Marine Management Organisation

Evaluating and advancing methodologies to validate the method of capture of fish / seafood (MMO1164)

MMO1164: Evaluating and advancing methodologies to validate the method of capture of fish / seafood

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Executive Summary

A proof of principle study was undertaken to assess if applying a biochemical analysis can assist in determining the method of capture in fish. Thirty-six mackerel (*Scomber scombrus)* that had been caught using either hanging net or line were subject to chemical analysis to identify a biochemical profile. The acquired data were then statistically analysed using both non-targeted multivariate analysis and targeted analysis to identify those chemicals that were significantly different between hanging net and line caught fish.

The results demonstrated that the biochemical profile of mackerel can distinguish between hanging net and line caught. This differentiation was established using both statistical approaches and is hypothesised to result from the different stress levels experienced by fish dependant on capture method.

The level of implementation of this analysis when assessed against the Technology Readiness Levels (TRL) as defined by the European Research Council (2014) (where the higher the number (1-9) means the more advanced and operationally applicable) suggests this approach is likely to be currently TRL 4 - Technology validated in lab.

Further work is required to validate this approach as the work produced here is proof of principle only. Although multiple samples from line and net caught were analysed, all line caught fish originated from the same location, which was different to the net caught fish. Therefore, although the biomarkers that have been tentatively identified correlate with the hypothesis that the stress experienced by the fish is different dependant on capture method, further samples from multiple locations are required to validate these assumptions. This type of analysis should only be used in complementation with other evidence by the MMO until robust validation of the hypothesis has been completed.

1. Introduction

As part of the Marine Management Organisation's (MMO) ongoing work looking at enhancing provenance and traceability the MMO sought an assessment of approaches available for confirming by which method fish sold ashore were originally caught, to potentially support and verify other systems for managing compliance and enforcement. These approaches could apply to fish caught domestically and fish products imported into the UK requiring catch certificates.

Among the fisheries regulations used to manage fishing the UK are those that permit the capture of particular species using only specific fishing gears at a certain time and or in a certain place.

In some cases the type and location of skin injuries such as skin punctures, bruising or scale loss can be indicative of capture from trawl, net, hook or subsequent handling. However, physical damage is not always apparent (Colotelo et al 2009). MMO sought approaches to help distinguish how fish were caught. This project assesses a non-targeted metabolomics approach.

Black et al. (2017) used a non-targeted metabolomics approach using Rapid Evaporative Ionisation Mass Spectrometry (REIMS) to address fish fraud regarding mis-labelling and geographic origin. This work also observed differences in the biochemical make up between line and trawler net caught haddock, which this project explored further for a different species / gear combination; Atlantic mackerel (*Scomber scombrus*) caught with hanging net or hook and line.

Metabolomics is the non-targeted and comprehensive study of small molecules, commonly known as metabolites, within living organisms. The detection of metabolites produced from cellular processes can provide a unique chemical fingerprint of a sample or set of samples under a given condition.

It is hypothesised that different capture methods will impact different stress responses in the fish caught. Metabolic markers of stress for fish have been reported in the literature for both cold stress in *Symphysodon aequifasciatus* (discus fish) (Wen et al. 2018) and geographical / diet stress in *Octopus vulgaris* (octopus) (Garrido et al. 2016) although the latter research did primarily focus on proteins / transcripts as biomarkers rather than the metabolome (the total number of metabolites present within an organism, cell, or tissue).

This proof of principle study investigated a non-targeted metabolomics approach using Mass Spectrometry to evaluate whether it was possible distinguish between line and hanging net caught mackerel. It was hypothesised that markers of stress, either up or down regulated, would be more prevalent in the net caught mackerel compared to the line caught. Cook et al. (2018) identify trawl or hanging net capture methods as the most harmful gear for fish capture which is expected to illicit high stress responses.

2. Sample description

A mackerel assignment captured by hook and line were received into Fera Science Ltd on 9th July 2019 from Falfish, Falmouth, UK. 23 mackerel arrived frozen and were stored at -80°C before preparation for analysis.

A mackerel assignment caught by hanging net were received on 8th August from Devon and Severn IFCA, Devon, UK. 18 mackerel arrived frozen and were stored at -80°C before preparation for analysis. All mackerel were visually assessed at around 30cm in length.

All samples were logged into the Fera Laboratory Information Management System (LIMS) and assigned a unique sample number. Each whole mackerel represented 1 sample. Samples from the hook and line assignment were allocated numbers S19-034317 to S19-034339. Samples from the hanging net assignment were allocated numbers S19-034340 to S19-034357.

As there was an uneven number of samples between the two capture groups, 5 mackerel samples were taken from the hook and line group for sample preparation evaluation. The main experiment was undertaken on 18 vs 18 samples from each group.

3. Methodologies

3.1. Sample preparation

Obtaining a representative sample to capture the metabolome in a whole mackerel is not straightforward. Within this study the whole fish was prepared and homogenised for analysis as follows.

Each fish weighing approximately 300g, from each sample group, was further frozen in liquid nitrogen before chopping into approximately 7 pieces. These pieces were then homogenised in a Retsch Grindomix GM300 Knife Mill (pre-cooled by liquid nitrogen). The subsequent wet sample powder was then immediately stored at -80°C overnight before freeze drying for approximately 4 days and further mixed as a dry powder.

Fish were prepared in a random order from the two capture groups to avoid any preparation bias which may affect the final results and subsequent conclusions. The resulting sample as a dried milled mixed powder was assumed to be homogenous, however from one of the “sample preparation evaluation” samples a small study was undertaken to check this before the main experiment. This involved taking 12 replicates from the same single sample through the methods described below and assessing the data for similarity, i.e. to understand variability associated with sampling from a large powdered fish sample.

3.2. Sample extraction

For each fish sample, a sub-sample was taken (150mg +/- 5mg). 1.5ml of methanol/water (1:1, v/v) was added to this sub-sample. The sample containers

were shaken for 20 minutes at high speed before being centrifuged for 10 minutes (20,800*g*, 20°C). The supernatant of each sample was diluted 10 fold with methanol/water (1:1, v/v) before a further centrifugation step for 5 minutes (20,800*g*, 20°C). 900µL (microliter) of the supernatants were transferred to vials for analysis by Liquid Chromatography - High Resolution Mass Spectrometry (LC-HRMS). A quality control (QC) sample was prepared by combining 100µL aliquots of each sample extract and briefly shaken.

All extracts were stored in the fridge at 4°C, prior to analysis.

3.3. Data acquisition and analysis

LC analysis was performed on an Accela High Speed LC system from Thermo Fisher Scientific. The column used was an ACE 3Q 150 x 3mm, 3µm (Advanced Chromatography Technologies). Mobile phases were 0.1% formic acid in water (Mobile Phase A, (MPA)) and 0.1% formic acid in acetonitrile (Mobile Phase B, (MPB)). Gradient applied was 100% MPA for 5 minutes before increasing to 100% MPB over 15 minutes. This was held for 10 minutes before reverting to 100% MPA and held for 2 minutes. Injection volume was 10µl, flow rate was 0.4 ml/min and column temperature was 25°C. The mass spectrometer used was a Thermo Exactive (Thermo Fisher Scientific). Data were acquired in two separate batches to cover both positive and negative ionisation modes.

Data were evaluated using Progenesis QI (Waters Corporation). Progenesis selected all potential peaks from each mass spectrometry data file (known as “peak picking”), aligned the files using retention time information and looked for significant differences in the peaks found and their relative abundances between sample types.

A principal component analysis (PCA) was produced to observe the data in a non-supervised fashion in order to associate potential trends and / or outliers within the whole data set. The list of peaks found to be significantly different between files depends on filters chosen by the analyst. In these analyses all peaks detected with a retention time <1 minute and with a width <0.06 seconds were firstly removed (>95% of these are background “noise”).

Two analyses were undertaken encompassing negative and positive ion mode, with comparison of samples caught by hanging net versus samples caught by hook and line. Each analysis systematically tested for peak by peak differences in the peak area (abundance) of compounds in a sample. Peaks were only included in a final significantly different list if $p<0.01$ (using Student’s t-test).

With a p-value threshold of 0.01, there is 1% chance of getting false positives i.e. differences defined as significant that are in reality, are not so. In single tests this is usually acceptable, but in metabolomics where there are a large number of statistical comparisons (~4000 here) within one analysis, multiple false positives are expected. P-values were thus corrected for the false discovery rate using an identical q-value of $q <0.01$) and mean fold change abundance between groups >10. All potential significantly different compounds from both ionisation mode experiments, were taken for tentative identification using the publicly available online library Metlin (Scripps Research) as described in Levin, et al (2016).

Further statistical analysis was undertaken using Matlab software (Mathworks). All the data acquired (after peak picking by Progenesis) were uploaded into the Matlab software and partial least squares linear discriminant analysis regression (PLS-LDA) undertaken. When there are more variables in a data set than observations such as this data set, where there are potentially thousands of metabolites (variables) and only 2 observations (line vs net caught) PLS is a useful statistical tool to evaluate / model if the two observations can be classified based on the whole data set, i.e. their metabolites and their abundances detected.

Xcalibur (Thermo Fisher Scientific) was used to assess the data in a targeted manner. As well as the non-targeted metabolomics approach described, a selected number of stress marker compounds identified in Wan et al. (2018) were targeted in the data set for evaluation. These were glucose, glyceric acid, levoglucosan and nicotianamine which were all described as decreasing in the stressed sample group, and spermidine, ornithine, 3-aminoisobutryic acid, eicosenoic acid and tartaric acid which were all described as increasing (from Wan et al. 2018).

Once peak abundances (peak areas) were obtained in Xcalibur, these were exported into Excel (Office 365, Microsoft) where fold change and t-test analysis was undertaken on net versus line sample data.

4. Results

4.1. Homogeneity assessment

From 12 replicates of the same powdered whole mackerel sample (LIMS: S19-034335) the variation was assessed in the positive MS data set. Table 1 summarises a list of example compounds and the % relative standard deviation (% RSD) of their responses over the 12 analyses. All were deemed acceptable (<10% variation) and provided an understanding of the possible variation associated with the sample preparation and analysis process.

Table 1 - Example compound % RSD monitored across 12 replicate analyses of the same powdered mackerel sample

Compound	Mass detected (*m/z*)	Retention time (minutes)	% Relative Standard Deviation
Arginine	175.1189	1.6	9.3
Proline	116.0706	2.0	6.3
Threonine	120.0656	1.9	4.6
Tryptophan	205.0971	9.8	9.0
Indole	118.0651	10.2	7.0

4.2. Metabolomics non-targeted study

From the positive mode data set, approximately 4,500 masses (potential compounds) were detected from all of the samples analysed. The negative data set provided approximately 3,600 masses giving a total of approximately 8,000 masses to further interpret.

The differences in the metabolite profiles between both sample groups are shown in Figures 1 and 2. Both PCA's show potential differences in the data set between the two capture groups (separating on PC 1, with some outliers) however in the positive data set (Figure 2) this is less apparent.

Figure 1- PCA of all samples in negative mode: line caught (purple) and net caught (blue)

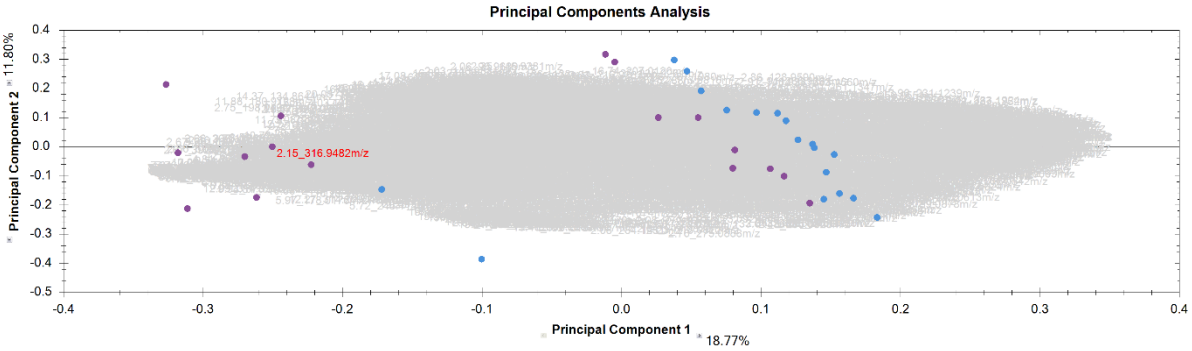


Figure 2 - PCA of all samples in positive mode: line caught (purple) and net caught (blue)

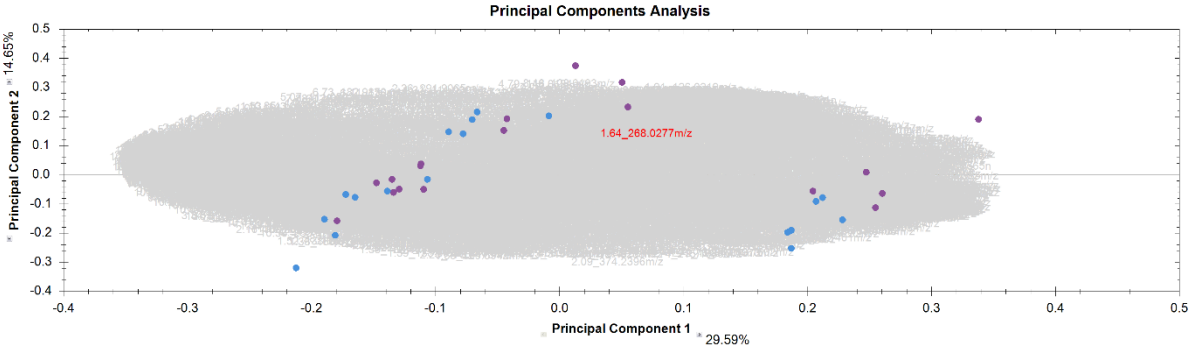
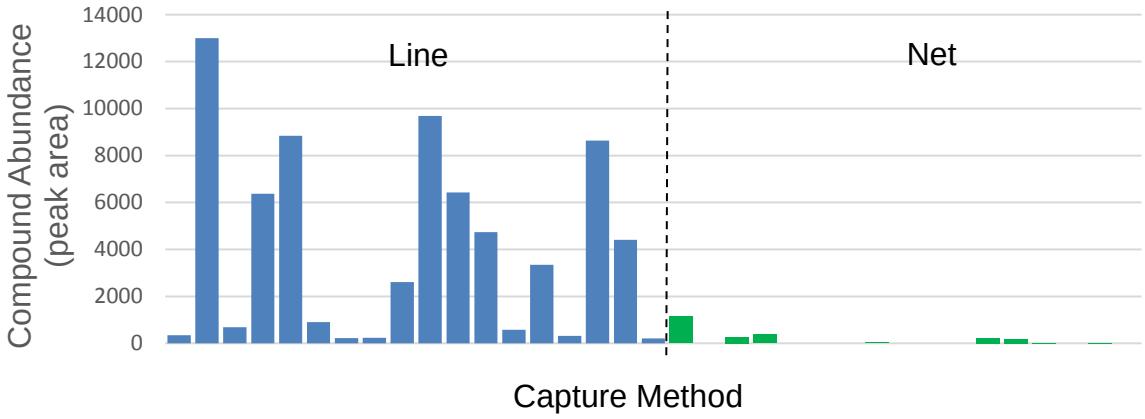


Table 2 provides combined information for the 16 peaks (potential compounds) across both mass spectrometry modes found to be significantly different between the sample groups when applying the filters as described in section 3.3. The table describes if the compound increases or decreases in abundance as a result of the assumed extra stress of capture by hanging net. The compound identifications are tentative only and have not been confirmed with a reference analytical standard.

Figure 3 provides a bar chart illustration for glutathione (tentatively identified) which was significantly lower in the net caught sample group, showing each individual sample's abundance in each group.

Figure 3 - Bar chart showing spread of glutathione response across samples within each group



Figures 4 and 5 provide box and whisker plots for 2 example compounds of significantly different abundances between the sample groups. These plots show the significantly different mean abundance for the compound between sample groups along with the abundance spread (variation) within each group.

Figure 4 - Box and whisker plot for the compound tentatively identified as Glutathione, which was discovered as significantly lower in the net caught sample group

Figure 5 - Box and whisker plot for the compound tentatively identified as Ascorbate 2-sulfate, which was discovered as significantly lower in the net caught sample group

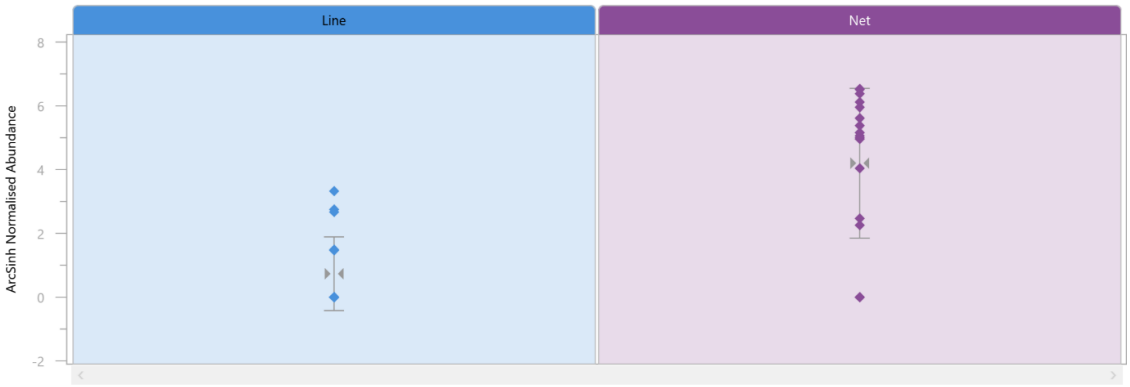


Figure 6 shows the PLS-LDA plot when plotted in Matlab, showing a clear difference between sample groups when evaluating the whole data set (metabolome).

Figure 6 – PLS-LDA plot (scores 1 versus 2, discriminating line from net caught) of all data acquired in negative ion mode.

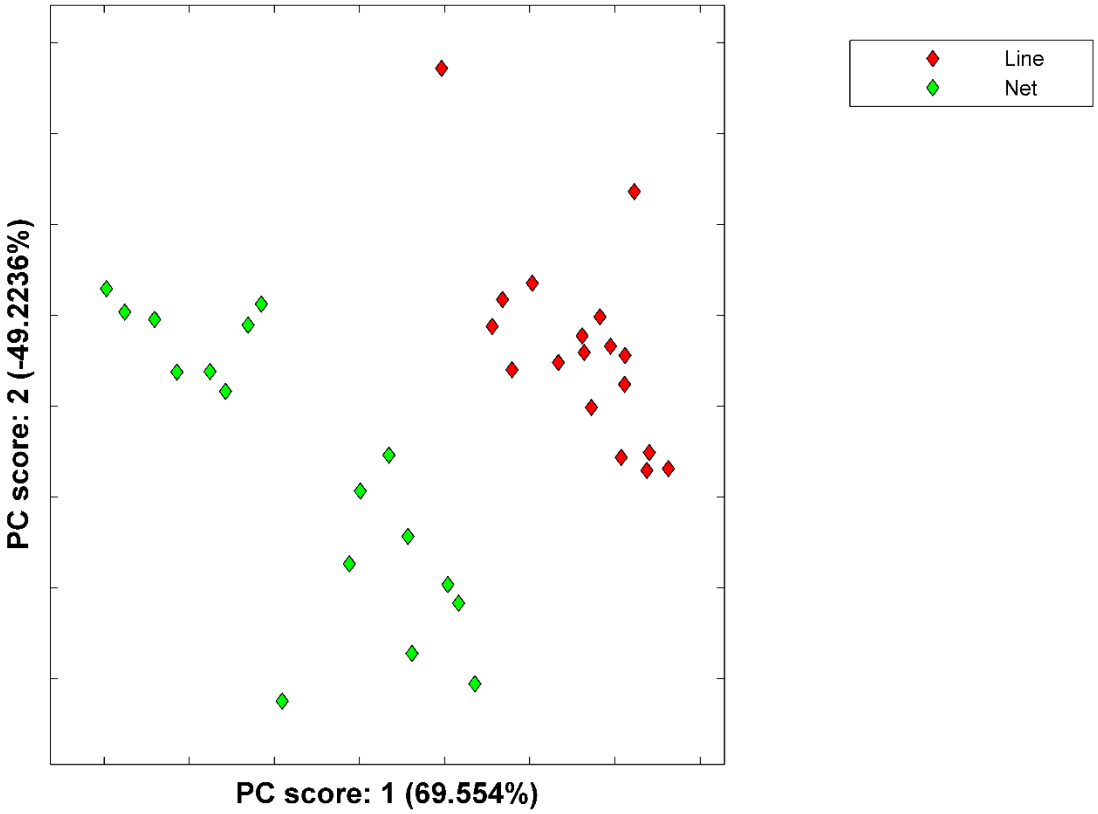


Table 2 - List of compounds showing significantly different abundances between sample groups, tentatively identified by LC-HRMS. A grey box indicates not identified.

No	Compound mass/z	Retention time	Trend in net caught	Mean fold change (net vs line)	Tentative identification	Suggested formula / Ion
1	125.99409	3.6	Increase	579		
2	168.06863	2.0	Increase	71	2-Hydroxy-4-(methylthio)butanoic acid	$C_5H_{10}O_3S$ $[M+NH_4]^+$
3	126.98662	3.6	Increase	60	Ascorbate 2-sulfate	$C_6H_8O_9S$ $[M-2H]^{2-}$
4	154.92778	3.2	Increase	41		
5	292.13096	11.5	Decrease	40	Phenylalanyl-Gamma-glutamate	$C_{14}H_{19}N_3O_4$ $[M-H]^-$
6	384.93579	2.1	Increase	39		
7	212.05973	2.7	Increase	39		
8	305.41463	9.6	Decrease	39		
9	306.07691	4.3	Decrease	30	Glutathione	$C_{10}H_{17}N_3O_6S$ $[M-H]^-$
10	166.97469	4.3	Decrease	29	Phosphonopyruvate	$C_3H_5O_6P$ $[M-H]^-$
11	293.14343	22.9	Decrease	27		

No	Compound mass/z	Retention time	Trend in net caught	Mean fold change (net vs line)	Tentative identification	Suggested formula / Ion
12	468.89715	2.2	Increase	27		
13	341.21203	21.4	Increase	23	Epoxy docosahexaenoic acid (DHA)	$C_{22}H_{30}O_3$ $[M-H]^-$
14	450.29015	20.5	Increase	13		
15	199.00112	4.3	Decrease	11	Erythrulose 1-phosphate	$C_4H_9O_7P$ $[M-H]^-$

4.3. Targeted analysis

From the non-targeted data sets, the compounds described in section 3.3 were scrutinised in a targeted manner. Table 3 summarises the results, including the p-values and fold change differences between sample groups.

Table 3. Summary of targeted compounds data (originally identified from Wan et al. 2018) Compounds in bold are significantly different between sample groups (p <0.01)

Compound	Expected trend under higher stress	Observed trend in stress	p-value	Mean fold change (net vs line
Eicosenoic acid	Increase	None	0.06	1.2
Tartaric acid	Increase	None	0.06	1.4
3-Aminoisobutryic acid	**Increase**	**Decrease**	**0.0007**	**1.5**
Spermidine	Increase	None	0.83	1.05
Ornithine	**Increase**	**Decrease**	**0.00007**	**1.7**
Nicotianamine	Decrease	None	0.23	1.1
Glucose	**Decrease**	**Decrease**	**0.003**	**1.9**
Glyceric acid	**Decrease**	**Decrease**	**0.0003**	**1.5**
Levoglucosan	**Decrease**	**Decrease**	**0.002**	**1.7**

5. Discussion

5.1. Metabolomics: non targeted analysis

From initial scrutiny of the PCA scores plots there is a discrete difference in the metabolomes between the hanging net and line caught sample groups. When the data is analysed using the supervised analytical technique PLS-LDA this is more pronounced.

From the filters applied to the data after the univariate statistics 7 example compounds have been tentatively identified that contribute to difference observed in

the PCA / PLS. There was a further 8 masses (potential compounds) detected that could not be identified by the database used in this study.

In this data analysis a relatively strict cut off of >10 fold mean response and a significance of $p < 0.01$ was applied. This strict approach was taken as when potentially looking for biomarkers discreet differences in one study are often unreproducible unless large.

From the compounds identified as significantly different between the two sample groups, a number agreed with other findings in the literature regarding stress response. Note: there is an assumption in this study that the hanging net caught method is more stressful to the fish than the hook and line method. For example, glutathione abundance was found to be significantly lower (mean response was 30 times lower, $p < 0.001$) in the net caught sample group compared to the line caught group, and this is visually apparent in Figure 3.

Glutathione (GSH) is known to be important in stress management in both plants and animals exposed to both abiotic and biotic stress. It is known to both increase (in humans (Maher, 2005) and plants (Cheng et al. 2015)) and decrease (in fish (Nakano et al. 2014)) in the metabolome when the organism is exposed to stress. Nakano et al. (2014) report a decrease in GSH when salmon undergo thermal environmental stress which agrees with the assumption of this study that net caught fish experience higher stress.

Other compounds tentatively identified as significantly decreasing in the net caught samples relative to line caught samples include erythrulose-1-phosphate ($p < 0.001$), phosphonopyruvate ($p < 0.001$) and the peptide phenylalanyl-gamma-glutamate ($p < 0.001$).

Erythrulose-1-phosphate and phosphonopyruvate are phosphorous containing carbohydrates. Wan et al, (2018) identified very chemically similar compounds as significantly changing in stress conditions in discus fish (6-phospho-gluconate, 3-phosphoglycerate), however these authors found the phosphate carbohydrates to increase due to stress, not decrease as found here.

The peptide phenylalanyl-gamma-glutamate would likely exist in the fish metabolome as an endogenous peptide. It has been reported (in plants) that small mobile peptides can regulate abiotic stress responses (Takahashi and Shinozaki, 2019).

Compounds tentatively identified as significantly increasing in the net caught sample were an epoxy docosahexaenoic acid (DHA) ($p < 0.001$) , the vitamin C related compound ascorbate-2-sulfate ($p < 0.001$) and 2-hydroxy-4-(methylthio) butanoic acid ($p < 0.001$).

2-Hydroxy-4-(methylthio)butanoic acid expressed the largest fold change difference between the sample groups from the compounds tentatively identified (71 times higher in the mean abundance of the net caught fish compared to the line caught). This compound is lipid based, being a fatty acid derivative obtained by insertion of a sulphur atom at a position on the chain. There is little reported in the literature relating this compound and a physiological stress response. Dibner, et al. (1992) did

associate increasing concentrations of this compound with having an ameliorative effect on poultry chicks that were exposed to heat stress.

Ascorbate-2-sulfate is involved in the metabolism of ascorbate (Vitamin C) and interestingly in fish acts uniquely (compared to other plants and animals) as the storage molecule for this vitamin (Tucker and Halver, 1986). There is a reported metabolic interaction (Wilson, 2002) between ascorbate (as dehydroascorbic acid) and glutathione (also reported in this project as significantly changing between sample groups).

Epoxy DHA's are a collection of lipid molecules. DHA is an omega-3 unsaturated fatty acid that exists in animal tissue. It is reported to have an important role in tissue regeneration after stress (McDaniel et al., 2008).

5.2. Targeted analysis

From the nine compounds evaluated in a targeted manner from the metabolomics data set, not one of the compounds had a mean fold difference in abundance between the two sample groups >2 fold. However, the sugar based molecules glucose ($p = 0.003$), glyceric acid ($p < 0.001$) and levoglucosan ($p = 0.002$) all were found to have a significantly lower abundance in the net caught sample group which agrees with the findings in Wen et al., (2018).

The non-proteinogenic amino acid ornithine ($p < 0.001$) and amino acid metabolite 3-aminoisobutyric acid ($p < 0.001$) likewise had significantly lower abundance in the net caught sample group, however Wan et al, (2008) reported these as significantly increasing in a stress related sample group in their study.

6. Conclusions and Recommendations

From this targeted and non-targeted proof of principle study the following conclusions can be drawn:

- On first principles in this controlled study, how they were caught and then how they were subsequently stored / transported for analysis), there is promising evidence to show metabolomics can determine if a fish sample has been exposed to a stressful capture such as hanging net.

- From a modelling approach, i.e. the use of the data set as a whole rather than using individual specific biomarkers, the PLS-LDA shows that this approach is promising.

- Furthermore, a raft of interacting metabolites have been tentatively identified as significantly different in the net caught fish compared to the line caught, with many of these already reported in the literature as stress related compounds.

- The ascorbate / glutathione metabolism changes alongside DHA (upregulated to possibly deal with tissue stress) potentially provides a promising set of

metabolites that could be measured in a targeted fashion to determine the physical / abiotic stress that may be induced by net capture.

- It is likely that the observations described above would be applicable to the majority of fish species, however the biomarkers may change within species.

- This approach may have serious drawbacks if used in a real world scenario. Once caught there will be little control in how the fish are stored / transported and if they will be exposed to any other stress in the meantime post capture. This extra variation would mask any stress response from the capture method potentially providing many false positives of stress (for example if line caught fish were then left to suffocate on a boat for a period of time before being killed).

- The completed study was performed using a small sample set, fish from the different capture groups were from different locations and collected at different times. All samples within a capture group were from the same location and were collected at the same time, therefore the variability within a capture sample group but caught at different locations has not been evaluated.

- It is this author's opinion that a metabolite analysis approach, whether using the non-targeted modelling solution or targeted biomarkers could be used in complementation with other intelligence to determine the type of capture. A larger study, containing all expected variability should be performed and used to show that the discrimination observed between capture groups is robust.

Potential next steps are proposed as follows:

1. Identification of the tentatively identified markers in Table 2, where analytical standards can be purchased, to undertake confirmation analysis of the potential biomarkers of capture stress.

2. A follow-on study to validate this technique in multiple species, including where possible trawl net as well as hanging net and line caught fish, using samples from the same capture method from different locations.

3. A follow-on study with greater sample numbers to validate the approach before it can be used for complementary enforcement purposes. This is a proof of principle study with a moderate statistical power. To obtain a high statistical power (> 0.8) it is suggested that ≥ 1000 sample replicates per capture type should be analysed (Power analysis from Progenesis QI) and that these samples should be sourced from a range of geographical locations over a time course study.

4. To undertake all 3 of the recommendations above, including the 1000 sample study, would be a further study of approximately 12 – 18 months costing in the region of £200,000.

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