MASTS Small Grants Report

Grant Application title: SASG9 Plasma proteome of an unwanted phenotype of steelhead trout
Recipient: Bernat Morro (PhD student. Institute of Aquaculture, University of Stirling) and Dr Mary Doherty (Lecturer. Department of Diabetes and Cardiovascular Sciences, University of Highlands and Islands)
Address: Institute of Aquaculture, University of Stirling, Stirling, Scotland. FK9 4LA
Email: bernat.morro@stir.ac.uk
Activities covered by grant: LC MS/MS (Gel electrophoresis liquid chromatography tandem mass spectrometry) proteome profiling service of blood plasma samples

Background

Over the last 30 years there has been an increase in the production of the sea-run phenotype of rainbow trout (*Oncorhynchus mykiss*), steelhead trout, reaching over 71000 tonnes in 2015 (Food and Agriculture Organization of the United Nations, 2015). This phenotype presents desirable attributes such as the resistance to infectious pancreatic necrosis (Okamoto et al., 1993; Ozaki et al., 2001) and its tolerance to brackish water (Altinok & Grizzle, 2001) where sea lice cannot survive. However, there is limited knowledge on the environmental control of steelhead smoltification and appropriate tools to assess trout smolt status are lacking. In consequence, when the all season Atlantic salmon smoltification protocol was applied to steelhead trout the industry faced numerous problems such as high mortalities and fish that experienced no growth after seawater transfer, known as 'pinne' fish (which account for up to 10% of the production), especially during the summer season, making this period (and one third of the production potential) mostly abandoned by the industry.

The development of 'pinne' fish is presumed to be the result of a mismatch between environmental conditions and the timing of smoltification. Hence, this developmental anomaly is likely related to a lack of understanding of vital intrinsic (*e.g*. critical size, genetically determined phenotypic plasticity) and extrinsic (light, temperature, salinity, etc.) factors governing the physiological development of the fish during the smoltification and subsequent seawater adaptation processes.

The aim of this project was to analyse the plasma proteome of 'pinne' steelhead trout and compare it to control steelhead trout that successfully smoltified and grew optimally in seawater. Identifying differences in protein abundance between the two groups could be used to understand the underlying physiological mechanisms leading to the development of 'pinne' fish.

Materials and methods

Fish

In September 2016, 242 post smolt steelhead trout were sampled, 2 months after being transferred to seawater. The fish were sacrificed by lethal overdose of benzocaine solution (Sigma-Aldrich). For each fish, weight and length were recorded and blood was sampled, among other organs (i.e. liver, spleen, head kidney and gills). Blood was extracted using heparinised syringes and centrifuged at 3500xg for 10 minutes to obtain blood plasma, which was frozen at -80^{o}C.

Sample preparation (Stirling University)

To carry out the proteomic analysis (Figure 1), pooled blood plasma samples (12+ individuals per condition) of both 'pinne' and well-grown individuals were used. Fish were selected based on their condition factor, the 'pinne' having the lowest values (1.21 ± 0.07) among the population and the well-grown the highest (1.56 ± 0.07).

Protein concentration in plasma was determined using the BCA assay (Interchim). Samples were then run in 1D-SDS PAGE directly or after enrichment with Proteominer™ (Bio-Rad), which reduces the abundance of the most abundant proteins and allows the detection of the less abundant ones (equaliser technology). Gels were run in a BIO-RAD Mini-PROTEAN Vertical Electrophoresis apparatus immersed in tris-glycine running buffer (24.7 mM Tris-Base, 191.8 mM glycine, 3.46 mM SDS, ultra-pure water, pH adjusted to 8.3) at 200V (400 mA) for 50 min. The run gels were washed 3 times (5 minutes each) with 200 ml distilled water. Then, they were fixed and stained in 20 ml of SimplyBlue™ SafeStain (Thermo Fisher Scientific) for 1 hour at room temperature with gentle shaking. Afterwards, the solution was discarded and the gel was washed 3 times (5 minutes for the first two and overnight for the last one) with 200 ml distilled water. Under a laminar flow cabinet, each gel lane was sliced in around 20 cuts (plugs) using a scalpel, and then processed independently in 1.5 ml reaction polypropylene tubes. For destaining, plugs were incubated twice (10 minutes each) in 100 µl of destaining solution (50 mM ammonium bicarbonate and acetonitrile (ACN) 50% v/v) at 37°C. Samples were then incubated in 50 µl of reducing solution (10 mM dithiothreitol and 100 mM ammonium bicarbonate) at 37°C for 30 min. Next, plugs were incubated in the dark with 50 µl of alkylation solution (55.1 mM iodoacetamide and 100 mM ammonium bicarbonate) at 37°C for 30 minutes, and then dehydrated in 100 µl of ACN at 37°C for 15 minutes. After complete evaporation of the ACN on a heat block (37°C), plugs were digested with 50 µl of trypsin (Promega) prepared in a solution of 5mM acetic acid and 45 mM ammonium bicarbonate. Initially, samples were incubated at 37°C for 1 hour. Then, 20 µl of 100 mM ammonium bicarbonate was added and the tubes were incubated for another 14 hours at 37°C. Finally, the digests were transferred into sterile 1.5 mL polypropylene tubes containing 2 µl of 10% formic acid, and completely dried using a vacuum drier (Savant DNA SpeedVac 110).

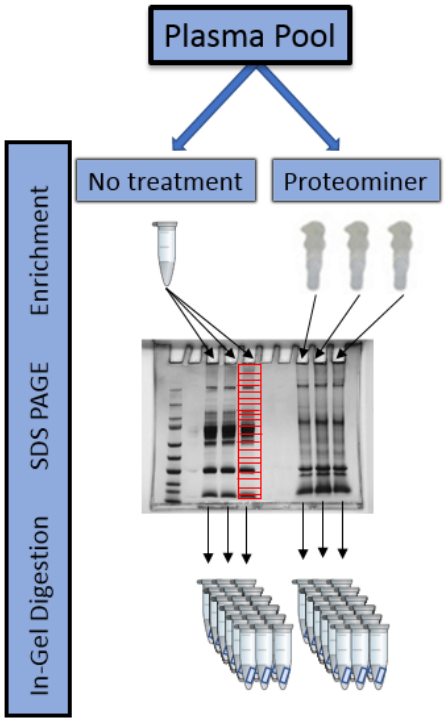

Figure 1. Overview of the methods prior LC-MS/MS analysis. These methods were applied to both plasma samples from 'pinne' and well-grown steelhead trout. The red squares indicate the approximate location of the cuts that divided each lane in small gel plugs.

LC MS/MS (University Highlands and Islands)

Tryptic digests were analysed with a LTQ-Orbitrap XL LC–MSn mass spectrometer (Thermo Fisher Scientific) equipped with a nanospray source and coupled to an Ultra High Pressure Liquid Chromatographer system (Waters nanoAcquity). Initially, 5 µL of sample resuspended in ultrapure water were loaded, desalted and concentrated in a BEH C18 trapping columns (Waters) with the instrument operated in positive ion mode. The peptides were then separated on a BEH C18 nanocolumn (1.7 µm, 75 µm × 250 mm, Waters) at a flow rate of 300 nL/min using an acetonitrile/water gradient; 1% ACN for 1 min, followed by 0−62.5% ACN over 21 min, 62.5− 85% ACN for 1.5 min, 85% ACN for 2 min and 1% ACN for 15 min.

MS spectra were collected using data-dependent acquisition in the range m/z 400−2,000 using a precursor ion resolution of 30,000, following which individual precursor ions (top 5) were automatically fragmented using collision induced dissociation with a relative collision energy of 35%. Dynamic exclusion was enabled with a repeat count of 2, repeat duration of 30 s and exclusion duration of 180 s.

Data analysis

The mass spectrometry data was analysed using Progenesis QIP against the Actinopterygii SwissProt database. The initial search parameters allowed for a single trypsin missed cleavage, carbamidomethyl

modification of cysteine residues, oxidation of methionine, acetylation of N-terminal peptides, a precursor mass tolerance of 10 ppm, a fragment mass tolerance of ±0.5 Da, and a FDR of 0.01. After normalization, the Hi-N approach was used for quantitation. For each protein, the 3 most abundant peptides have their abundances averaged to provide a reading for the protein signal. The ranking of peptide abundance is based on the integrated value across all the runs, allowed by the lack of missing values and accurate alignment. This gives added confidence in the peptide selection, taking all runs into account to make the ranking robust. An ANOVA test was used to compare the abundance of each detected protein in both conditions.
All plots were created using R software.

Results

Overall, 475 different proteins were identified. Of these, 469 were found in both whole (untreated) and enriched plasma (treated with Proteominer^{TM}).

Setting the cut-off at p-value ≤ 0.05 and fold change of 2, a total of 182 and 102 proteins were found to be significantly different in abundance between well-grown and 'pinne' fish, in enriched and whole plasma, respectively (Figure 2a and 2b). Forty-three of them were found to be consistently found in both methods, while 139 proteins were obtained only in enriched plasma and 59 only in whole plasma (Figure 2c). Of these, 130 were higher in 'pinne' fish while 111 were higher in well-grown fish.

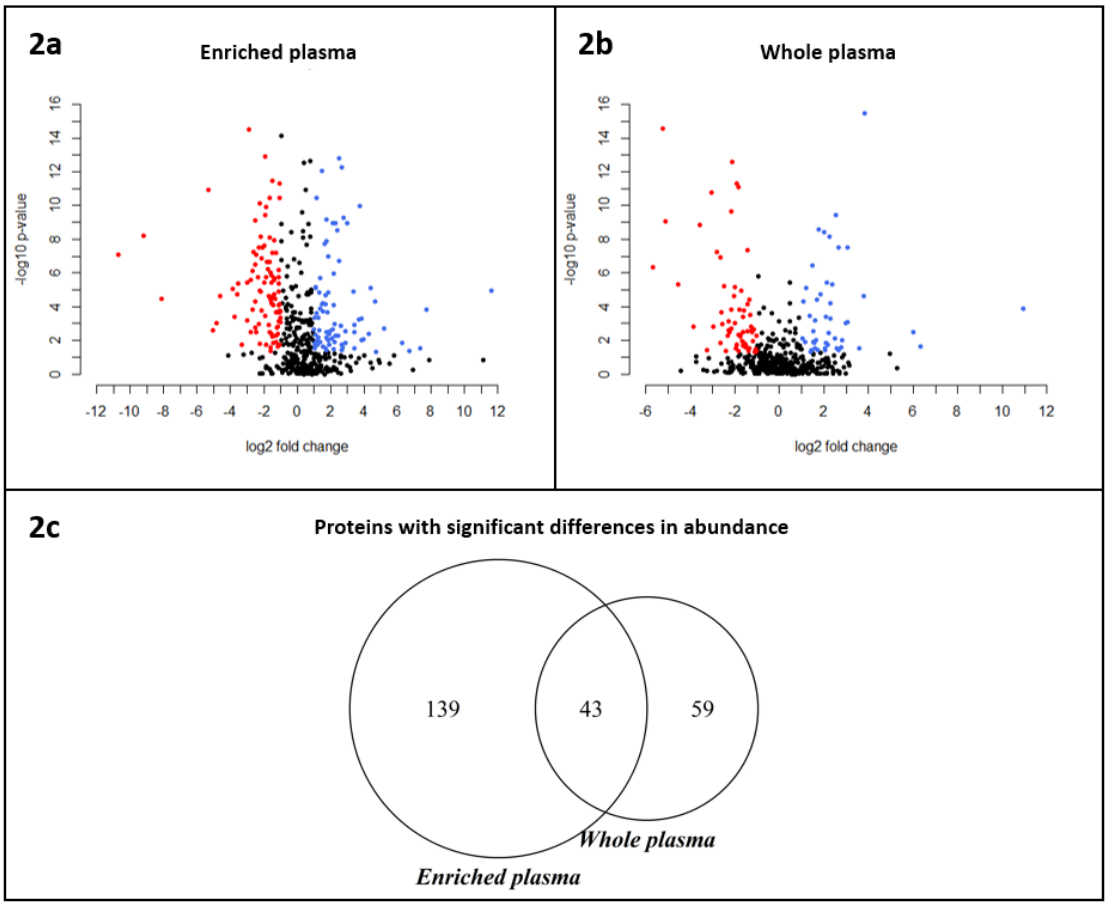

Figure 2. Detected proteins by GeLC MS/MS. Volcano plots of the detected proteins in **(2a)** enriched plasma and **(2b)** whole plasma. Coloured dots represent those proteins with a p-value below 0.05 and a fold change above 2. Red dots indicate proteins that are higher in 'pinne' while the blue ones are higher in well-grown fish. **(2c)** Venn diagram of the proteins with a p-value below 0.05 and a fold change above 2 detected in enriched and whole plasma.

Conclusions and future analysis

A vast database of plasma proteins detected in steelhead trout under different physiological conditions is now available. Many proteins from this list were found to be significantly different in abundance according to the condition of the fish (well-grown versus 'pinne'). This information will be used to gain a better understanding of the processes that lead to the 'pinne' phenotype in steelhead trout.

The data that has been gathered will be further analysed using both holistic tools such as gene ontology and related software (e.g. CytoScape) and a more targeted approach that will involve studying specific proteins independently.

This data, along with growth and morphometric data, sodium-potassium ATPase activity of the gill, insulin-like growth factor 1 measurements in blood plasma, qPCR data of genes in the somatotropic axis in liver, and metabolomics of liver will be submitted to a peer-reviewed journal in 2018.

References

Altinok, I., & Grizzle, J. M. (2001). Effects of brackish water on growth, feed conversion and energy absorption efficiency by juvenile euryhaline and freshwater stenohaline fishes. *Journal of Fish Biology, 59*(5), 1142-1152. doi:10.1006/jfbi.2001.1722

Food and Agriculture Organization of the United Nations. (2015). *FishStatJ - software for fishery statistical time series*

Okamoto, N., Tayama, T., Kawanobe, M., Fujiki, N., Yasuda, Y., & Sano, T. (1993). Resistance of a rainbow trout strain to infectious pancreatic necrosis. *Aquaculture, 117*(1-2), 71-76. doi:10.1016/0044-8486(93)90124-H

Ozaki, A., Sakamoto, T., Khoo, S., Nakamura, K., Coimbra, M. R. M., Akutsu, T., & Okamoto, N. (2001). Quantitative trait loci (QTLs) associated with resistance/susceptibility to infectious pancreatic necrosis virus (IPNV) in rainbow trout (oncorhynchus mykiss). *Molecular and General Genetics, 265*(1), 23-31.