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Serum enolase: a non-destructive biomarker of white skeletal myopathy during pancreas disease (PD) in Atlantic salmon *Salmo salar* L.

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Abstract

Diseases which cause skeletal muscle myopathy are some of the most economically damaging diseases in Atlantic salmon, *Salmo salar* L., aquaculture. Despite this, there are limited means of assessing fish health non-destructively. Previous investigation of the serum proteome of Atlantic salmon, *Salmo salar* L., during pancreas disease (PD) has identified proteins in serum that have potential as biomarkers of the disease. Amongst these proteins, the enzyme enolase was selected as the most viable for use as a biomarker of muscle myopathy associated with PD. Western blot and immunoassay (ELISA) validated enolase as a biomarker for PD, whilst immunohistochemistry identified white muscle as the source of enolase. Enolase was shown to be a specific marker for white muscle myopathy in salmon, rising in serum concentration significantly correlating with pathological damage to the tissue.

Keywords: Atlantic salmon, biomarkers, enolase, health, myopathy, pancreas disease.

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Introduction

Atlantic salmon, *Salmo salar* L., are susceptible to a number of diseases of known and unknown aetiology, including a number of viral, bacterial and amoebic pathogens which significantly reduce profitability of the industry, thus limiting its economic growth. For instance, the diseases, such as infectious pancreatic necrosis (IPN), infectious salmon anaemia (ISA), heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and pancreas disease (PD), which all have viral aetiology, are detrimental to salmon production. Indeed, HSMI and PD have been shown recently in Norway to be spreading to new areas (Bornø *et al.* 2010) and, whilst they usually cause variable mortality rate (Yousaf *et al.* 2013), they also result in significant economic losses due to reduced growth and, as a result of their pathogenesis (causing skeletal muscle myopathy), a reduction in fillet quality (Kongtorp, Taksdal & Lyngoy 2004; McLoughlin & Graham 2007; Larsson *et al.* 2012; Lerfall *et al.* 2012). However, there is currently a lack of non-destructive means of diagnosing and monitoring general fish health including PD. In addition, whilst well-established methodologies, such as virus neutralization antibody test, are widespread means of infection detection, it fails to detect infections of unknown aetiology (i.e. they are disease specific) and whilst sites may have infection this may never develop

Journal of Fish Diseases

into a pathological disease outbreak. In the light of this, and the fact that there has been a recent identification of melanization and lesions appearing on fillets at time of harvest (causing economically expensive downgrading) of unknown cause, there is a real demand for non-destructive markers of skeletal muscle myopathy.

A recent proteomic study of salmon serum during experimental PD identified a number of potential biomarkers of the disease, including the enzymes enolase, aldolase and 3-phosphoglyceraldehyde dehydrogenase in serum (Braceland *et al.* 2013). An initial biomarker validation study to identify usable antisera for immunoassay development revealed that enolase would be the most appropriate to use as a biomarker of PD. Classically, this enzyme is defined as a glycolytic enzyme catalysing the conversion of 2-phospho glycerate (2-PG) to phosphoenolpyruvate (PEP) in the ninth and penultimate step of glycolysis (Pancholi 2001). In mammalian species, there have been three paralogs; enolase 1 (α enolase), 2 (γ enolase) and 3 (β enolase). Tracy & Hedges (2000) clearly defined which have differing tissue distribution, although these have the ability to form heterodimers. However, the enolase enzyme and its distribution are poorly defined in teleost species. Although its usefulness as a serum biomarker of disease has been widely studied in mammalian species little is known of its pathophysiology in any fish species. In human medicine, enolase 1 (α enolase) has been shown to increase in patients with hepatic fibrosis (Zhang *et al.* 2013), enolase 2 (γ enolase) is used as a biomarker for a number of human diseases including ishaemic stroke (Singh *et al.* 2013), and a significant correlation of enolase 3 (β enolase) concentration with progressive muscular dystrophy (Mokuno *et al.* 1984) has been reported.

This study aims to investigate the potential of enolase as a non-destructive marker of pathology, and more specifically myopathy, in Atlantic salmon using an experimental model of PD. In addition, using this proposed marker, the sensitivity of a number of histopathological scoring systems to assess myopathy was examined, thus investigating the relationship of myopathy and serum enolase levels and comparing continuous non-destructive means of diagnosis (serum enolase concentrations) and a categorical destructive method (histopathology).

Materials and methods

Fish husbandry and challenge

A cohabitation trial, described in detail in Braceland *et al.* (2013), was carried out using the introduction of Trojan fish (infected with salmonid alphavirus subtype 3) to induce PD in trial populations. Cohabitant fish were sampled at 0, 2, 3, 4, 5, 6, 8, 10 and 12 weeks post-challenge (wpc). At each time point, 9 fish per tank were killed by lethal overdose of anaesthetic (MS-222, Pharmaq) and blood collected in non-heparinized vacutainers for analysis of serum biochemistry. From 6 of these fish, pyloric caecae and pancreas (hereafter referred to as pancreas), heart and skeletal muscle tissue were processed from standardized locations for histology. Fish sampled at time point 0 were removed from the tanks before the addition of Trojan shedders. The experiment was approved by the Norwegian National Animal Research Authority (NARA) prior to the trial commencing.

Histopathology

Tissues for histology from the salmon were immediately fixed in 3.5% v/v formaldehyde in buffered saline pH 7.0 (4.0 g NaH2PO4.2H2O, 6.5 g Na2HPO4.2H2O) prior to further processing by standard paraffin wax techniques, sectioned and stained with haematoxylin and eosin (H & E). Tissue sections were examined by an experienced pathologist, and a scoring system was used to semi-quantify the distribution and severity of the tissue lesions in the pancreas, heart and skeletal muscle as used in previous studies (McLoughlin *et al.* 2006; Christie *et al.* 2007; Braceland *et al.* 2013).

Preparation of tissue lysate

Whole skeletal muscle was collected from adult fish, independent of the trial described above, with an average weight of 3 kg at a site in Ardnish (UK). Fish were killed using a lethal overdose of anaesthetic (MS-222, Pharmaq), and tissues removed and frozen in dry ice and then stored until use at −80 °C. Protein was extracted into lysate from tissue samples by grinding one gram of tissue with a mortar and pestle kept frozen by the periodic addition of liquid nitrogen. Once a fine powder was formed, it was transferred to another mortar, 10 mL of cell lysis buffer (20 mM Tris-HCL, pH 7.5) added, and the tissue buffer

mix ground for a further 5 min. The resulting extract was then transferred into a 15-mL universal tube and centrifuged at 10 000 × ***g*** at 4 °C for 10 min, and the supernatant removed, transferred into another tube and the centrifugation repeated. The solid residue was discarded after each centrifugation. Finally, the supernatant was passed through a 0.45-μm syringe-driven filter and stored at −80 °C.

Protein concentrations were determined for tissue lysates and serum samples via the Bradford assay (Sigma-Aldrich) with protein concentrations being determined against a bovine serum albumin protein standard. Serum samples were pooled according to week post-challenge (Wpc).

Western blotting

The suitability of the three selected enzymes as markers of PD pathogenesis was initially screened by Western blotting. Aldolase (aldolase A) was probed using a rabbit anti-N-terminal region sequence polyclonal (Aviva Systems Biology) at an optimized concentration of 1:2000 (diluted in 5% skimmed milk TTBS). A mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Millipore) raised against GAPDH immunogen from rabbit muscle was used at a dilution of 1:500.

Three separate commercially available rabbit polyclonal primary antibodies were used to investigate serum enolase in Atlantic salmon sera by Western blot analysis. The first of these was produced against a peptide sequence designed using human enolase 1 sequence (Aviva Systems Biology) and is referred to as ENO1a herein. The others were purchased from GeneTex and were produced using peptides based on zebrafish enolase 1 and enolase 3 sequences and are herein referred to as ENO1b and ENO3 antibodies, respectively. In the first instance, these were found to cross-react with salmon enolase and then used for Western blotting using optimized concentrations of: 1:2000 (in 5% skimmed milk TTBS) for ENO1a and ENO1b antibodies and 1:5000 for ENO3 antibody. Samples for Western blotting were pools made up determined by week from the cohabitation trial with one microlitre being taken from every individual sample and pooled together for each time point. This resulted in nine pools being made from week 0 to week 12 post-challenge.

Protein in samples were separated by one-dimensional electrophoresis (1D-E) using Criterion XT precast gels 4–12% Bis-Tris (Biorad). Samples were prepared in sample buffer (Biorad) and XT reducing agent (Biorad) and then diluted in water until the final protein loading was 8 μg per sample. The samples were then heated on a heating block for 5 min at 95 °C. Gels were run at 200V in a 3-(N-morpholino) propanesulfonic acid (MOPS) running buffer (Biorad) for 1 h, with one well containing 8 μL of the protein molecular weight standard 'all blue' (Biorad). Gels were then removed and protein transferred to a 0.2-μm pore size nitrocellulose membrane using the iBlot® dry blotting system (Novex Life Technologies). Nitrocellulose was then placed into Ponceau S stain for 10 min to ensure the transfer of protein to the membrane was successful and the global protein profile intensity was comparable for each sample. The stain was subsequently washed off using TTBS buffer which comprised of 50 mM Tris, 150 mM NaCl and 1% polyethylene glycol sorbitan monolaurate. The membrane was then blocked for 1 h in 10% w/v powdered skimmed milk (dissolved into TTBS) and washed three times in TTBS. Primary antibody was diluted in TTBS containing 5% w/v powdered milk and left rocking overnight at 4 °C. Antibody was then removed, and the membrane washed three times before treatment with HRP linked donkey polyclonal secondary antibody to rabbit IgG (Abcam) at a 1:10 000 dilution (in TTBS containing 5% w/v powdered milk) for 1 h at room temperature. After washing, the blot was then developed using Pierce enhanced chemiluminescence (ECL) Western Blotting Substrate (Thermo Scientific). This ECL substrate binds HRP on the membrane, thus allowing for band detection after exposure to a photographic emulsion-coated film, in this case Hyperfilm™ Film (GE Healthcare) and development with a X-ray film developer. Exposure times were optimized, and films then scanned (Umax Poerlook III) and saved in .TIFF format for analysis, and band intensity analysis of Western blot band intensities was carried out using Image J (http://rsb.info.nih.gov/ij/).

Western blots of the same samples were run and then stained with a rabbit polyclonal antisera to aldolase at 1:2000 dilution (Aviva Systems Biology) and a monoclonal antibody to 3-phosphoglyceraldehyde dehydrogenase at a 1:250

dilution (Millipore). Donkey polyclonal secondary antibody (HRP linked) at a 1:10 000 dilution and a donkey polyclonal antibody to mouse IgG (Abcam) at 1:5000 were used to detect antigen-bound aldolase and GAPDH antibodies, respectively.

Western blots were also carried out using anti-enolase 3 antibody (ENO3) following two-dimen sional electrophoresis (2D-E) and subsequent transfer, to examine the specificity of the antibody. In brief, 2D-E was carried out as in Braceland *et al.* (2013) with pooled W0pc and W8pc serum to see whether binding was in agreement with previous proteomic discovery of the enzyme in terms of molecular weight and isoelectric point (pI) and differential as indicated by 1D-E Western blots and previous proteomic information.

Enzyme-linked immunosorbent assay for enolase (ELISA)

For ELISA determination of enolase concentrations, the primary antibodies used were ENO1a and ENO3 at dilutions (in TTBS) of 1:500 and 1:1000, respectively, chosen on the basis of Western blot results and optimized for ELISA.

Serum samples and muscle lysate containing enolase and used as standard were diluted to specified dilutions using an ELISA plate coating buffer (0.2 M sodium carbonate/bicarbonate pH 9.4). To each well, 100 μL of either diluted sample, standard or blank (buffer alone, unless otherwise stated) was added and the plate left overnight at 4 °C. Wells were emptied, washed 3 times using 250 μL TTBS, blocked with 10% w/v (in TTBS) powdered skimmed milk and left on a shaker for 1 h at room temperature. The plate was washed again three times, and then, 100 μL of primary antibody at appropriate dilution in TTBS added to each well and the plate left on a shaker for 1 h. Another series of washes were carried out, and then, 100 μL of a HRP linked donkey polyclonal secondary antibody to rabbit IgG (Abcam) at a 1:10 000 dilution added for 1 h with the plate on a shaker. Finally, wells were washed with TTBS three times and then developed using 100 μL (per well) of a 3, 3′, 5, 5′ – tetramethylbenzidine (TMB) Microwell Peroxidase Substrate Kit (Insight Biotechnology) which by peroxidase reaction catalysed by HRP forms a blue by-prod uct. After incubation, on a shaker, of 10 min, the reaction was stopped by the addition of 50 μL of 1 M hydrochloric acid. The addition of HCl causes the colour of solution to change to yellow, thus enabling accurate measurement of the intensity at 450 nm using a plate reader (FLUOstar OPTIMA, BMG Labtech). The ELISA was validated by the assessment of performance characteristics. Purified salmon enolase was not available to use as a calibrator, therefore a pool of whole muscle lysate which is known to possess a high concentration of the enzyme and the presumed source of increasing serum concentration during PD and given an arbitary unit of (AU) 100. Accuracy was determined by parallel curves of dilutions of serum sample with a high enolase content versus the calibrator. Precision was established by calculating the intra- and intercoefficients of variance. The limit of detection was assessed as the amount in AU of enolase detectable at 3SD from the mean of blank. Specificity depends on antibody cross-reactivity.

Two sample sets were analysed by ELISA. First, enolase content of the pooled samples used as for Western blots was determined. Second, serum from fifty individual fish (see Table 1) from the PD trial were selected on the basis of skeletal muscle pathology due to the significant correlation of enolase abundance with skeletal muscle myopathy (see results). This was carried out to investigate the relation of the histopathological scoring system to this non-destructive biomarker and by examining serum concentrations from fish with a range of spectrum of muscle pathology severity.

Immunohistochemistry

Tissues for histology from salmon were immediately fixed in 3.5% v/v formaldehyde in buffered

Table 1 Mean white muscle histopathological score for each time point of sampling and corresponding enolase 1 and enolase 3 concentrations

Week	Average white muscle histopathology scores	Enolase 1 concentration (Au)	Enolase 3 concentration (Au)
0	0	18.325	0
2	0	16.915	0
3	0.0139	24.967	9.062
4	0.3194	30.258	25.051
5	1.0278	43.966	41.763
6	1.6389	52.093	49.6
8	2.0972	55.499	60.905
10	1.0833	37.024	47.042
12	0.0833	27.42	20.607

saline pH 7.0 (4.0 g $NaH_2PO_4.2H_2O$, 6.5 g $Na_2HPO_4.2H_2O$) prior to further processing by standard paraffin wax techniques and then sectioned in preparation for IHC. In the first instance, sections were stained with haematoxylin and eosin (H & E). IHC was carried out using muscle sections which were subjected heat-induced epitope retrieval (HIER) using Menarini Access Retrieval Unit using a sodium citrate (pH 6) buffer at 125 °C with full pressure for 1 min and 40 s. Slides were then loaded on to a Dako autostainer and rinsed with a tris-tween (pH 7.5) buffer (herein buffer A) and then treated with Dako Real™ peroxidase blocking solution for 5 min before being rinsed twice with buffer A for a further 5 min. Primary antibodies were diluted to 1:1000 (a number of concentrations were initially tested for optimization) using Dako universal diluent and incubated for 30 min. Sections were then rinsed again with buffer A twice for 5 min before treatment with an anti-rabbit IgG HRP labelled polymer (Dako) secondary for 30 min. Post-washing (buffer A 5 min twice) development was carried out using Dako K5007 DAB and then rinsed with distilled water, counterstained with Gills Haematoxylin for 30 s and washed in water again. Finally, they were dehydrated by treatment with 70% meth spirits then absolute alcohol, cleared with xylene and then mounted in DPx mounting media (Cell Path cat # SEA-0302-00A).

Results

1D-E Western blotting

Western blots were carried out using all three commercially available antibodies to enolase anti-ENO1a (Fig. 1a), anti-ENO1b (Fig. 1b) and anti-ENO3 (Fig. 1c). All of these antisera detected a dominant protein with a molecular weight (Mw) of just under 50 KDa with some lesser abundant proteins apparent with ENO1b and ENO3. These could be due to non-specific cross-reactions or in the case of protein with low Mw, could be degradation product. Semiquantification of band intensities of the Western blots with antibody to enolase was carried out using image J (Fig. 1a, b and c) with similar band profiles being seen in all three blots. Some variation in the specificity and reactivity was observed between these antibodies which may account for a failure to detect enolase in W3 with ENO3 (at a concentration above the sensitivity of the test). It was not until W3pc that a band could be detected by these Western blots, whilst a band was still clearly observable in W12pc sample. These profiles agreed, in the main, with pathology scores of

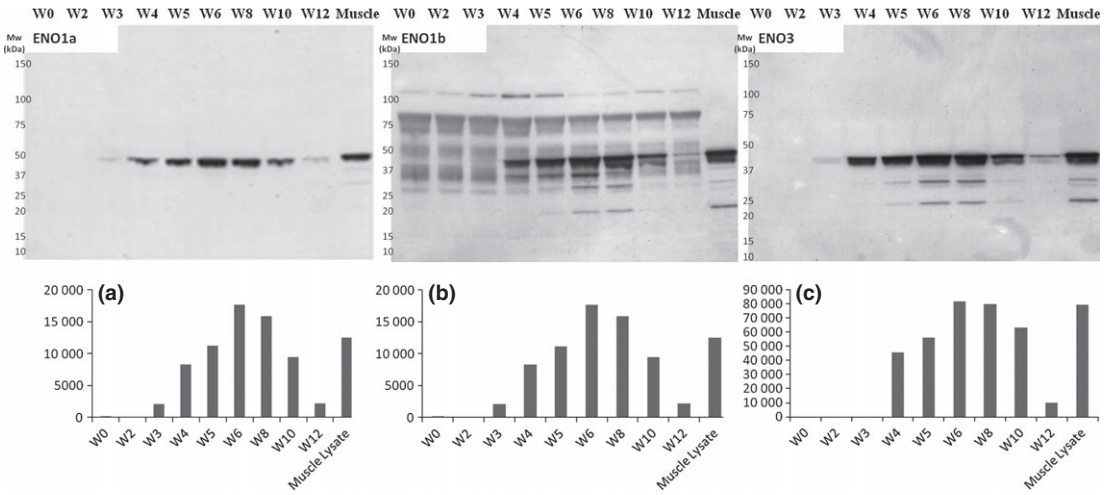

Figure 1 Western blots of serum using (a) anti-ENO1a (Aviva systems biology) antibody at 1:2000 concentration. (b) ENO1b (GeneTex) antibody at 1:2000 and (c) anti-ENO3 (GeneTex) antibody at 1:5000. Sample identifications for each lane can be found at the top of each with 'W' indicating serum from the relevant week post-introduction and 'Muscle' indicating in which lane a muscle lysate was used, with 8 μg of protein loaded per lane. Band intensities from image J analysis of Western blot are given below respected blots.

white muscle (Fig. 4 and Table 1) with a higher band intensity being observed where there is an increase in lesion severity. In addition, the observation of a band at the same Mw and of high intensity with muscle lysate indicated further that pathogenesis of PD may lead to the raised concentrations of enolase in serum. However, blots using ENO1a and ENO3 gave a higher intensity in band when using W6 serum compared with W8 serum despite the latter having a greater pathology score. It may be that the release of enolase from tissue into the serum precedes the maximum observable pathology or this may be due to Western blots being at best semiquantitative. To enable a better estimate of serum enolase, an immunological assay in the form of an ELISA was developed out using ENO1a and ENO3 antibodies (ENO1b omitted due to previously discussed background staining) to investigate in a more quantitative manner the relationship between serum enolase concentrations, Wpc and the pathogenesis of PD.

Western blot with antiserum to aldolase (Fig. 2a) and to GAPDH (Fig. 2b) confirmed that these enzymes were also raised in serum during the PD infection. In comparison with the Western blot with antibody to enolase, the aldolase blot showed more background staining suggestive of some non-specific interaction, whilst the blot with antibody to GAPDH revealed a shorter time window in the infection where the protein could be detected and enolase was selected for immunoassay development.

2D-E Western blotting

Figure 3 shows the antibody to ENO3 antibody to be specific for enolase which was identifiable from Mw (~48kD) and pI (7 to 8) in comparison with its position in the salmon serum proteome as described by Braceland *et al.* (2013). Whilst there is some binding in other areas, these proteins are most likely to be albumin and apolipoprotein, based on previous investigations, which are the most abundant proteins in the salmon serum proteome. Moreover, this non-specific binding does not appear to alter depending on disease state, having similar intensity in both W0pc and W8pc blots. In addition, there appears to be an absence of any specific ENO3 binding in W0pc with high intensity with W8pc serum, thus in agreement with 1D-E Western blots.

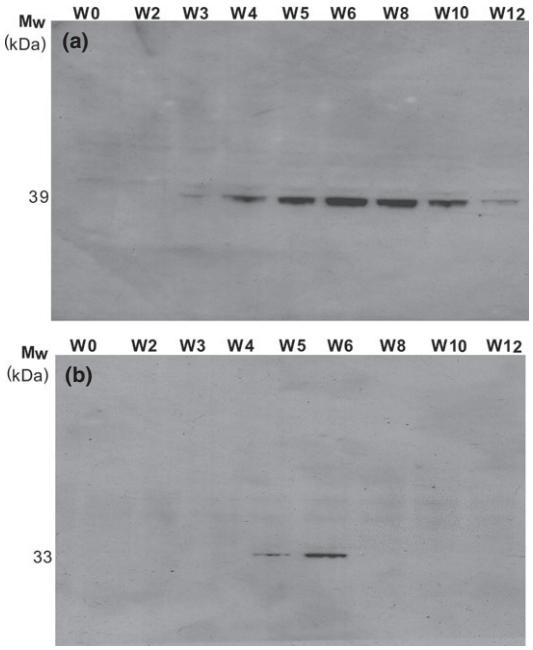

Figure 2 Western blot using (a) anti-Aldolase (Aviva systems biology) antibody at 1:2000 concentration. (b) antibody at 1:250 concentration. Sample identifications for each lane can be found at the top of each with 'W' indicating week post-introduction and 'Muscle'.

Histopathology

The mean histopathological pathology scores using the semiquantitative scoring system as described by McLoughlin *et al.* (2006) can be seen in Fig. 4. The scores were used for comparison between pathology and enolase levels determined by ELISA.

ELISA

The ELISA was developed to quantify the serum concentrations of enolase with the use of ENO1a and ENO3 antibodies being compared.

Average intra-assay %CV using ENO1a antibody was 3.4% and ENO3 3.5% ($n = 98$). Interassay %CV of low standard (W4pc pool) was 10.8%, and high standard (W8pc) = 6% when using antibody ENO1a, whilst ENO3 antibody gave interassay %CV results of 12.4 and 4.3% for low and high standards, respectively ($n = 30$). Limit of detection was found to be 2 AU (arbitrary units) for ENO1a and 2.1 AU when the ELISA was carried out using ENO3 antibody. When using W0pc, sera as blank limit

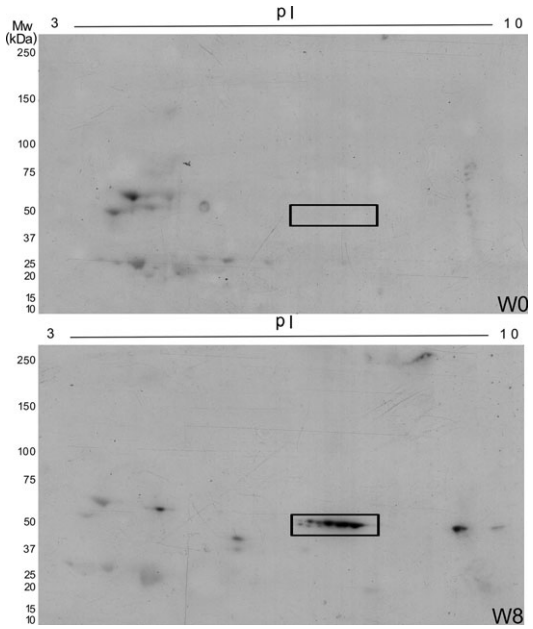

Figure 3 Two-dimensional Western blot using anti-ENO3 (GeneTex) antibody at 1:5000. The top image shows the staining post-development when week 0 post-challenge pool was used, and the bottom shows week 8 post-challenge. A black box is around the area where the antibody is shown to be specific ENO3 staining at the correct Mw and pI.

of detection were found to be 4.5 AU (ENO1a) and 4.7 AU (ENO3) (Fig. 5). The same pooled sera, used in Western blots, were used in an indirect ELISA which used a whole muscle lysate 'standard' to give a sample concentration in arbitrary units. Optical density (OD) values were corrected against a foetal calf serum (FCS) blank. Quantification of serum enolase using both ENO1a and ENO3 antibodies by ELISA resulted in a similar profile of change after infection and validated Western blot results (Fig. 6). In addition, the concentration of enolase 1 and enolase 3 concentrations significantly correlated with mean white muscle histopathology scores (Table 1) with Pearson's coefficient values of 0.9639 and 0.945, respectively. Concentrations of enolase did not correlate significantly with the pathology of any other tissue (white muscle, heart and pancreas) in salmon with PD.

Fifty fish from the trial were selected to give a range of histopathological scores (Table 2) and their enolase 3 concentrations assayed. In a modification the ELISA pooled W0, sera were used as the blank so concentrations could be corrected against those of a 'healthy fish', thus giving more actual relevance. These concentrations were then compared to all tissue pathological scores by linear regression procedure GLM. Results again verified those previously with the only significant correlation being found with white muscle pathology ($P \leq 0.0001$ and 38% variation explained). Figure 7 illustrates this relationship with the enolase 3 concentrations of specific fish plotted against their corresponding histopathological score of white muscle damage. A clear trend of higher sera enolase concentrations can be seen in fish with worse histopathological damage.

Immunohistochemical staining

Immunohistochemistry (IHC) was carried out using the antibodies utilized for Western blot and ELISA results. Figure 8 illustrates IHC staining of

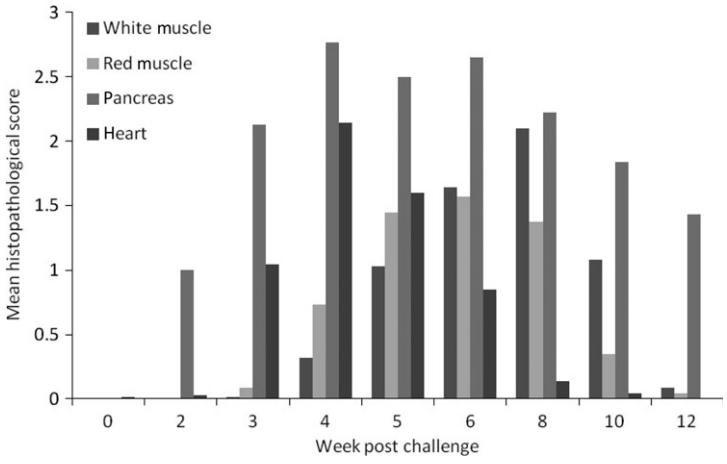

Figure 4 Mean histopathological score (using a semiquantitative scoring system) of each tissue at the nine sampling time points from week 0 to week 12 post-challenge. (*n* per time point = 108).

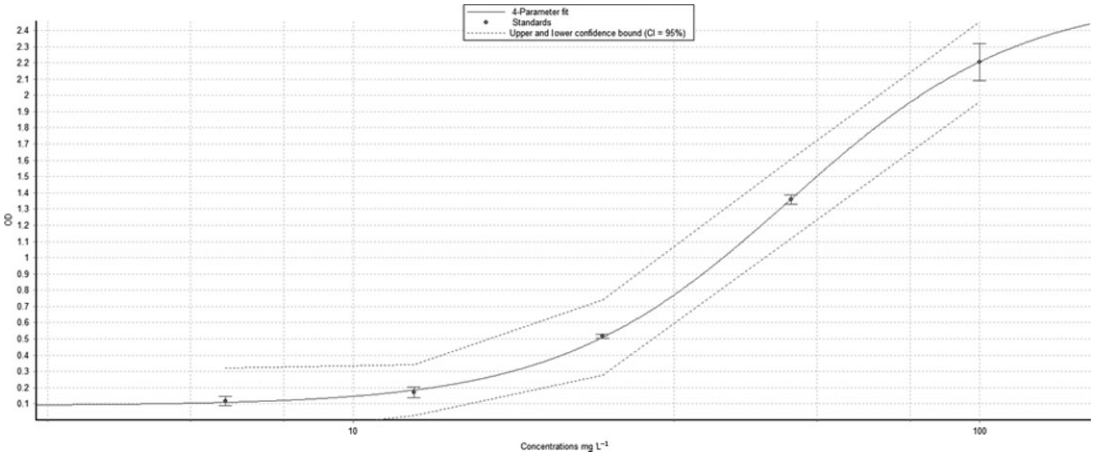

Figure 5 Standard curve of arbitrary unit standard.

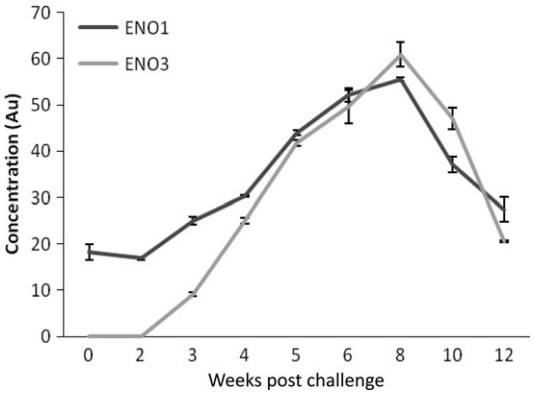

Figure 6 Enolase concentrations of pooled sera from the nine sampling time points (*x* axis) post-challenge. Arbitrary units are given as sera optical densities (ODs) were read against the OD of whole skeletal muscle lysate.

a muscle section comprising both red and white muscle fibres. A clear differential staining pattern can be observed, with little or no staining seen in red muscle indicating a low concentration or absence of antigen but with clear staining in the white muscle.

Discussion

Through the use of histopathology and serum protein analysis, a significant relationship between white muscle pathology and serum enolase content has been demonstrated. Serum enolase quantification may therefore be a useful biomarker of this myopathy. This study has provided validation for the biomarker discovery using proteomics previously described (Braceland *et al.* 2013). Western blotting demonstrated that aldolase and enolase shared very similar profiles, whilst GAPDH abundance shows a sharp rise at W5pc, peaking at W6pc and then falling rapidly as described in Braceland *et al.* (2013). Enolase was chosen as the most appropriate marker of PD pathogenesis, rather than aldolase or GAPDH, due to lower background binding, a greater fold increase in intensity and being at detectable levels for longer in the sera. Such a biomarker may be useful as a non-destructive and cost-effective health monitoring tool in Atlantic salmon as it may detect myopathy in apparently normal fish and predict flesh quality issues at harvest. In addition, enolase also holds promise in assessing the sensitivity of histopathological scoring systems. Semiquantitative scoring systems such as the one used in this study (McLoughlin *et al.* 2006), whilst extremely useful in describing the extent or degree of pathology, are limited in a number of ways. First, due to the categorical nature of any semiquantitative scoring system, there will always be a level of subjectivity, as to where one sample is placed, and given that there will in nature always be heterogeneity, this can lead to the reduction in accuracy. For instance, whilst there was a significant correlation of white muscle histopathological scores and enolase 1 and 3 sera abundance, the level of variance between concentrations of fish placed in the same category (or score) was reasonably high. This was reflected in the fact that whilst the probability of a correlation being at $P \leq 0.0001$, the amount of variance explained was only 38%. Whilst this is

Table 2 Samples used with their identification (ID) histopathological damage scores of all tissues examined (heart, pancreas, red and white muscle) and enolase concentrated in arbitrary units (AU)

ID (tank. fish.week)	Heart	Pancreas	Red muscle	White muscle	ENO 3 CONC. (AU)
1.1.0	0	0	0	0	5
8.6.12	0	2	0	0	0
3.3.0	0	0	0	0	0
4.4.5	2	3	0	0	12
12.1.12	0	3	0	0	0
2.3.3	2	3	1	0	0
1.5.3	3	3	1	0	0
4.2.6	2	3	1	0	25
6.6.3	2	3	1	0	1
11.6.3	2	3	1	0	12
4.3.4	1	3	2	0	8
2.1.4	3	3	2	0	1
2.2.4	2	3	2	0	6
7.4.5	3	3	2	0	20
1.3.4	3	3	2	0	0
1.6.4	2	3	3	0	9
10.2.5	1	3	3	1	18
4.5.5	3	3	3	1	12
8.2.6	1	3	3	1	26
1.6.6	2	3	3	1	17
3.5.10	0	1	0	1	19
3.2.8	0	2	0	1	0
7.1.5	3	2	0	1	0
9.4.12	0	3	0	1	2
11.5.4	2	3	0	1	0
1.6.10	1	3	0	2	33
5.3.10	0	2	0	2	12
6.2.8	1	3	0	2	23
8.3.8	0	2	0	2	18
7.4.10	0	2	0	2	0
8.2.8	0	2	0	3	30
9.1.8	0	3	0	3	29
10.4.8	0	1	0	3	33
9.5.10	0	3	0	3	8
11.4.10	0	3	0	3	28
11.6.5	1	3	3	3	21
6.5.5	0	3	3	3	15
2.2.6	2	3	3	3	17
11.3.6	0	3	3	3	22
8.1.8	0	3	3	3	26
2.6.10	0	2	2	2	37
6.6.10	0	2	2	2	26
1.6.5	2	2	2	2	23
3.6.8	0	2	2	2	19
1.6.8	0	3	2	2	36
5.5.4	2	3	1	1	8
3.6.4	2	3	1	1	15
11.2.12	0	3	1	1	26
H3.6.6	0	2	1	1	12
6.1.10	0	2	1	1	17

still a reasonably high degree of variance being explained, it is probable that the use of a less categorical scoring system would increase sensitivity and thus accuracy. However, the use of such a system would take longer to carry out using more resource and require an even higher level of skill.

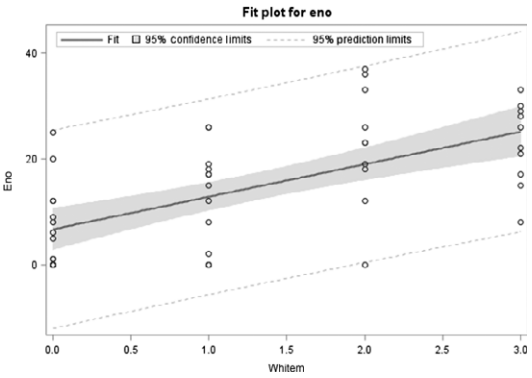

Figure 7 Individual fish enolase three concentrations plotted against corresponding white muscle histopathological score.

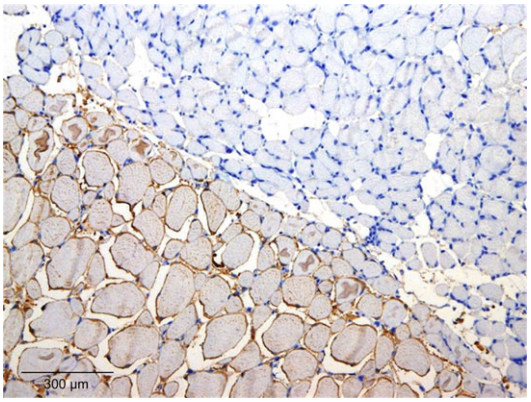

Figure 8 Immunohistochemical staining of enolase using ENO1a antibody and subsequent staining with haematoxylin. Presence of antigen is indicated brown staining.

In addition, humoral non-destructive markers can give an indication on the extent of pathological damage in terms of the whole organism rather than just in the small area sampled for histopathological analysis.

It is important to note that currently, in salmon disease, other non-destructive markers of skeletal muscle myopathy are used, with the most common being creatine kinase (CK) (Ferguson, Rice & Lynas 1986; Rodger *et al.* 1991; Yousaf & Powell 2012). However, the assay relies on the enzyme activity of CK whilst a reliable and well-established method has some disadvantages over the enolase ELISA described here. For instance, the range of concentrations in healthy sera (due to a high and variable activity) is large (Ferguson *et al.* 1986; Rodger *et al.* 1991; Yousaf & Powell 2012), thus making it hard to identify subtle

changes in concentrations on a site to base health management decisions on at a given time point. This is especially a problem when a site has an outbreak of HSMI and CMS where changes may be quite small and fall within this range. In addition, so far the literature has only investigated total CK concentrations, which is ubiquitous, thus not indicating a specific pathology.

Unless a biomarker is truly tissue specific, histopathology remains the most reliable means of identifying specific pathogenesis. Enolase in vertebrates is characterized as possessing three paralogs; enolase 1 (α enolase), 2 (γ enolase) and 3 (β enolase) (Tracy & Hedges 2000). Enolase 2 is regarded as neuron specific, thus enolase 1 and enolase 3 were chosen in this study for immunoassay development with enolase 3 being chosen as the best marker due to information on interassay CV and reliability in the ELISA and also with its specificity being demonstrated in both one- and two-dimensional electrophoresis and Western blot. Both correlate with white muscle pathology. Interestingly, enolase 1 did not correlate with heart pathology despite enolase activity (via an enzyme kinetics assay) has been shown in heart lysate from coho salmon, *Oncorhynchus kisutch* (Landrey, Applegate & Cardenas 1978). Moreover, α-enolase (enolase-1) has been proposed as an early indicator of myocardial infarction in humans (Mair 1997). An explanation as to why enolase concentrations were not altered in serum due to cardiac myopathy is that either the limit of sensitivity of the Western blot and ELISA is too high and thus the amount of enolase being released (passively by pathological damage) into the humoral system is too low and therefore cannot be detected. Despite this, even when mean pathological damage is low (0.0139) at week 3 post-challenge, there is a detectable increase from previous sampling points in enolase concentration. However, this may be due to the quantity of muscle when compared to heart. A similar issue was found by Yousaf & Powell (2012) when trying to analyse the effect of the pathogenesis of CMS where they found no significant correlation of pathological damage to the heart on creatine kinase (CK) and lactate dehydrogenase (LDH). However, in the same study, they showed heart pathology in fish with HSMI significantly correlated with CK and LDH levels. However, as future studies attained samples from sites of natural outbreak, it could be that the sampling had simply missed the effect of pathology on the proteome, with the enzymes leaked already being turned over. Despite this, the lack of correlation (between heart pathology and enolase concentration) in this current study is interesting, and future work will aim to identify the distribution of specific isoforms of enolase in salmon. This would be of great use as if isoforms are found, as in mammals, to be tissue specific, they may be used to differentiate between pathologies of different tissues in a non-destructive manner as is routinely carried out in human medicine and for other livestock species. In fact studies where the direct effect of specific tissue pathologies on the humoral proteome or enzyme concentrations is distinctly limited, in salmon infectious disease. Creatine kinase has also been studied in terms of sera levels in fish with PD (Ferguson *et al.* 1986; Rodger *et al.* 1991). However, the relationship between sera levels and histopathology was not directly investigated.

Conclusion

In conclusion, this study has identified, through the use of commercial antibodies, significant correlations of enolase 1 and 3 serum concentrations with muscle pathology in PD. The enolase ELISA has been shown to have potential uses as a non-destructive health monitoring tool for this and possibly other diseases involving myopathy in salmon and other teleostei species. Such markers, currently lacking in aquaculture, may be beneficial to the industry by being an early stage indicator of a problem developing at a given site. Monitoring in such a way may have economic benefits as such an early diagnosis of a disease problem therefore limiting intra- and intersite spread of aetiological agents and allowing for the implementation of other disease management strategies such as functional feeds at the most optimal time. In addition, this marker may also be useful, due to its correlation with white muscle pathology, a means of assessing the sensitivity of histopathological scoring systems such as the one used in this study. Monitoring may also help lower the ecological impact of the industry in terms of farmed to wild stock disease interactions. Future work on this enzyme aims to examine tissue distribution of specific isoforms and the establishment of a reference range of enolase in healthy salmon production site and change during against natural outbreaks. This will involve a real expansion of study number (n) and the validation of this marker using

samples from fish suffering from diseases other than PD which cause muscle myopathy.

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