A pattern. The bands may indicate cellular disruption and release of fragmented actin from cells. It is also possible that actin degradation may have occurred prior to sampling of mucus. In cases where actin was intact, there may be an absence of significant protease activity or a lack of access of proteases to actin as seen in human cells (Song *et al.*, 1997). Fragmentation patterns of the actin control may indicate actin degradation, which may reflect endogenous protease activity in fish epidermal mucus.

Though the sampling of mucus is less invasive than blood sampling, the recording of a significant stress response in fish is not unexpected as it is difficult to maintain a negligible stress level in caged fish. However, reliance on cortisol to evaluate the impact of stress on organisms may miss subtle physiological effects. The lack of an increase in cortisol levels for the long-term stress study suggests that either the fish were minimally stressed by the daily stressor, such that fish

recovered fully between stress events, or that cortisol may not be the most sensitive indicator of handling stress. In the current study, the timing of the sampling periods may have been outside of the window when a potential cortisol response would have been measured. It is possible that the daily stress may have raised cortisol levels differentially in the treatment groups that may have affected enzyme levels or actin degradation differentially within treatment groups. The observed increase in actin fragmentation in the stressed groups with no concurrent increase in cortisol levels suggests that protein indicators may provide evidence of a prior stressor or perturbation to a fish in the absence of elevated cortisol levels.

The current study examines how handling stress may lead to a change in Atlantic salmon mucus constituents. It is possible that actin banding patterns generated in response to handling stress suggest evidence of activation of proteases which may have been triggered by short-term elevated cortisol levels. However, protease activation may also result from physical disturbances such as abrasion due to netting or overcrowding. Protein fragments generated by protease activity could trigger or prime an immune response as seen previously by the induction of the nitrous oxide response of goldfish macrophages by transferrin cleavage products (Stafford & Belosevic, 2003). Transferrin fragments have been identified in the mucus of Atlantic salmon infected with sea lice (*Lepeophtheirus salmonis*) suggesting the presence of proteases that may arise from either the host or the pathogen (Easy & Ross, 2009). Proteolytically derived fragments of vitellogenin in zebrafish embryos have been used as a diagnostic

tool to identify stress and thus overall fitness at the molecular level in zebra fish (Gündel *et al.*, 2007). In the current study, the shift towards increased fragmentation of actin over time suggests a possible link in Atlantic salmon mucus molecular indices and the response to handling stress. Thus examination of changes in mucus proteins may provide insight into different aspects of the stress response in Atlantic salmon. However, the regulation of actin fragmentation and specific proteases involved remain to be elucidated.

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Figure Captions

Figure 1. Banding patterns used for characterization of Immunoblots. Numbers indicate molecular mass in kDa.

Figure 2. Immunoblot of Atlantic salmon epidermal mucus from control fish short-term stress study. Lanes 1-3 T0, lanes 4-6 3 hour, lanes 7-8 24 hours. Numbers on side indicate molecular mass in kDa (kDa). Numbers on top indicate individual fish. Ac = actin at ~ 42 kDa.

Figure 3. Immunoblot of Atlantic salmon epidermal mucus from stressed fish - short-term stress study. Lanes 1-3 indicate time 0, lanes 4-6 are at 3 hour and lanes 7-9 are at 24 hours post-stress. Numbers on side indicate molecular mass in kDa. Numbers on top indicate individual fish. Ac = actin at ~ 42 kDa

Figure 4. Immunoblot of Atlantic salmon epidermal mucus at time 0 using anti-bovine actin antibody – long-term stress study. Letters indicate banding patterns. Numbers on side indicate molecular mass in kDa. Numbers on top indicate individual fish. Ac = actin at ~ 42 kDa

Figure 5. Immunoblot of Atlantic salmon epidermal mucus at day 7 using anti-bovine actin antibody – long-term stress study. Lanes 1-5 indicate mucus from stressed fish and lanes 6-11 is mucus from control fish. Letters indicate banding patterns. Numbers on side indicate molecular mass in kDa. Numbers on top indicate individual fish. Ac = actin at ~ 42 kDa

Figure 6. Immunoblot of Atlantic salmon epidermal mucus at day 14 using anti-bovine actin antibody – long-term stress study. Lanes 1-5 shows mucus from stressed fish and lanes 6-11 are from control fish. Letters indicate banding patterns. Numbers on side indicate molecular mass in kDa. Numbers on top indicate individual fish. Ac = actin at ~ 42 kDa

Figure 7. Immunoblot of Atlantic salmon epidermal mucus at day 21 using anti-bovine actin antibody – long-term stress study. Lanes 1-3 and 9-12 is mucus from stressed fish. Lanes 4-8 is mucus from control fish. Letters indicate banding patterns. Numbers on side indicate molecular mass in kDa. Numbers on top indicate individual fish. Ac = actin at ~ 42 kDa

Figure 8. Digestion of mucus and actin by cathepsin B. A; SDS-PAGE, Lane 1 Bovine actin. Lane 2 Bovine actin with cathepsin. B; Immunoblot with anti-bovine actin antibody. Lane 1 Bovine actin. Lane 2 Bovine actin with cathepsin B. C; SDS-PAGE: Lane 1 Atlantic salmon epidermal mucus. Lane 2 Atlantic salmon epidermal mucus with cathepsin. Lane 3 Cathepsin alone. D; Immunoblot with anti-bovine actin antibody. Lane 1 Atlantic salmon epidermal mucus. Lane 2

Atlantic salmon epidermal mucus with cathepsin. E; Immunoblot with anti-bovine actin antibody Lanes 1-2 Cathepsin alone. Molecular mass is given in kDa.

Table Captions

Table 1 Comparison of enzyme activity (mean ± SD) between mucus isolated from short-term stressed fish. Number of replicates is provided in brackets.

Table 2 Comparison of enzyme activity (mean ± SD) between mucus isolated from long-term stressed fish. Number of replicates is provided in brackets.

Table 3 Pearson moment correlations between actin band density and cortisol levels in mucus from stressed and control fish.

Figure 1

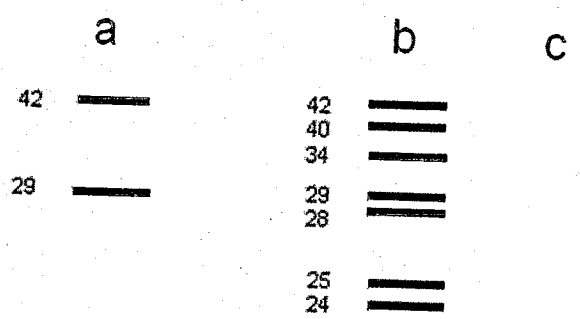

Figure 2

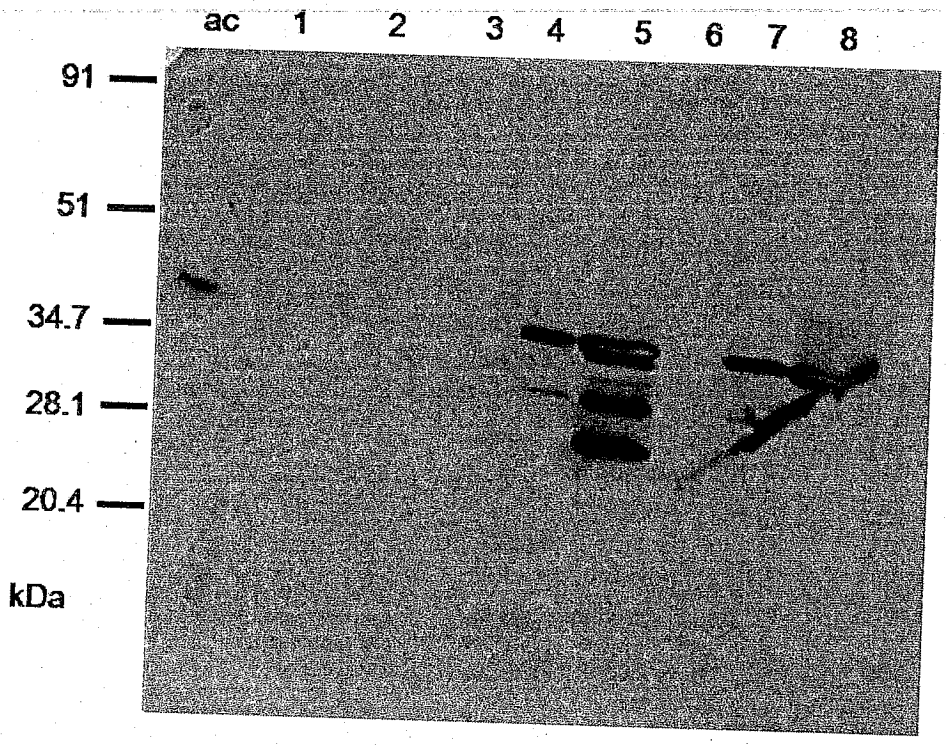

Figure 3

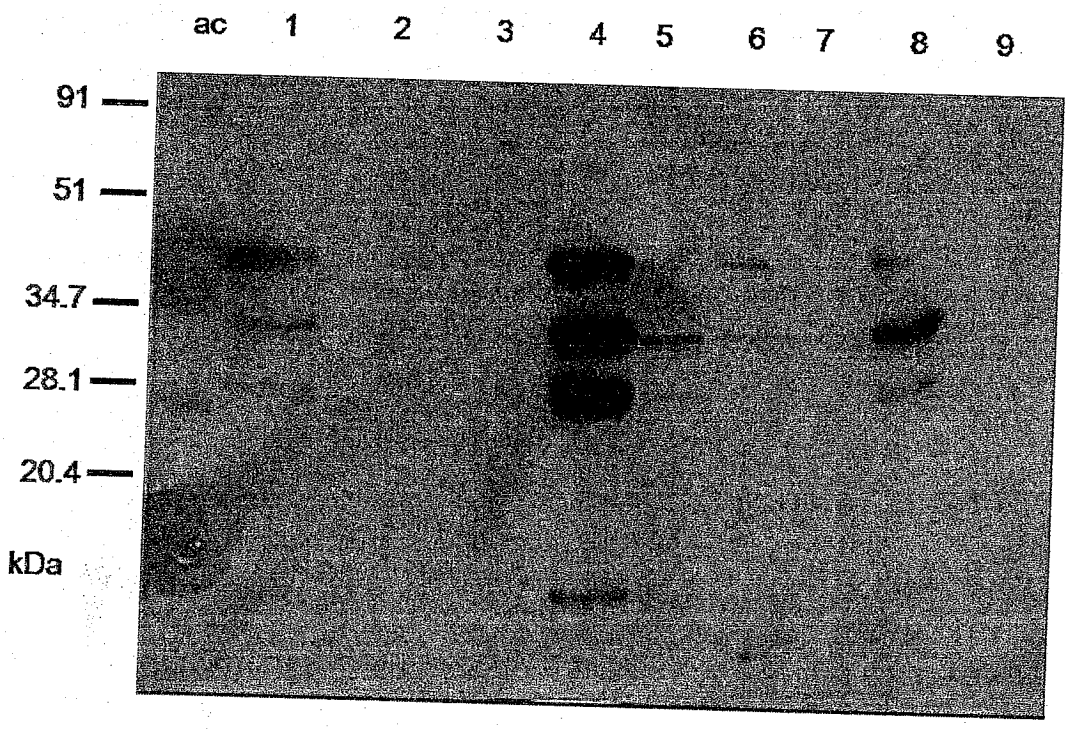

Figure 4

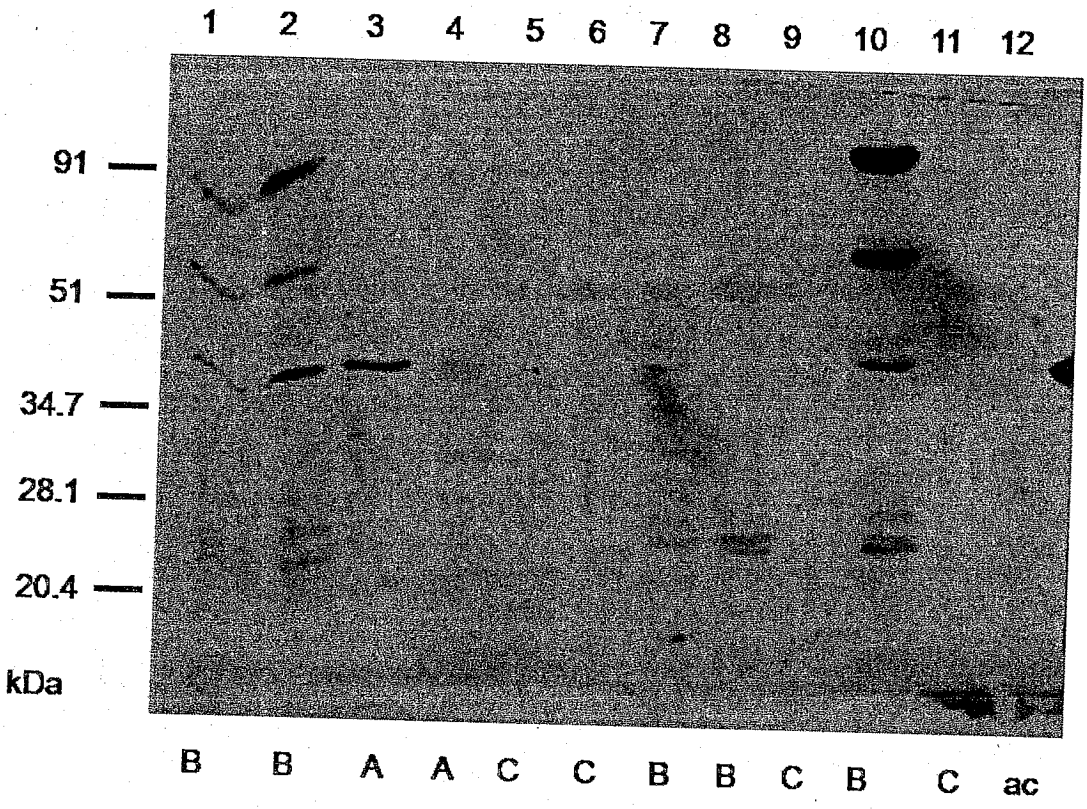

Figure 5

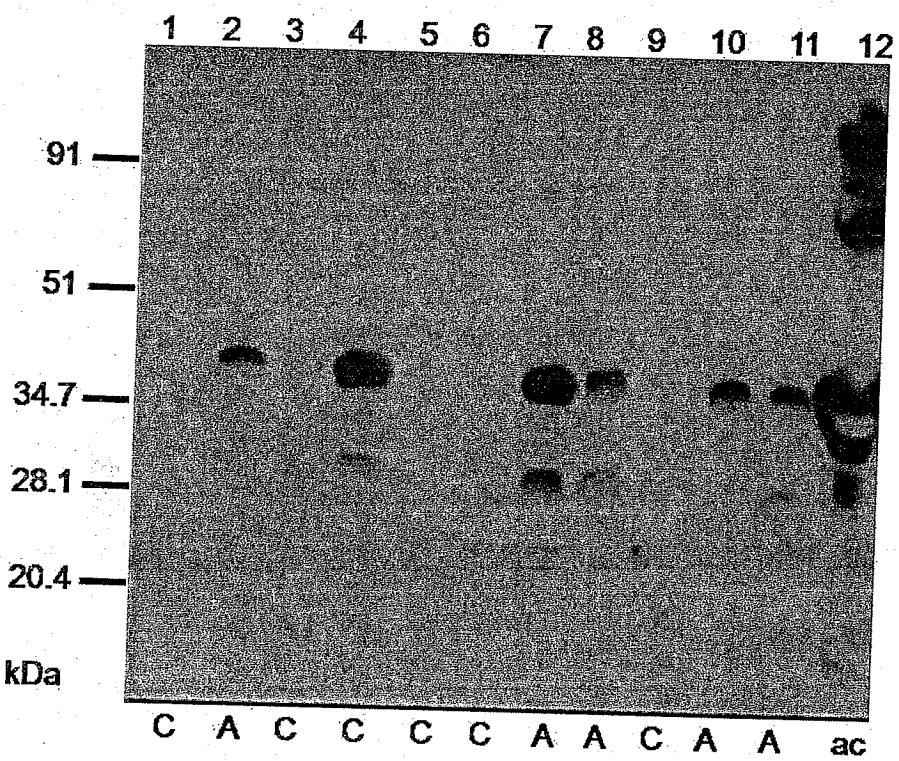

Figure 6

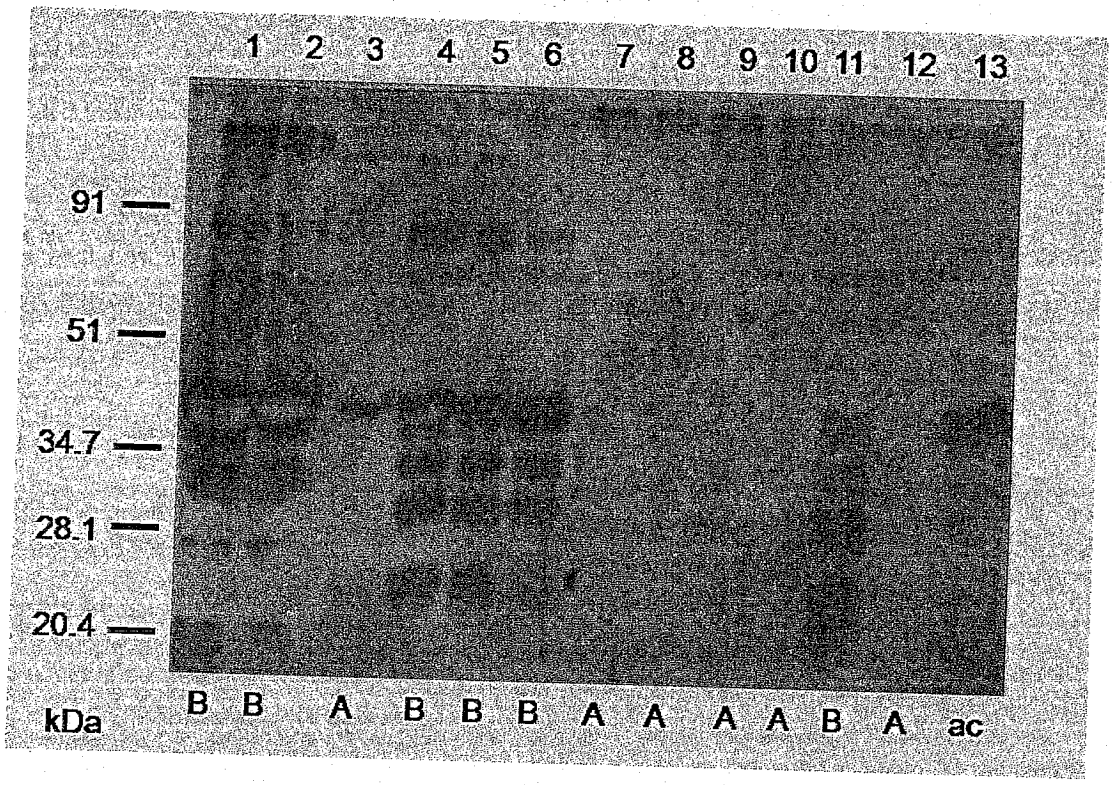

Figure 7

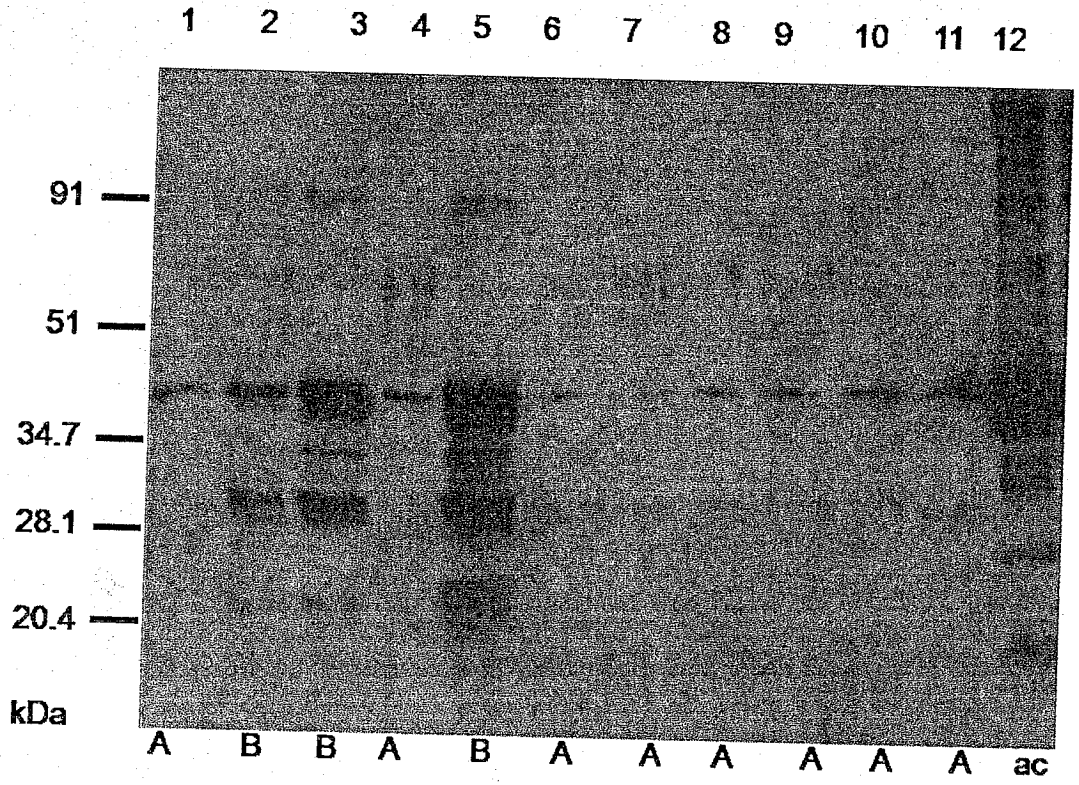

Figure 8

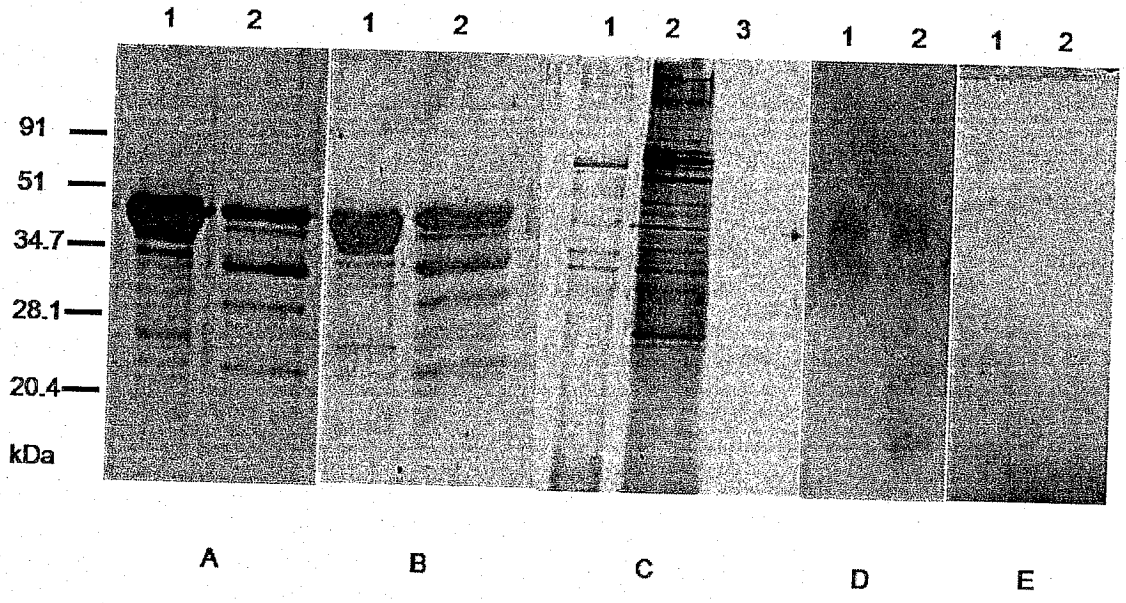

Table 1

Lysozyme activity vs. Cortisol levels - Pearson's Values (=n) Short-term stress study	
Control	
Time	Lysozyme
0	0.27 (6)
3hr	0.41 (6)
24 hour	0.77 (5)

Table 2

Enzyme activity vs. Cortisol levels - Pearson's Values Long-term stress study (=n)			
Time	Lysozyme	Alkaline phosphatase	Cathepsin
0	-0.24 (12)	-0.12 (12)	-0.21 (6)
7 day	-0.02 (12)	0.16 (12)	0.19 (11)
14 day	0.52 (11)	-0.22 (5)	-0.11 (8)
21 day	0.07 (11)	-0.25 (10)	0.32 (11)

Table 3

Long-term stress	Band density: cortisol levels (=n)
Time Point	Pearson's Value
d-1	0.25 (6)
7d	-0.18 (6)
14d	0.42 (11)
21d	-0.29 (11)