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Differential protein expression profile in the liver of pikeperch (*Sander lucioperca*) larvae fed with increasing levels of phospholipids

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Abstract:

A comparative proteomic approach was used to assess the protein expression profile in the liver of 34 days old pikeperch larvae fed from day 10 post hatching, with three isoproteic and isolipidic formulated diets varying by their phospholipid (PL) contents (% dry diet weight): 1.4% (PL1), 4.7% (PL5) and 9.5% (PL9). Using 2D-DIGE minimal labelling of liver extracts, we were able to show 56 protein spots with a differential intensity ($p < 0.05$) depending on the dietary PL content. Among these spots, 11 proteins were unambiguously identified using nanoLC-MS/MS tandem mass spectrometry. In the PL9 larvae, our results indicate that the glycolytic pathway could be down-regulated due to the under-expression of the fructose biphosphate aldolase B and the phosphoglucomutase 1. Meanwhile, propionyl coenzyme A carboxylase (a gluconeogenic enzyme) was under-expressed. In addition, another gluconeogenic and lipogenic enzyme, pyruvate carboxylase, was identified in 3 different spots as being under-expressed in fish fed with the intermediate PL level (PL5). A high PL content increased the expression of sarcosine dehydrogenase, an enzyme involved in methionine metabolism, along with vinculin, a structural protein. Moreover, several stress proteins (glutathione S-transferase M, glucose regulated protein 75 and peroxiredoxin-1) were modulated in response to the dietary PL level and fatty acid composition. In the larvae fed with the lowest dietary PL content (PL1), over-expression of both GSTM and GRP75 might indicate a cellular stress in this experimental treatment, while the under-expression of Prx1 might indicate a lower defence against oxidative stress**.** In conclusion, this nutriproteomic approach showed significant modifications of protein expression in the liver of pikeperch larvae fed different PL contents, highlighting the importance of these nutrients and their influence on metabolism processes and on stress response.

Keywords: 2D-DIGE; Metabolism; Phospholipid; Pikeperch; Proteomics

Abbreviations: AldoB, fructose biphosphate aldolase B; ATP, adenosin triphosphate; FDR, false discovery rate; GRP75, glucose regulated protein 75; HUFA, highly unsaturated fatty acids; NKEF, natural killer enhancement factor; Prx1, peroxiredoxin-1; PGM1, phosphoglucomutase 1; PUFA, polyunsaturated fatty acids; PCCA, propionyl coenzyme A carboxylase; PL, phospholipids; PC, pyruvate carboxylase; SrDH, sarcosine dehydrogenase; V, vinculin

1. Introduction

Pikeperch (*Sander lucioperca*) is an economically important species, interesting for aquaculture and sport fishing in Europe and several Mediterranean countries where it was introduced. As for a large number of species, the larval rearing remains a critical period particularly in terms of feeding and nutritional requirements (Barnabé, 1989). Feeding larvae with an artificial diet allows limiting or even avoiding the costly production of live preys (rotifers, *Artemia* nauplii). However, it poses a problem of satisfying the nutritional requirements and of providing the best performances.

It is well known that lipids constitute a major energy source for fish (Bell and Tocher, 1989), and play a critical role in larvae development (Rainuzzo et al., 1997; Sargent et al., 1999). Furthermore, phospholipids (PL) have been demonstrated to significantly affect survival, growth and deformities in several fish species (Kanazawa, 1985; Geurden et al., 1995; Cahu et al., 2003; Gisbert et al., 2005). They play a major role in maintaining the structure and function of cellular membranes (Kanazawa, 1985; Geurden et al., 1995; Cahu et al., 2003; Gisbert et al., 2005; Tocher, 2003). They also stimulate lipoprotein synthesis in intestinal enterocytes (Fontagné et al., 1998; Geurden et al., 1998), enhance the transport of dietary lipids (Kanazawa, 1991; Teshima et al., 1986), and improve the intestinal absorption of long chain fatty acids (Fontagné et al., 2000). A previous study demonstrated a positive effect of dietary PL on the pikeperch larvae development, especially regarding their growth rate and digestive capacities (Hamza et al., 2008). To date, little is known about their role in metabolic processes in fish species, even if several studies evidenced the effect of the nutritional status on some enzyme activities involved in the intermediary metabolism (Cowey et al., 1977; Moon et al., 1980; Suarez et al., 1995; Metón et al., 1999; 2003; Caseras et al., 2002).

Proteomics aims at analysing theoretically all expressed proteins and their interaction in a given cell or tissue, including all protein isoforms and modifications. Proteomics and genomics have been extensively used, especially in the medical and clinical fields (Gharbi et al., 2002; Zhou et al., 2002), where they help identifying proteins or genes involved in pathologies such as obesity, diabetes or cancer. In environmental sciences, proteomics focuses on the dynamics of the proteome of organisms in response to environmental changes. It offers a powerful tool to understand the effects of pollutants on protein expression in an organism (López-Barea et Gómez-Ariza, 2006; Monsinjon and Knigge, 2007; Nesatyy and Suter, 2007). More recently, nutritional genomics has also been the focus of much interest, aiming at understanding how the diet influences gene transcription (nutritranscriptomics), protein expression (nutriproteomics) and metabolites synthesized by an organism (nutrimetabolomics) (Zhang et al., 2008). In reviews, Kussmann et al., (2006; 2008), considered that proteomics applied to nutritional

research should contribute to the identification of bioactive food components, for assessing their biological efficacy, and to elucidate biomarkers for defining an individual's susceptibility to diet in nutritional interventions.

To date, only a limited number of studies have explored the dietary regulation of protein or gene expression (Cousins, 1999; Cheung et al., 2001; Schmid et al., 2003; Zeisel et al., 2005; De Roos et al., 2005), especially in fish species (Panserat et al., 2001; Martin et al., 2001; 2003; Vilhelmsson et al., 2004; Brunt et al., 2008; Murray et al., 2009; Sissener et al., 2009). In addition to understand the metabolic processes involved in the adaptive responses, these studies help to improve the animal health status and growth performances.

The present study aimed to assess changes in the protein expression profile in the liver of pikeperch larvae to address the metabolic processes at a cellular level. The liver was chosen because it is the principal site of proteins, lipids and carbohydrates metabolism. We used the 2D-DIGE (two dimensional differential in-gel electrophoresis) technique (Unlu et al., 1997), to analyze the effects of phospholipid supplementation in the diet on the liver proteome of 34 days old pikeperch larvae.

2. Materials and methods

2.1. Fish and diets

Pikeperch (*Sander lucioperca*) larvae were obtained from a private hatchery (Viskweekcentrum Valkenswaard, The Netherlands) and transferred to INSTM (Institut National des Sciences et Technologies de la Mer, Tunisia). The larvae were acclimated in two 500 L tanks (20-22°C) and fed from mouth opening (day 4 post hatching, ph) *ad libitum* each hour from 8:00 to 20:00 h, with newly hatched small size *Artemia* nauplii (AF, INVE Belgium). On day 10 ph, the larvae were transferred to the experimental unit in a recirculating system containing 12 cylindroconical tanks of 60 L each (20 larvae L^{-1}). Four tanks were randomly assigned to each experimental group. Temperature and dissolved O_2 were maintained at 21-23°C and above 6 mg L^{-1}, respectively, with water exchange of up to 100% h^{-1}.

From day 10 to day 34 ph, larvae were fed one of three isoproteic and isolipidic microdiets (Table 1) formulated according to the patent WO0064273 and containing modified levels of soybean lecithin and cod liver oil to obtain three diets with increasing PL content: 1.5 (PL1), 4.7 (PL5), and 9.5% (PL9). The fatty acid composition of the three diets PL1, PL5 and PL9 were detailed in table 2.

2.2. Sampling

Growth was monitored by sampling 30 larvae per tank on days 4 and 10 ph, and 10 larvae per tank on days 16, 22, 28 and 34 ph. The larvae were weighed collectively from day 0 to day 22, and individually on days 28 and 34. Growth was estimated as follows:

Instantaneous specific growth rate (SGR, % day^{-1}) = $100(LnWf - LnWi)\ \Delta T^{-1}$

where Wf, Wi= final and initial weight of larvae (mg), T= time (days)

For the proteomic analysis, 10 larvae per tank (40 per treatment) were collected on day 34 and immediately stored at -80 °C. Later, the larvae were dissected on a glass maintained at 0°C and whole liver was extracted and immediately frozen in liquid nitrogen and kept at -80°C until analysis. The individual liver weights were 2.3±0.3, 2.8 ±0.4 and 3.2±0.5 mg, for PL1, PL5 and PL9 larvae, respectively.

2.3. Protein extraction and CyDyes labelling

Proteins were extracted in 1:10 w:v lysis buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 30 mM Tris), and cells were lysed by 2 x 10 s sonication on ice. Following lysis of samples, the soluble protein fractions were harvested by centrifugation at 12 000 x *g* for 15 min at 4°C. The pH of the protein extract was adjusted to 8.5 by addition of the appropriate volume of 50 mM NaOH and protein concentration was evaluated using the BioRad protein assay as described by the manufacturer (BioRad, UK). For DIGE minimal labelling, 25 µg of protein were labelled with 200 pmol of fluorescent amine reactive Cyanine dyes freshly dissolved in anhydrous dimethyl formamide following the manufacturer's recommended protocols (GE Healthcare). Labelling was performed on ice for 30 min in the dark and quenched with 1mM lysine for 10 min on ice. Cy3 and Cy5 were used to label samples as described in table 3, while a mix sample composed of equal amounts of proteins from each replicate was minimally labelled with Cy2 and was used as the internal standard. The 3 labelled mixtures were combined and the total proteins (75 µg) were added v: v to reduction buffer (7 M urea, 2 M thiourea, 2% DTT, 2% CHAPS, 2% IPG 4-7 buffer) for 15 min at room temperature.

2.4. Protein separation by 2 dimensional electrophoresis

Prior to the first dimension separation of proteins, IPG strips (24 cm, pH 4-7 ; GE Healthcare) were rehydrated overnight with 450µl of a rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG 4-7 buffer, 2% DTT). The sample sets containing the labelled mixtures were then cup-loaded onto the IPG strips and isoelectric focusing was performed on an Ettan™ IPGphor II isoelectric focusing unit (GE Healthcare). Electrophoresis was run at 20°C for a total of 68 000 Vh at 50 µA per strip. Prior to SDS-PAGE, focused IPG strips were equilibrated in buffer (50 mM Tris, 6 M urea, 30% glycerol, 2% SDS, pH 8.8) containing 1 % DTT and then

2.5% iodoacetamide for 2x15 min. Strips were then loaded onto a 14 % 24 cm, 1mm thick, acrylamide gel. The strips were overlaid with 1 % agarose in SDS running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS) and run in an ETTAN™ DALTsix electrophoresis unit (GE Healthcare) at constant 3W/gel at 15°C until the blue dye front had run off the bottom of the gels.

2.5. Image analysis and statistics

Labelled proteins were visualized using a Typhoon 9400 Imager (GE Healthcare) at three specific wavelengths (488 nm for Cy2, 532 nm for Cy3, 633 nm for Cy5). Resolution was of 100 µm. The PMT was set to ensure a maximum pixel intensity between 40 000 and 60 000 pixels. Image analysis was performed using the DeCyder BVA 5.0 software (GE Healthcare). Briefly, the Differential In-Gel Analysis (DIA) module co-detected (about 2500 spots for each co-detection) and differentially quantified the protein spot intensity in each image, using the internal standard sample as a reference to normalize the data. At a second step, the Biological Variation Analysis (BVA) was used to calculate ratios between samples and internal standard abundances by performing a gel-to-gel matching of the internal standard spot maps for each gel. Protein spots with intensities analyzed as significantly different ($p<0.05$) using a Student's t-test for independent samples, were considered to correspond to proteins differentially expressed between PL1/PL5, PL1/PL9 and PL5/PL9 treatments.

2.6. Preparative gels

To identify the differentially expressed proteins, preparative gels were run prior to spot excision and mass spectrometry analysis. The same protocol as described above was followed except that each gel was loaded with 350 µg proteins of mix samples and that gels were post-stained with ruthenium II tris (bathophenanthroline disulfonate) RuBP overnight (7 µL ruthenium per 1 L ethanol 20%) after six hours fixation in ethanol/acetic acid 30 %/10 % and 3 x 30 min in ethanol 20 % at 20 °C.

2.7. Protein identification by ESI-MS/MS and database searching

Spots were excised from preparative gels using the Ettan™ Spot Picker (GE Healthcare) and proteins were digested with trypsin by in-gel digestion. Briefly, the gel pieces were twice washed with distilled water and then shrunk with 100 % acetonitrile. The proteolytic digestion was performed by the addition of 3 µl of modified trypsin (Promega) suspended in 100 mM NH_4HCO_3 cold buffer. Proteolysis was performed overnight at 37°C. The supernatant was collected and combined with the eluate of a subsequent elution step with 5 % formic acid. The

eluates were kept at – 20°C prior to analysis.

The digests were separated by reverse-phase liquid chromatography using a 75 μm X 150 mm reverse phase NanoEase Column (Waters, USA) in a CapLC (Waters, USA) liquid chromatography system. Mobile phase A was 95 % of 0.1 % formic acid in water and 5 % acetonitrile. Mobile phase B was 0.1 % formic acid in acetonitrile. The digest (1μl) was injected and the organic content of the mobile phase was increased linearly from 5 % B to 40 % in 40 min and from 40 % B to 100 % B in 5 min. The column effluent was connected to a PicoTip emitter (New Objective, USA) inside the QTOF source. Peptides were analysed in the DDA mode on a Q-TOF2 (Waters, USA) instrument. In survey scan, MS spectra were acquired for 1s in the m/z range between 450 and 1500. When intensity of 2^+ or 3^+ ions rose above 20 counts/second there was an automatic switch to the MS/MS mode. The collision-induced dissociation (CID) energy was automatically set according to mass to charge (m/z) ratio and charge state of the precursor ion. Acquisition in MS/MS was stopped when intensity fell below 5 counts/second or after 15 s. Q-TOF2 and CapLC were piloted by MassLynx 4.0.(Jasko, USA). For the electrospray survey, background was subtracted with a threshold of 35 %, polynomial 5. For smoothing, we used the Savitzky-Golay method with 2 iterations and a window of 3 channels. Finally, we assigned the mass of peaks with 3 % of threshold, a minimum peak width of 4 channels and a centroid top method at 80 %. For MS/MS raw data, we performed a rigorous deisotoping method with a threshold of 3 %.

Peptides were analyzed using a nano-LC-ESI-MS/MS (Waters Ltd, USA) instrument on a CapLC Q-TOF2 mass spectrometer (Waters Ltd). Mascot Distiller Workstation (MatrixScience) processed the native mass spectrometry raw data into high quality, de-isotoped peak lists for use with Mascot 2.2 (MatrixScience). Enzyme specificity was set to trypsin, and the maximum number of missed cleavages per peptide was set at one. Carbamidomethylation was allowed as fixed modification and oxidation of methionine as variable modification. Mass tolerance for monoisotopic peptide window and MS/MS tolerance window were set to ± 0.2 Da. The peak lists were searched against the full NCBInr database (9694989 sequences downloaded on September the 15^{th} 2009).

In all protein identifications, only significant hits as defined by Mascot probability analysis were considered initially, with a significant threshold set at 0.01. With this threshold, identity or extensive homology were indicated by individual ions scores higher than 58-61. The false discovery rate (FDR) was calculated for each identification in Mascot software. As suggested by Gupta and Pevzner (2009), single hit proteins were kept for identification as they argued that the "single-peptide" approach must be used in conjunction with control of the FDR. The correlation

between theoretical isoelectric point (*pI*) and molecular mass of the protein with the position of the corresponding spot in the 2-D gel was also taken into account.

3. Results

3.1. Growth

At the end of the experiment, final mean weights of larvae ranged from 160 to 240 mg (table 4). Both mean weights and SGR were significantly influenced by the dietary PL content (P=0.0002), the phospholipid-rich PL9 diet clearly favouring the growth of the larvae.

3.2. Protein expression profile

The effects of the 3 tested PL contents on protein expression profiles were assessed by running a total of 6 gels as described in Table 3. The number of spots detected on the gels varied between 2111 and 2391. Changes in the protein expression pattern in pikeperch larvae fed different dietary PL levels are presented in figure 1. Significant differences ($p<0.05$) in intensity were observed for 56 protein spots in all gels, corresponding to a change in protein abundance. In order to identify the proteins of interest, 23 protein spots were excised from preparative gels for MS analysis. The remaining spots were not excised either because of their low abundance in the gel or because of the possible contamination by neighbouring protein spots. Among the excised spots, 11 proteins were identified using nano-LC-ESI Q-TOF2 MS/MS. The results of the MS analysis of the protein spots are summarized in table 5 and changes in the protein expression pattern are presented in table 6. Under our stringent conditions, the FDR remained at 0 % for each identification, and 7 proteins were identified on the basis of a single hit peptide at $p<0.01$. All Mascot scores were highly significant and allowed a reliable identification.

Among these identified proteins, 4 decreased and 2 increased in abundance with variations of 1.2 to 2.4 for PL5 *vs* PL1. For PL9 *vs* PL1, 3 proteins were under-expressed (1.1 to 3.6 fold) and 4 were over-expressed (1.2 to 1.5 fold). Lastly, for PL9 *vs* PL5, only one protein decreased in abundance (1.3 fold) while 4 increased in abundance (1.2 to 1.7 fold).

In PL9 treatment, over-expression was observed for both sarcosine dehydrogenase (SrDH) and vinculin (spots number 416 and 518, respectively) compared to PL5 and PL1 larvae. Propionyl Coenzyme A carboxylase (PCCA) and peroxiredoxin-1 (Prx1), (spots 596 and 1918, respectively), were over-expressed in PL9 (1.37-fold and 1.49-fold, respectively) and in PL5 (1.20-fold and 2.42-fold, respectively) compared to PL1. Three other spots were identified as pyruvate carboxylase (PC) (spots 193, 216, 504). All of them were between 1.33 and 1.68-fold over-expressed when comparing PL9 to PL5 treatment ($p<0.01$ except for spot 193, not

significant), while no significant difference was observed when comparing PL9 to PL1 treatment. Correlatively, a decrease ranging from 1.32 to 1.61-fold ($p<0.05$ for spot 193, not significant for spots 216 and 504) occurred in PL5 compared to PL1.

On the other hand, some protein spots showed lower abundance in larvae fed with high PL levels. Fructose biphosphate aldolase B (AldoB) and glutathione S transferase M (GSTM) were under-expressed in PL9 (3.60-fold and 1.17-fold, respectively, $p<0.01$) and PL5 (2.34-fold and 1.24-fold, respectively, $p<0.05$) compared to PL1, while no significant difference was pointed out between PL9 and PL5. A similar pattern of expression occurred for a phosphoglucomutase 1 (PGM1), identified on the basis of 5 highly significant peptides. For this latter protein, high PL levels seem to decrease its abundance compared to PL1, even if only PL5 showed significant results ($p<0.05$). Lastly, larvae fed with PL9 showed a decrease in abundance for a protein identified as glucose regulated protein 75 (GRP75). This under-expression appeared in comparison with both PL5 (1.31-fold, $p<0.05$) and PL1 (1.42-fold, $p<0.01$).

4. Discussion

In a previous study we already showed a positive effect of dietary PL on growth and maturation of the digestive tract as well as fatty acid incorporation in pikeperch larvae (Hamza et al., 2008). In order to get a better insight in the mechanisms involved, we compared liver protein expression profiles in young pikeperch (day 34) fed with increasing levels of phospholipids during their larval development. Proteome analysis using the quantitative 2D-DIGE method revealed differential expression of 56 proteins. Among them, 11 protein species were identified for a total of 9 different proteins. Some of them were related to the primary metabolism: AldoB (glycolysis), PC (3 spots) and PGM1 (glycogenolysis-glycogenesis), whereas amino-acid metabolism seems to be also affected as indicated by the differential expression of SrDH. Another group of proteins was related to the cellular response to stress in general and to oxidative stress in particular (GSTM, GRP75 and Prx1). Finally, one structural protein, vinculin, was also identified with success. All identified proteins are normally present in the liver. Vinculin, which is usually expressed in muscle, has already been shown to be expressed in liver (Kawai et al., 2003).

4.1. Proteins involved in energy metabolism

In all species, the liver is the major organ of energy metabolism and particularly gluconeogenesis (Knox et al., 1980). In fish liver, glycolysis is mostly concerned by providing precursors for biosynthesis of varied molecules rather than by producing pyruvate for oxidation

(Guillaume et al., 1999).

In the liver of PL1 larvae, AldoB was highly over-expressed compared to PL5 and PL9 larvae. This glycolytic enzyme, found in liver and kidney, converts fructose 1,6 biphosphate into dihydroxyacetone phosphate and glyceraldehyde phosphate characterized by a net gain of energy. This result may reflect that the energy allocated to the primary metabolism was relatively more important in PL1 larvae, which were the smallest larvae. In a similar study, an over-expression of aldolase in rainbow trout was reported with a soyprotein rich diet (Martin et al., 2003). The authors considered that this was most likely a reflection of increased metabolism and general turnover of the proteins as well as energy demand occurring in these fish compared to fish fed with fish protein diet. In a same way, the PL9 larvae of the present work showed the highest growth, even though the three experimental diets were iso-energetic. This indicates that PL9 larvae had the ability to allocate a larger amount of nutrients and energy into tissue formation, which may reflect that higher PL level in the diet could improve its assimilation. A study conducted with shrimp suggested that the beneficial effects of dietary PL may be due to a more efficient transport and utilization of dietary neutral lipids through a better lipid mobilization, which results in an increase of the energy available for growth (Kontara et al., 1997). Moreover, it is generally admitted that protein synthesis does not require enhanced glycolytic capacity (Pelletier et al., 1994; Lemieux et al., 2003). This assumption of a decrease of the glycolytic pathway in larvae fed with high PL levels might also be strengthened by the observation of a similar expression profile in the pikeperch liver for the PGM1. This enzyme facilitates the interconversion of glucose 1-phosphate and glucose 6-phosphate. The resulting glucose 6-phosphate can travel down the glycolysis or pentose phosphate pathway. However, the under-expression was very weak and not significant for PL9 vs PL1 (1.13-fold) and significant at 0.041 only for PL5 vs PL1, which urge us to take care when interpreting this expression pattern. In fish species, several authors showed that glycolysis is depressed by high fat diets and particularly polyunsaturated fatty acids (PUFA) (Cowey et al., 1977; Suarez et al., 1995; Likimani and Wilson, 1981). Thus, we can assume that the effect of PL on the metabolism of the pikeperch may also be related to their fatty acid composition. The highest levels of PUFA (n-6 and n-3) contained in PL5 and PL9 diets (Hamza et al., 2008), could result in a decreased glycolysis in the larvae of these treatments.

On the other hand, PCCA showed a significant over-expression in the liver of PL9 and PL5 larvae compared to PL1 group. PCCA is a mitochondrial carboxylase, which catalyzes key reactions in gluconeogenesis, fatty acid synthesis and amino acid catabolism. It is responsible for the carboxylation of propionyl coenzyme A to methylmalonyl coenzyme A which can thus integrate the Krebs cycle (Pacheco-Alvarez et al., 2002). Afterwards, export of Krebs cycle

products to the cytosol leads to formation of gluconeogenic intermediates. Our results indicate that gluconeogenesis could be depressed in liver of pikeperch larvae fed with low level of PL. It has been demonstrated that PCCA is also responsible for the carboxylation of acetyl CoA to malonyl CoA used for the synthesis of long-chain fatty acids during development of bactaeria (*myxococcus xanthus*) (Kimura et al., 1998). PCCA is also known to play a central role in mammalian fatty acid synthesis (Jiang et al., 2005). In our study, we can assume that PL1 larvae, fed with the richest diet in long chain fatty acids ($C \geq 20$) require less synthesis of these fatty acids which could explain the under-expression of PCCA in these larvae. Furthermore, we previously demonstrated that PL1 larvae contained higher level of long chain fatty acids (Hamza et al., 2008).

However, the identification of three spots as PC avoids any conclusion about possible effects of PL level on gluconeogenesis. For two spots (216 and 504), PC was significantly over expressed in PL9 larvae compared to PL5 while no difference appeared between PL1 vs PL9 on the one hand, and PL1 vs PL5 on the other hand. We observed that another spot (193) was significantly under-expressed in PL5 compared to PL1 group. PC is a gluconeogenic and lipogenic enzyme that catalyzes the ATP-dependent and irreversible carboxylation of pyruvate to oxaloacetate which can be utilized for glucose, fat or amino acids synthesis as well as for neuronal transmitters (Wallace et al., 1998). A reduction of PC activity in PL5 larvae might be expected to decrease gluconeogenesis and Krebs cycle as it was reported for juvenile hybrid tilapia (Shiau and Chin, 1999). In the absence of a clear trend concerning the reduction of PC activity (significant only in PL5 larvae) it is difficult to conclude about PL role on this enzyme. Further studies are required to understand the interaction between dietary PL level and PC activity.

4.2. Proteins involved in oxidative and cellular stress

It is known that fish tissue and commercial diets formulated with marine fish oils (rich in n-3 PUFA) can be altered by peroxidation (Stephan et al., 1995). PL1 diet was the richest in n-3 PUFA. Nevertheless, peroxidation of all the diets seems unlikely due to the supply of vitamin E and C known for their antioxidant effect, the good condition of storage and use of the diets (frozen and used within a month as suggested by the manufacturer). The effect of oxidative stress due to oxidized lipids could have an effect on antioxidant enzymes as demonstrated in several fish species (Mourente et al., 2002; Tocher et al., 2003; Ketterer et al., 1983). We previously reported a significantly higher level of n-3 PUFA in the larvae fed PL1 diet (Hamza et al., 2008). The observed over-expression of GSTM in these larvae could reveal a stress status of these larvae. GSTs belong to a family of multifunctional enzymes, known by their role in

protecting cellular components against various toxic effects and oxidative stress (Ketterer et al., 1983). The involvement of this enzyme in the detoxification of lipid peroxide products, has been notably pointed out in goldfish (*Carassius auratus*) (Bagnyukova et al., 2006). GSTs have been purified and characterized in the liver of several fish species such as sea bass (*Dicentrarchus labrax*) (Angelucci et al., 2000), Atlantic salmon (*Salmo salar*) and brown trout (*Salmo salar*) (Novoa-Valiñas et al., 2002).

The mitochondrial GRP75, a protein belonging to the HSP70 family was significantly over-expressed in PL1 and PL5 larvae compared to PL9. This family of proteins is known to regulate the correct assembly of newly synthesized proteins as well as the refolding of denatured proteins and thus are often referred as molecular chaperones (Fink, 1999). The induction of HSP expression due to biotic and abiotic stress has been shown for several fish species like zebrafish (*Danio rerio*) (Yeh and Hsu, 2000; Krone et al., 2005), and rainbow trout (*Oncorhyncus mykiss*) (Iwama et al., 1998). The over-expression of GRP75 in PL1 and PL5 larvae may point out an inverse relation between stress and PL level. Previous studies demonstrated that the supplementation of phospholipids to the diet reduced sensitivity to stress in shrimp (Kontara et al., 1997; Coutteau et al., 2000). We may assume the same hypothesis for pikeperch larvae. Moreover, the oxidative stress seems to be also related to the fatty acid composition of the diet. Indeed, high n-3 diets increased incidence of oxidative stress in the liver of Atlantic salmon (*Salmo salar*) (Kjaer et al., 2008). In our study, PL1 diet, which is the richest in n-3 fatty acids, may induce an oxidative stress in the liver of PL1 larvae.

In the same way, Prx1 is another antioxidant enzyme that exhibits peroxidase and chaperone activities. In teleost fishes, Prx1 gene have been identified and characterized as Natural Killer Enhancement Factor (NKEF) genes in common carp, (*Cyprinus carpio*) (Shin et al., 2001),[67] and channel catfish, (*Ictalurus punctatus*) (Li and Waldbieser, 2006). Functions of NKEF are to enhance the cytotoxic capacity of natural killer cells and to prevent DNA and protein from being damaged by oxidative stress (Shin et al., 2001). In the liver of PL5 and PL9 larvae, we showed that Prx1 was significantly over-expressed compared to PL1 larvae. The over expression of Prx1 in high PL content fed animals may be related to an enhanced protection against phospholipid peroxidation in the cell membrane as it has been shown for 1-cys Prx in the lung epithelial cells of the rat (Pak et al., 2002).

4.3. Other proteins

SrDH (spot 416) was significantly over-expressed in PL9 larvae compared to the other groups. This mitochondrial enzyme is involved in the oxidative degradation of choline to glycine and related to the metabolism of methionine (Finkelstein, 1998). Sarcosine is reported to be formed

from dietary intake of choline. Knowing that phosphatidylcholine is the essential component of phospholipids in these diets, and that PL9 diet contained the highest level of phosphatidylcholine, we could expect a higher expression of SrDH in PL9 larvae.

Finally, phospholipid level in the diet has affected the expression of a structural protein. Vinculin is a protein responsible of cellular adhesion, rarely reported in fish species (Costa et al., 2003). Its over-expression in PL9 larvae (compared to PL5 and PL1 larvae) may be related to their higher growth and with the major role of PL in the membrane structure. Moreover, it seems that acidic phospholipids (phosphatidic acid, phosphatidylinositol, phosphatidylserine) may play a role in regulating the activity of vinculin (Weekes et al., 1996).

5. Conclusions

To our knowledge, this study is one of the first to rely on a proteomic approach to unravel some of the molecular effects of diet composition in a commercial fish, the pikeperch. This global approach highlighted the alteration in the protein expression profile due to different dietary PL contents and fatty acid profiles. The identified proteins are involved in several cellular processes, with a majority of them implicated in intermediary metabolism (glycolysis and gluconeogenesis). Methionine metabolism was also concerned, as well as structure and stress status. These results can be related to the growth and priorities in the energy expenditure in larvae, but can also depend upon their fatty acid composition (related to the diet composition). In nutritional studies, proteomics appeared as a useful tool to elucidate the effect of diet composition on metabolic processes in fish with the view to better understanding physiological processes during ontogenesis. Further proteomic and transcriptomic studies should investigate the nutritional regulation of the expression of genes and proteins involved in lipid metabolism (lipogenesis, fatty acid elongation and desaturation).

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Figure legends

Figure 1. Representative 2-D gel showing the identified 11 protein spots differentially expressed between two or three treatments (PL1, PL5 and PL9). Proteins of the samples obtained for the different experimental conditions were differentially labelled with Cy3 and Cy5. An internal standard composed of equal amount of each sample and labelled with Cy2 was added. Labelled samples (25µg of each of the Cy3 and Cy5 labelled samples and of the Cy2 labelled internal standard) were loaded on 24 cm 4-7 IPG-strips and subjected to IEF. Proteins were further separated by SDS-PAGE (12.5%) in the second dimension.

653 Table 1. Composition of the three experimental diets corresponding to different

654 phospholipid (PL) contents.

Diet Ingredients (%)	PL1	PL5	PL9
Fish meal	60	60	60
Hydrolyzed fish meal CPSP G	14	14	14
Cod liver oil	13	7	0
Soybean lecithin	0	6	13
Vitamin mixture	8	8	8
Mineral mixture	4	4	4
Betaine	1	1	1
Lipid composition(% DM)			
Neutral lipids	20.6	14.2	8.5
Phospholipids	1.4	4.7	9.5
Proximal composition (% DM)			
Crude protein	58	58	58
Crude lipids	22	20	20
Ash	12.0	12.5	13.2
% dry matter (DM)	96.2	95.3	90.5
Protein energy + lipid energy (kJ kg^{-1})	18.0	17.2	17.2

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656

656 Table 2. Fatty acid composition (% of total fatty acids) in the PL and NL fractions of the
657 experimental diets PL1, PL5 and PL9
658

	PL1		**PL5**		**PL9**	
Fatty acid	NL	PL	NL	PL	NL	PL
14:0	5.5	2.1	5.4	0.6	4.3	0.3
16:0	15.0	21.4	14.9	20.6	15.4	20.7
18:0	2.8	4.3	2.6	3.7	2.6	3.7
Σ Sat	23.3	27.8	22.9	24.9	22.3	24.7
16:1n-7	6.7	2.8	6.8	0.8	6.1	0.4
18:1n-7	3.5	3.8	3.6	1.9	4.0	1.7
20:1n-7	0.4	0.3	0.4	0.1	0.4	0.0
18:1n-9	14,6	13,8	14,7	11,3	15,3	10,9
20:1n-9	7.6	2.9	7.6	0.8	8.7	0.4
22:1n-11	7.8	1.3	7.7	0.4	9.1	0.2
Σ Mono	40.6	24.9	40.8	15.3	43.6	13.6
18:2n-6	2.4	1.6	2.5	44.0	3.5	50.1
20:2n-6	0,3	0,2	0,3	0,1	0,3	0,1
20:4n-6	0.6	1.7	0.6	0.4	0.6	0.2
Σ n-6	3.3	3.5	3.4	44.5	4.4	50.4
18:3n-3	1.0	0.5	1.1	4.0	1.1	4.5
18:4n-3	2.5	0.8	2.7	0.2	2.5	0.1
20:4n-3	1.1	0.6	1.1	0.2	0.7	0.1
20:5n-3	10.6	12.2	10.9	3.1	9.7	1.7
22:5n-3	2.0	1.4	1.7	0.3	0.9	0.2
22:6n-3	11.0	24.8	11.0	6.3	10.0	3.5
Σ n-3 (PUFA)	28.2	40.3	28.5	14.1	24.9	10.1

659 Sat : Saturated, Mono: monounsaturated, PUFA : Polyunsaturated fatty acid
660
661
662

Table 3. Labelling of the samples with Cy3 and Cy5 Cyanines

	Cy3	Cy5
Gel 1	S 1 (PL1)	S 2 (PL5)
Gel 2	S 4 (PL9)	S 7 (PL1)
Gel 3	S 3 (PL5)	S 5 (PL9)
Gel 4	S 8 (PL1)	S 9 (PL5)
Gel 5	S 6 (PL9)	S 11 (PL1)
Gel 6	S 12 (PL5)	S 10 (PL9)

S 1 to S12 : samples from tank 1 to 12. Four of them correspond to each treatment (PL1, PL5, PL9)

Table 4. Final weight and specific growth rate (SGR), in the three experimental groups of pikeperch larvae fed with diets differing by their phospholipid content (day 34).

	PL1	**PL5**	**PL9**
Final mass (mg)	160±9.6a	190±22.1b	238±12.5c
SGR (% day^{-1})	14.1±0.2a	14.8±0.5b	15.8±0.2c

Means±SD (n=4) Values with different letters in the same line are significantly different ($p<0.01$)

Table 5. MS data of the 11 proteins identified after the 2D-DIGE analysis comparing the protein expression profile in the liver of 34 days old larvae fed with different dietary PL (1.5, 4.7 and 9.5 %) content.

Accession	Spot	Protein	MW (kDa)	p*I*	Score	Expect	Peptide
Q8AYN3	193	Pyruvate carboxylase (PC) (*Pagrus major)*	129.4	6.2	73	0.00095	LDTGIALEK
					74	0.00054	DAHQSLLATR
Q8AYN3	216	Pyruvate carboxylase (PC) (*Pagrus major)*	129.4	6.2	74	0.00084	LDTGIALEK
Q64380	416	Sarcosine dehydrogenase (SrDH) (*Rattus norvegicus*)	101.4	6.2	80	0.00012	DGTMDPAGTCTTLTR
Q8AYN3	504	Pyruvate carboxylase (PC) (*Pagrus major)*	129.4	6.2	63	0.0085	ALALGDLNK
Q64727	518	Vinculin (*Mus musculus*)	116.7	5.8	95	4.5e-006	MTGLVDEAIDTK
					80	0.00013	AQQVSQGLDVVTAK
Q6DGE2	596	Propionyl Coenzyme A carboxylase, alpha polypeptide (PCCA) *(Danio rerio*)	77.8	6.8	91	1e-005	MADEAVCVGPAPTSK
A9CD13	633	Glucose regulated protein 75 (GRP75) (*Sparus aurata*)	68.8	5.6	67	0.0029	MKETAENYLGTK
					65	0.0047	VQQTVQDLFGR
Q7SXW7	816	Phosphoglucomutase 1 (PGM1) (*Danio rerio)*	61.1	5.7	96	4e-006	LSGTGSAGATIR
					64	0.0068	FFGNLMDAGK
					71	0.0013	SMPTSGALDNVAK
					101	9.7e-007	YDYEEVDSDAANK
					79	0.00016	TKPYTDQKPGTSGLR
O73866	1557	Fructose-biphosphate aldolase B (AldoB) *(Salmo salar)*	39.2	8.1	91	1.1e-005	GILAADESTGTMGK
Q6PFJ6	1824	Glutathione S transferase M (GSTM) (*Danio rerio)*	26.1	5.6	68	0.0023	LLLEYTGTK
C3KHC7	1918	Peroxiredoxin-1 (Prx1) (*Anoplopoma fimbria*)	22.2	6.3	73	0.00063	QITINDLPVGR

Accession N°, number in Uniprot database (www.uniprot.org); M, methionine methylation; p*I*, Isoelectric point; MW, molecular weight
Score, Mascot probability based on Mowse Score calculated for MS/MS results, significance threshold was set at 0.01; Expect, expect value is the probability that the sequence match is a random event. For all identifications, the FDR has been calculated as 0 %.

Table 6. Fold change in abundance and *P* value for 11 proteins identified by MS according to the diet

682

Spot	Protein	PL5vsPL1	*P*	PL9vsPL1	*P*	PL9vsPL5	*P*
1557	AldoB	-2.34	0.026	-3.60	0.006	-1.54	NS
816	PGM1	-1.60	0.041	-1.13	NS	+1.42	NS
193	PC	-1.61	0.029	-1.21	NS	+1.33	NS
216	PC	-1.45	NS	+1.16	NS	+1.68	0.006
504	PC	-1.32	NS	+1.21	NS	+1.60	0.008
596	PCCA	+1.20	0.003	+1.37	0.015	+1.15	NS
416	SrDH	-1.08	NS	+1.25	0.015	+1.16	0.019
1824	GSTM	-1.24	0.038	-1.17	0.009	+1.05	NS
633	GRP75	-1.09	NS	-1.42	0.003	-1.31	0.017
1918	Prx1	+2.42	0.018	+1.49	0.02	-1.62	NS
518	Vinculin	-1.28	NS	+1.24	0.026	+1.58	0.005

683 Data were analysed by Student's *t* test. - and + indicate an under- and over-expression of the protein. NS : non significan

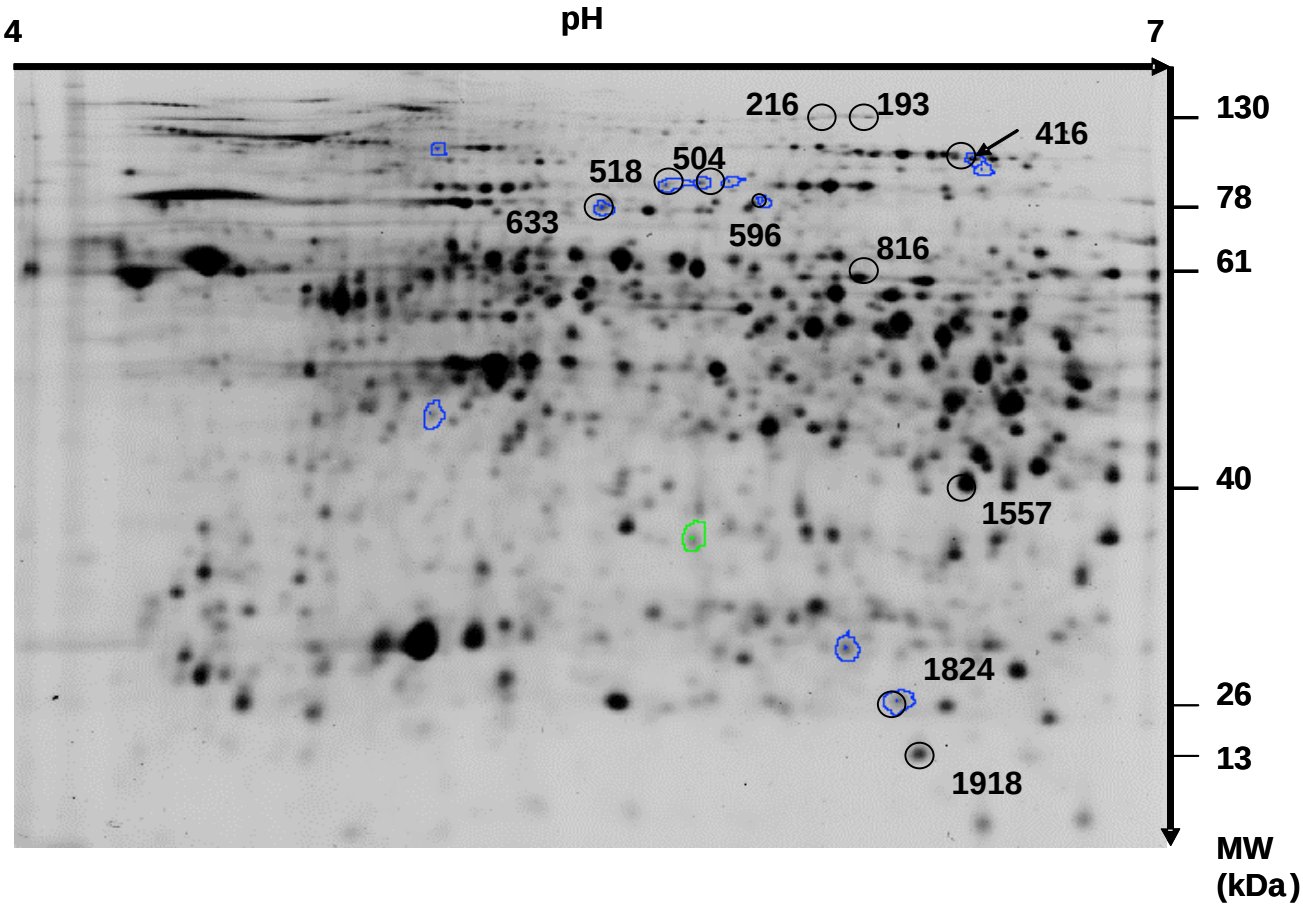
4
pH
7
216
193
416
518
504
633
596
816
130
78
61
40
1557
1824
26
13
1918
MW
(kDa)

Figure 1