Masts Small Grants Report SG392 Effect of mycotoxins in fish gills using targeted transcriptomics and untargeted phosphoproteomic approaches.

Researchers involved in this project:

Ms Anne Greulich, Dr Amaya Albalat and Dr Simon MacKenzie (Institute of Aquaculture, University of Stirling)

Dr Emma Carrick and Dr William Mullen (Institute of Cardiovascular and Medical Sciences, University of Glasgow)

Address: Institute of Aquaculture, University of Stirling, Stirling, Scotland. FK9 4LA
Corresponding email: amaya.albalat@stir.ac.uk

Activities covered by grant: Analysis of phosphorylated peptides using a non-targeted MS approach in a rainbow trout gill cell line.

Background

Mycotoxins are toxic secondary metabolites produced by microscopic filamentous fungi. Dexoxynivalenol (DON) commonly known as vomitoxin is one of the most frequent mycotoxins associated with fungi of the genus *Fusarium* found in cereal-based food throughout the world. This mycotoxin has become a major concern due to its shown toxic effects in humans and terrestrial farmed animals. Studies have shown that chronic dietary exposure of mycotoxins in pigs, poultry and trout leads to decreased feed intake and weight gain and immune dysregulation (Bryden 2012; Matejova et al., 2016).

Aquaculture is a rapidly growing industry that has the potential to play a key role in food security with current scenarios predicting an increase in population and global demand for food for at least another 40 years (Godfray et al. 2009). Feed supply to ensure growth and sustainability of the aquaculture sector is, as in all animal production systems, of paramount importance. Traditionally, aquaculture feed production has been heavily reliant in fish meal and fish oil provided by pelagic fisheries. However, intense research for the substitution of such finite natural resources has allowed a very significant reduction in the average inclusion of fish meal and fish oil in commercial feed. Indeed FAO has predicted that reduction of fish meal in the next decade for marine fish will be from 26 to 12% and from 3 to 1% in tilapia (Tacon et al., 2011). This approach although desirable from both fisheries and aquaculture perspectives in turn demands for more research to fully understand the impact of plant protein and oil use for fish feeds within the context not only of fish nutritional requirements *per se* but also from a fish health and welfare perspective. Research in this area has shown that mycotoxin contamination of grains is very prevalent with between 36-100% of feed samples destined for aquatic and terrestrial livestock tested being contaminated by at least one type of mycotoxin (Pietsch et al., 2013; Greco et al., 2015). The impact of such in-feed exposure in fish species is currently very sparse. Furthermore, another source of exposure highlighted in the literature and little studied is the one from water-exposure due to the presence of considerable concentrations of mycotoxins being discharged from agriculture run-offs that could be affecting aquaculture sites near farming sites. However, within this context little is known about the ecotoxicological impact of mycotoxins at levels found in the environment and the consequence of such exposure to aquatic organisms (Schwartz et al., 2011). Currently, the European Commission has set the maximum concentration of dexoxynivalenol (DON) at 5 mg/kg for fish which is 5.5 times greater than the maximum allowed for pig feed (0.9 mg/kg). This discrepancy indeed could be related to the limited amount of literature available on the effects of mycotoxins such as DON in fish.

Recent research carried out in Stirling using a Rainbow Trout gill cell line (RTG-W1) has shown that DON weaken gill cells tight junctions and increase both cell mortality and cell proliferation at the same time.

On the other hand, recent studies by other groups working in mycotoxins in other non-fish biological systems, have reported that phosphorylation of mitogen-activated protein kinases (MAPKs) play a key role in the regulation of downstream events (Zhang et al. 2016). However, there is a lack of overall detail on the signalling networks that might be involved mycotoxin-mediated cellular responses.

The aim of this research was to gain access to cutting edge MS-technology and expertise available at the Institute of Cardiovascular and Medical Sciences (Univ Glasgow) to develop a methodological pipeline using MS-based phosphoproteomics. Method development was carried out in RT gill-W1 epithelial cells challenged with poly-IC for 30 min and thereafter once the protocol was defined the same protocol was used but cells were challenged with DON for 30 and 60 min.

Materials and methods

Cell culture: In this project, the RT gill-W1 epithelial cell line was used. This is a cell line developed from rainbow trout (*Oncorhynchus mykiss*) gill tissue. The RTgill-W1 cells were grown in 25 cm^2 tissue culture flasks in Leibovitz's Medium supplemented with GlutaMAX™ (L-15, Invitrogen) and 10% Fetal Bovine Serum (FBS) at 22°C without CO_2 using a LEEC Precision Cooled Incubator. Cells were checked daily for the desired monolayer formation and confluence using an OLYMPUS® IMT-2 inverted microscope. RTgill-W1 cells were grown to ca. 90% confluence and then passaged with a split ratio of 1:3.

Cells used for phosphoproteomics analysis were seeded in six well plates containing inserts (Falcon™ Cell Culture Inserts: For Use With 6-Well Plates, pore size: 0.4 µm).

Trials: A six-well culture plate with inserts was grown to near confluence and the spent medium in the inserts was removed. For the control, 2 mL of fresh L-15 medium was added. For challenging the cells 2 mL of L-15 per well were supplemented with 2 µL of 1 µg mL-1 poly I:C (used as positive control for method development trial) or 100 ng mL-1 of Deoxynivalenol (DON, CAS: 51481-10-8, Romer Labs). The supplemented L-15 was added and the plate incubated at 22°C for 30 min in case of poly I:C and at 22°C for 30 min or 60 min in case of DON.

Cell collection: Medium was removed and cells were washed twice with either DPBS or HEPES (1M, Invitrogen). Then 400 µL of Lysis buffer (sodium deoxycholate (1% w/v), ammonium bicarbonate 50 mM, Halt™ Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, 10 µL per 1 mL Lysis-buffer)) was added to each well. Cells were carefully scraped off the insert membrane using a pipette tip. Then three wells per sample were pooled together in a 15 mL Falcon tube and stored at -80°C until further use.

Protein quantification and SDS-PAGE: To quantify the protein concentration in the cell lysates a BCA assay was carried out using the INTERCHIM UPTIMA BC Assay Protein Quantitation Small Kit. A standard curve was prepared with BSA (2 - 0.05 µg µl-1). Standard curve and sample dilutions were analysed in a BioTek Synergy HT 96-well plate spectrophotometer (BioTek, USA) at a wavelength of 562 nm. Then 15 µg of protein per sample were separated in a 1D-SDS-PAGE (GTX 4-20% Mini-protean gel, tris-glycine SDS-buffer (1x)). For estimation of molecular weight, protein markers were loaded in each gel (Precision Plus Protein™ Standards, 10-250 kDa from Bio-Rad, UK). Separated proteins were visualised using a MS-compatible Invitrogen™ SimplyBlue™ SafeStain (Thermo

Fischer Scientific, USA) and de-stained overnight with dH_2O. The gel was imaged using the Syngene Ultra Violet Transilluminator (Syngene; InGenius LHR Gel Imaging System).

Trypsin digestion: Proteins from cell lysates were resuspended in 100 µL of DTT (1 mM) in 100 mM of ammonium bicarbonate. The samples were vortexed until all protein was dissolved and then aliquots combined and incubated for 30 min at 37°C. 5.5 mM iodoacetamide was added and again samples incubated for 30 min at 37°C. Finally trypsin was added in a trypsin to protein ratio of 1:75 and incubated at 37°C overnight. The digestion was stopped by adding 1% of formic acid. Then the HyperSep™ SpinTip (Thermo Fisher Scientific) was used to clean up the digest following the manufacturer's instructions.

Phosphopeptide Enrichment: Phosphopeptide enrichment was done using the Pierce TiO2 Phosphopeptide Enrichment and Clean-up Kit from Thermo Fisher Scientific, following the manufacturer's instructions. Afterwards samples were dried in Savant DNA 110 SpeedVac® Concentrator and stored at -80°C.

LC-MS/MS: Digested proteins were analysed with a Dionex Ultimate 3000 RSLS nano-flow system (Dionex, Camberley UK). Samples were reconstituted in 10 µL of water and a volume of 5 µL were loaded onto a Dionex 100 µm × 2 cm 5 µm C18 nano-trap column at a flowrate of 5 µL min-1 by an Ultimate 3000 RS autosampler (Dionex, Camberley UK). The composition of the loading solution was 0.1% formic acid and ACN (98:2). Once loaded onto the trap column the sample was washed off into an Acclaim PepMap C18 nano-column 75 µm × 15 cm, 2 µm 100 Å at a flowrate of 0.3 µL min-1. The trap and nano-flow column were kept at 35°C in a column oven in the Ultimate 3000 RSLC. The samples were eluted with a gradient of solvent A: 0.1% formic acid and ACN (98:2) versus solvent B: 0.1% formic acid and ACN (20:80) starting at 5% B rising to 50% B over 100 min. The column was washed using 90% B before being equilibrated prior to the next sample being loaded. The eluant from the column was directed to a Proxeon nano-spray ESI source (Thermo Fisher Hemel UK) operating in positive ion mode then into an Orbitrap Velos FTMS. The ionisation voltage was 2.5 kV and the capillary temperature was 200°C. The mass spectrometer was operated in MS–MS mode scanning from 380 to 2000 amu. The top 20 multiply charged ions were selected from each full scan for MS–MS analysis, the fragmentation method was HCD at 30% collision energy. The ions were selected for MS2 using a data dependant method with a repeat count of 1 and repeat and exclusion time of 15 s. Precursor ions with a charge state of 1 were rejected. The resolution of ions in MS 1 was 60,000 and 7500 for MS2.

Data processing: LC-MS/MS data was processed initially uploading the raw spectra data into Thermo Proteome Discoverer 1.4 Thermo Scientific (Hemel Hempstead, UK). Peak picking was performed under default settings for FTMS analysis such that only peptides with signal to noise ratio higher than 1.5 and belonging to precursor peptides between 700-8000 Da were considered. Peptide and protein identification was performed with SEQUEST algorithm. An in house compiled database containing proteins from the latest version of the UniProt SwissProt database (2017) was compiled to include only *Danio rerio* entries (method development dataset) or *Oncorhynchus mykiss* (DON-trial). The search parameters were: Tryptic cleavage with 2 missed cleavages; static modification was Carbamidomethyl of Cysteines; dynamic modifications was oxidation of Methionine and phosphorlyation of S,T and Y. Precursor tolerance was 10 ppm with MS2 tolerance of 0.05 Da. Resulting peptides and protein hits were further screened by excluding peptides with an error tolerance higher than 10 ppm and by accepting only those hits listed as high and medium confidence by Proteome Discoverer software.

Results

Method development: Since to date, there is no established protocol to enrich phosphopeptides from RTgill-W1 protein samples a method was developed. Cells were grown using cell culture inserts. These inserts have an apical side and a basolateral side. In the method development experiment cells were challenged with poly I:C, which has been reported to interact with the toll-like receptor 3 (TLR3) that is part of the innate immune system and consequently the immune response. The interaction with TLR3 triggers a signalling cascade during which proteins are phosphorylated. For challenging the cells a concentration of 1 µg mL-1 poly I:C in L-15 medium was used for 30 min. Before collecting the cells, two buffers were tested on the final wash step of the cells, DPBS and HEPES. According to published literature, mostly DPBS or PBS is used for washing the cells. Even though there is cleaning step before the phosphopeptide enrichment, there still might be some phosphate which competes with phosphorylated peptides for binding spaces on the column. As an alternative HEPES was tested as a phosphate free buffer to increase phosphopeptide recovery. Tryptic digestion and the clean-up thereof were done using standard methods. For the Phosphopeptide enrichment the Pierce TiO_2 Phosphopeptide Enrichment and Clean-up Kit from ThermoFisher Scientific was used. A literature review revealed that Titanium Dioxide (TiO_2) at present is the best and most commonly used method to enrich phosphopeptides from protein samples. The binding buffers in the enrichment kit contain trifluoroacetic acid (TFA) but the paper by Kettenbach and Gerber (2011) revealed that TFA might be detrimental in terms of phosphopeptide recovery. That is why alongside TFA also formic acid (FA) was tested as a binding buffer. The kit was used following the manufacturer's instructions. The only deviation from the instructions was a 60 min incubation time instead of 10 min (Kettenbach and Gerber, 2011). The overall set up of the experiment resulted in four different conditions testes: HEPES / TFA, HEPES / FA, DPBS / TFA and DPBS / FA. The LC-MS/MS revealed the highest peptide recovery for samples treated with FA irrespective of the cell wash buffer used (Fig. 1). However, TFA in combination with HEPES as a cell wash buffer selectively enriched the most phosphopeptides. That is why for further experiments HEPES in combination with TFA was chosen.

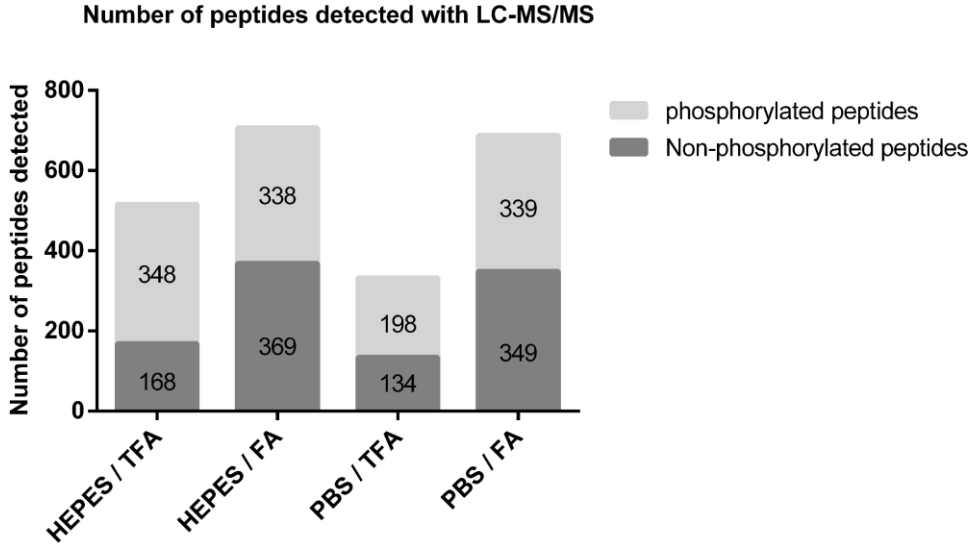

Fig. 1 Number of phosphorylated and non-phosphorylated peptides detected with LC-MS/MS, testing different conditions.

Effect of DON on the Phosphoproteome: As shown in Fig. 2, the LC-MS/MS detection of peptides (and also proteins) was much lower (around 100 peptides maximum) than it had been for the method development (around 600 peptides using the same sample preparation). The overall peptide recovery was 25 peptides for the 30 minutes control, 98 peptides for 30 minutes DON treatment, 89 peptides for the 60 minutes control and 91 peptides for 60 minutes DON treatment, with the majority of the peptides being phosphorylated (Fig. 2).

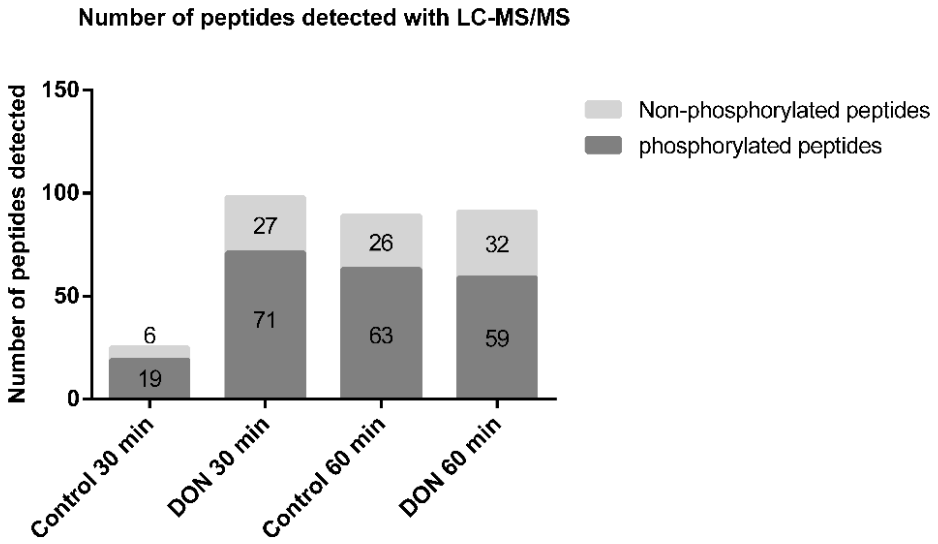

Fig. 2 Number of phosphorylated and non-phosphorylated peptides detected with LC-MS/MS, in un-treated RT-gill cells (control) and treated RT-gill cells with DON for 30 and 60 min.

Cells treated with DON for 30 min only shared 9.8 % of phosphorylated peptides with control cells, while cells treated with DON for 60 min shared a higher percentage (43.5%) of phosphorylated peptides with their control (Fig. 3).

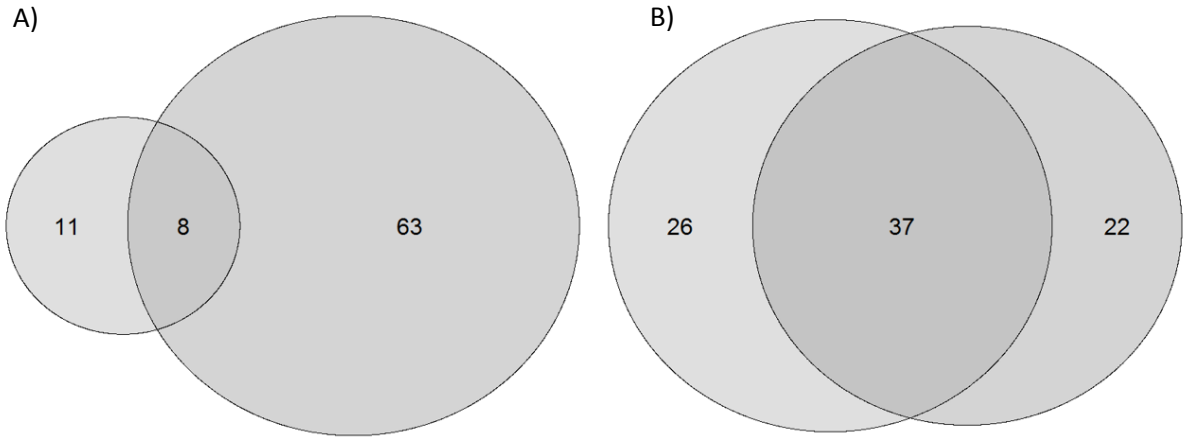

Fig. 3 Venn diagram showing the number of phosphorylated peptides shared between control and DON-challenged RT-gill cells for A) 30 min and B) 60 min.

In total only 20 proteins (18 containing phosphorylated peptides) were recovered summarizing all conditions. Of these proteins 4 were detected in the 30 minutes control, 14 in the 30 minutes DON treatment, 14 in the 60 minutes control and 12 in the 60 minutes DON treatment (Fig. 4).

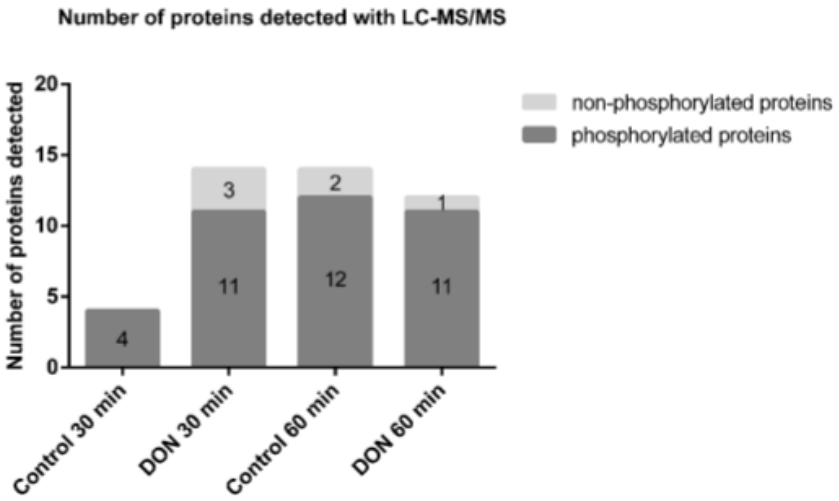

Fig. 4 Number of phosphorylated and non-phosphorylated proteins detected with LC-MS/MS, in un-treated RT-gill cells (control) and challenged RT-gill cells with DON for 30 and 60 min.

Cells treated with DON for 30 min shared 25% of phosphorylated proteins with their control, while cells treated with DON for 60 min shared twice as many phosphorylated proteins (53.3%, Fig. 5).

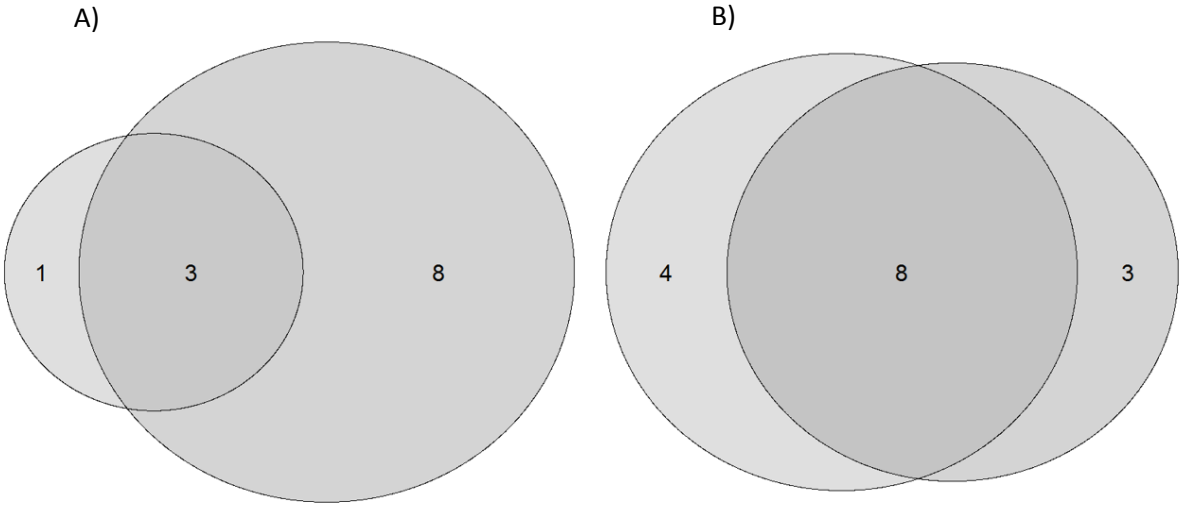

Fig. 5. Venn diagram showing the number of phosphorylated proteins shared between control and DON-challenged RT-gill cells for A) 30 min and B) 60 min.

Since the number of proteins was so low a closer examination was conducted on those that contained phosphorylated detected peptides (Table 1). Four heat shock proteins linked to stress response were found in all conditions tested except the 30 minutes control (Table 1). Only one peptide linked to the Heat shock 90kDa protein 1 beta isoform was found in the 30 min control. Another protein found was Sequestosome-1 which is suspected to regulate the activation of NFKB1 by TNF-alpha, nerve growth factor (NGF) and interleukin-1 in the cells. It probably also has a part in titin/TTN downstream signalling in muscle cells. This would correlate with the two titin-like isoforms found. Yet another protein found was “ras GTPase-activating protein-binding protein 2-like isoform X6” which is part of the negative regulation of the Wnt-signalling pathway and as a result changes transcription of target genes.

Discussion and Further work

This is the first time an untargeted approach to detect phosphorylated peptides has been conducted in RTgill-W1 cells. The method development indicated that both HEPES and PBS are suitable buffers for washing the cells before cells are harvested and lysed.

When cells were challenged with DON using the same protocol the number of phosphopeptides was lower than during the method development when cells had been challenged with Poly-IC. When comparing this data with published literature it would appear that in fact the number of phosphopeptides detected was very low suggesting a methodological problem most likely during the enrichment process (technical issue). However, as no control cells (un-challenged) were analysed during method development it is also possible that in the conditions tested (DON concentration and time) phosphorylation events are not being triggered to the same extend compared with Poly-IC treatment. Even with the limited dataset presented here a number of interesting candidate proteins were identified. Therefore, further research is currently being conducted to further characterise the phosphorylation that takes place in RTgill-W1 cells when challenged with both Poly-IC and mycotoxins such as DON.

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Bryden WL. 2012. *Anim. Feed Sci. Technol*. 173, 134–158; **Godfray** HCJ et al. 2010. *Science* 327, 812; **Greco** M, et al. 2015. *Toxins* 7, 4595-4609; **Kettenback** and Gerber SA. 2011. Anal Chem 83, 7635-7644 **Matejova** et al. 2017. World Aquaculture Soc 48, 2; **Pietsch** C et al. 2013. *Toxins* 5, 184–192; **Schwartz** P. 2011. *Environ. Toxicol.* 276–289; **Smith** et al. 2009. PNAS 106, 12: 4584-4590; **Tacon** AGJ et al. 2011. FAO: Rome, Italy, 2011; **Zhang** ZQ et al. 2016. *Toxins* 8, 270.

Table 1: Proteins detected containing phosphorylated peptides in DON challenge trial. Proteins were annotated using the UniProt KB database. Also depicted are peptides that were associated to the proteins and under which treatment conditions they were detected. The numbers associated to the different treatment conditions show in how many replicates the peptides were detected. The controls for 30 min and 60 min were done in duplicate (n=2) and the DON treated samples had four replicates (n=4).

Accession Number	**Protein Name**	**Organism**	**Molecular Function**	**Biological Process**	**Peptide sequences**	**Control**	**DON**	**Control**	**DON**
						30 min		**60 min**	
P26351	Thymosin beta-11	*O. mykiss*	actin monomer binding	actin filament organisation	KTETQEKNPLPTK	1	4	2	2
					TETQEKNPLPTK	0	4	2	4
C1BGK3	FXYD domain-containing ion transport regulator 3	*O. mykiss*	Ion channel activity	Ion transport transmembrane	SGSNAAQPLNDQAR	0	1	1	1
A0A1S3NMZ0	MARCKS-related protein-like	*S. salar*	Calmodulin binding	Control cell movement	EGETDSIEAAPAAEGEVAK	0	3	1	4
					KFSLKNSFK	0	3	1	4
Q5DW66	Heat shock 90kDa protein 1 beta isoform a	*O. mykiss*	ATP binding	Response to stress	EKEISDDEAEEEEK	1	4	2	4
					IEDVGSDDEEDSKDK	1	4	2	4
					IEDVGSDDEEDSK	0	4	2	4
					EISDDEAEEEEK	0	2	0	2
					YIDQEELNK	0	2	0	0
					EISDDEAEEEEKAEK	0	1	0	0
					ELISNASDALDK	0	0	0	1
C0HAK5	Heat shock cognate 70 kDa protein	*S. salar*	ATP binding	Response to stress	VEIIANDQGNR	0	1	1	1
Q5DW65	Heat shock 90kDa protein 1 beta isoform b	*O. mykiss*	ATP binding	Response to stress	EISDDEAEEEKEEK	0	4	1	4
					IEDVGSDDEEDSKDK	0	4	2	4
					IEDVGSDDEEDSK	0	4	2	4
					YIDQEELNK	0	2	0	0
					ELISNASDALDK	0	0	0	1
Q58EL4	Dbx2 protein	*D. rerio*	Sequence-specific DNA binding	Regulation transcription	EKEISNTHTHMEK	1	0	0	0

A0A1S3SL16	ras GTPase-activating protein-binding protein 2-like isoform X6	*S. salar*	RNA binding	Stress granule assembly	APSPVPVESPPSTQEAPK	0	1	0	0
B5X3Q9	Sequestosome-1	*S. salar*	Zinc ion binding	Protein degradation	DAGGSGDEEWTHVTSK	0	2	1	2
A5H1I0	Heat shock protein 90 alpha	*P. olivaceus*	ATP binding	Response to stress	DKEVSDDEAEEEEEKK	0	4	2	3
					DKEVSDDEAEEEEEK	0	2	0	1
					YIDQEELNK	0	2	0	0
B9EMR5	Splicing factor, arginine/serine-rich 7	*S. salar*	RNA binding		KSPIPDAD	0	0	0	1
A0A1S3RZJ3	B-cell receptor CD22-like	*S. salar*	Receptor	Immune system	SESYSGSPGVR	0	0	0	1
A0A1S3LAU1	LIM domain and actin-binding protein 1-like isoform X1	*S. salar*	Actin filament binding	Actin filament bundle assembly	TASQSEGEDGSLK	0	0	2	0
A0A1S3RQQ6	neuroblast differentiation-associated protein AHNAK-like	*S. salar*	Cadherin binding, RNA binding	Protein oligomerisation source, regulation of RNA splicing source, regulation of voltage-gated calcium channel activity	LKSEDLAEGVDVR	0	0	1	0
A0A1S3SNI6	serine/arginine repetitive matrix protein 1-like isoform X5	*S. salar*	RNA binding	mRNA processing	GHAADPSLSPSR	0	0	1	1
A0A1S3RAQ6	myosin-9-like isoform X1	*S. salar*	actin binding Source, ATP binding Source, motor activity		AGIESDEESEPK	1	4	2	4
A0A1S3KSW1	titin-like isoform X3	*S. salar*	molecular spring		KPETAPTPSPK	0	0	0	1
A0A1S3KSS0	titin-like isoform X5	*S. salar*	molecular spring		YTLTLENSSGSK	0	1	0	0