UNIVERSIDADE DO ALGARVE

Dietary Nitrogen Utilisation and Protein Expression in Fish

Mahaut Diane Marie Stéphanie de Labrouë de Vareilles Sommières

Tese para obtenção de grau de

Doutoramento em Ciências da Terra, do Mar e do Ambiente

(Ramo de Aquacultura; Especialidade em Nutrição)

Trabalho efectuado sob a orientação de:

Doutor Luis Eugénio Castanheira Conceição, Centro de Ciências do Mar do Algarve (CCMAR) and Sparos Lda, Faro, Portugal,

Professor Doutor Pedro Miguel Leal Rodrigues, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Faro, Portugal,

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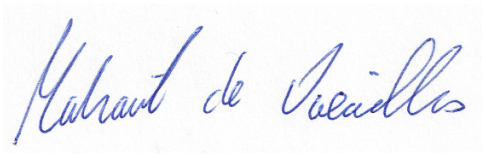
Declaração de autoria do trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.



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Summary

Aquaculture is one of the most promising sources for food protein for humans. However, it still faces many challenges concerning its ecologic-, economic- and socially sustainable development. One main issue is the dependency on limited marine resources for the production of aquafeeds. Concerning protein ingredients in aquafeeds, finding alternative sources to fishmeal is necessary. Thus it is crucial that research be further invested towards understanding of dietary nitrogen utilisation by fish, making use of novel tools in molecular biology ("*omics*" approaches). This thesis aimed to further explore how the growth of early-life stages of fish can be affected by the availability of dietary nitrogen through a comparative proteomics approach (2-DE and MALDI-ToF-ToF mass spectrometry), focusing particularly on skeletal muscle. Two important Mediterranean mariculture species (*Diplodus sargus* and *Sparus aurata*), and the model organism zebrafish (*Danio rerio*), were used for evaluating the effects of dietary inclusion level and peptide size distribution profile of fish protein hydrolysates (FPH) in larval stages (Chapters 2 - 4), and juvenile fish (Chapter 5), respectively. Juvenile zebrafish were also used for evaluating the effect of dietary content of an indispensable amino acid (AA), lysine, on growth and muscle proteome (Chapter 6).

It is seen that a moderate dietary inclusion level of FPH improve larval growth but has no effect on juvenile fish compared to an intact-protein based diet. Also, the importance of peptide distribution profile of dietary FPH is evinced. A dietary excess of short peptides and free AA is confirmed as detrimental to protein anabolic efficiency in both larvae and juveniles. Dietary lysine is conformed as essential for muscle growth of fish, possibly influencing muscle growth pattern in zebrafish. This thesis contributes to improving our understanding of the possible metabolic and regulatory pathways in the early-life stages of fish affected by the dietary nitrogen source, including a critical evaluation of a comparative proteomics approach in this endeavour.

Keywords Aquaculture, fish larva nutrition, protein hydrolysates, lysine, proteomics, skeletal muscle

Resumo

A aquacultura constitui hoje em dia uma das fontes mais promissoras de proteína alimentar para o Homem. No entanto, este sector depara-se ainda com numerosos desafios relativamente ao seu desenvolvimento ecológica-, económica- e socialmente sustentável. Uma das principais questões é a dependência de recursos marinhos já limitados, para a produção de rações alimentares para os peixes cultivados. No que diz respeito à fonte proteica dos alimentos, torna-se cada vez mais necessário encontrar ingredientes alternativos à farinha de peixe habitualmente utilizada. Nesse sentido, é fundamental que a investigação científica aposte na compreensão da utilização alimentar do azoto pelas espécies cultivadas, recorrendo às novas ferramentas da biologia molecular (tecnologias "*omics*"), além do uso de técnicas mais clássicas. As ferramentas "*omics*" oferecem metodologias compreensivas para estudar sistemas bioquímicos, expandindo o nível de investigação do estudo de moléculas únicas ao estudo simultâneo de amplas gamas de moléculas presentes numa célula ou tecido, em termos de presença e abundância relativas, sem ser necessário possuir conhecimento *a priori*. Tendo isto em conta, esta tese pretendeu explorar como o crescimento de fases iniciais da vida do peixe pode ser afectado pela disponibilidade de azoto alimentar, utilizando proteómica comparativa, com foco particular no músculo-esquelético, um tecido *per se* excelente indicador de crescimento e acreção proteica. Mais concretamente, procurou-se aprofundar o conhecimento das possíveis vias metabólicas e regulatórias afectadas pela inclusão de hidrolisados protéicos de pescado (HPP) na ração de larvas e juvenis, e pelo conteúdo em lisina nas dietas para juvenis. Os HPP foram escolhidos porque constituem uma fonte alternativa à farinha de peixe pura, já que têm potencial como forma económica e prática de converter subprodutos de pescado em ingredientes proteicos aceitáveis para a indústria de rações de aquacultura. O efeito no crescimento da inclusão da lisina no alimento foi estudado visto que a lisina é um amino ácido essencial e frequentemente limitante quando são incorporadas fontes proteicas alternativas nos alimentos, como por exemplo, de origem vegetal.

Assim sendo, nos Capítulos 2, 3 e 4 da presente tese, estudou-se o efeito dos HPP alimentares no crescimento, expressão de proteínas e utilização de azoto em larvas de peixe. No capítulo 1, utilizou-se larvas de sargo (*Diplodus sargus*), uma espécie

relativamente nova e promissora na aquacultura de países Mediterrânicos, para avaliar três alimentos: uma dieta de referência para larvas, contendo 10 % de um HPP amplamente utilizado e disponível comercialmente (CPSP-90, Sopropêche, France) e duas dietas experimentais, contendo diferentes fracções de HPP, diferindo na distribuição do tamanho dos péptidos, a um nível de inclusão de 20 %. Para além de uma avaliação zootécnica (crescimento, sobrevivência), recorreu-se a uma análise clássica de electroforese bidimensional (2-DE) para separação de proteinas do corpo larvar inteiro, junto com espectrometria de massa por MALDI-ToF-ToF para identificação de proteínas cuja expressão fora alterada pelas dietas experimentais. Nos capítulos 3 e 4, utilizando desta vez larvas de uma das espécies mais importantes na aquacultura Mediterranica, a dourada (*Sparus aurata*), a abordagem proteómica foi feita recorrendo à técnica altamente sensível 2-D DIGE e focou-se no proteoma da carcaça, em vez de corpo inteiro. Adicionalmente, apenas um tipo (fonte, método de hidrólise) de hidrolisado proteico foi comparado, com diferentes níveis de inclusão no alimento (Capítulo 3) e com diferentes fracções de tamanhos de péptidos (Capítulo 4). De modo a trazer informação complementar ao "*screening*" obtido pela análise proteómica comparativa e compreender melhor como o crescimento e sobrevivência larvar são afectados pelo azoto alimentar, recorreu-se à análise de excreção larvar de amónia e a um ensaio de marcação radioactiva do alimento vivo, *Artemia* sp., com hidrolisados proteicos radioactivos para estudar a capacidade digestiva das larvas e o seu metabolismo de azoto. No capítulo 5, a análise por proteómica comparativa foi mais apurada ainda, através da utilização do peixe-zebra (*Danio rerio*) como organismo modelo (para o qual o genoma se encontra inteiramente sequenciado, o que gera uma muito melhor fiabilidade na identificação proteica) e através da redução da complexidade da amostra proteica estudada (isto é, focando o proteoma sarcoplasmático do músculo esquelético epaxial do tronco). Pretendeu-se desta forma estudar os efeitos do perfil de distribuição dos tamanhos peptídicos dos HPP usados nas dietas no crescimento e proteoma do músculo de peixes juvenís. No Capítulo 6, procurou-se aprofundar o conhecimento sobre os efeitos do conteúdo alimentar em lisina no crescimento, através da análise do proteoma do músculo-esquelético epaxial do tronco de peixe-zebras juvenis.

Foi observado que uma inclusão moderada (10-20 % da matéria seca) de HPP nas dietas larvares, tanto de sargo como de dourada, pode beneficiar o crescimento larvar, desde que o

conteúdo total de proteína bruta, bem como o conteúdo de di- e tripéptidos e amino ácidos livres sejam tidos em consideração e mantidos abaixo de determinados limites máximos. Por outro lado, no caso dos juvenis de peixe-zebra (agástricos), o aumento da solubilidade e digestibilidade da proteína alimentar através da inclusão de 30 % (matéria seca) de HPP, com uma distribuição equilibrada do perfil de tamanho dos péptidos, não resulta num efeito benéfico em termos de crescimento, quando comparada com uma dieta baseada em proteína intacta (i.e. não hidrolisada).

Independentemente do estado de desenvolvimento, condições ambientais e espécie (marinha, de águas temperadas, como as larvas de sargo e dourada, ou de água doce e tropical como os juvenis de peixe-zebra), fornecer um excesso de péptidos curtos e amino ácidos livres na dieta resulta em efeitos negativos no crescimento e/ou sobrevivência e utilização geral do azoto alimentar, que se reflectem principalmente em alterações do "*turnover*" proteico no músculo, com aumento do catabolismo. Este efeito foi também induzido por uma deficiência alimentar em lisina em juvenis de peixe-zebra, confirmando a observação geral de que os desequilíbrios em amino ácidos alimentares resultam na redução da eficiência anabólica em peixes.

Foi também visto que é necessário ter em atenção a estrutura molecular dos péptidos constituintes dos HPP e a sua concentração global nas dietas. Este facto é particularmente evidente no Capítulo 4, onde o aumento do nível de inclusão das fracções de maior tamanho da proteína hidrolisada, sob a suposição de que a inclusão resultaria num aumento da biodisponibilidade do azoto nas dietas larvares sem elevar de forma abrupta o afluxo de amino ácidos no sistema, não resultou em melhorias no crescimento, e pelo contrário teve o efeito oposto.

A utilização da tecnologia da proteómica comparativa (2-DE/2D-DIGE e MALDI-ToF-ToF MS) foi uma abordagem útil, já que as proteínas cuja expressão foi afectada pelos tratamentos experimentais complementaram os resultados do crescimento e utilização do azoto, e ajudaram à sua interpretação. Adicionalmente, o uso desta abordagem inovadora permitiu a visualização de padrões de resposta às dietas experimentais que não foram detectadas pela análise do crescimento e da sobrevivência apenas, e que demostra a utilidade desta técnica para estudos futuros. É também relevante referir que o uso desta

abordagem permitiu a identificação de candidatos específicos para estudos futuros mais dirigidos, o que sublinha a sua utilidade. No entanto, a principal mensagem a reter da presente tese, que é inerente ao dinamismo e sensibilidade do proteoma a sinais ambientais e mudanças subtis do estado fisiológico, é que estudos adicionais irão beneficiar grandemente da redução da complexidade das amostras (heterogeneidade dos tecidos) e da obtenção e utilização de tanta informação contextual quanto possível (incluindo observações obtidas através de métodos clássicos e/ou de outras abordagens "*omics*"), de forma a melhorar a interpretação dos resultados da proteómica.

Palavras-chave Aquaculture, nutrição de larvas de peixe, hidrolisados protéicos, lisina, proteómica, músculo esquelético

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Chapter 1. General Introduction

1.1. Food security and sustainable aquaculture

A fundamental question for mankind is if and how agriculture will be able to supply the nutritious food needed by the global human population beyond 2050, when the population is predicted to reach 9.3 billion (UN, 2011). There are signs of sustained, increased food prices in international food markets and the suggested causes are, among others, rising and changing patterns of consumption in large and fast-growing developing countries such as China and India, an increasing trade-off between biofuels and food, and the effects of climate. There is also a growing concern about the supply of freshwater for agricultural production in the decades to come. There are in all events reasonable doubts about the ability to produce the food needed in the near future, and this is a major challenge for society (Olsen, 2011, references therein).

In the light of these questions, and given that total captures from fisheries plateaued in the 1990's, the Food and Agriculture Organization (FAO) has pointed to aquaculture as the most promising future source for food protein for humans. In 1980, aquaculture production represented 9% of fishery resources; by 2010, it had increased to 43%. It is thought that such a production will need to double in the next 25 years. The FAO is promoting aquaculture because it is an important source of income and employment and also because of its great contribution to food security and the development of many countries (FAO, 2011). However, to become productive and environment-, economic- and socially sustainable, the aquaculture industry faces several challenges, which can be roughly grouped into three main topics: 1) diversification of the ingredients used for feeds, with decreased reliance on sources from high trophic levels, particularly marine, 2) resolution of problems derived from stressful conditions, diseases and/or deterioration of environmental conditions, and 3) introduction of new species to make this industry less vulnerable to market demand (COM, 2013).

1.2. Alternative protein sources for aquafeeds: amino acid

In the 1980's, the majority of feed resources required for producing carnivorous and omnivorous fish and crustaceans originated from pelagic forage fisheries (FAO, 2011), thus greatly compromising the sustainability of this industry. However, owing to major investments in research, the tendency over the last decade is moving towards a greater use of agricultural feed resources for both fish and crustacean production (Gatlin et al., 2007, Naylor et al., 2009). This change has been driven by the limited availability of marine feed resources and the lower production costs that can be obtained with their replacement, such as by plant resources from agriculture. The strategy of increasing the fraction of non fish oil/meal products in formulated pellet feeds has most likely mitigated a feed resource crisis in global fish and shrimp mariculture and supported a continuous increase in production volumes (Olsen, 2011).

However, to mention but one of the numerous issues concerning the replacement of fish/shellfish meal with other resources in aquafeeds (for a more detailed description see *e.g.* Matos, 2013, and references therein), in order to make feeds of similar nutritional quality to those composed mainly by fish meal, it is usually necessary to use supplemental amino acids (AA) to ensure that nutritional needs are met, particularly in the case of plant protein-sources, which often show deficiencies in some indispensable AA (IAA). The dietary supply of IAA in the right amounts and balance is of particular importance since animals cannot synthesize them (Wilson, 1994). Dietary AA are mostly absorbed as peptides and free AA (FAA) and used either for synthesis of proteins or otherwise catabolised and transaminated into other AA, as well as used in gluconeogenesis, lipogenesis and in the synthesis of other nitrogen-containing molecules. In addition, AA are major fuel compounds during the early life stages of fish (Rønnestad et al., 1999; 2003). AA imbalances in the feed can lead to increased AA oxidation resulting in decreased food conversion efficiencies and, subsequently, growth (Gómez-Requeni et al., 2003). Correction of dietary AA profiles using moderate levels of crystalline AA has shown good results in fish (Rodehutscord et al., 2000) and is a common practice in the formulation of practical aquafeeds (Espe et al., 2007; Silva et al., 2009a). Nevertheless, the efficiency of

such supplementation is variable between species, and also within a given species (Espe et al., 2007; Saavedra et al., 2008a,b; Pinto et al., 2010).

1.2.3. Lysine and muscle growth

Thus, better understanding the metabolic effects of AA supplementation is paramount in improving the effectiveness of such supplements. Lysine (Lys) is the first limiting IAA in most plant protein sources available for fish feed production and it is a common practice to supplement with Lys–HCl those dietary formulations that aim to replace fish meal in aquafeeds (Wilson, 1994; Gómez-Requeni et al., 2004; 2005; Espe et al., 2007; Hansen et al., 2007; Silva et al., 2009a). In addition, Lys is of particular importance because it is almost completely utilised for body protein accretion and it has been demonstrated in fish that Lys supplementation may enhance protein synthesis and deposition, and reduce nitrogen losses (Conceição et al., 2003; Espe et al., 2007; Hevrøy et al., 2007; Abimorad et al., 2009). More recently, the metabolic effects of different dietary Lys levels were examined in juveniles of an agastric cyprinid, the zebrafish (*Danio rerio*) using a comparative proteomics approach (Gómez-Requeni et al., 2011). It was seen that Lys deficiency was accompanied by a down-regulation of muscle proteins and up-regulation of proteins associated to fasting, energy deficit, growth arrest and apoptosis. Additionally, it was found that excess Lys was accompanied by an up-regulation of proteins related to glycolysis, steroidogenesis and sexual maturation (Gómez-Requeni et al., 2011).

However, analysing the proteome of an entire organism is a very complicated task and the metabolic pathways by which dietary Lys influences growth performance require further investigation. Perhaps focusing on a specific organ or tissue could further improve data interpretation, such as analysing the skeletal muscle proteome. In skeletal muscle, a significant fraction of the synthesized protein accumulates as growth, unlike in other tissues, such as liver or gills, in which high fractional rates of protein synthesis are matched by similarly high rates of protein degradation, with the effect of an inherently low rate of growth efficiency. As a result, the contribution of these tissues to overall growth of the fish is minute (Houlihan et al., 1995). Growth or an increasing body size is thus directly linked to an increase in myotomal muscle, which can comprise from 40 to over 60 % of the body mass in teleosts (Zimmerman and Lowery, 1999). Also, the myotomal muscle has an

important ancillary metabolic function, its sarcomeric proteins constituting a reservoir of AA that can be mobilised as an energy source by other tissues or for new protein synthesis such as in gonad generation (Johnston et al., 2011). Additionally, in fish aquaculture, muscle is fundamentally important since it generally represents the final commercialised product.

1.2. Alternative protein sources for aquafeeds: protein hydrolysates

Another way to meet future demands for an increase in the aquaculture industry is to focus on the replacement of the economic- and ecologically costly use of fish meal and -oil in aqua feeds by by-products of the poultry and meat industries (Anastasiou and Nengas, 2005). Additionally, a current research trend is the utilisation of marine by-products such as fishery by-catches and discards, and improvement of meal quality produced from fish not used for human consumption (Hardy et al., 2001). Large quantities of protein-rich fish processing by-products are discarded as waste annually and this may pose serious disposal and pollution problems, especially in developing countries. By developing technologies for protein recovery and modification, production of valuable food ingredients and industrial products will be possible. By-product and discards need to be preserved to maintain quality (Espe and Lied, 1999). Fish protein hydrolysate (FPH) production by use of industrial enzymes is considered to be more predictable, reproducible and possibly more gentle in separating soluble nitrogen compounds from the nonsoluble ones, than autolysis resulting from the more traditional method of preservation, fish silage (Liaset et al., 2002). Thus FPH production has a potential as an economical and practical way of converting fish by-products into acceptable protein ingredients for the fish feed industry and also for human consumption (Hevrøy et al., 2005; Chalamaiah et al., 2010).

Furthermore, there have been reports of beneficial effects from including FPH in artificial feeds for larviculture, which constitutes to date one of the main bottlenecks of marine finfish aquaculture. A principal goal in marine larviculture is the replacement of live preys, normally rotifers and *Artemia*, by inert formulated feeds (Cahu and Zambonino-Infante, 2001; Engrola et al., 2009a; Kolkovski, 2001; 2008; Gisbert et al., 2012). The development

of high-quality artificial microparticulate feeds may potentially ameliorate water quality and overcome some disease problems, as well as reduce the high cost of live feed production, since rotifers and brine shrimp production and their enrichment procedures require considerable space, manpower and labor (Gisbert et al., 2012). In contrast, microfeeds potentially have a high and constant nutritional value, are easier to maintain and potentially have lower production costs. These advantages have significant implications for the future sustainability of marine fish larvae production (Kolkovski, 2008). Although the formulation and manufacturing of these feeds have improved in the past few years and several commercial microdiets exist in the market (Holt et al., 2011), artificial feeds still result in poor larval performance compared to live preys and their successful replacement has only been fully or partially achieved in a very limited number of marine fish species (López-Alvarado and Kanazawa, 1995; Zambonino-Infante et al., 1997; Cahu and Zambonino-Infante, 2001; Koven et al., 2001; Yúfera et al., 2005; Fernández-Diaz et al., 2006; Seliez et al., 2006; Kvåle et al., 2009; Saavedra et al., 2009a).

The problems that hamper further progress in the use of microfeeds for the early larval stages of fish include: low attractiveness and consequent lower ingestion rates; poor digestibility; high leaching losses of soluble molecules such as FAA, peptides, vitamins and minerals; and difficulties in formulating complete and well balanced feeds due to lack of knowledge on nutritional requirements (Kvåle et al., 2006; Conceição et al., 2011). The problem of microfeed attractiveness has been partly overcome by the inclusion of protein hydrolysates or FAA, which are known to serve as attractants (Kolkovski et al., 1997; Cahu and Zambonino-Infante, 2001; Koven et al., 2001). Additionally, the inclusion of specific nutrients such as FPH can enhance the digestibility and nutritional value of the microfeed (Kolkovski, 2008).

Protein hydrolysates are promising as core materials in microfeeds as they typically consist of low molecular-weight peptides resulting from protein pre-digestion (Gisbert et al., 2012). As live prey typically contain large amounts of FAA and are thought to undergo significant autolysis (Kolkovski 2001), the digestive functions of the stomach are not necessary for efficient digestion of live prey in early stages of marine fish larvae (Nankervis and

Southgate, 2009). Indeed, it has been suggested that the proteolytic activity is one of the factors that limit growth in altricial fish larvae, which initially assimilate simple forms of AA more efficiently than more complex forms, such that the hydrolysis of proteins prior to feeding can compensate for the absence of a complete gastric digestive system in these larvae (Rønnestad et al., 2003; Tonheim et al., 2005, Zambonino-Infante and Cahu, 2007; Önal and Langdon, 2009; Conceição et al., 2011). Additionally, di- and tripeptides are absorbed quickly and efficiently by the intestine without initial pancreatic digestion (Zambonino-Infante et al., 1997).

Moreover, the inclusion of protein hydrolysates in aquafeeds can constitute an alternative, and perhaps more efficient way of AA supplementation. Peptides offer an advantage over those FAA that are unstable or insoluble (Dabrowski et al., 2003). Also, the use of a peptidic mix such as protein hydrolysates reduces the hypertonicity that results from a FAA feeding solution (Gilbert et al., 2008). Furthermore, AA transporters appear at different times during ontogenesis in vertebrates, including fish (Buddington, 1992) and thus it can be assumed that the supply in some IAA in larvae is ensured by peptides, which have their own transporters (Cahu et al., 2003)

In this sense, different types of experimental and commercial protein hydrolysates differing on their original raw material (i.e. casein, krill, squid, shrimp, mussel, fish meal, yeast, pig blood), their production system (i.e. silage, enzymatic digestion, fermentation, among others) and their biochemical characteristics (i.e. AA profile, molecular weight of peptides) have shown that protein hydrolysates enhanced larval and fry growth and/or survival performance in several freshwater and marine species (Gisbert et al., 2012), such as common carp, *Cyprinus carpio* (Carvalho et al., 1997), rainbow trout, *Oncorhynchus mykiss* (Dabrowski et al., 2003), Atlantic salmon, *Salmo salar* (Bergen and Storebakken, 1996), European seabass, *Dicentrarchus labrax* (Zambonino-Infante et al., 1997; Cahu et al., 1999), Atlantic cod, *Gadus morhua*, and Atlantic halibut, *Hippoglossus hippoglossus* (Kvåle et al., 2009), gilthead seabream *Sparus aurata* (Gisbert et al., 2012). In contrast, high levels of protein hydrolysate inclusion in microfeeds may not or negatively affect larval growth as it has been reported in European seabass (Cahu et al., 1999), turbot,

Scophthalmus maximus (Oliva-Teles et al., 1999), gilthead seabream (Kolkovski and Tandler, 2000), Atlantic halibut (Kvåle et al., 2002) or common carp (Carvalho et al., 2004). In addition, protein hydrolysates have been reported to likely enhance the immune response of European sea bass (Kotzamanis et al., 2007), Atlantic halibut (Hermannsdóttir et al., 2009), and Japanese seabass, *Lateolabrax japonicas* (Liang et al., 2006), and to promote normal skeletogenesis (Zambonino-Infante et al., 1997). Although the positive effect of protein hydrolysates on fish and particularly larval development is currently well acknowledged, such that most of commercial microfeeds designed and manufactured include a moderate level of protein hydrolysate in their formulations (Holt et al., 2011; Gisbert et al., 2012), much still remains to be elucidated on why this is so.

1.3. Proteomics in aquaculture

With respect to the development of optimised formulated feed in fish nutrition research, the application of novel tools in molecular biology will be necessary to detect various aspects of metabolism and influences on physiological responses to dietary treatments. Cutting edge technologies from "*omics*" approaches offer a comprehensive method to study biochemical systems by expanding the level of investigation from single biomolecules to a wide range of molecules present in a cell or tissue at once, in terms of their presence and relative abundance, without any *a priori* knowledge (Alves et al., 2010). *Omics* includes *genomics* to study DNA variations, *transcriptomics* for genome-wide characterisation of gene expression by measuring mRNAs, *proteomics* to assess the cell and tissue-wide expression of proteins, and *metabolomics* for global assessment of metabolite concentrations, at a given time and under defined conditions, and their application in aquaculture research has steadily increased in recent years (Forné et al., 2010; Rodrigues et al., 2012; Zhou et al., 2012).

1.3.1. Strategies for proteome analysis[1]

Regardless of the chosen strategy, proteome analysis requires quantification and identification of all (or a specific subset) of the proteins present in a given biological sample. For this purpose, the most commonly used approaches apply techniques based on chromatography (e.g. RP-HPLC, SCX-HPLC, affinity chromatography), electrophoresis (e.g. SDS-PAGE, BN-PAGE, IPG-IEF/SDS-PAGE, DIGE) and mass spectrometry (e.g. ESI-IonTrap, ESI-OrbiTrap, ESI-QToF, MALDI-ToF). Depending on whether quantification is performed at the protein or the peptide level, these approaches are usually classified as top-down proteomics or bottom-up/"shotgun" proteomics, respectively. Due to the central role of IPG-IEF/SDS-PAGE, another classification of proteomic approaches is according to whether electrophoretic methods are employed or not, that is, gel-based proteomics or gel-free proteomics, respectively.

Given the difficulty of applying mass spectrometric methods directly on full-length proteins, gel-free approaches usually consist in “shotgun” approaches, with proteins being digested with a known protease (most frequently trypsin) prior to analysis. This step greatly increases the complexity of the protein mixture, which is why most of these approaches require online coupling of liquid chromatography methods, in order to reduce the number of peptides entering the mass spectrometer at any given time. The biggest advantage of these approaches is the reduced number of sample processing steps, greatly increasing their throughput and automation and thus, reproducibility. Another advantage is that quantification and identification is performed simultaneously. On the other hand, application of gel-free approaches can be challenging, as reliable identification and quantification can only be achieved for proteins that display several easily-ionizable proteotypic peptides. Furthermore, although "label-free" methods exist, the majority of quantitative gel-free approaches require prior labelling of proteins/peptides with either stable isotopes (e.g. ICAT, SILAC) or isobaric tags (e.g. TMT, iTRAQ). Finally, since quantification and identification is performed at the peptide level, extrapolating that information to the protein level can be a challenging task.

[1] for an extended review see Silva, 2013

Gel-based methods are the classical approach for proteome analysis, usually following a top-down strategy based on two-dimensional gel electrophoresis (i.e. IPG-IEF/SDS-PAGE), for protein separation and quantification, and mass spectrometry, for protein identification (at the peptide level). The main advantages of these approaches are their relative low cost and straightforwardness, along with the fact that post-translationally modified proteins are readily separated due to induced shifts in isoelectric point and molecular weight. However, their application for large-scale studies can be labour-intensive and challenging, compared to purely gel-free strategies.

1.3.2. Typical gel-based proteomic workflow

Although there are many possible approaches for gel-based proteome analysis, almost all of them follow a common multi-step workflow that begins with extraction and solubilisation of the proteins present in a specific tissue, followed by separation and quantification based on two-dimensional gel electrophoresis. Identification of the proteins is usually achieved by post-electrophoretic digestion and subsequent mass spectrometry-based analysis of the resulting peptides.

1.3.2.1. Protein extraction and solubilisation

This step consists in mechanical homogenisation of the biological sample in an appropriate lysis/extraction buffer, in order to release the proteins and ensure that they are maintained in solution, avoiding as much as possible that they be subjected to post-extraction modifications, such as oxidation or proteolysis. For this purpose, lysis/extraction buffers usually contain chaotropes (e.g. urea and thiourea), nonionic/zwitterionic detergents (e.g. CHAPS or Triton X-100), reducing agents (e.g. DTT or beta-mercaptoethanol) and proteolytic inhibitors (e.g. EDTA). Carrier ampholytes are usually also added to ensure a stable pH gradient during IEF.

Another important issue is the application of fractionation procedures. Although it is common to attempt a "full extraction", with the aim of extracting and solubilising all proteins in a given tissue, particularly high-abundance proteins (such as myosin and actin,

in the case of muscle, or albumin and immunoglobulins, in blood plasma) can hamper the detection of low-abundance, but possibly highly relevant, proteins like transcription factors and other regulatory proteins. Fractionation procedures can also be applied if the focus of the study are proteins from a specific cellular location, such as mitochrondrial, nuclear or membranar proteins, or with specific post-translational modifications like phosphoproteins or glycoproteins. For these purposes, fractionation procedures can be applied in order to perform a selective extraction, often using techniques such as (ultra)centrifugation and affinity chromatography.

1.3.2.2. Separation of proteins by electrophoresis

Once proteins have been solubilised, separation is performed using two-dimensional gel electrophoresis (2DE), based on the application of isoelectric focusing in an immobilised pH gradient (IPG-IEF) and polyacrylamide gel electrophoresis in denaturing conditions for separation according to molecular weight (MW) (SDS-PAGE).

Modern isoelectric focusing, performed on immobilized pH gradients, allows for reproducible electrophoretic separation of polypeptides according to their isoelectric point (p*I*), since the application of charge induces movement of the polypeptides along the pH gradient until their charge is null. Isoelectric focusing is followed by polyacrylamide gel electrophoresis in denaturing conditions, by coating proteins in SDS, which both denatures them and confers them a negative charge proportional to their mass, thus smaller polypeptides move faster along the gel medium than bigger proteins and separation by MW is achieved. Reduction and alkylation of cysteine residues is usually performed to ensure that no intra or intermolecular dissulfide bridges between polypeptides occur during this step and influence protein migration in the gel.

The combination of two electrophoretic separations based on two uncorrelated physical properties of proteins (isoelectric point and molecular weight) allows for the high-resolution separation of hundreds/thousands of proteins in a single run, which explains the usefulness of 2DE (IPG-IEF/SDS-PAGE) as the classical separation method in proteomics.

1.3.2.3. Staining of proteins and gel imaging

After proteins are separated according to their MW and p*I*, they need to be visualised and quantified. The classical way is through non-selective protein stains, such as Coomassie Brilliant Blue (CBB) or silver staining. CBB is particularly favoured nowadays because of its higher reproducibility and its wide linearity range. Other possible staining methods include the use of fluorescent dyes like SYPRO Ruby (non-selective), Pro-Q Diamond (selective for phosphoproteins), Pro-Q Emerald (selective for glycoproteins) and Pro-Q Amber (selective for transmembrane proteins). Stained gels are then imaged using an appropriate scanner and the resulting images can be analysed with specific softwares, such as PDQuest™ 2-D Analysis (Bio-Rad) or Progenesis SameSpots (Nonlinear Dynamics). These software packages perform semi-automated protein spot detection and matching across all gels, providing an estimate of the abundance of each detected protein through integration of each spot volume.

A more recently developed technique is the pre-electrophoretic labelling of proteins with cyanine dyes, which allows up to 3 different protein mixtures to be run in parallel on a single gel (i.e. multiplexed 2-DE). The common strategy, designed as DIGE or differential gel electrophoresis, is to run a common internal standard sample across all the gels of an experiment, thus increasing the reliability of protein spot detection, matching and quantification. Not only are a greater number of samples able to be run on the same sets of gels, but also the impact of gel-gel variations is minimised, justifying its current popularity for gel-based studies.

1.3.2.4. Mass spectrometry-based protein identification

The classical identification of proteins separated by polyacrylamide gel electrophoresis is the use of immunoblotting/Western blotting, which consists in the use of antibodies that recognise specific proteins. For proteomic approaches, this strategy is often impractical given it requires prior production and validation of antibodies for each specific protein to be identified. Therefore, mass spectrometry-based methods are applied for protein identification. Typical mass spectrometers used for this purpose use ionisation methods

such as electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI), coupled to many different types of mass selection approaches (e.g. ToF, LTQ, QToF, Orbitrap).

Protein identification begins by the excision of protein spots of interest from a preparative polyacrylamide gel, followed by enzymatic proteolytic digestion. Trypsin is usually chosen because it tends to generate peptides with an average length of about 10 AA residues, an appropriate size for analysis with common mass selection methods. Mass spectrometry (MS) analysis is therefore performed at the peptide level, and then extrapolated to obtain the identity of the precursor protein.

In the case of organisms with well characterised genomes/transcriptomes, protein identity can be obtained directly from a simple MS spectrum of the resulting peptide mixture, since the masses of tryptic peptides are highly characteristic of each specific protein, constituting a reproducible and unique "fingerprint". This approach, peptide mass fingerprinting (PMF), exploits known information on the nucleic acid/protein sequences of a given organism to predict the expected mass fingerprint of each expressible protein, enabling the experimenter to assign a "most probably identity" to each protein spot.

On the other hand, the genome and transcriptome of most organisms is not yet well-characterised to the point where PMF can be reliably performed. In this case, the masses of tryptic peptides is not enough to infer protein identity and further information must be obtained from the peptides besides their mass using tandem mass spectrometry (MS/MS). These approaches involve random fragmentation of the peptides inside the mass spectrometer, for instance by collisions with an inert gas. Because peptide bonds are generally weakest, the resulting fragments are usually highly characteristic of a peptide's AA sequence, enabling approaches such as *de novo* sequencing and peptide fragment fingerprinting (PFF). *De novo* sequencing consists in attempting to directly infer the peptide sequence from the obtained fragmentation spectrum. Because this is an extremely complex task, PFF is more ofter used. This consists in generating theoretical fragmentation spectra for all the possible tryptic peptides known from phylogenetically-related organisms and comparing them with the obtained MS/MS spectra for each protein spot. Since the

fragmentation spectrum is highly characteristic of each specific peptide, it becomes possible to identify proteins using genomic/transcriptomic information of other organisms, as long as there are some highly-conserved tryptic peptides in common.

The challenges and limitations of MS-based identification of protein spots are related with the need for supporting genomic/transcriptomic information, which is currently still scarce unless one works with model organism; with the difficulty in identification of low abundance protein spots; and with the fact that not all peptides are easily ionisable. Nevertheless, technical advances in mass spectrometry and next-generation DNA/RNA sequencing, as well as improved statistical and bioinformatic approaches for peptide/protein identification, are expected to mitigate some of these issues in the near future and it is foreseeable that MS-based approaches will remain the central approach for protein characterisation in the near future.

1.4. Zebrafish as a model in aquaculture

The zebrafish (*Danio rerio*) is a vertebrate multidisciplinary model organism that is used in such fields as developmental biology, reproduction, immunity, disease and drug discovery, physiology, toxicology, among others (indeed it has even been sent to outer space). Today, an important topic in aquaculture research is the utility of zebrafish as a model for fish nutrition research. The zebrafish has several advantages that allow it to be used as a model organism for nutritional and growth studies. Compared with most farmed finfish, zebrafish possess the most developed genomic program ((http://www.ensembl.org/Danio_rerio/index.html), they are easy to maintain and breed, have short generation time (~4 months) and produce a large number of offspring. Additionally, it has recently been demonstrated that mosaic hyperplasia (characteristic of indeterminate muscle growth) is the main mechanism of muscle fibre expansion in zebrafish, as is the case for larger fish of commercial importance such as Atlantic salmon (Patterson et al., 2008; Johnston et al., 2009). In the last decade, several studies have highlighted the possibility of considering zebrafish as a model species for nutritional and growth studies (Goolish et al., 1999; Meinelt et al., 2000; Carvalho et al., 2006; De-Santis and Jerry, 2007; Johnston et al., 2009;

Siccardi et al., 2009; Plastra et al., 2010; Gómez-Requeni et al., 2010; 2011; Enyu and Shu-Chien 2011; Ulloa et al., 2011).

By using zebrafish as a model, a large number of dietary factors could be evaluated in far less time and at lower cost, and their effect studied more readily at a molecular level than in many other aquacultured finfish species (Dahm and Geisler, 2006). Conversely the formulation of optimised diets would greatly benefit zebrafish researchers and facilitate further metabolic studies in zebrafish as a model for other fields (Penglase et al., 2012). However, whether knowledge gained with zebrafish can be applied to other teleosts, especially considering the hundreds of species of finfish that are farmed in the world, is questionable. For instance, zebrafish, like other cyprinids, are functionally stomachless and knowledge on the distribution of digestive enzymes, their activities, and functional roles in zebrafish is also limited. Nevertheless, given the particularities of early development and ease with which they can be reared or maintained and with which we can intervene at the cellular or molecular levels within the whole organism, zebrafish remains a model of choice for basic research (Kaushik et al., 2011).

1.5. Objectives

The purpose of the research performed in the framework of this PhD was to further explore how the growth of early-life stages of fish can be affected by the availability of dietary nitrogen through a comparative proteomics optic, with particular focus on skeletal muscle tissue. Using a two-dimensional electrophoresis approach for protein expression analysis, followed by tandem mass spectrometry for protein identification, the general aim was to increase our understanding of the possible metabolic and regulatory pathways affected by the dietary nitrogen source; namely, the dietary inclusion level and peptide size distribution profile of FPH in both larval and juvenile fish, and the dietary content of the IAA Lys in juvenile zebrafish.

1.6. Thesis outline

In order to meet its objectives, this thesis is structured into 7 Chapters, including 5 experimental ones. **Chapters 2**, **3** and **4** determine how dietary FPH affect larval growth, protein expression and nitrogen utilisation in marine altricial larvae. In **Chapter 1**, a relatively new and promising species in Mediterranean aquaculture, the white seabream, *Diplodus sargus*, is used to assess larval growth and survival and whole body proteome via a classical 2DE approach as affected by three experimental microfeeds: a reference larval feed containing a 10 % inclusion level of a widely-used, commercially available fish protein hydrolysate (CPSP-90™) and two experimental feeds containing a 20 % inclusion level of different fractions of an FPH, differing in the peptide size distribution. In **Chapters 3** and **4**, using larvae of the important Mediterranean aquaculture species, gilthead seabream, the proteomics approach is further refined by use of 2-D DIGE and by focusing on just the larval carcass proteome. Additionally, only the same type (source, method of hydrolysis) of hydrolysed protein is compared at different inclusion levels (**Chapter 3**) and then different peptide size fractions of the same type of FPH (**Chapter 4**). To complement the general screening achieved by the proteomics approach, additional contextual information is obtained by a tracer study (*Artemia* radiolabelling) to evaluate larval digestive capacity and N-metabolism and analysis of larval nitrogen excretion, with the final aim of understanding how growth and survival are affected by the dietary nitrogen source. In **Chapter 5**, the proteomics approach is further refined by using zebrafish, for which the genome has been entirely sequenced and thus yielding higher reliability on protein identification, and focusing on one particular tissue, the trunk epaxial white muscle, and more specifically, the sarcoplasmic proteome. This is performed with the ultimate aim of analyzing the effects of the dietary peptide distribution profile of FPH on growth and muscle proteome of juvenile fish. In **Chapter 6**, it is attempted to further understand the effects of dietary Lys content, an IAA often limiting in practical diets, and known to play a crucial role in growth, on muscle growth by analyzing the entire trunk epaxial white muscle of juvenile zebrafish. Finally, **Chapters 7** and **8** discuss how the growth of early-life stages of fish can be affected by the availability of dietary nitrogen, how this thesis contributed to improve our understanding of the possible metabolic and regulatory pathways affected by

the dietary nitrogen source, including a critical evaluation of a comparative proteomics approach in this endavour, and point to some future perspectives in this field.

Chapter 2. Impact of dietary protein hydrolysates on skeleton quality and proteome in *Diplodus sargus* larvae

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2.1. Abstract

In order to investigate the effects of dietary protein hydrolysates (PH) on larval growth performance, skeleton quality and proteome expression, triplicate groups of white seabream (*Diplodus sargus*) larvae were co-fed from first-feeding with live feed and three microencapsulated diets differing in the molecular weight of their PH fraction (Control – inclusion of CPSP-90™; H – inclusion of a high amount in 0.5-30 kDa hydrolysates; L – inclusion of a high amount in <500 Da hydrolysates). At 15 days after hatching (DAH), proteome expression changes were assessed in entire larvae by two-dimensional gel electrophoresis and the quality of larval skeleton was analysed at 28 DAH through double staining of cartilage and bone. Dietary PH fractions tested affected growth, the larvae fed diet H being significantly larger than those fed diet L, but it did not affect the incidence of deformed larvae, nor the number of deformities per fish. Two-dimensional analysis of larvae proteome allowed the detection and the comparative quantification of a total of 709 protein spots having a pI between 4 and 7, around half of which had an expression significantly affected by dietary treatment, the main difference being between proteome of Control larvae with those of both groups L and H. From these spots, 52 proteins involved in diverse processes such as cytoskeletal dynamics, energetic, lipoprotein, amino acid (AA), and nucleotide metabolisms, protein chaperoning and degradation, and signal transduction, were identified. This study revealed that the molecular weight of the dietary protein hydrolysate fraction had a minor impact on skeletal deformities in white seabream larvae, but affected growth performance and had a strong impact on larvae whole body proteome.[2]

2.2. Introduction

The production of live feed for marine fish larviculture is costly, labour intensive and its replacement by the development of high quality artificial microdiets has been of major focus in recent years (Yúfera et al., 1999; 2005; Murray et al., 2010). Although early life stages have high potential growth rates (Conceição et al., 1997), generally poor growth performance has been achieved in marine fish larviculture, unless live feed is used. High growth rates require an abundant supply of dietary amino acids (AA) for anabolic as well as

[2] The analysis of white seabream larval skeletal development as affected by dietary PH was omitted from this chapter since this was not within the scope of this thesis.

energetic purposes (Finn et al., 2002). However, the early life stages of fish seem to have a limited capacity for digesting complex proteins (Rønnestad et al., 2000; 2003). Modulating the solubility and molecular size of proteins presented in artificial diets has been shown to affect larval quality of different fish species (Zambonino-Infante et al., 2005). For instance survival, growth, development of the digestive tract and/or the occurrence of skeletal deformities were improved by the inclusion of dietary protein hydrolysates (PH) in larvae of various fish species such as European seabass (Zambonino-Infante et al., 1997; Cahu et al, 1999), rainbow trout (Dabrowski et al., 2003), Atlantic cod and Atlantic halibut (Kvåle et al., 2009), and gilthead seabream (Gisbert et al., 2010). However, including PH in aquafeeds deserves further attention as some inclusion levels of dietary peptides and/or free AA (FAA) have been shown to have no effect, or even a detrimental one on growth performance and/or survival (Cahu et al., 1999, Kolkovski and Tandler, 2000, Carvalho et al., 2004, Tonheim et al., 2005; Savoie et al., 2006).

In the last decade, proteomics approaches have been increasingly used in fish biology research, primarily to investigate the physiology, development biology and the impact of contaminants in fish model organisms, such as zebrafish (*Danio rerio*), as well as in some commercial species produced in aquaculture, mainly salmonids and cyprinids. More recently, nutritional genomics has also been the focus of much interest, aiming at understanding how diet influences gene transcription (nutritranscriptomics), protein expression (nutriproteomics) and metabolites synthesized by an organism (nutrimetabolomics) (Zhang et al., 2008). Using comparative proteomics, a wide range of molecules and biochemical pathways involved in fish nutrition can be identified, without any *a priori* knowledge (Martin et al., 2001; 2003; Vilhelmsson et al., 2006; Sveinsdóttir et al., 2008; Sissener et al., 2009; Hamza et al., 2010; Sveinsdóttir and Gudmundsdóttir, 2010).

White seabream (*Diplodus sargus*) is a relatively new species in marine aquaculture production, partly due to its market value and also because it seems that its availability in the wild has been decreasing (Pousão-Ferreira et al., 2001, Santos et al., 2006). In order to verify to what extent growth performance of larval *D. sargus* can be affected by the

availability of dietary nitrogen, graded levels of fish PH (FPH) were introduced in the microdiets of the larvae and the whole-body proteome analysed.

2.3. Material and methods

2.3.1. Husbandry, experimental set-up and feeding protocol

White seabream (*Diplodus sargus*) eggs were obtained from the Aquaculture Research Station of CRIPSul/IPIMAR (Olhão, Portugal). After yolk sac absorption, the larvae were randomly distributed into nine 200 l cylindro-conical fibreglass tanks (80 larvae l^{-1}) so as to test three dietary treatments in triplicate. This semi-closed re-circulating system consisted of natural sea water subjected to biophysical filtration and UV-irradiation prior to entering the tanks at an initial rate of 0.4 l min^{-1}, slowly increased to a maximum of 1 l min^{-1} (30 % h^{-1} water renewal) from 12 days after hatching (DAH). Minimal aeration was supplied through an air tube with outlet at the bottom of the tank. Dissolved oxygen, water temperature and salinity (6.7 ± 0.6 mg l^{-1}, 19.2 ± 0.8ºC, 35 ± 0.5, respectively) were closely monitored and adjusted as necessary, and all tanks were cleaned on a daily basis, the surface layer by a skimmer. As long as live feed was given to the larvae, the water was kept green with the microalgae *Nannochloropsis oculata* and *Isochrysis galbana*. The diurnal light:dark cycle was set at 14:10 hours from controlled illumination provided by overhead fluorescent lamps. From 8 DAH, the photoperiod was extended to 24 hours of light until the end of the experiment.

Larvae were fed with *Brachionus plicatilis* enriched with Protein Selco (INVE Aquaculture, Belgium) from 3 to 20 DAH. *Artemia* nauplii (AF, Salt Lake) from 12 to 20 and then *Artemia* metanauplii (EG, Salt Lake) enriched with Easy DHA Selco (INVE, Aquaculture, Belgium) from 21 to 27 DAH. The amount of live prey supplied was gradually reduced and replaced by inert diets in this period, being of little dietary significance after 9 DAH. Live feed was supplied twice a day, 20 to 30 minutes after administrating the inert diets. From first-feeding, larvae were co-fed with one of three microencapsulated diets (Table 2.1.) formulated to differ in the type of PH and tested in triplicates: diet Control (C), with a commercially available hydrolysed fish meal, CPSP-90™ (composed approximately by 50 % molecules < 0.5 kDa and 50 % between 0.5 and 30

kDa), at an inclusion level of 10 %, as commonly used in larval diets; diet High (H), containing 10 % more larger molecular weight hydrolysates (0.5 – 30 kDa) than the other diets; and diet Low (L), containing 10% more smaller molecular weight hydrolysates (< 0.5 kDa, *i.e.*, FAA, di- and tripeptides) than diets C and H. These two hydrolysates fractions were obtained from a commercial fish protein hydrolysate kindly provided by COPALIS. They were obtained by ultrafiltration in a Pellicon system using Biomax membranes (Millipore) with molecular weight cut-off of 30 and 0.5 kDa. The size of the dietary microparticles was < 200 µm during the first 15 days, then 200–400 µm. The diets were given manually four times a day until 8 DAH, and then eight times a day to the end of the trial with the help of automatic dispensers at night.

Table 2.1. Composition of the experimental diets (%, DM basis)

	Diets		
Ingredients	**Control (C)**	**High (H)**	**Low (L)**
Aglonorse micro feed[a]	28	21	21
CPSP – 90[b]	10	0	0
Cuttle fish meal[c]	10	10	10
Casein[d]	11	6	6
Na-alginate[e]	8	8	8
Bread yeast	6	6	6
Dextrin	6	6	6
Soy lecithin	6	6	6
Fish oil	7	9	9
Vitamin and mineral premix[f]	4	4	4
Vitamin C[g]	3	3	3
Vitamin E[h]	1	1	1
HYDROL_0,5	0	5	15
HYDROL_0,5_30	0	15	5
Proximate composition (%)			
Dry matter	95.09	95.25	97.02
Crude protein	47.62	46.65	46.72
Crude fat	18.69	21.93	22.82
Ash	6.24	6.80	6.53

[a] AgloNorse, Norway; [b] CPSP-90, Soprepêche, France; [c] Squid Powder 0278, Rieber & Søn ASA, Norway; [d] ICN 901633; [e] ICN 154724; [f] Following the requirements reported by the National Research Council (1993); [g] Rovimix Stay C-35, Roche; [h] ICN 100555.

2.3.2. Growth and survival

Samples of twenty larvae per tank were sampled at 2 and 15 DAH for length and dry weight measurements. Survival was assessed as the number of larvae alive at the end of the trial, after withdrawing those removed for sampling.

2.3.3. Analysis of larval proteins by two-dimensional electrophoresis (2-DE)

At 15 DAH, an equivalent amount of larvae from each tank of a same dietary treatment was pooled to obtain 400 mg of entire larvae per treatment. Samples were lysed by sonication in 2 volumes of ice-cold buffer (7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 0.3 % (w/v) DTT, 0.6 % (v/v) protease inhibitor cocktail (Sigma-Aldrich)). Homogenates were centrifuged twice at 12000 *g* for 10 min at 4 °C to pellet insoluble material. The resulting supernatants were depleted of non-protein contaminants using a ReadyPrep™ 2-D Cleanup Kit (Bio-Rad) and resuspended in ReadyPrep 2-D rehydration/sample buffer (Bio-Rad). All protein quantifications were performed using Quick Start™ Bradford Protein Assay (Bio-Rad).

Larval proteins were first separated according to their isoelectric point with ReadyStrip™ IPG Strips, 11 cm long and with a linear pH 4-7 (Bio-Rad). For each strip, 300 µg of protein in a final volume of 200 µL were loaded overnight by passive rehydration. Isoelectric focusing was performed using an Ettan IPGphor (GE Healthcare), at 20 °C, for a total of 30000 Vhrs. The remaining equilibration, second electrophoresis, staining and image analysis steps were performed as in Alves et al. (2010), using Criterion™ XT Precast 10.5-14 % Tris-HCl gels (Bio-Rad) and an electrophoresis buffer containing 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3. For each experimental condition, the 2-D gel electrophoresis was performed in quadruplicates simultaneously.

2.3.4. Protein identification by mass spectrometry

Protein spots differentially expressed between experimental conditions that could be manually excised from preparative gels were identified after tryptic digestion by either LC-MS/MS (ESI-Ion Trap) at the Aberdeen Proteomics facilities (University of Aberdeen, UK) or MALDI Tof/ToF MS at the Mass Spectrometry Laboratory, Analytical Services Unit

(ITQB, UNL, Portugal) and the Proteomics laboratory, Molecular Biology Department (University of Bergen, Norway). Details for the identification by LC-MS/MS have been published elsewhere (Alves et al., 2010), as have those by MALDI ToF/ToF MS at the Proteomics laboratory (Gómez-Requeni et al., 2011). At the Mass Spectrometry Laboratory, the tryptic digests were dissolved in 2% acetonitrile / 5% formic acid and the extracted peptides loaded onto an R2 microcolumn (RP-C18 equivalent) where they were desalted, concentrated and eluted directly onto a MALDI plate using α-ciano-hidroxycinamic (CHCA) as the matrix solution in 50% acetonitrile / 5% formic acid. Mass spectra of the peptides were acquired with positive reflectron MS and MS/MS modes using a 4800plus MALDI Tof/ToF™ analyser (AB Sciex) with exclusion list of trypsin autolysis peaks (842.51, 1045.56, 2211.11 and 2225.12). For both MALDI ToF/ToF MS results, the collected MS and MS/MS spectra were analysed in combined mode by using Mascot search engine and NCBI nr (ftp://ftp.ncbi.nih.gov/blast/db/README) database restricted to Actinopterygii taxonomy, 50 ppm peptide mass tolerance and 0.5 Da MS/MS mass tolerance, up to one missed cleavage allowed, formation of singly, positively charged peptides, carbamidoethylation of cysteine residues and possible oxidation of methionine residues.

2.3.5. Data analysis

Growth (dry weight, total length and growth rate) and survival data were subjected to one-way ANOVA after confirming the normality and homoscedasticity assumptions and after arcsin transforming all data expressed as percentages. When statistically significant variations were found ($p < 0.05$), Tukey's multiple comparison tests were subsequently applied ($p < 0.05$). Growth rate was calculated as $100*(e^{(\ln DWf - \ln DWi)/t} -1)$, with DW_f and DW_i as final and initial dry weight (mg) of larvae, respectively, and t as number of days. For length data, growth rate was obtained as $(TL_f-TL_i)/t$, with TL_f and TL_i as final and initial total larval length (mm), respectively, and t as number of days.

A one-tailed non-parametric Wilcoxon-Mann-Whitney U-test was used to assess differentially expressed proteins between experimental groups. Protein spots that exhibited a difference in normalised volume superior to 1.5-fold between experimental conditions, at $p < 0.05$, were considered to be significantly differentially expressed. In addition, an

exploratory multivariate analysis was performed on this reduced set containing only protein spots identified as differentially expressed between experimental groups.

All statistical analyses were performed using the R software environment, version 2.11.1 (R Development Core Team, 2011).

2.4. Results

2.4.1. Growth and survival

There was a significant effect of diet on growth of white seabream larvae at 15 DAH but no differences were observed between survival rates by the end of the trial (Table 2.2). Fish larvae fed diet H showed a higher growth rate, both in terms of weight and total length, than those fed diet L, which showed the lowest values among all groups.

Table 2.2. Growth and survival of *Diplodus sargus* larvae fed a balanced diet with a commercially based fish protein hydrolysate (Control), a balanced diet rich in larger polypeptides – 0.5-30 kDa peptides – (High), and a balanced diet rich in free amino acids, di- and tripeptides – < 0.5 kDa – (Low). Values are mean ± S.E.M (n=20). Different letters in the same row indicate significant differences (One-way ANOVA and Tukey's multiple comparison tests, $p < 0.05$).

	Control (C)	**High (H)**	**Low (L)**
2 DAH			
Dry weight (mg)	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Total length (mm)	3.59 ± 0.03	3.59 ± 0.03	3.59 ± 0.03
15 DAH			
Dry weight (mg)	0.06 ± 0.02ab	0.07 ± 0.02 a	0.05 ± 0.00 b
Total length (mm)	4.54 ± 0.09 a	4.61 ± 0.08 a	4.29 ± 0.09 b
RGR (2-15 DAH)			
DW (% day^{-1})	7.76 ± 0.91ab	9.42 ± 0.92 a	7.01 ± 0.69 b
TL (mm day^{-1})	0.07 ± 0.01 a	0.08 ± 0.01 a	0.05 ± 0.01 b
Survival (%)	2.2 ± 0.3	2.7 ± 0.4	2.8 ± 0.2

2.4.2. White seabream larval proteome response

The two-dimensional analysis of the white seabream larvae proteome performed at 15 DAH for each dietary treatment allowed the detection and the comparative quantification of a total of 709 protein spots, of which 339 spots showed significant variation depending on the diet (> 1.5-fold or < 0.67-fold, $p < 0.05$) (Fig. 2.1.). Among these spots, 126 had a significant differential expression between group C and both groups L and H (100 spots under-expressed and 26 spots over-expressed in group C), 98 spots displayed significant variations between groups C and L (45 spots under-expressed and 53 spots over-expressed in group L) and 84 between groups C and H (15 spots under-expressed and 69 spots over-expressed in group H).

Proteome of larvae from groups H and L were significantly differentiated by the expression of 93 spots, of which 44 were over-expressed in larvae fed the diet L and 49 were under-expressed. Principal component (PC) analysis of protein spot expression data revealed a clear separation of samples into clusters according to dietary treatment (Fig. 2.2.), showing that the protein expression profiles of white seabream larvae fed with the experimental PH (diets H and L) were rather similar, comparatively to that of larvae fed with the control diet.

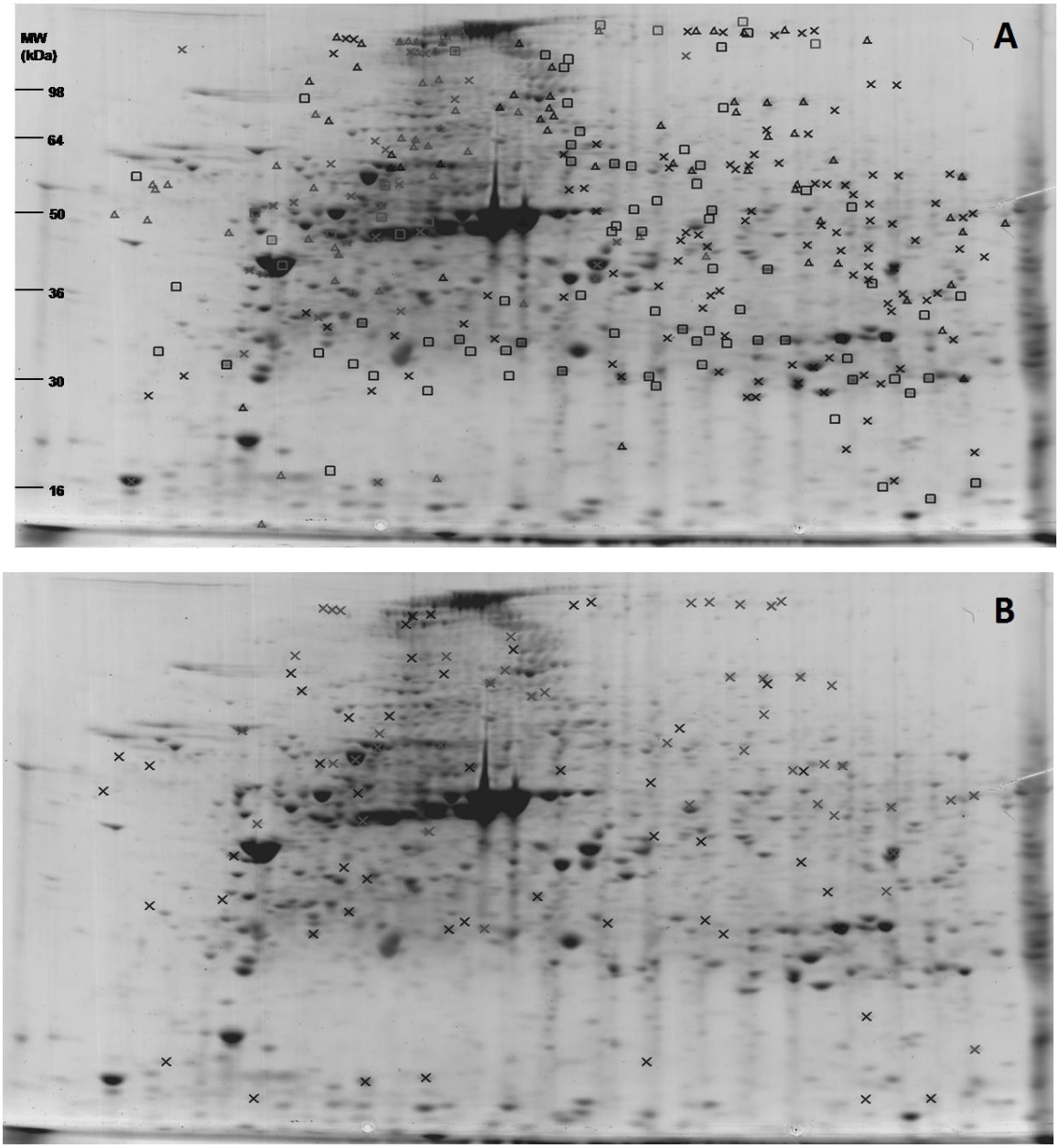

Figure 2.1. Two-dimensional gel electrophoresis of white seabream larval whole-body proteome showing protein spots which displayed statistically significant variation between (A) larvae fed the control diet and larvae fed diets L and H; and (B) larvae fed diet L and diet H. A molecular weight scale in kiloDalton (kDa) is shown to the left in (A). The pH range of the gel is of 4 to 7, from left to right. (B) Protein spots differentially expressed in group L compared to group H were marked with a cross.

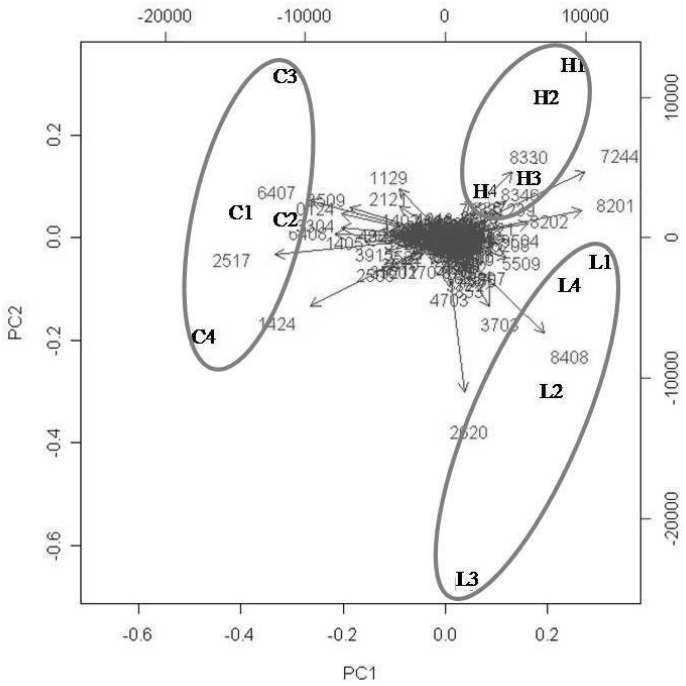

Figure 2.2. Principal component analysis biplot obtained after removal of spurious variables. Samples from white seabream larvae fed the control diet, diet Low or diet High are labelled with C, L and H, respectively. The first principal component (PC1) accounted for 56 % of the observed variance and the second (PC2) for 18 %.

This was further evidenced by a heatmap generated from the relative abundance of identified proteins for all samples, in which protein spots were clearly grouped into two main clusters, over- or under-expressed in group C compared to remaining groups, and a fainter cluster within a main cluster, in which protein spots from group L were clearly over-expressed compared to group C (Fig. 2.3.).

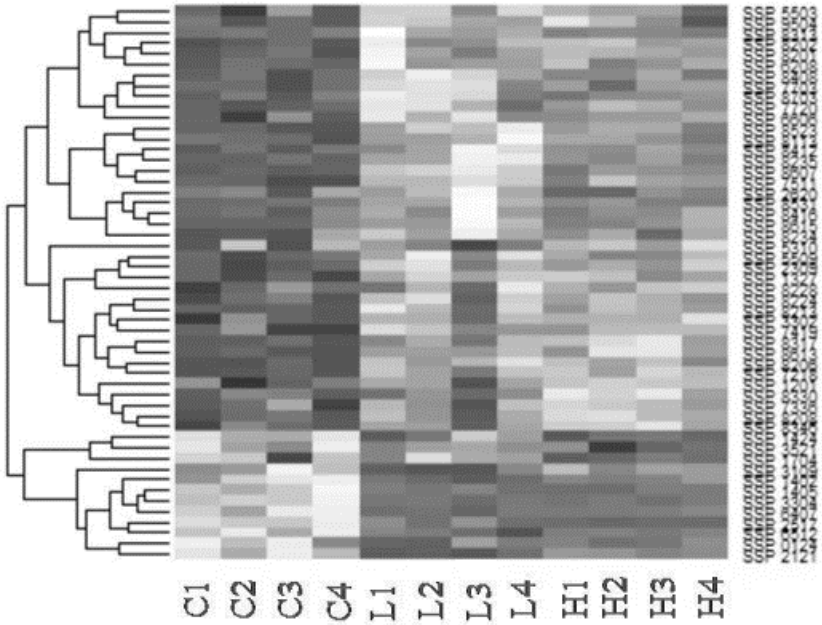

Figure 2.3. Heatmap showing relative abundance of identified proteins for all samples. Spots were grouped using Euclidian distance by agglomerative hierarchical clustering (complete linkage method). Dark shades indicate a lower than average expression of protein spots and light shades a higher than average expression. Samples from white seabream larvae fed the control diet, diet Low or diet High are labelled with C, L and H, respectively. "SSP"s indicate protein spot number identification.

Among the 52 altered proteins that could be excised and identified (Table 2.3. and Fig. 2.3.), 16 protein spots were characterised as cell cytoskeleton proteins, 9 of which were over-expressed in group C (alpha and beta actins, tropomyosin alpha chains, myosin light chain 3 and type II keratins) and 7 under-expressed in this group (alpha actins, cofilin 2, myosin-binding protein H-like –MyBPH – and beta tubulin). The 13 protein spots related to energy metabolic processes were either over-expressed in group L (fructose-bisphosphate aldolase –aldolase, glyceraldehyde-3-phosphate dehydrogenase –GAPDH, and enolases (-1 and -2), or in both groups H and L (ATP synthase subunits, muscle-type creatine kinase isoforms and/or PTMs and enolase-3). Also over-expressed in groups H and L were 2 AA metabolism proteins (glutamine synthetase and antiquitin) and 2 proteins in nucleotide metabolism (nucleoside diphosphate kinase – NDK, and Apurinic/apyrimidinic

endonuclease – Ape, nuclear). The remaining 19 protein spots identified were diverse functional proteins such as crystallins (structure formation), proteasome subunits (proteasomal degradation pathway), pre-mRNA processing factor 19 (RNA splicing factor, member of E3 ubiquitin ligase family), heat shock proteins (response to stress and chaperone activity), 14-3-3 proteins (phosphoserine/phosphothreonine binding, involved in many cellular pathways and stress response), protein disulfide isomerise – PDI (formation, reduction and isomerisation of disulphide bonds, chaperone activity, lipoprotein metabolism and redox homeostasis), and calbindin (calcium binding, many physiological processes). These were under-expressed in the control group compared to groups H and L, except for one proteasome subunit (alpha type 3) over-expressed in group C, protein disulfide isomerise –PDI over-expressed in group L relative to H, and apolipoprotein A-IV4 (lipoprotein metabolism) over-expressed in group H.

Table 2.3. Protein spots identified by LC-MS/MS or MALDI-ToF/ToF MS, using peptide fragment fingerprinting (PFF) in the option MS/MS Ion Search from the bioinformatics application Mascot. The PFF was made in the non redundant NCBI nr database for the Actinopterygii taxonomic level (p-value<0.05). Caption: a) theoretical isoelectrical points (pI_t) and molecular weights (Mw_t) based on the best result's sequence; experimental isoelectric points (pI_e) and molecular weights (Mw_e) estimated from the position of the spots in the gels; Mw is given in kDa; b) number of peptides matched (E-value<0.05), "n.a." when PFF gave no significant results; c) a non-probabilistic protein score, derived from the ions score (the Mowse score and corresponding E-value from peptide mass fingerprinting (PMF) is given when PFF gave no significant result). For data provided by the Mass Spectrometry Laboratory, ASU, ITQB, UNL, the confidence interval (%) is given instead of the E-value.

Spot N.	**Highest score hit**	**GI number (species)**	**pI_t/Mw_t pI_e/Mw_e (a)**	**Coverage (%)**	**PM (b)**	**Best peptide match: sequence, charge state, E-value**	**Combined mowse score (c)**
0124	Fast skeletal myosin light chain 3	gil5852836 (*Sparus aurata*)	4.3/13.2 4.4/17.0	45	6	GTYDDYVEGLR, +2, 1.5E-04	400
1201	Apolipoprotein A-IV4	gil74096419 (*Takifugu rubripes*)	4.8/28.5 4.6/27.2	8	2	LDPYAQDLQAR, +1, 2.6E-06	132
1327	14-3-3 protein	gil46326988 (*Oncorhynchus mykiss*)	4.7/29.2 4.7/28.9	28	2	YLAEFATGNDR, +1, 1.9E-02	76
1402	Tropomyosin alpha-1 chain	gil60390740 (*Liza aurata*)	4.6/40.4 4.7/32.8	42	6	TIDDLEDELYAQK, +2, 3.4E-06	644
1405	Tropomyosin alpha-1 chain	gil60390740 (*Liza aurata*)	4.7/38.7 4.7/32.8	40	6	TIDDLEDELYAQK, +2, 2.0E-06	554
1424	Tropomyosin	gil295792268 (*Epinephelus coioides*)	4.7/32.7 4.7/41.1	56	3	KLVIIEGDLER, +1, 3.3E-08	235
1704	Protein disulphide isomerase (similar to)	gil47223959 (*Tetraodon nigroviridis*)	4.6/76.1 4.7/55.2	20	5	VDATEETELAQEFGVR, +2, 1.0E-05	486
2121	Cytoplasmic beta actin	gil166202369 (*Oncorhynchus kisutch*)	5.6/11.2 5.0/16.7	21	n.a.	n.a.	80 (98.2%)
2309	14-3-3 protein gamma	gil237769613 (*Thunnus orientalis*)	4.9/28.3 4.9/31.9	18	1	ELEAVCQDVLNLLDNFLIK, +1, 100%	122

Spot N.	Highest score hit	GI number (species)	pI_t/Mw_t pI_e/Mw_e (a)	Coverage (%)	PM (b)	Best peptide match: sequence, charge state, E-value	Combined mowse score (c)
2517	Type II keratin E3-like protein	gil48476437 (*Sparus aurata*)	4.9/38.6 4.9/39.4	58	1	VDALQDEINFLR, +1, 1.0E-05	72
2620	ATP synthase subunit beta, mitochondrial	gil198285477 (*Salmo salar*)	4.9/52.9 5.0/58.8	14	2	IVAVIGAVVDVQFDEGLP, +1, 1.6E-17	315
3109	ATP synthase subunit beta, mitochondrial	gil198285477 (*Salmo salar*)	4.9/52.9 5.1/14.5	44	3	IPVGPETLGR, +1, 3.2E-08	315
3302	Calbindin 2a	gil41152295 (*Danio rerio*)	5.0/31.1 5.1/30.5	16	2	IEMSELAQILPTEENFLLC, +1, 8.0E-04	152
3304	Proteasome subunit alpha type-3	gil229366168 (*Anoplopoma fimbria*)	5.2/28.4 5.1/33.9	8	1	AFELELSWVGEVTNGR, +1, 1.6E-10	98
3521	Type II keratin E3-like protein	gil48476437 (*Sparus aurata*)	4.9/38.6 5.0/40.8	63	3	VDALQDEINFLR, +1, 7.1E-05	167
5310	Skeletal alpha actin	gil291167454 (*Cobitis choii*)	5.2/42.3 5.5/29.7	48	1	SYELPDGQVITIGNER, +1, 3.0E-02	103 (9.0E-06)
5503	Skeletal alpha actin	gil6653228 (*Sparus aurata*)	5.3/42.2 5.3/43.0	49	2	AVFPSIVGRPR, +1, 4.8-04	136
5509	Alpha actin	gil8489855 (*Salmo trutta*)	5.2/41.9 5.5/51.5	27	1	AGFAGDDAPR, +1, 100%	190
6208	Small heat shock protein	gil226439776 (*Epinephelus coioides*)	6.4/27.1 5.7/25.8	59	2	WDTWSNSYR, +1, 1.0E-03	93
6407	Skeletal alpha-actin type-2b	gil30268609 (*Coryphaenoides yaquinae*)	5.7/37.5 5.2/42.2	42	13	DLYANNVLSGGTTMYPGIADR, +2, 2.2E-07	469
6512	Skeletal alpha actin	gil291167454 (*Cobitis choii*)	5.2/42.3 5.6/38.8	48	n.a.	n.a.	135 (5.7E-09)
6606	Glutamine synthetase	gil18252824 (*Bostrychus sinensis*)	5.7/41.3 5.7/51.8	25	n.a.	n.a.	87 (3.3E-04)

Spot N.	Highest score hit	GI number (species)	pI_t/Mw_t pI_e/Mw_e (a)	Coverage (%)	PM (b)	Best peptide match: sequence, charge state, E-value	Combined mowse score (c)
7218	ATP synthase subunit alpha, mitochondrial	gil213512628 (*Salmo salar*)	9.0/57.2 6.1/25.5	20	1	TGAIVDVPVGEELLGR, +1, 1.9E-04	78
7336	Myosin-binding protein H-like	gil317418695 (*Dicentrarchus labrax*)	5.8/57.8 6.1/30.1	2	1	TGDWFTVLEHYHR, +1, 100%	97
7419	Proteasome, subunit beta type 8	gil315518855 (*Oryzias celebensis*)	8.0/31.0 6.2/40.5	41	n.a.	n.a.	67 (3.6E-02)
7511	Beta tubulin	gil10242162 (*Notothenia coriiceps*)	4.8/50.2 6.0/44.8	56	1	FPGQLNADLR, +1, 1.2E-05	71
7703	Heat shock protein 9	gil28278640 (*Danio rerio*)	7.0/74.2 5.9/73.7	18	4	VLGQFTLVGIPPAPR, +1, 3.2E-08	306
7720	Antiquitin	gil188036012 (*Acanthopagrus schlegelii*)	5.9/55.1 6.1/56.6	30	n.a.	n.a.	73 (8.4E-03)
8113	Nucleoside diphosphate kinase	gil194500331 (*Sparus aurata*)	6.4/17.1 6.5/16.8	40	2	TFIAIKPDGVQR, +1, 3.5E-04	107
8201	Beta A2 crystallin	gil77024823 (*Dissostichus mawsoni*)	6.3/23.8 6.0/23.7	48	9	CEFMLECQNIMER, +2, 2.3E-06	572
8202	GammaN2 crystallin	gil222522581 (*Poecilia reticulata*)	6.3/21.6 6.3/24.1	22	3	VFGDGAWVMYEEPNFR, +1, 100%	92
8206	Cofilin-2	gil213515222 (*Salmo salar*)	6.6/18.7 6.2/15.8	36	1	YGLYDATYETK, +1, 8.6E-04	53
8208	Beta A1 crystallin	gil72535901 (*Dissostichus mawsoni*)	6.1/23.0 6.4/25.5	25	n.a.	n.a.	88 (99.8%)
8213	Beta A1-2 crystallin	gil221048019 (*Epinephelus coioides*)	6.1/24.9 6.5/25.1	45	2	NWGSHCQTPQIQSIR, +1, 100%	172

Spot N.	Highest score hit	GI number (species)	pI_t/Mw_t pI_e/Mw_e (a)	Coverage (%)	PM (b)	Best peptide match: sequence, charge state, E-value	Combined mowse score (c)
8224	Beta A4 crystallin	gil77024825 (*Dissostichus mawsoni*)	6.4/23.0 6.6/24.1	29	1	IIVFDEECFQGR, +1, 100%	86
8228	Beta A1-2 crystallin	gil221048019 (*Epinephelus coioides*)	6.1/24.9 6.7/25.5	50	1	HSGDYQHWR, +1, 7.5E-04	83
8234	Beta A4 crystallin	gil77024825 (*Dissostichus mawsoni*)	6.8/22.3 6.4/23.7	50	6	IIVFDEECFQGR, +2, 2.4E-07	410
8235	Cofilin-2	gil229366360 (*Anoplopoma fimbria*)	6.8/18.9 6.8/18.8	15	1	VTDEVIAVFNDMK, +1, 100%	82
8313	Proteasome subunit alpha type 1	gil62079624 (*Oreochromis mossambicus*)	9.1/22.8 6.4/30.5	37	n.a.	n.a.	68 (2.7E-02)
8317	Beta B1 crystallin	gil77024827 (*Dissostichus mawsoni*)	6.4/26.4 6.7/30.1	28	2	SIIVECGPFVAFEQTNFR, +1, 3.2E-11	209
8330	Creatine kinase isoform a	gil156972295 (*Hippoglossus hippoglossus*)	6.9/27.2 6.9/27.5	26	6	GGDDLDPNYVLSSR, +2, 6.3E-06	387
8346	Muscle-type creatine kinase M1	gil21694041 (*Oreochromis mossambicus*)	6.6/27.6 7.0/43.3	16	5	TFLVWVNEEDHLR, +2, 4.4E-06	339
8408	Glyceraldehyde 3-phosphate dehydrogenase	gil15146358 (*Pagrus major*)	6.6/39.5 6.4/36.4	43	9	VPVADVSVVDLTCR, +2, 6.0E-07	624
8412	Creatine kinase M2	gil4027927 (*Cyprinus carpio*)	6.2/42.9 6.7/36.9	9	1	TFLVWVNEEDHLR, +1, 99.9%	77

Spot N.	Highest score hit	GI number (species)	pI_t/Mw_t pI_e/Mw_e (a)	Coverage (%)	PM (b)	Best peptide match: sequence, charge state, E-value	Combined mowse score (c)
8416	Creatine kinase isoform a	gil156972295 (*Hippoglossus hippoglossus*)	6.9/27.5 6.5/33.2	27	n.a.	n.a.	82 (1.2E-03)
8523	Chain A, crystal structure of zebrafish Ape	gil162329921 (*Danio rerio*)	5.5/32.3 6.6/45.7	28	1	ITSWNVDGLR, +1, 5.0E-02	35
8531	Muscle type creatine kinase CKM1	gil268315573 (*Platichthys stellatus*)	6.2/43.2 6.6/44.5	34	1	GFTLPPHNSR, 1+, 1.6E-04	80
8607	Enolase-1 (alpha)	gil37590349 (*Danio rerio*)	6.2/47.4 6.4/57.6	43	1	IGAEVYHNLK, +1, 7.9E-05	91
8613	Enolase-2 (gamma)	gil6624237 (*Lethenteron reissneri*)	6.3/43.3 6.7/53.6	30	1	EVILPVPAFNVINGGSHAGNK, +1, 99.2%	63
8615	Beta-enolase (3)	gil295792264 (*Epinephelus coioides*)	6.3/47.8 6.8/59.6	39	1	GNPTVEVDLWTAK, +1, 5.4E10-06	74
8703	Pre-mRNA processing factor 19	gil291190276 (*Salmo salar*)	6.1/54.8 6.4/64.1	9	1	SLVFDQSGTYLAVGGSDI, +1, 100%	111
9504	Fructose-bisphosphate aldolase C	gil46849419 (*Acipenser baerii*)	5.4/35.9 6.7/42.6	2	1	ALQASALNAWR, +1, 2.7E-06	77

2.5. Discussion

2.5.1. Effect of dietary protein hydrolysates on larval development

It has been suggested that including PH in inert diets for larviculture can improve performance and quality because the dietary nitrogen source thus provided is more readily available than intact proteins to finfish larvae, which generally do not have a fully developed and functional digestive system (Cahu and Zambonino-Infante, 1995; Carvalho et al., 1997; Zambonino-Infante et al., 1995; Savoie et al., 2011). Our results showed for the first time that a 15 % inclusion of larger polypeptides (0.5- 30 kDa) together with a 5 % inclusion of FAA and di- and tripeptides (diet H) improved growth of white seabream larvae at 15 DAH, whereas including only 5 % larger polypeptides but 15 % FAA, di- and tripeptides (diet L) reduced the growth rate of larvae, compared to the control larvae (approximately 5% larger polypeptides and 5 % FAA, di- and tripeptides). It could be that diet L presented an excess of FAA, di- and tripeptides that saturated the peptide and AA intestinal transport mechanisms, resulting in a reduced early-stage performance, as suggested by Cahu et al. (1999) and Carvalho et al. (2004), and also discussed in Tonheim et al. (2005).

The growth rates and survival observed in this study were slightly lower than those previously reported for this species (Saavedra et al., 2009a; 2010; Guerreiro et al., 2010) and this is most probably due to the very early weaning of larvae onto microdiets. Substitution of live feed for inert diets in marine larviculture has been difficult to achieve with success in terms of survival and growth. White seabream weaning is usually performed after 20 DAH, when the digestive tract development is complete and the stomach becomes functional (Cara et al., 2003; Ortiz-Delgado et al., 2003; Guerreiro et al., 2010). In the present study, larvae were in contact with the microdiets from first feeding onwards so as to enable the study of their effects on development as early as possible, but this may have lead to an increase in the mortality rate, especially for smaller larvae.

2.5.2. Whole body proteome response

Feeding early white seabream larvae with different compositions of FPH clearly affected the expression profile of soluble, abundant proteins with molecular weight between 10 and 150 kDa and a pI value between 4 and 7. Interestingly, the proteome of the control diet-fed larvae was clearly more dissimilar to those of larvae fed the experimental FPH (diets L and H), demonstrating that the inclusion level, source and peptide profile of the FPH affected more than what was directly reflected in growth at 15 DAH.

A large number of protein spots identified corresponded to cytoskeletal proteins, that play key roles in the maintenance of cell architecture, cell motility, proliferation, differentiation and organelle transport. Alpha actin, a major constituent of muscle tissue, corresponded to almost a third of these protein spots and seemed to be both up-and down-regulated across dietary treatment. This can probably be attributed to the existence of multiple isoforms and/or post-translational modifications whose occurrence seems to be regulated by numerous and complex mechanisms. Myosin light chain isoforms have also been shown to be closely regulated, particularly during fish development (Silva et al., 2010a and others therein) and interestingly, a similar isoform, along with beta actin, were also shown to be down-regulated in cod larvae fed with fish PH (Sveinsdóttir and Gudmundsdóttir, 2010). Type II keratins expression has also been shown to be developmental stage-specific in some telesost larvae, being down-regulated in later stages of development (Sarropoulou et al., 2005; Sveinsdóttir et al., 2008; Infante et al., 2011) and thus could indicate a better development of larvae fed diet H, in which they were under-expressed, which is consistent with the growth results.

Of the remaining cytoskeletal proteins, cofilin-2 and beta tubulin were over-expressed in groups L and H relative to the control group, possibly indicating increased cytoskeletal reorganisation in larvae fed a higher dietary content of PH.

Consistent with, but not restricted to, enhanced cytoskeletal dynamics, all proteins identified as belonging to energy metabolic processes were over-expressed in the larvae fed experimental fish PH compared to control larvae. In the case of larvae fed diet H, the increase in muscle creatine kinase expression could simply be due to the high energy requirements of an increased growth rate, as seen for example by Guderley et al. (2001) in

threespine stickleback. The over-expression of muscle creatine kinase, mitochondrial ATP synthase subunits and proteins belonging to the glycolytic/gluconeogenic pathways in larvae fed diet L may reflect a more important energy allocation to primary metabolism and higher protein catabolism. Studies show that dietary AA imbalances increase protein catabolism (Aragão et al., 2004a) and indeed, regulation of protein synthesis is a promising means to limit energy expenditures under unfavourable feeding conditions (Salem et al., 2007). If we assume that diet L presented an excess of FAA, di- and tripeptides, thereby impairing normal dietary nitrogen absorption and utilisation, then larvae from this group might have been suffering from enhanced protein catabolism to meet basal energy requirements. In agreement with this are the over-expression of glutamine synthetase and antiquitin in this group. Glutamine synthetase catalyses the ATP-dependent conversion of glutamate and ammonium to glutamine, playing a key role in nitrogen metabolism and is critical for the detoxification process of ammonia, a common product of protein catabolism and gluconeogenesis (Essex-Fraser et al., 2005, and others therein). Antiquitin, or aldehyde dehydrogenase 7, is a multifunctional enzyme that protects cells from oxidative stress by metabolising a number of lipid peroxidation-derived aldehydes, has been shown to act as an osmo-protectant and is directly involved in lysine catabolism (Tang et al., 2008, references therein), thus its increased abundance could be pointing to a more active AA metabolism.

The protein pre-mRNA splicing factor 19, a conserved eukaryotic RNA splicing factor and member of WD40 repeat family of E3 ubiquitin ligases was over-expressed in group L, along with the proteasome subunit alpha type 1, which could also be pointing to an increased activation of the proteasome degradation pathway and thus higher protein catabolic activity. However, other subunits of the 20S core particle structure of the proteasome were observed with different expression patterns, which is not surprising given the variety of intervention points of the proteasome, suggesting that the role of each subunit should be further investigated.

The protein disulfide isomerase, essential for correct folding, stability and/or multimerisation of many proteins, was also over-expressed in group L, relative to group H, possibly indicating a higher demand from the unfolded protein response pathway in the ER lumen of these larvae. However, other proteins with chaperone activity were also over-

expressed in both groups H and L, relative to the control group, such as the small heat shock protein and HSP9. Indeed, quite a number of proteins (nuclear Ape, NDK, 14-3-3, calbindin) implicated in cell-cycle progression, stress response, survival pathways and metabolic regulatory pathways, were over-expressed in both groups L and H relative to the control group and shows the significant impact that changing the dietary fraction of fish PH has on whole body proteome of white seabream larvae.

Interestingly, apolipoprotein A-IV4 was over-expressed in group H. Studies show that this protein may play a unique role in integrating appetite regulation, intestinal lipid absorption and energy storage in mammals (Simon et al., 2011). Assuming Apo A-IV4 has a similar role in developing white seabream, we interpret the signs of increased expression of this protein in the group which displayed higher weight gain as a sign that the dietary PH molecular size fraction and percentage in diet H might have enhanced appetite regulation and triglyceride absorption in these larvae through an induced increase in Apo A-IV4 expression.

Finally, a number of crystallin isoforms and/or PTMs were identified, under-expressed in the control group compared to those fed the experimental PH fractions. Crystallins were initially characterised as structural proteins of the vertebrate lens, however, it is now believed that many crystallins may play a number of non-lens biology-related roles (Weadick and Chang, 2009, references therein) and further research is necessary for establishing the specific roles played by crystallins.

2.6. Concluding remarks

The dietary FPH inclusion level and peptide size distribution affects growth and whole-body proteome of *D. sargus* larvae at an early stage. A dietary inclusion level of 15 % (DM basis) in FAA and di- and tripeptides (diet L) negatively affected growth of larvae. The whole-body proteome at 15 DAH clearly responded to each dietary treatment and interestingly, proteins of larvae receiving the experimental FPH had a more similar expression pattern among each other than with those of the control larvae, thus indicating a response to dietary treatment that was not directly reflected in growth at the time sampled. To our best knowledge this is the first study using comparative proteomics with such a high

number of proteins identified, in the teleost larval proteome. The proteins identified were involved in diverse processes such as, cytoskeletal dynamics, energy and carbohydrate metabolism, lipoprotein metabolism, AA metabolism, nucleotide metabolism, protein chaperoning and degradation, and signal transduction. The increased abundance of proteins implicated in cell-cycle progression, stress response, survival pathways and metabolic regulatory pathways, in both groups L and H relative to the control group indicate the significant impact that changing the dietary fraction of fish PH has on whole body proteome of white seabream larvae and this provides a good starting point to further investigate specific roles of dietary PH on the development of teleost larvae.

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Chapter 3. The dietary inclusion level of hydrolysed protein (CPSP-90™) affects gilthead seabream (*Sparus aurata*) larval protein utilisation

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3.1. Abstract

The dietary inclusion level of protein hydrolysates in larval microfeeds can affect larval performance and their capacity to wean to formulated inert feeds. This study aimed to evaluate the effect that the dietary inclusion level of a commonly available fishmeal hydrolysate (CPSP-90, Sopropêche, France) has on gilthead seabream (*Sparus aurata*) larval growth and survival. Two inclusion levels of CPSP-90 (10 and 20 %, DM basis) were tested, in triplicate. To further understand the dietary PH utilisation by larvae, a comparative analysis of the larval carcass proteome (using 2D-DIGE followed by MALDI-ToF-ToF mass spectrometry) was performed at 27 days after hatching (DAH) in combination with analysis of nitrogen excretion by larvae (at 20 and 28 DAH) and a tracer study using radiolabelled *Artemia* to assess the larval capacity for digesting and absorbing proteins at different developmental stages (21 and 30 DAH).

Results obtained showed that a 20 % dietary inclusion level (30 % of dietary protein) of CPSP-90 had a detrimental effect on performance of early life-stage *S. aurata* compared to a 10 % (15 % of dietary protein) inclusion level. This detrimental effect could have been caused by protein overloading of digestive and metabolic capacities, reflected in the very low survival, higher catabolism of absorbed dietary protein and nitrogen loss. It remains to be elucidated if the surprisingly higher growth rate recorded for these larvae is an effect of size-selective survival. A 10 % dietary inclusion level of CPSP-90 seems to be more adequate for rearing gilthead seabream larvae, who showed better survival, elevated efficiency for dietary protein retention and higher expression of skeletal muscle growth-related proteins by the end of the trial.

3.2. Introduction

Live feeds, such as rotifers, *Artemia* nauplii and copepods, are used extensively worldwide as the main feed item in commercial freshwater and marine larviculture (Conceição et al., 2010). However, the cost in infrastructure, labor and energy to mass culture these zooplankters, added to their variable supply and often sub-optimal nutritional quality, have generated over the past decades an increased interest in aquaculture research for their substitution by artificial diets (see review by Koven et al., 2001). Besides providing an

economical alternative, inert microdiets offer the possibility for a more controlled manipulation of nutritional composition (Kolkovski, 2008). Despite many recent improvements in the formulation and production of artificial microdiets (Holt et al., 2011), these still generally lead to poor larval performance compared to live feed.

Since larvae of many species of commercial interest initiate exogenous feeding when their digestive system is still at a rudimentary stage of development, in particular limited by the lack of a stomach and associated pepsin and acidic protein digestion (Rønnestad et al., 2007; Yúfera et al., 2011, references therein), digestive processes, and especially protein digestion, have been pointed out as major limitations to the efficient utilisation of artificial diets by larvae (Carvalho et al., 2004; Nankervis and Southgate, 2009; Conceição et al., 2011; Rønnestad et al., 2013). A sufficient supply of dietary amino acids (AA) is a prerequisite for high growth rates. Additionally, fish larvae rely on the extensive combustion of AA in their energy metabolism. Not only do inert microdiets usually have high nutrient density and a high protein content, but a lot of attention has been given to understanding AA metabolism and the requirements of marine fish larvae (e.g. Rønnestad et al., 2000; 2003; Aragão et al., 2007; Saavedra et al., 2009b; Conceição et al., 2011), such that adequate supplementation and balance of AA have greatly improved formulated microdiets. So it is unlikely that inferior larval performance reared on artificial feed results from inadequate protein content or a poor AA profile. Instead, poorer performance is more probably linked to the digestibility of the dietary protein, among other important factors (Kotzamanis et al., 2007; Tonheim et al., 2007; Conceição et al., 2011).

Protein solubility may, in part, explain the difficulties of altricial fish larvae in using inert microdiets, since commonly used live feeds, unlike formulated feeds, contain a high proportion of water-soluble protein, short peptides and free AA (FAA) (Conceição et al., 2010; 2011, see references therein). In these early life stages, AA from dietary proteins are absorbed mainly as short peptides or FAA from the intestine (Rønnestad et al., 2000; 2001) and so the provision of a more digestible protein source should improve both their growth and survival. Protein hydrolysates (PH) are pre-digested proteins and in several species, their inclusion in the diet has been reported to have a positive effect on larval performance compared to formulations containing unprocessed fish meal. PH supplementation enhanced

growth of salmon (*Salmo salar*) fry (Berge and Storebakken, 1996) and carp (*Cyprinus carpio*) larvae (Carvalho et al., 2004); improved growth and survival of sea bass (*Dicentrarchus labrax*) larvae, also promoting normal skeletogenesis (Zambonino-Infante et al., 1997) and additionally facilitated the onset of their adult mode of digestion (Cahu et al., 1999), besides likely enhancing their immune response (Kotzamanis et al., 2007). Moreover, it was seen that a moderate inclusion of PH in the diet tended to improved growth and survival of Atlantic halibut (*Hippoglossus hippoglossus*) larvae (Kvåle et al., 2002) and survival of larval Atlantic cod (*Gadus morhua*) (Kvåle et al., 2009). The positive effects of dietary PH in these studies generally resulted from formulations with ca. 5-15 % of PH inclusion (dry matter (DM) basis) (20-25 % total protein), with higher inclusion levels resulting in reduced growth and/or survival of larvae, such as for sea bass (Zambonino-Infante et al., 1997; Cahu et al., 1999), gilthead seabream (*Sparus aurata*) (Kolkovski and Tandler, 2000), Altantic halibut (Kvåle et al., 2002; 2009) and common carp (Carvalho et al., 2004).

It seems then that determining an optimal dietary inclusion level of PH for larval microdiets is of interest for improving larval performance and their capacity to wean to formulated inert feeds. With this in mind, the present study aimed to evaluate the effect that the dietary inclusion level of a commonly available fishmeal hydrolysate (CPSP-90, Sopropêche, France) has on larval growth and survival. Using larvae of gilthead seabream, an important Mediterranean aquaculture species, two inclusion levels of fish PH (FPH) (10 and 20 %, DM basis) were tested. To further understand the utilisation of the dietary PH we used comparative analysis of the larval carcass proteome (using two-dimensional fluorescence difference gel electrophoresis – 2D-DIGE – followed by MALDI ToF tandem mass spectrometry) in combination with a tracer study using radiolabelled *Artemia* to assess the larval capacity for digesting and absorbing proteins at different developmental stages and nitrogen excretion to assess the utilisation of the different PH.

3.3. Materials and Methods

3.3.1. Husbandry, experimental setup and feeding protocol

Gilthead seabream eggs were obtained from the commercial hatchery Maresa SA (Ayamonte, Spain). Hatching rate was around 70 %. Newly hatched larvae were distributed into six 100 L cylindrical conical fibreglass tanks, at an initial density of 100 larvae per litre, so as to test two inert microdiets in triplicate. Tanks were connected via a semi-closed seawater recirculating system, with mechanical, biological and UV filter, and each tank possessed an additional water up-welling circuit and continuous aeration. The initial water flow rate was of 0.04 L min^{-1} and was gradually increased after 8 days after hatching (DAH) to a final 0.50 L min^{-1} (30 % h^{-1} water renewal) from 20 DAH onward. The tanks were siphoned every other day, purged from the bottom every morning and equipped with a skimmer to keep the surface layer clean. Water was checked for ammonia and nitrites every other day. Photoperiod was of 16 h light : 8 h dark provided by controlled illumination from overhead fluorescent lights, with 500 lux measured at tank water surface. The water temperature, salinity and oxygen level were 18.6 ± 0.3 ºC, 36.2 ± 0.2 ‰, and 93.9 ± 2.3 % (mean ± SD), respectively, throughout the study period.

Larvae were reared from hatching to 30 DAH. They were fed with *Brachionus plicatilis* enriched with Protein Selco (INVE Aquaculture, Belgium) from first-feeding to 25 DAH, at an initial density of 10 rotifers mL^{-1}. As long as live feed was given to the larvae, the microalgae *Nannochloropsis oculata* and *Isochrysis galbana* were added to the water to keep "green water" conditions. Experimental microdiets were introduced at 9 DAH. Before being given to the larvae, the microdiets were hydrated by passing through a hydrated 50 mL falcon tube, from the automatic feeder into the tank. From this point, the amount of live prey supplied was gradually reduced and replaced by the inert diets, becoming of little dietary significance approximately one week after the introduction of the microdiets, that is, when about 75 % of larvae had inert feed in their digestive tract (as observed at the binocular microscope).

3.3.2. Experimental feed

The two microdiets tested were formulated to be iso-energetic, -nitrogenous and –lipidic, differing by the inclusion level of FPH (Table 3.1.). Diet CONT was a reference diet for gilthead seabream larvae, based on previous work by Yúfera et al. (2005). In diet CPSP, the PH content was increased from 15 % (diet CONT) to 30 % of dietary protein (10 and 20 %

DM basis, respectively), using a commercially available source of FPH (CPSP-90, Sopropêche). The microdiets were prepared following a method described by Yúfera et al. (2005) (Patent ES P200201435), which consists in a modification of the method of internal gelation described by Poncelet et al. (1992). Briefly, an emulsification and internal gelation, using alginate, produces the microcapsules which are then hardened at the surface by externally gelling the remaining non-bound alginic monomers in a calcium citrate solution, thereby entrapping the dietary ingredients in a matrix of Ca-alginate. After preparation, the microdiets were washed with freshwater on an 80 µm sieve, freeze dried and subsequently sieved to obtain particle sizes of diameter 80-200 µm and 200-400 µm.

Table 3.1. Composition of the experimental microdiets with different inclusion levels of fish protein hydrolysates.

Ingredients (% DM)	**CONT**	**CPSP**
Aglonorse micro feed [a]	13.0	13.0
CPSP – 90 [b]	10.0	20.0
Cuttlefish meal [c]	38.0	28.0
Casein [d]	5.0	5.0
Sodium-alginate [e]	7.0	7.0
Bread yeast	3.0	3.0
Cod liver oil	10.0	10.0
Marine PL [f]	6.0	6.0
Vitamin and mineral premix [g]	4.0	4.0
Vit C [h]	3.0	3.0
Vit E [i]	1.0	1.0
Proximate composition (%)		
Protein	66.7	63.9
Lipids	18.6	20.7
Ash	3.2	3.3
Dry matter	99.1	98.8

[a] AgloNorse, Norway; [b] CPSP-90, Soprepêche, France; [c] Squid Powder 0278, Rieber & Søn ASA, Norway; [d] ICN 901633; [e] ICN 154724; [f] Marine Lecithin LC40, Phosphotech, France; [g] Following the requirements reported by the National Research Council (1993); [h] Rovimix Stay C-35, Roche; [i] ICN 100555.

Crude protein (N×6.25) in diets was determined by the Kjeldahl method, total lipid with the Soxhlet method, moisture and ash gravimetrically after drying for 24 h at 105 °C and after combustion for 24 h at 550 °C (AOAC, 1990), respectively (Table 3.1.). All analyses were carried out on freeze dried and grounded samples, in duplicates for all diet types. If differences between parallels exceeded standardised values, new duplicate analyses were performed according to accredited procedures (AOAC, 1990). To determine total AA content in diets, dietary samples were hydrolysed in 6 M HCl at 108 °C for 24 h in nitrogen-flushed vials and analysed by reversed-phase high pressure liquid chromatography (HPLC) in a Waters Pico-Tag amino acid analysis system, using norleucine as an internal standard. The Breeze software (Waters, USA) was used to study the resulting chromatograms. Tryptophan was not determined, since it is partially destroyed by acid hydrolysis. Asparagine is converted to aspartate and glutamine to glutamate during acid hydrolysis, so the reported values for these AA represent the sum of the respective amine and amino acid in the proteins (Table 3.2.).

Table 3.2. Total (free plus protein-bound) amino acid (AA) composition and peptide molecular weight distribution of the soluble protein fraction of experimental microdiets. Values are expressed in weight percentage of all AA (mean ± standard deviation; n = 3). Different letters within rows denote significant differences between diet CONT and diet CPSP for a given AA (Welch's Two Sample t-test, $P < 0.01$). IAA—indispensable (and conditionally indispensable) AA; DAA – dispensable AA; Ala-alanine; Arg-arginine; Asp+Asn-aspartate+asparagine; Cys-cysteine; His-histidine; Glu+Gln-glutamine+glutamate; Gly-glycine; Ile-isoleucine; Leu-leucine; Lys-lysine; Met-methionine; Phe-phenylalanine; Pro-proline; Ser-serine; Thr-threonine; Tyr-tyrosine; Val-valine.

	CONT	**CPSP**
IAA		
Arg	7.27 ± 0.08 **a**	6.98 ± 0.05 **b**
Cys	0.63 ± 0.08	0.68 ± 0.04
His	2.25 ± 0.02	2.56 ± 0.06
Ile	3.70 ± 0.11	3.64 ± 0.11
Leu	8.81 ± 0.15	8.78 ± 0.85
Lys	8.24 ± 0.19	8.24 ± 0.09
Met	3.49 ± 0.01	3.54 ± 0.03
Phe	4.77 ± 0.09	4.88 ± 0.02
Thr	4.90 ± 0.09	4.89 ± 0.06
Tyr	4.37 ± 0.03 **b**	4.58 ± 0.01 **a**
Val	5.16 ± 0.22	5.12 ± 0.17
% IAA	53.79 ± 0.55	53.90 ± 0.14
DAA		
Ala	5.40 ± 0.03	5.27 ± 0.05
Asp+Asn	10.06 ± 0.26	9.84 ± 0.05
Glu+Gln	16.13 ± 0.15	16.15 ± 0.14
Gly	4.64 ± 0.08	4.46 ± 0.00
Pro	4.82 ± 0.08	5.07 ± 0.07
Ser	5.17 ± 0.19	5.31 ± 0.09
Peptide molecular weight (% soluble protein)*		
< 200 Da	0.15	0.30
200-500 Da	3.7	7.3
> 500 Da	6.2	12.4

* Data on peptide molecular weight distribution of CPSP-90™ was obtained from Kotzamanis et al. (2007).

3.3.3. Growth and survival

Total length and dry weight was measured at 3, 10, 20 and 28. For total length, individual photographs of 20 larvae per tank were taken and analysed with the UTHSCSA Image-Tool software (University of Texas, Health Science Center, San Antonio, TX, USA). These larvae were then frozen in liquid nitrogen in groups of 5 larvae and weighed after freeze drying for dry weight measurement. Survival was assessed as the number of larvae alive at the end of the trial, after withdrawing those removed for sampling from the initial number of larvae in the tank. Growth in dry weight was calculated as $100(e^{(\ln DWf - \ln DWi)/t} - 1)$, with DW_f and DW_i as final and initial dry weight (mg) of larvae, respectively, and t as number of days. For length data, growth was obtained as $(TL_f - TL_i)/t$ with TL_f and TL_i as final and initial total larval length (mm), respectively, and t as number of days.

3.3.4. Ammonia excretion

Ammonia excretion was measured in larvae fasted overnight, at 20 and 28 DAH. Ten larvae from each tank were enclosed in 45 mL spherical glass vials for 2 hours after gently being briefly rinsed in filtered seawater. The vials were filled with oxygen saturated, filtered sea water and sealed underwater so as to remove all air bubbles. Temperature and salinity were the same as tank water. At the end of the trials, fish larvae were frozen for dry weight measurement and the seawater was transferred to 50 mL plastic flasks, where 45 µL of sulphuric acid 25 % was added, and then frozen for ammonia quantification. Ammonia concentration was determined according to Berthelot (Grasshoff, 1983). Samples were treated with alkaline citrate, sodium hypochloride and phenol in the presence of sodium nitroprussiate which catalyses the reaction. The blue colour formed by indophenol plus ammonia reaction was measured at 630 nm. Mass-specific ammonia excretion (expressed as g NH_4^+ g DW^{-1} h^{-1}) was calculated as

$$MNH_4^+ = \Delta[NH_4^+]V_{H2O}DW^{-1}\,\Delta T^{-1}$$

where $\Delta[NH_4^+]$ is the difference in concentration between the sample and the blank water, V_{H2O} is the volume of water in the incubator vial (mL), DW is the larvae dry weight (g), and ΔT is the time duration of the trial (h).

3.3.5. Protein utilisation

Capacity of larvae to digest and metabolise *Artemia* proteins was studied in 21 and 30 DAH larvae fed *Artemia* metanauplii radiolabelled with a [U-^{14}C] protein hydrolysate (1.85 MBq mL; Amersham Pharmacia Biotech Ltd, Buckinghamshire, UK), according to the method developed by Morais et al. (2004a) and adapted by Engrola et al. (2010). The afternoon prior to the measurements of *Artemia* intake and protein utilisation, seabream from each treatment (n = 30) were randomly collected and transferred to the radiolabelling laboratory where they were stocked in 1 L tanks with clean seawater and fasted overnight. *Artemia* nauplii were enriched at a density of 200 *Artemia* mL^{-1} in a sealed incubation system at 28 ºC, with a dose of 3.3 µL of [U–^{14}C] protein hydrolysate per mL of seawater. The radiolabelling process was done overnight. After incubation *Artemia* metanauplii were washed in seawater several times, counted and samples (n = 4; 3 mL each sample) were taken to measure the incorporated radiolabel. Samples of the incubation seawater were also taken (n = 4; 3 mL each sample) to correct for the ^{14}C present.

On the day of the trial, seabream larvae were allowed to eat the radiolabelled *Artemia* during 1 hour. Fed larvae were then carefully transferred individually with Pasteur pipettes through two baths of clean seawater (to eliminate any possible non-ingested ^{14}C AA) to an incubation vial. The incubation setup was described previously by Rønnestad et al., (2001). Briefly, it consisted of sealed vials containing 7.5 mL of seawater with gentle air flow, the larvae being placed individually into these vials. The air is pumped through a capillary from the incubation vial to a chemical $^{14}CO_2$ trap (5.0 mL of KOH, 0.5 M). After a 24 h incubation period each larva was rinsed with clean seawater and sampled for further analysis. The incubation vials were then resealed and HCl injected gradually (1.0 mL of HCl, 1 M), in order to release the remaining CO_2 in the seawater to the KOH trap.

Scintiliation cocktail (Ultima Gold XR, Packard Bioscience, Milan, Italy) was added to the incubation seawater and KOH containing vials and counted for radioactivity (DPM, disintegrations per minute). All counts were corrected for quench and lumex. Larvae and *Artemia* were solubilised with a tissue solubiliser (Solvable, Perkin-Elmer, Milan, Italy) and incubated at 50 ºC during 24 h. After cooling, scintiliation cocktail (Ultima Gold XR; Packard Bioscience) was added and samples were counted for radioactivity (DPM).

Seabream larvae that had not ingested any live prey were discarded from further analysis. The Artemia intake (AI; % of body DW) after a single meal of radiolabelled *Artemia* was determined as:

$$AI = 100[(R_{total} / SR_{Artemia}) / DW_{larvae}]$$

where R_{total} is the sum of radioactivity (DPM) in the incubation seawater, CO_2 trap and larval body, and $SR_{Artemia}$ is the specific radioactivity per *Artemia* samples (DPM mg^{-1} *Artemia* DW) and DW_{larvae} is the dry weight of *S. aurata* larvae (mg) .

Protein utilisation was determined based on protein digestibility (D, %), retention efficiency (R, %) and catabolism (C, %). These estimates were determined as:

$$D = 100[(R_{larva} + R_{CO2trap}) / (R_{larva} + R_{CO2trap} + R_{water})],$$

$$R = 100[R_{larva} / (R_{larva} + R_{CO2trap})],$$

$$C = 100[R_{CO2trap} / (R_{larva} + R_{CO2trap})],$$

where R_{larva} is the total radioactivity in larval body (DPM), $R_{CO2trap}$ is the total radioactivity per CO_2 trap (DPM) and R_{water} is the total radioactivity in the incubation seawater (DPM).

3.3.6. Comparative analysis of larval carcass proteome

At 27 DAH, approximately 200 larvae from each tank were rinsed in distilled water, immediately dissected on ice for removal of head and visceral parts, and the carcasses were then frozen in liquid nitrogen. A sample of 40 mg (wet weight) from each tank was lysed by sonication in 200 µL of ice-cold buffer [7 M urea, 2 M thiourea, 4% (w / v) CHAPS, 30 mM Tris, 1 % (v / v) protease inhibitor cocktail (Sigma-Aldrich)]. Homogenates were centrifuged twice at 12000 g for 10 min at 4 ºC to pellet insoluble material. The resulting supernatants were depleted of non-protein contaminants using a ReadyPrep™ 2-D Cleanup Kit (Bio-Rad) and resuspended in the lysis buffer. The pH of the protein extract was adjusted on ice to 8.5 by addition of the appropriate volume of dilute NaOH. All protein quantifications were performed using Quick Start™ Bradford Protein Assay (Bio-Rad).

For DIGE minimal labelling, 50 µg of protein was labelled with 400 pmol of fluorescent amine reactive cyanine dyes freshly dissolved in anhydrous dimethyl formamide following the manufacturer's recommended protocol (Amersham CyDye DIGE Fluor, minimal labelling kit, GE Healthcare). Labelling was performed on ice for 30 min in the dark and quenched with 1 mM Lys for 10 min. For each dietary treatment, an additional "replicate" was created, by pooling equal amounts of protein extraction from each replicate (tank) of the same dietary treatment. Thus, two replicates per dietary treatment were labelled with Cy3 and two with Cy5 to normalise for label differences, while an internal control consisting of equal quantities of protein from all replicates was labelled with Cy2.

For gel electrophoretic separation, 50 µg of muscle protein of one replicate from each dietary treatment plus 50 µg of internal standard were diluted in ReadyPrep™ 2-D Rehydratation Buffer (Bio-Rad) to a final volume of 450 µl and loaded overnight on to a 24 cm Immobiline™ Drystrip pH 4-7 (GE Healthcare), by passive rehydration. In the first dimension, proteins were isoelectric focused using an Ettan™IPGphor™ 3 isoelectric focusing unit (GE Healthcare), at 20 ºC for a total of 60040 Vhours at maximum 75 µA per strip. After the first dimension, strips were equilibrated (cysteine sulfhydryl groups reduced then carbamidomethylated) using standard Bio-Rad reducing (2 % w/v DTT) and alkylating (2.8 % w/v iodoacetamide) buffers (6 M urea, 20% v/v glycerol, 2% w/v SDS and 0.5 M Tris- HCl pH 8.8), 40 min each step, at room temperature. The proteins were then separated according to their molecular mass in 12.5 % SDS-PAGE gel using a standard TGS running buffer (25 mM Tris, 192 mM Glycine, 0.2 % SDS).

After electrophoresis, gels were immediately scanned with a Typhoon Trio™ Variable Mode Imager (GE Healthcare) using a resolution of 100 µm and PMT value set to ensure that maximum pixel intensity was below saturation. Image analysis was performed using the Progenesis Same-Spots software (Nonlinear Dynamics, Newcastle upon Tyne, UK), which computed multiplication (fold-change) and p-values of all spots applying Student's t-test.

To identify the selected differentially expressed proteins, preparative gels were run prior to spot excision following the same protocol as described above except that labelling was not

performed, each gel was loaded with 1 mg of protein from mixed samples and Colloidal Coomassie Blue G250 staining was used.

Proteins of interest were excised and subjected to in-gel trypsin digestion. Briefly, the spots were successively washed with washing solution [25 mM NH4HCO3 in 50 % v/v H2O/acetonitrile (ACN)]. The proteins were submitted to a reduction and alkylation step using 10 mM DTT at 56 ºC for 45 min, followed by 55 mM iodoacetamide in the dark at room temperature for 45 min. Finally, gel pieces were thoroughly washed as before and dried in a SpeedVac Concentrator 5301 (Eppendorf, Germany). The dried gel spots were rehydrated by addition of digestion buffer (40 mM NH4HCO3 and 4 ng/µl of trypsin (Trypsin Gold, MS grade, Promega) during 30 min incubation on ice and digestion was performed overnight at 37 ºC. Following tryptic digestion, the supernatant was collected and 1 % trifluoroacetic acid (TFA) was added to the gel plugs, followed by 60 % ACN/0.1 % TFA in order to extract the peptides more efficiently. Both these extracts were combined with the first supernatant and these samples were dried again. The tryptic peptides were desalted and further concentrated using small disks of C18 3M Empore Disks (Varian A.B., Solna) placed into 10 µl pipet tips, and were eluted with saturated matrix solution (α-cyano-4-hydrozy-cinnamic acid (12 mg/ml, Bruker Daltonics, Bremen) in 60 % ACN/0.2 % TFA) onto the target plate. Matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) (Autoflex and Ultraflex, Bruker Daltonics) and MALDI with MS/MS (Ultraflex with a LIFT module, Bruker Daltonics) was used for mass analyses of the peptide mixtures. The spectra were externally calibrated using Peptide Calibration Standard II (Bruker Daltonics, Bremen), while the internal calibration was performed with the trypsin autolytic products. FlexAnalysis 3.0 (Bruker Daltonics, Bremen) was used to create the peak list and Bio-Tools 3.0 (Bruker Daltonics, Bremen) was used for interpretation of MS and MS/MS spectra, and proteins were identified by peptide mass fingerprinting (PMF) via the database search program MASCOT (http://www.matrixscience.com), using the Actinopterygii and Chordata NCBInr and SWISSPROT protein databases. MS/MS analysis and repeated MASCOT database searches of a minimum of two precursor ions recognised in the PMF search were performed to confirm the PMF-based protein identification, whenever possible. It was assumed that the peptides were monoisotopic, cysteine residues were carbamidomethylated and

methionine residues possibly oxidised. The fingerprinting method allowed for a maximum of one missed tryptic cleavage per protein. The maximum deviation permitted in matching the parent ion/fragment ion mass values was 100 ppm/0.8 Da, respectively. Following the database searches, the MOWSE score, number of peptide matches, sequence coverage, molecular weight, and pI value were used to evaluate the results.

3.3.7. Data analysis

Welch's Two Sample t-test was used to analyse the effect of dietary treatment on growth, survival, mass-specific ammonia excretion, *Artemia* intake and protein utilisation of *S. aurata* larvae at the same age, as well as for comparing the AA composition of experimental microdiets. Differences were considered significant when $P < 0.05$, or $P < 0.01$ for AA data. All percentage data were arcsine ($x^{1/2}$)-transformed prior to analysis. All statistical analysis was carried out using the R statistical computing environment (R Development Core Team, 2011).

3.4. Results

3.4.1. Larval growth and survival

Growth of *S. aurata* larvae was similar until the point when most larvae (> 75 % of larvae) were ingesting the microdiets (around 18 DAH), after which larvae fed diet CPSP grew more than the control larvae in terms of total length (Fig. 3.1.).

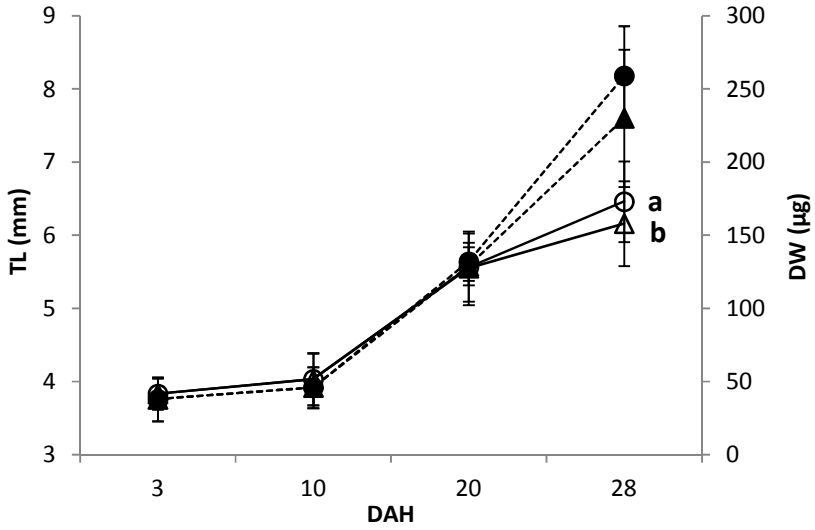

Figure 3.1. Growth of *S. aurata* larvae fed different dietary CPSP-90™ inclusion levels . Total length in mm (TL, solid line)and dry weight in µg (DW, hatched line) for diet CONT (triangle) and diet CPSP (circle). Results are expressed as means ± standard deviation. For TL n = 60 larvae and for DW n = 12 pools of 5 larvae. Different letters for a selected DAH denotes significantly different mean values (Welch's Two Sample t-test, $P < 0.05$).

Independent of dietary treatment, growth seems to have slowed down from 20 to 28 DAH (Table 3.3.). On the other hand, survival at the end of the experiment was significantly lower in larvae administered diet CPSP compared to those reared with the control diet (Table 3.3.).

Table 3.3. Growth and survival of *S. aurata* larvae fed expreimental feed containing different inclusion levels of CPSP-90™. Values are means ± standard deviation (n = 3). Mean values for a selected period not sharing a common letter are significantly different (Welch's Two Sample t-test, $P < 0.05$).

	CONT	**CPSP**
Growth in length (mm day^{-1})		
3-10 DAH	0.03 ± 0.06	0.03 ± 0.06
10-20 DAH	0.15 ± 0.01	0.15 ± 0.02
20-28 DAH	0.08 ± 0.00 **b**	0.11 ± 0.03 **a**
Growth in weight (% DW day^{-1})		
3-10 DAH	3.33 ± 6.91	3.33 ± 6.91
10-20 DAH	11.51 ± 0.18	11.76 ± 0.87
20-28 DAH	7.30 ± 0.93	8.72 ± 0.70
Survival (%)	5.16 ± 0.95 **a**	1.70 ± 0.46 **b**

3.4.2. Ammonia excretion and protein utilisation trial

The mass-specific ammonia excretion of fasted *S. aurata* larvae tended to be higher for those administered the diet with a higher inclusion level of FPH, and this difference was significant at 20 DAH (Fig. 3.2.). Mass-specific ammonia excretion was lower at 28 DAH than at 20 DAH for both dietary treatments.

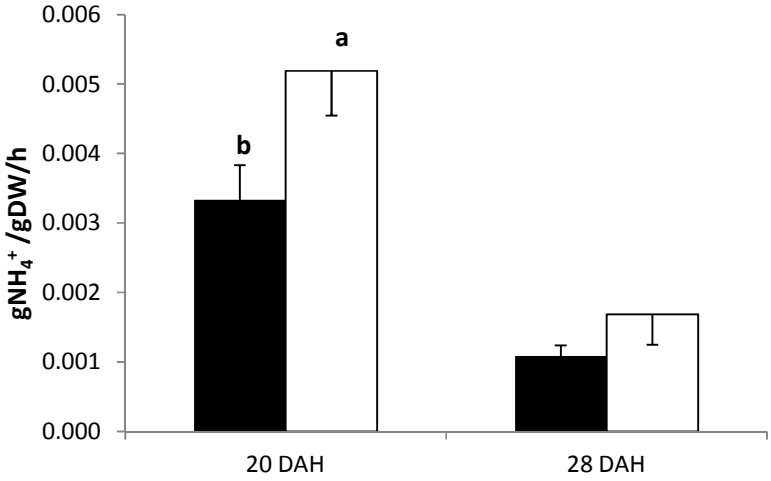

Figure 3.2. Mass-specific ammonia excretion of fasted 20 and 28 DAH *S. aurata* fed diets with different dietary CPSP-90™ inclusion levels. Values are mean ± standard deviation (n = 3 pools of 10 larvae). Diet CONT – black, diet CPSP – white. Different letters for a given DAH represent significant differences (Welch's Two Sample t-test, $P < 0.05$).

At 21 DAH, larvae proved to be unfit for the metabolic trial, showing a high mortality rate (60 – 80 %) during the 24 h incubation period and a non-detectable ingestion of *Artemia.* Those data were thus discarded from further analysis. On the other hand, at 30 DAH, larval survival was high both during overnight acclimation in the laboratory (93 % for CONT larvae and 90 % for CPSP larvae, n= 30) and during the 24 h incubation period (80 % for CONT larvae and 87 % for CPSP larvae, n = 15). Though the amount of larvae ingesting *Artemia* was the same for both treatments, *Artemia* intake per larva was quite variable, with 28.56 ± 12.40 (% BDW, mean ± SD) for larvae fed the control diet and 35.14 ± 21.67 (% BDW, mean ± SD) for those fed diet CPSP (Fig. 3.3.).

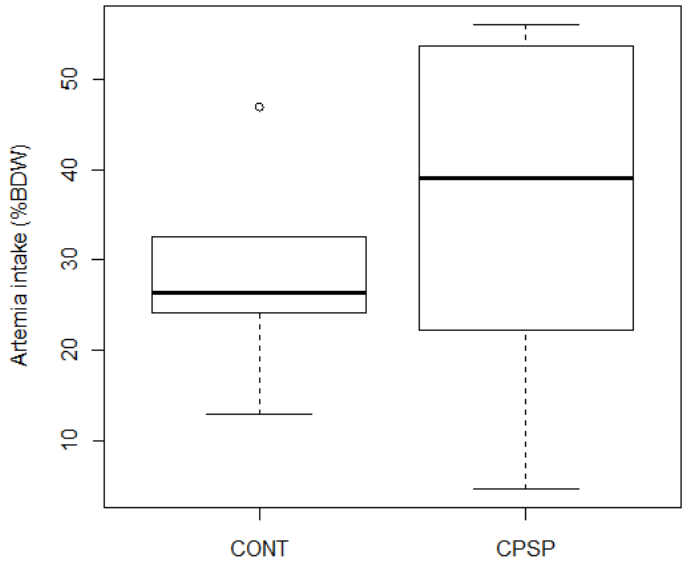

Figure 3.3. Artemia intake (% body dry weight (BDW)) of *S. aurata* at 30 DAH. CONT –control diet; CPSP – diet CPSP. *Artemia* intake was not significantly different between treatments (n = 5, Welch's Two Sample t-student, $P < 0.5$).

Concerning protein utilisation, digestibility ranged from 70-80 % of total ingested protein for S. aurata larvae at 30 DAH and was unaffected by dietary treatment (Fig. 3.4.). However, larvae fed the control diet showed almost complete retention of the digested protein with little catabolism after 24 h post-feeding, compared to those fed the diet with a higher inclusion level of PH, which had catabolised a significant portion of digested label.

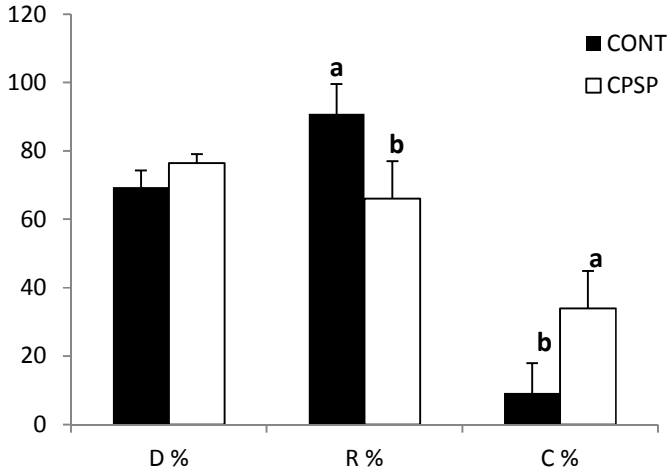

Figure 3.4. Protein digestibility (D, % of radiolabel in the larva and metabolic trap in relation to total radiolabel ingested), protein retention (R, % of radiolabel in the larva in relation to digested label) and catabolism (C, % of radiolabel in the metabolic trap in relation to digested label) in *S. aurata* at 30 DAH, after 24 h incubation. Values are means ± standard deviation (n = 5). Different letters indicate statistical differences (Welch's Two Sample t-test, $P < 0.05$) between larvae from different dietary treatments.

3.4.3. Comparative analysis of larval carcass proteome

Comparative 2D-DIGE analysis of seabream larval carcass, using the Progenesis Same-Spots software (Nonlinear Dynamics, Newcastle upon Tyne, UK), enabled the detection and quantification of 839 protein spots across all spot maps, covering a molecular mass range of ~10 kDa to 200 kDa and pI values between 4 and 7 (Figure 3.5.).

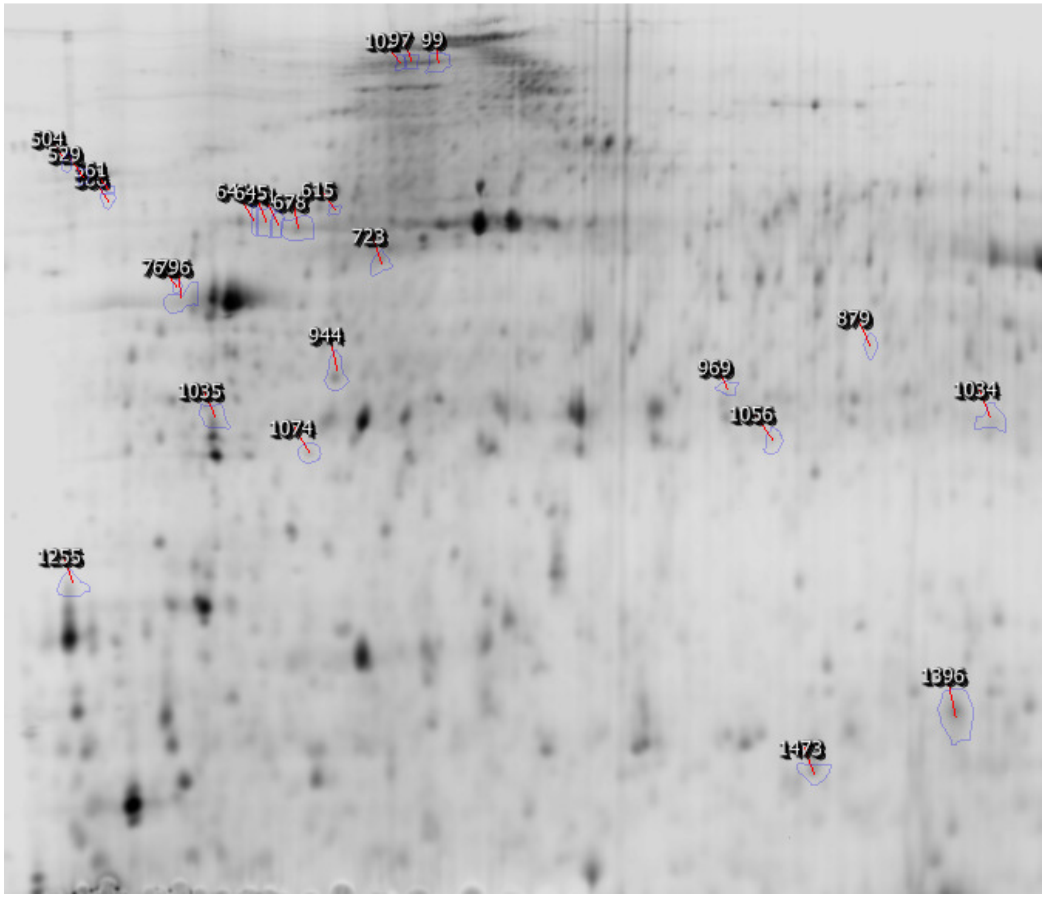

Figure 3.5. Minimal CyDye™ DIGE Fluor-stained 2D gel (pH 4-7 linear, 12.5 % Tris-HCl) from larval carcass of *S. aurata* at 27 DAH. Reference image representing those protein spots whose expression was significantly affected by the dietary treatment (Student's t-test, $P < 0.05$, fold-change > 1.2).

Analysis of the normalised protein quantity using Student's t-test revealed 25 protein spots with significantly altered expression according to dietary inclusion level of PH, 19 spots being over-expressed and 6 under-expressed in Control (fold-change > 1.2; $P < 0.05$). Of the protein spots whose expression was significantly affected by dietary fish PH inclusion level, 9 could be excised and subjected to in-gel tryptic digestion, 6 of which were successfully identified, corresponding to cytoskeletal proteins (actin, myosin light chains 1 (MyLC1) and 3 (MyLC3) and myosin heavy chain (MyHC)((Table 3.4.). The remaining 3 protein spots could not be identified, mainly due to a low abundance in the gels.

Table 3.4. . Protein spots identified by MALDI-ToF-ToF MS, using peptide fragment fingerprinting (PFF) in the option MS/MS Ion Search from the bioinformatics application Mascot. The PFF was made in the non redundant NCBI nr database for the Actinopterygii taxonomic level.

Spot #	p-value[a]	Fold-change	Protein identification	NCBI accession number	MW/pI E MW/pI T[b]	Coverage (%)	PM[c]	Best matched peptide sequence and E-value	Combined MOWSE score
Higher abundance in CONT									
97	0.044	1.3	Myosin Heavy Chain	ABC42922	75/5.1 203/5.6	21	36	3(3) ANSEVAQWR 2.2e-05	184
504	0.032	1.5	No protein match		59/4.2				
723	0.041	1.2	Skeletal alpha actin	AAF22646.1	34/4.7 42/5.3	35	14	2(3) SYELPDGQVITIGNER 1.5e-08	164
1035	0.030	1.4	Myosin light chain 1	P82159	30/4.6 20/4.5	36	7	1(2) AGFEDYVEGLR 1.9e-04	100
1255	0.014	1.2	Myosin light chain 3	AAD54228	20/4.2 17/4.4	50	10	2(2) GTYDDYVEGLR 0.0032	86

Spot #	p-value[a]	Fold-change	Protein identification	NCBI accession number	MW/pI E MW/pI T[b]	Coverage (%)	PM[c]	Best matched peptide sequence and E-value	Combined MOWSE score[d]
1396	0.023	1.7	Skeletal alpha actin	AAF22646.1	14/6.6 42/5.3	24	10	2(3) QEYDEAGPSIVHR 1.7e-07	187
1473	0.049	1.2	No protein match		13/6.2				
Higher abundance in CPSP									
944	0.024	1.3	Skeletal alpha actin	AAF22646.1	31/4.9 42/5.3	25	12	3(3) AGFAGDDAPR 2.1e-07	194
1074	0.032	1.2	No protein match		28/4.8				

[a] p-value obtained from comparative analysis (Student's t-test) of protein spot expression between groups using Progenesis Same-Spots software (Nonlinear Dynamics, Newcastle upon Tyne, UK);
[b] theoretical (T) isoelectrical points (pI) and molecular weights (MW) based on the best result's sequence; experimental (E) isoelectric points (pI) and molecular weights (MW) estimated from the position of the spots in the gels; MW is given in kilo Daltons (kDa);
[c] number of peptides matched (E-value < 0.05), "n.a." when PFF gave no significant results;
[d] a non-probabilistic protein score, derived from the ions score (the Mowse score and corresponding E-value from peptide mass fingerprinting (PMF) is given when PFF gave no significant result).

3.5. Discussion

To study how the inclusion level of dietary FPH influences growth performance in gilthead seabream larvae, it was necessary to work with inert microdiets because the live feed commonly used to rear teleost altricial early life stages is difficult to manipulate in terms of AA and hydrolysed protein products due to the metabolism of the live feeds (Aragão et al., 2004a, Conceição et al., 2010). A better control of dietary content is achieved with microencapsulated feed. However, despite recent advances in the production of microdiets for gilthead seabream larvae (Yúfera et al., 2005, Sandel et al., 2010), it has not been possible to successfully rear Sparidae larvae solely with inert feed, for reasons such as low attractiveness compared to live feed; poorer digestibility; high leaching losses of soluble molecules such as FAA, peptides, vitamins and minerals; among others (e.g. Langdon, 2003; Yúfera et al., 2011). Despite current limitations, the feeding protocol used in the present study (combining the use of rotifers and alginate microdiets until *ca.* 18 DAH, and then essentially just microdiets until 28 DAH) enabled growth and survival of gilthead seabream larvae throughout the study period. The larval acceptance of the alginate microdiet was quite high, with most larvae actively feeding and ingesting the particles about one week after its introduction.

The overall larval performance in terms of growth and survival were lower than that usually observed for *S. aurata* when reared solely on commonly enriched live feed (Moyano et al., 1996; Aragão et al., 2004a) but growth results were similar or better than those reported in previous nutritional studies attempting to rear gilthead seabream larvae directly, or with an early weaning scheme, onto inert microdiets (Fernández-Díaz and Yúfera, 1997; Yúfera et al., 1999; Robin and Vincent, 2003; Robin and Peron, 2004; Yúfera et al., 2005; Seiliez et al., 2006). Survival is more difficult to compare due to the different trial durations of the reported studies. Though the group weaned onto the CPSP diet had a very low survival at the end of the trial, survival of the control group is within the range of values commonly reported in these types of studies, where higher mortality rates have been associated with the difficult adaptation phase of marine fish early life stages to artificial diets (e.g. Kolkovski et al., 1997; Cahu and Zambonino-Infante, 2001; Saavedra et al., 2009a; de Vareilles et al., 2012 – Chapter 2 of present thesis).

In both dietary treatments, the growth rate decreased after 20 DAH, possibly caused by temporary factors associated with the adaptation period from live to inert microdiets. Whilst studying different weaning regimes in red porgy (*Pagrus pagrus*) larvae, Andrade et al. (2012) also observed a decrease in growth rate during the initial weaning period. These authors attributed the decreased growth rate to a reduction in rotifer consumption induced by the increased ingestion of microdiets. Together with the limited capacity for early life stages to digest inert feed, this would leave larvae with less energy and nutrients available for growth. However, Aragão et al. (2004a) too observed a decrease in growth rate of *S. aurata* larvae reared solely on enriched live feed, after 16 DAH, when *Artemia* were introduced in the feeding protocol. Therefore, caution must be taken in interpreting these results as the slowed growth could simply be developmental, or both developmental and nutritionally related.

Both growth rates and final total length were significantly higher for seabream larvae reared on the diet with a 20 % inclusion level of CPSP-90. Moderate inclusion levels of dietary PH (generally up to ca. 15 % dietary inclusion level, DM basis) have been shown to improve larval performance, such as maturation of digestive tract, proper skeletal development, growth and survival, in several species (e.g. Zambonino-Infante et al., 1997; Cahu et al., 1999; Kvåle et al., 2002; Carvalho et al., 2004). It is suggested that the hydrolysis of proteins prior to feeding can compensate for the absence of a complete gastric digestive system in altricial fish larvae, which initially assimilate simple forms of AA and peptides more efficiently than the more complex forms (Rønnestad et al., 2000; 2003; Tonheim et al., 2005, Zambonino-Infante and Cahu, 2007, Conceição et al., 2011; Rønnestad et al., 2013). However, in this study, survival was significantly lower in the diet containing a 20 % inclusion level of FPH. In a previous study with white seabream (*Diplodus sargus*) larvae, the best growth results were obtained with a 20 % inclusion level of a FPH, though growth was not significantly different with that of larvae reared on a 10 % dietary inclusion level of CPSP-90 and survival was similar in all dietary treatments (de Vareilles et al., 2012 – Chapter 2 of present thesis). These results do not contradict the present ones because although the PH molecular size distribution of the diets with 20 % inclusion was similar for both studies (dietary inclusions of 5 % vs. 7.6 % of MW < 500 Da and 15 % vs. 12.4 % of MW >500 Da in the previous vs. current study, respectively), many

other factors differed, such as the species under study, the type of microencapsulated diet used, total protein content of diet, protein source of hydrolysates and possibly method of hydrolysis. Indeed it seems that the influence of PH inclusion level on fish larval development will depend on the species and developmental period under study, rearing and weaning conditions, the protein source and molecular weight distribution of hydrolysates, total dietary protein content and composition in other nutrients, among other factors. For example, a 19 % inclusion of FPH (CPSP-G, Sopropêche, France) was considered a moderate dietary inclusion level in a study with sea bass larvae, where best results in terms of growth, digestive tract maturation and survival were obtained with this inclusion level (Cahu et al., 1999). Additionally, it was found that larval *D. labrax* growth was more than twice as high in groups fed diets that incorporated native protein and low FPH levels (CPSP-G, 14 %) compared to the group with a 46 % PH inclusion level, and that survival at day 42 of this latter group was significantly lower (Cahu et al., 2004). However, just a 10 % dietary inclusion of CPSP-90 (similar to the control group in this study) improved growth and immunological status of larval sea bass compared to a 19 % inclusion level (Kotzamanis et al., 2007).

Thus for the present study, increasing the dietary hydrolysate inclusion level from 10 % to 20 % did not constitute an improvement for gilthead seabream larval development to 30 DAH. In previous studies where poor results in terms of survival were obtained for early life stages fed diets with a high inclusion of low molecular weight PH, it was suggested that these diets ended up having a reduced nutritional value at time of ingestion due to high leaching rates of small peptides and FAA from the microdiets (López-Alvarado and Kanawaza, 1995; Kvåle et al., 2009; Nankervis and Southgate, 2009). However, in the present study, the leaching rate was similar for both alginate microdiets, that is, approximately 10 % of crude protein after 5 minutes and up to one hour agitation in autoclaved seawater with the same temperature and salinity as experimental tanks (determined by the Kjeldahl method, data not shown). Nonetheless, leaching rates can be specific to individual AA and their molecular form in the diet (Yúfera et al., 2002). Taking this into account, it cannot be excluded that the final AA composition of diets at time of ingestion might have differed, which in turn could have influenced larval performance.

Lower survival and general performance of fish larvae reared on high PH inclusion level diets may also be caused by the overloading of metabolic systems by the sudden AA and short peptides flux into the digestive tract (Cahu et al., 1999; Aragão et al., 2004b; Kolkovski and Tandler, 2000; Kvåle et al., 2002; Kolkovski, 2008). The FAA pool in feeding fish larvae is small and kept within narrow limits so absorbed dietary AA are either used for protein synthesis or are processed otherwise (Conceição et al., 2011; Rønnestad and Conceição, 2012). Additionally, the absorption of individual AA by the different transport systems can proceed at different rates, selective catabolism of individual AA may occur, and the excess or deficiency in one AA is able to affect the metabolic fate of another (see reviews Wu, 2009; Conceição et al., 2011). So a higher dietary inclusion of low molecular weight PH may alter normal AA metabolism and also cause a rapid elevation of plasma FAA levels, disturbing whole body homeostasis, impairing growth and development, and sometimes even having toxic effects for the larval organism, eventually leading to mortalities (Wu, 2009; references therein).

The increased nitrogen excretion observed in the CPSP diet-fed larvae compared to the control group is indicative of a higher catabolism of AA for energy production. An increased mass-specific ammonia excretion was also seen for Senegalese sole (*Solea senegalensis*) post-larvae (Aragão et al., 2004b) and white seabream larvae (Saavedra et al., 2009a) when fed unbalanced dietary AA profiles. It was suggested that these diets would have disturbed the AA metabolism, leading to enhanced AA loss and protein catabolism for energy production. The lower mass-specific ammonia excretion observed at 28 DAH compared to 20 DAH, independent of dietary treatment, could mean that larvae rely less on AA for energy production and relatively more dietary nitrogen is being retained for protein synthesis as they develop. However, as could be assessed from the metabolic trial with labeled *Artemia*, catabolism continued to be greater for CPSP diet-fed larvae at 30 DAH compared to control larvae, which in turn showed higher protein retention (see below). So it appears that in this study, gilthead seabream larvae between 20 and 30 DAH had difficulties in handling a 20 % inclusion level (30 % of dietary protein) of dietary PH compared to 10 % (15 % of dietary protein) inclusion level (DM basis).

In order to study if adaptations to the diet´s PH content affected the larval capacity to digest and absorb proteins, larvae from different treatments were fed radiolabelled *Artemia* and assessed for protein utilisation. *Artemia* constitutes a protein source with high digestibility in fish larvae, thus enabling the study of protein utilisation with very high potential (Rønnestad and Conceição, 2005; Engrola et al., 2010). Although the determination of protein utilisation using radiolabelled microdiets might have been more appropriate, the incorporation of radioactive tracers in microdiets is technically difficult, for security reasons, and short peptides and AA leaching can also easily occur (see Engrola et al., 2009b and references therein).

Gilthead seabream larvae were possibly too fragile at 21 DAH (*ca.* 130 µg DW and 5.6 mm TL) to enable the use of this method for studying protein utilisation at this stage, but larvae of 30 DAH (*ca.* 245 µg DW and 6.3 mm TL) showed a good resilience for transportation and acclimation to the laboratory, and had a high survival rate throughout the 24 h study period. The dietary treatment did not have a significant influence on *Artemia* intake, which showed quite an inter-individual variability. All groups had a high capacity for digesting *Artemia* protein, with label absorption in larvae varying between 70-80 %. These values are comparable to those obtained using radiolabelled live-feed for Senegalese sole early life stages (Morais et al., 2004b; Engrola et al., 2009b; 2010) and larval spot (*Leiostomus xanthurus*) (Govoni et al., 1982).

Contrarily to *Artemia* intake and digestive capacity of larvae, the metabolic fate of absorbed dietary nitrogen was affected by dietary treatment. The control larvae showed elevated retention of absorbed label in the body (*ca.* 90 % of absorbed labelled protein), indicating a high efficiency for dietary AA retention, as has previously been observed for young fish larvae (Conceição et al., 1998; Morais et al., 2004b). This may point to an enhanced growth potential in these larvae. In comparison, CPSP diet-fed larvae showed significantly lower label retention in the body (66 % of absorbed labelled protein) and higher catabolism which, as previously discussed, might be explained by a reduced anabolic efficiency; that is, a possible saturation of the intestinal transport systems with a consequent AA imbalance affecting AA oxidation, which can lead to decreased food conversion efficiencies and have been suggested to explain some morphophysiological disorders (Aragão et al., 2007). A

tendency for increased oxidation and reduced assimilation relative to absorption was also found for increasing degree of hydrolysis of the administered protein to larval Atlantic halibut (*Hippoglossus hippoglossus*) by Tonheim et al. (2005). It is surprising then that in the CPSP diet-fed larvae, the significantly lower survival, higher nitrogen loss and catabolism of absorbed dietary protein, were not reflected in a lower growth rate. It could be that these seabream, fed with the higher inclusion level of FPH, compensated for nutritional deficiencies (i.e., loss of AA due to fast absorption and high subsequent excretion/catabolism) through a higher feed intake, and/or that the smaller, weaker larvae showed higher mortality and thus the final growth parameters measured are an over-estimation due to a size-selective mortality.

Finally, a comparative protein profiling of the larval carcass was performed in an attempt to further explore possible components of biological processes involved in gilthead seabream larvae's response to the different inclusion level of FPH in their diets. The results indicate a low impact of the inclusion level of FPH on muscle metabolism, mainly (given this tissue is the main constituent of larval carcass), as only a very reduced set of proteins (25 out of 839) were identified as being affected by the dietary treatment. Of the spots identified, those belonging to myosin, the major protein component of skeletal muscle tissues, had a higher abundance in larvae fed the diet with a 10 % inclusion level of CPSP-90 and could be indicative of enhanced protein synthesis for growth in these larvae at 27 DAH; though further protein identification is necessary to gain a better understanding.

3.6. Final considerations

Taken together, the results obtained in this study show that a 20 % dietary inclusion level (30 % of dietary protein) of CPSP-90 had a detrimental effect on performance of early life-stage gilthead seabream compared to a 10 % (15 % of dietary protein) inclusion level. This detrimental effect could have been caused by protein overloading of digestive and metabolic capacities, reflected in the very low survival, higher nitrogen loss and catabolism of absorbed dietary protein. It remains to be elucidated if the surprisingly higher growth rate recorded for these larvae is an effect of size-selective survival. A 10 % dietary inclusion level of CPSP-90 seems to be more adequate for rearing gilthead seabream larvae,

who showed better survival, a very high efficiency for dietary protein retention and a higher expression of skeletal muscle growth-related proteins by the end of the trial.

Acknowledgements

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Chapter 4. Influence of the dietary peptide size distribution profile on *Sparus aurata* larval protein utilisation: a comparative 2D-DIGE analysis

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4.1. Abstract

On the basis that increasing the digestibility of protein in feeds for teleost larvae can improve performance, but that detrimental effects can result from providing excess dietary free amino acids (FAA) and short peptides, this study aimed to analyse how increasing the dietary inclusion of the larger size peptide fraction from the same fish protein hydrolysate (FPH) influenced larval growth performance of gilthead seabream (*Sparus aurata*). Next, we further tried to elucidate to what extent dietary inclusions of different peptide sizes affected N-metabolism and the larval capacity to digest and absorb the same standard live prey animal. N-metabolism was assessed by analysis of nitrogen excretion in fasted animals while digestive capacity was assessed by analysing protein utilisation, and metabolism after the larvae were fed one standard meal (^{14}C- protein labelled *Artemia*) to satiation. Additionally, a comparative proteomic approach using the highly discriminatory 2D-DIGE technique was used to try to shed more light on metabolic processes involved in the larval physiological responses to the diets.

The alginate microencapsulated feed used in this experiment with a 20 % dietary inclusion level of a FPH composed of 25 % FAA, di- and tripeptides and 75 % peptides with MW between 500 and 30000 Da (diet LPH), was suitable for growth of *S. aurata* larvae. Increasing the PH dietary inclusion level to 35 % by adding the FPH fraction of MW between 500 and 30000 Da (diet HPH) did not improve growth performance. N-metabolism in fasted larvae and larval capacity to digest and absorb *Artemia* protein was not significantly affected by dietary treatment. However, the inferior growth performance and enhanced expression of proteins related to sarcomeric protein degradation and energy metabolism point to impaired anabolic efficiency induced by diet HPH, which had a clear effect on protein expression in larval carcass.

4.2. Introduction

The early life stages of many commercially cultivated marine fish species cannot be successfully reared solely on inert formulated feed as evidenced by their generally inferior growth and survival when compared to fish larvae fed live feed (Conceição et al., 2010). To some extent, this can be explained by the elevated amino acid (AA) requirements of these

rapidly developing organisms (Conceição et al., 2011), combined with their initially immature gastrointestinal tract (Rønnestad and Conceição, 2005; Zambonino-Infante et al., 2008; Naz and Türkman, 2009) and with the more complex protein sources generally incorporated in inert feeds, compared to that available in live feeds (Conceição et al. 2011).

With the aim of improving protein digestibility in artificial microdiets for larvae, a number of studies have focused on the dietary incorporation of protein hydrolysates (PH) (Hardy, 1991). These constitute a form of pre-digested protein and as such are believed to provide a more available dietary nitrogen source since fish larvae initially absorb FAA and short peptides into the intestinal enterocytes more easily than more complex nitrogen forms (see reviews by Cahu et al., 2003; Rønnestad et al., 2003; Zambonino-Infante and Cahu, 2007; Conceição et al., 2011; Rønnestad et al., 2013). Whilst a moderate dietary PH inclusion level has generally improved larval performance in diverse species (e.g. Berge and Storebakken, 1996; Carvalho et al., 1997; Zambonio-Infante et al., 1997; Kvåle et al., 2009), there appears to be a certain threshold of inclusion level above which no or negative results are obtained (e.g. Cahu et al., 1999; Kolkovski and Tandler, 2000; Kvåle et al., 2002). However, none of the above studies mention the peptidic molecular weight (MW) profile of the PH incorporated.

Along with their inclusion level, the peptide MW profile of PH seems to play a crucial role in how dietary PH affects larval development. In a study on how the solubility and peptide profile affect dietary protein utilisation, Carvalho et al. (2004) found that an excess of di- and tripeptides detrimentally affected performance in early-feeding life stages of common carp (*Cyprinus carpio*). These authors hypothesised that the reduced larval performance was either due to the direct saturation of the peptide transport mechanism or to the rapid hydrolysis of low MW peptides, which produced an excess of AA that subsequently saturated their intestinal transport mechanisms. As free AA (FAA) and dipeptides are absorbed faster than protein-bound AA, a precocious absorption of some essential FAA comparatively to those protein-bound may lead to AA imbalances and consequent decrease of protein utilisation and larval performance (Hardy, 1991; Rønnestad et al., 2000; 2007; Tonheim et al., 2005).

Additionally, the peptidic chain structure itself can play a role on regulation of different digestive enzymes' activities, as was seen for gilthead seabream (*Sparus aurata*) larvae fed PH of different protein source and dietary inclusion level (Gisbert et al., 2012). Moreover, studying the effect of different dietary levels of fish PH (FPH) on European sea bass (*Dicentrarchus labrax*) larval development, digestive tract enzymatic data indicated that a higher dietary inclusion level of PH or higher proportion of short peptides for the same inclusion level led to delayed larval development (Kotzamanis et al., 2007). We also reported inferior performance in white seabream (*Diplodus sargus*) larvae when fed a higher dietary inclusion level of PH and proportion of short peptides and FAA (de Vareilles et al., 2012 – Chapter 2 of the present thesis).

On the basis that increasing the digestibility of protein in feeds for teleost larvae can improve performance, but that detrimental effects can result from providing excess dietary FAA and short peptides, this study aimed to analyse how increasing the dietary inclusion of the larger size peptide fraction from the same FPH influenced larval growth performance of gilthead seabream, a commercially important cultured species in the Mediterranean. Next, we further tried to elucidate to what extent dietary inclusions of different peptide sizes affected N-metabolism and the larval capacity to digest and absorb the same standard live prey animal. N-metabolism was assessed by analysis of nitrogen excretion in fasted animals while digestive capacity was assessed by analysing protein utilisation, and metabolism after the larvae were fed one standard meal (^{14}C- protein labelled *Artemia*) to satiation. Additionally, a comparative proteomic approach using the highly discriminatory 2D-DIGE technique was used to try to shed more light on metabolic processes involved in the larval physiological responses to the diets.

4.3. Materials and Methods

4.3.1. Husbandry, experimental setup and feeding protocol

Gilthead seabream eggs were obtained from the commercial hatchery Maresa SA (Ayamonte, Spain). Newly hatched larvae were distributed into six 100 L cylindrical conical fibreglass tanks, at an initial density of 100 larvae per litre, so as to test two inert microdiets in triplicate. Tanks were connected via a semi-closed seawater recirculating

system, with mechanical, biological and UV filter, and each tank possessed an additional water up-welling circuit and continuous aeration. The initial water flow rate was of 0.04 L min^{-1} and was gradually increased after 8 days after hatching (DAH) to a final 0.50 L min^{-1} (30 % h^{-1} water renewal) from 20 DAH onward. The tanks were siphoned and water checked for ammonia and nitrites every other day, purged from the bottom every morning and equipped with a skimmer to keep the surface layer clean. The diurnal light:dark cycle was set at 16:8 hours from controlled illumination by overhead fluorescent lights, with 500 lux measured at tank water surface. The water temperature, salinity and oxygen level were 18.6 ± 0.3 ºC, 36.2 ± 0.2 ‰, and 93.9 ± 2.3 % (mean ± SD), respectively, throughout the study period.

Larvae were reared from hatching to 30 DAH. They were fed with *Brachionus plicatilis* enriched with Protein Selco (INVE Aquaculture, Belgium) from first-feeding to 25 DAH, at an initial density of 10 rotifers mL^{-1}. As long as live feed was given to the larvae, the microalgae *Nannochloropsis oculata* and *Isochrysis galbana* were added to the water to keep "green water" conditions. Experimental microdiets were introduced at 9 DAH. Before being given to the larvae, the microdiets were hydrated by passing through an irrigated 50 mL falcon tube, from the automatic feeder into the tank. From this point, the amount of live prey supplied was gradually reduced and replaced by the inert diets, becoming of little dietary significance approximately one week after the introduction of the microdiets, that is, when about 75 % of larvae had inert feed in their digestive tract (as observed at the binocular microscope).

4.3.2. Experimental feed

The experimental microdiets were formulated to be iso-energetic, -nitrogenous and –lipidic, according to the patent ES P200201435 and containing modified levels of FPH, so as to obtain two diets with increasing inclusion level of high MW PH (Table 4.1.).

Table 4.1. Composition of the experimental microdiets containing different levels of high molecular weight fish protein hydrolysates.

Ingredients (% DM)	**LPH**	**HPH**
Aglonorse micro feed [a]	10.0	5.0
Cuttlefish meal [b]	29.0	17.0
Casein [c]	5.0	5.0
Sodium-alginate [d]	7.0	7.0
Bread yeast	3.0	3.0
Cod liver oil	12.0	14.0
Marine PL [e]	6.0	6.0
Vitamin and mineral premix [f]	4.0	4.0
Vit C [g]	3.0	3.0
Vit E [h]	1.0	1.0
HP < 0.5 [i]	5.0	5.0
HP 0.5-30 [i]	15.0	30.0
Proximate composition (%)		
Protein	55.3	47.7
Lipids	22.7	25.7
Ash	4.4	5.0
Dry matter (% wet weight)	97.9	97.7

[a] AgloNorse, Norway; [b] Squid Powder 0278, Rieber & Søn ASA, Norway; [c] ICN 901633; [d] ICN 154724; [e] Marine Lecithin LC40, Phosphotech, France; [f] Following the requirements reported by the National Research Council (1993); [g] Rovimix Stay C-35, Roche ; [h] ICN 100555; [i] Fish protein hydrolysates smaller than 0.5 kDa MW (HP<0.5; FAA, di- and tripeptides) or of 0.5-30 kDa MW (HP 0.5-30; oligo- and polypeptides), produced at IPIMAR (Lisboa, Portugal).

Diet LPH had a low (20 %, dry matter (DM) basis) inclusion level of FPH, of which 5 % were FAA and di- and tripeptides (MW < 0.5 kDa) and 15 % were oligo- and polypeptides (0.5 kDa < MW < 30 kDa), whilst in diet HPH, there was a higher inclusion (35%, DM basis) of PH that resulted in 30 % inclusion level of oligo- and polypeptides) (Table 4.2.).

Table 4.2. Total (free plus protein-bound) amino acid (AA) composition and peptide molecular weight distribution of the soluble protein fraction of experimental microdiets. Values are expressed in weight percentage of all AA (mean ± standard deviation; n = 3). There were no differences between diet LPH and diet HPH for a given AA (Welch's Two Sample t-test, P < 0.01). IAA—indispensable (and conditionally indispensable) AA; DAA – dispensable AA; Ala-alanine; Arg-arginine; Asp+Asn-aspartate+asparagine; Cys-cysteine; His-histidine; Glu+Gln-glutamine+glutamate; Gly-glycine; Ile-isoleucine; Leu-leucine; Lys-lysine; Met-methionine; Phe-phenylalanine; Pro-proline; Ser-serine; Thr-threonine; Tyr-tyrosine; Val-valine.

	LPH	**HPH**
IAA		
Arg	7.44 ± 0.44	6.77 ± 0.16
Cys	0.68 ± 0.01	0.57 ± 0.03
His	2.46 ± 0.03	2.58 ± 0.01
Ile	3.58 ± 0.15	3.56 ± 0.01
Leu	9.03 ± 0.05	9.34 ± 0.21
Lys	8.35 ± 0.14	8.04 ± 0.07
Met	3.41 ± 0.03	3.61 ± 0.05
Phe	4.94 ± 0.22	5.05 ± 0.01
Thr	5.02 ± 0.31	5.01 ± 0.03
Tyr	4.52 ± 0.04	4.92 ± 0.05
Val	5.18 ± 0.31	5.14 ± 0.05
% IAA	54.62 ± 0.35	54.57 ± 0.32
DAA		
Ala	5.44 ± 0.05	5.25 ± 0.25
Asp+Asn	8.98 ± 0.08	6.98 ± 0.27
Glu+Gln	16.47 ± 0.05	16.59 ± 0.37
Gly	4.65 ± 0.07	4.52 ± 0.20
Pro	5.13 ± 0.13	6.19 ± 0.18
Ser	5.31 ± 0.24	5.91 ± 0.06
Peptide molecular weight (% DM of diet)		
< 500 Da	5.00	5.00
500 - 30000 Da	5.00	30.00

Crude protein (N×6.25) in diets was determined by the Kjeldahl method, total lipid with the Soxhlet method, moisture and ash gravimetrically after drying for 24 h at 105 °C and after

combustion for 24 h at 550 °C (AOAC, 1990), respectively (Table 4.1.). All analyses were carried out on freeze dried and grounded samples, in duplicates for all diet types. If differences between parallels exceeded standardised values, new duplicate analyses were performed according to accredited procedures (AOAC, 1990). To determine total AA content in diets, dietary samples were hydrolysed in 6 M HCl at 108 °C for 24 h in nitrogen-flushed vials and analysed by reversed-phase high pressure liquid chromatography (HPLC) in a Waters Pico-Tag amino acid analysis system, using norleucine as an internal standard. The Breeze software (Waters, USA) was used to study the resulting chromatograms. Tryptophan was not determined, since it is partially destroyed by acid hydrolysis. Asparagine is converted to aspartate and glutamine to glutamate during acid hydrolysis, so the reported values for these AA represent the sum of the respective amine and amino acid in the proteins (Table 4.2.).

4.3.3. Growth and survival

Total length and dry weight was measured at 3, 10, 20 and 28. For total length, individual photographs of 20 larvae per tank were taken and analysed with the UTHSCSA Image-Tool software (University of Texas, Health Science Center, San Antonio, TX, USA). These larvae were then frozen in liquid nitrogen in groups of 5 larvae and weighed after freeze drying for dry weight measurement. Survival was assessed as the number of larvae alive at the end of the trial, after withdrawing those removed for sampling from the initial number of larvae in the tank. Growth in dry weight was calculated as $100(e^{(\ln DWf - \ln DWi)/t} - 1)$, with DW_f and DW_i as final and initial dry weight (mg) of larvae, respectively, and t as number of days. For length data, growth was obtained as $(TL_f - TL_i)/t$ with TL_f and TL_i as final and initial total larval length (mm), respectively, and t as number of days.

4.3.4. Ammonia excretion and protein utilisation trial

Mass-specific ammonia excretion was measured in larvae fasted overnight, at 20 and 28 DAH, using 10 larvae from each tank according to method previously described in Chapter 3 of the present thesis.

Capacity of larvae to digest and metabolise *Artemia* proteins was studied in 30 DAH larvae fed *Artemia* metanauplii radiolabelled with a [U-^{14}C] protein hydrolysate (1.85 MBq mL;

Amersham Pharmacia Biotech Ltd, Buckinghamshire, UK), according to the method developed by Morais et al. (2004a) and adapted by Engrola et al. (2010). Thirty seabream larvae from each dietary treatment were randomly collected and transferred to the radiolabelling laboratory for overnight acclimation prior to the trial, which consisted in being allowed to feed for 1 h on radiolabelled *Artemia* metanauplii before being gently transferred to metabolic chambers for 24 h, as previously described in Chapter 3 of the present thesis. Briefly, these consisted in sealed vials containing 7.5 mL of seawater with gentle air flow, the air being then forced through a capillary from the incubation vial to a chemical $^{14}CO_2$ trap (5.0 mL of KOH, 0.5 M), as previously described by Rønnestad et al., (2001).

Seabream larvae that had not ingested any live prey were discarded from further analysis. Artemia intake (% body dry weight (BDW)) after a single meal of radiolabelled *Artemia* was determined as:

$$AI = 100[(R_{total} / SR_{Artemia}) / DW_{larvae}]$$

where R_{total} is the sum of radioactivity (DPM) in the incubation seawater, CO_2 trap and larval body, and $SR_{Artemia}$ is the specific radioactivity per *Artemia* samples (DPM mg^{-1} *Artemia* DW) and DW_{larvae} is the dry weight of *S. aurata* larvae (mg) .

Protein utilisation was determined based on protein digestibility (D, %), retention efficiency (R, %) and catabolism (C, %). These estimates were determined as:

$$D = 100[(R_{larva} + R_{CO2trap}) / (R_{larva} + R_{CO2trap} + R_{water})],$$

$$R = 100[R_{larva} / (R_{larva} + R_{CO2trap})],$$

$$C = 100[R_{CO2trap} / (R_{larva} + R_{CO2trap})],$$

where R_{larva} is the total radioactivity in larval body (DPM), $R_{CO2trap}$ is the total radioactivity per CO_2 trap (DPM) and R_{water} is the total radioactivity in the incubation seawater (DPM).

4.3.5. Comparative analysis of larval carcass proteome

At 27 DAH, approximately 200 larvae from each tank were rinsed in distilled water, immediately dissected on ice for removal of head and visceral parts, and the carcasses were then frozen in liquid nitrogen. Protein from samples of 40 mg (wet weight) from each tank was extracted and prepared as described in Chapter 3 of this thesis.

DIGE minimal labelling was performed on 50 µg of protein from each sample according to the experimental protocol previously described in Chapter 3 of the present thesis. Briefly, one sample from each group plus the internal standard were focused in the first dimension on individual 24 cm Immobiline™ Drystrips pH 4-7 (GE Healthcare) at 20 ºC for a total of about 60000 Vhours, and then proteins were focused in the second dimension in 12.5 % SDS-PAGE gels using a standard Tris-Glycine-SDS running buffer. After electrophoresis, gels were immediately scanned with a Typhoon Trio™ Variable Mode Imager (GE Healthcare) using a resolution of 100 µm and PMT value set to ensure that maximum pixel intensity was below saturation. Image analysis was performed using the Progenesis Same-Spots software (Nonlinear Dynamics, Newcastle upon Tyne, UK), which computed multiplication (fold) and p-values of all spots applying a Student's t-test.

To identify the selected differentially expressed proteins, preparative gels were run prior to spot excision following the same protocol as described above except that labelling was not performed, each gel was loaded with 1 mg of protein from mixed samples and Colloidal Coomassie Blue G250 staining was used. Proteins of interest were excised and subjected to in-gel trypsin digestion, followed by matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) (Autoflex and Ultraflex, Bruker Daltonics) and MALDI with MS/MS (Ultraflex with a LIFT module, Bruker Daltonics) for mass analyses of the peptide mixtures, as previously described in Chapter 3 of this thesis. The mass spectra were externally calibrated using Peptide Calibration Standard II (Bruker Daltonics, Bremen), while the internal calibration was performed with the trypsin autolytic products. FlexAnalysis 3.0 (Bruker Daltonics, Bremen) was used to create the peak list and Bio-Tools 3.0 (Bruker Daltonics, Bremen) was used for interpretation of MS and MS/MS spectra, and proteins were identified by peptide mass fingerprinting (PMF) via the database search program MASCOT (http://www.matrixscience.com), using the Actinopterygii and

Chordata NCBInr and SWISSPROT protein databases. MS/MS analysis and repeated MASCOT database searches of a minimum of two precursor ions recognised in the PMF search were performed to confirm the PMF-based protein identification, whenever possible. It was assumed that the peptides were monoisotopic, cysteine residues were carbamidomethylated and methionine residues possibly oxidised. The fingerprinting method allowed for a maximum of one missed tryptic cleavage per protein. The maximum deviation permitted in matching the parent ion/fragment ion mass values was 100 ppm/0.8 Da, respectively. Following the database searches, the MOWSE score, number of peptide matches, sequence coverage, molecular weight, and pI value were used to evaluate the results.

4.3.6. Data analysis

Welch's Two Sample t-test was used to analyse the effect of dietary treatment on growth, survival, mass-specific ammonia excretion, *Artemia* intake and protein utilisation of *S. aurata* larvae at the same age, as well as for comparing the AA composition of experimental microdiets. Differences were considered significant when $P < 0.05$, or $P < 0.01$ for AA data. All percentage data were arcsine ($x^{1/2}$)-transformed prior to analysis. All statistical analysis was carried out using the R statistical computing environment (R Development Core Team, 2011).

4.4. Results

4.4.1. Larval growth and survival

Growth of *S. aurata* larvae was similar until the point when most larvae (> 75 % of larvae) were ingesting the microdiets (around 18 DAH), after which the dry weight of larvae fed diet LPH was higher than that of those fed diet HPH (Figure 4.1.). Survival at the end of the trial was similar among groups, with 4.12 ± 0.58 % for LPH and 4.03 ± 1.83 % for HPH (mean ± SEM, n = 3).

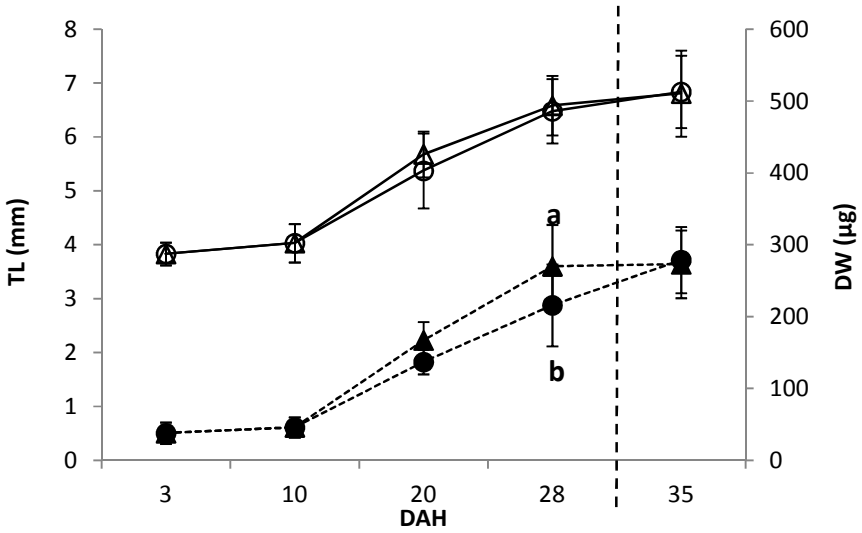

Figure 4.1. Growth performance of *S. aurata* larvae fed experimental feed containing different inclusion levels and peptide size distribution of fish protein hydrolysates. Total length in mm (TL, solid line)and dry weight in µg (DW, hatched line) for diet LPH (triangle) and diet HPH (circle). Results are expressed as means ± standard deviation. For TL n = 60 larvae and for DW n = 12 pools of 5 larvae. Different letters for a selected DAH denotes significantly different mean values (Welch's Two Sample t-test, $P < 0.05$). Vertical hatched line represents 30 DAH, the point from which all larvae were fed a control diet (see end of section 4.5. Discussion).

4.4.2. Ammonia excretion and protein utilisation trial

The mass-specific ammonia excretion of fasted *S. aurata* larvae was lower at 28 DAH than at 20 DAH for both dietary treatments but was not significantly different between dietary treatments at either age (Fig. 4.2.).

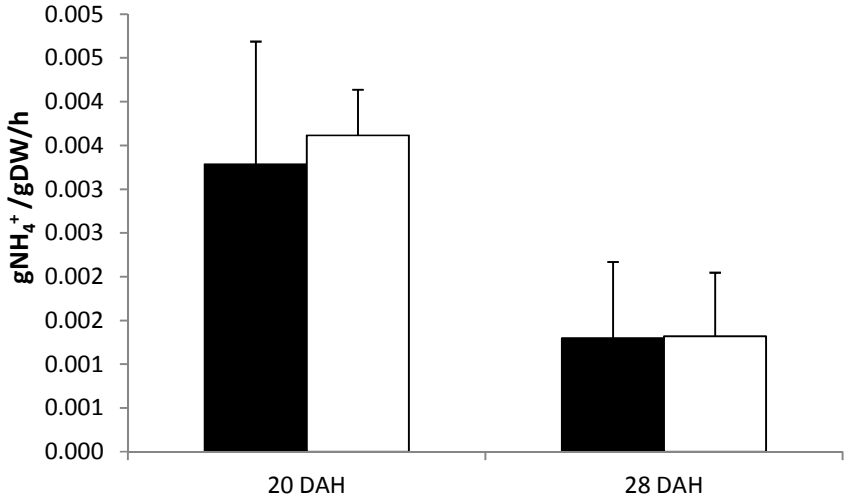

Figure 4.2. Mass-specific ammonia excretion of fasted 20 and 28 DAH *S. aurata* fed fed experimental feed containing different inclusion levels and peptide size distribution of fish protein hydrolysates. Values are mean ± standard deviation (n = 3 pools of 10 larvae). Diet LPH – black, diet HPH – white. There were no differences between dietary treatments for a given DAH (Welch's Two Sample t-test, $P < 0.05$).

At 30 DAH, the number of larvae ingesting *Artemia* metanauplii was quite different between treatments; from the 15 larvae from each group, 3 larvae reared on diet LPH ingested *Artemia* and 8 from treatment HPH. *Artemia* intake showed very high inter-individual variability, ranging from about 10 to 90 % BDW (Fig. 4.3.). Concerning protein utilisation, protein digestibility ranged from 65-80 % of total ingested protein for *S. aurata* larvae at 30 DAH, retention of absorbed protein ranged from 60-80 % and catabolism of absorbed protein from 20-40 % (Figure 4.4.).

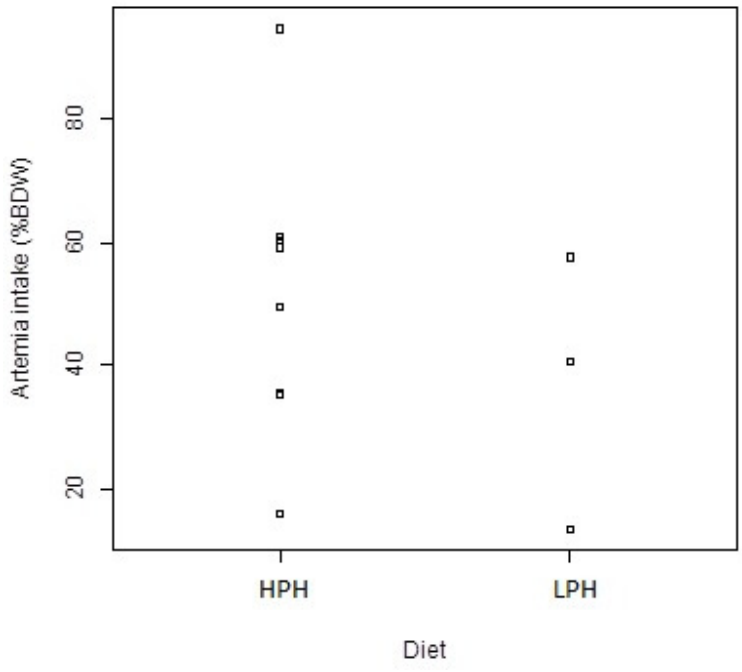

Figure 4.3. *Artemia* intake (% body dry weight (BDW)) of *S. aurata* at 30 DAH.

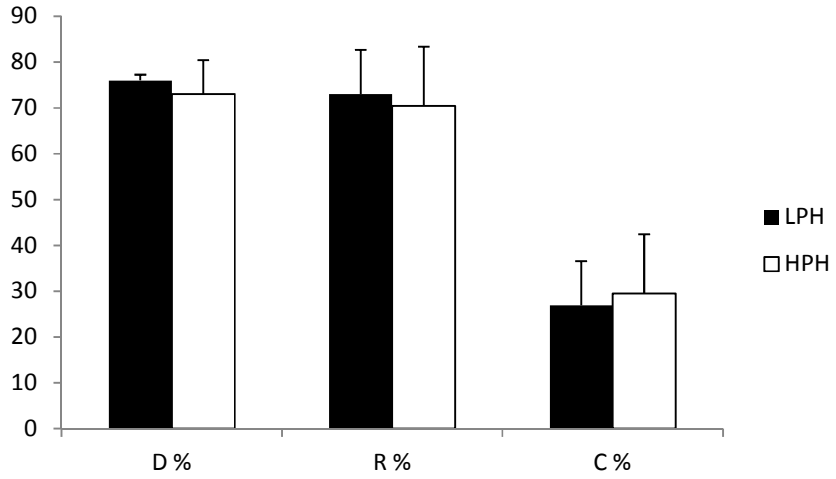

Figure 4.4. Protein digestibility (D, % of radiolabel in the larva and metabolic trap in relation to total radiolabel ingested), protein retention (R, % of radiolabel in the larva in relation to digested label) and catabolism (C, % of radiolabel in the metabolic trap in relation to digested label) in gilthead seabream at 30 DAH, after 24 h incubation. Values are means ± standard deviation (n = 3 for LPH and n = 8 for HPH).

4.4.3. Comparative analysis of larval carcass proteome

Comparative 2D-DIGE analysis of seabream larval carcass, using the Progenesis Same-Spots software (Nonlinear Dynamics, Newcastle upon Tyne, UK), enabled the detection and quantification of 845 protein spots across all spot maps, covering a molecular mass range of ~10 kDa to 200 kDa and pI values between 4 and 7 (Fig. 4.5.).

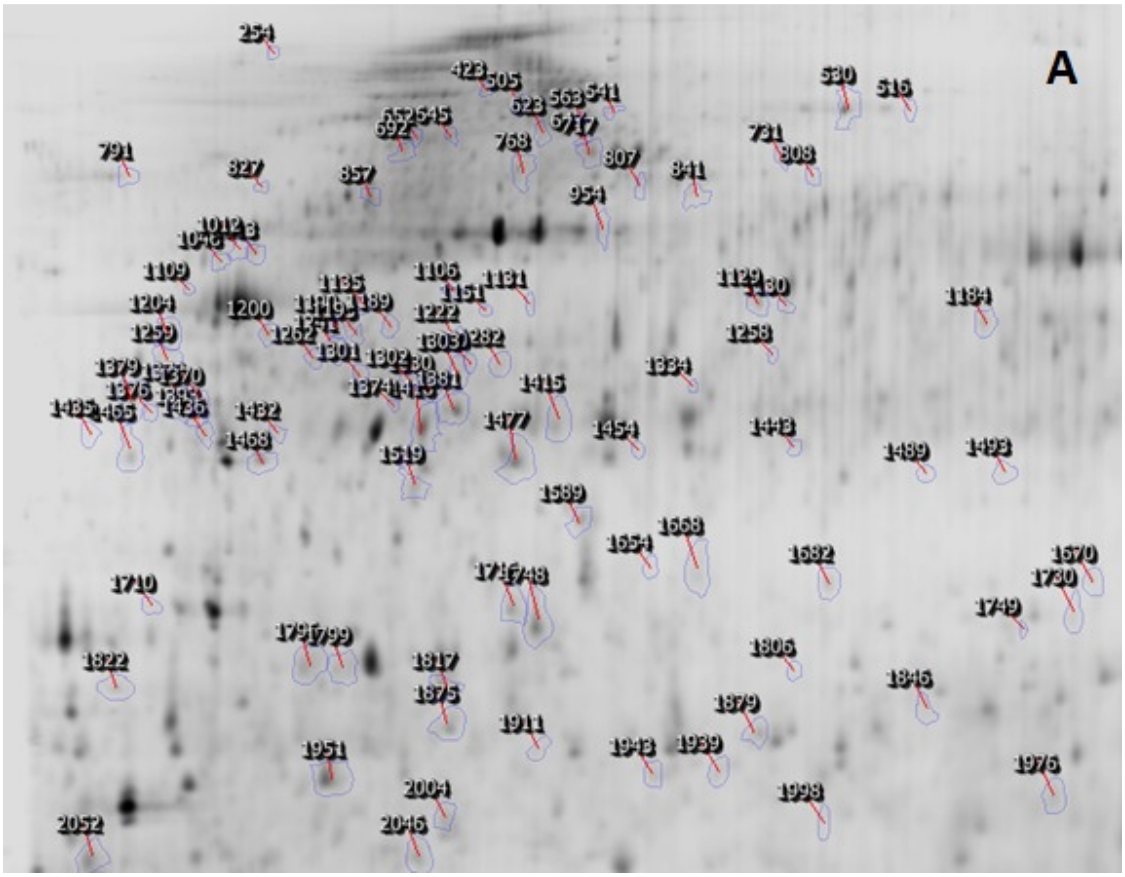

Figure 4.5. Minimal CyDye™ DIGE Fluor-stained 2D gel (pH 4-7 linear, 12.5 % Tris-HCl) from larval carcass of *S. aurata* at 27 DAH. Reference image representing protein spots whose expression was significantly affected by the dietary treatment (Student's t-test, $P < 0.05$). **A** – protein spots with fold-change from 1.2 to 1.5; **B** – most visible protein spots with fold-change from 1.5 to 2.0; **C** – protein spots with fold-change above 2.0.

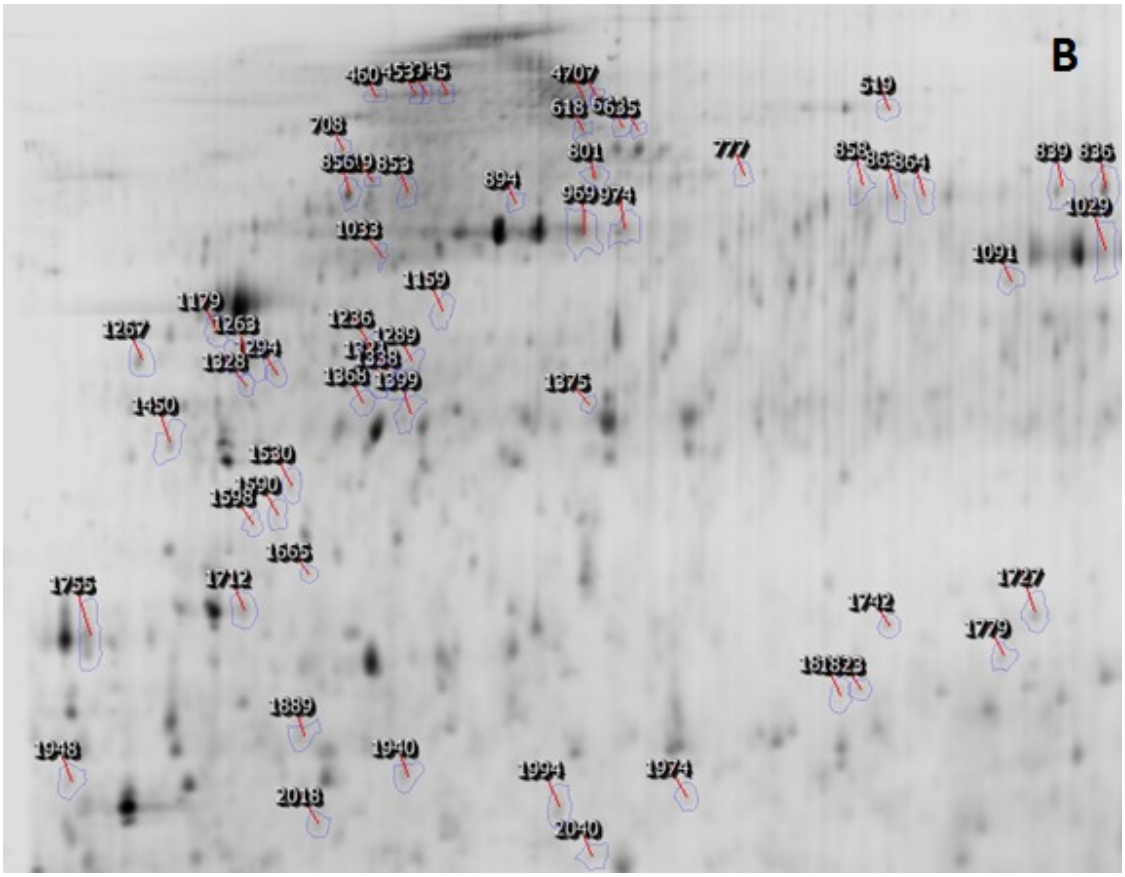

Figure 4.5. Minimal CyDye™ DIGE Fluor-stained 2D gel (pH 4-7 linear, 12.5 % Tris-HCl) from larval carcass of *S. aurata* at 27 DAH. Reference image representing protein spots whose expression was significantly affected by the dietary treatment (Student's t-test, $P < 0.05$). **A** – protein spots with fold-change from 1.2 to 1.5; **B** – most visible protein spots with fold-change from 1.5 to 2.0; **C** – protein spots with fold-change above 2.0.

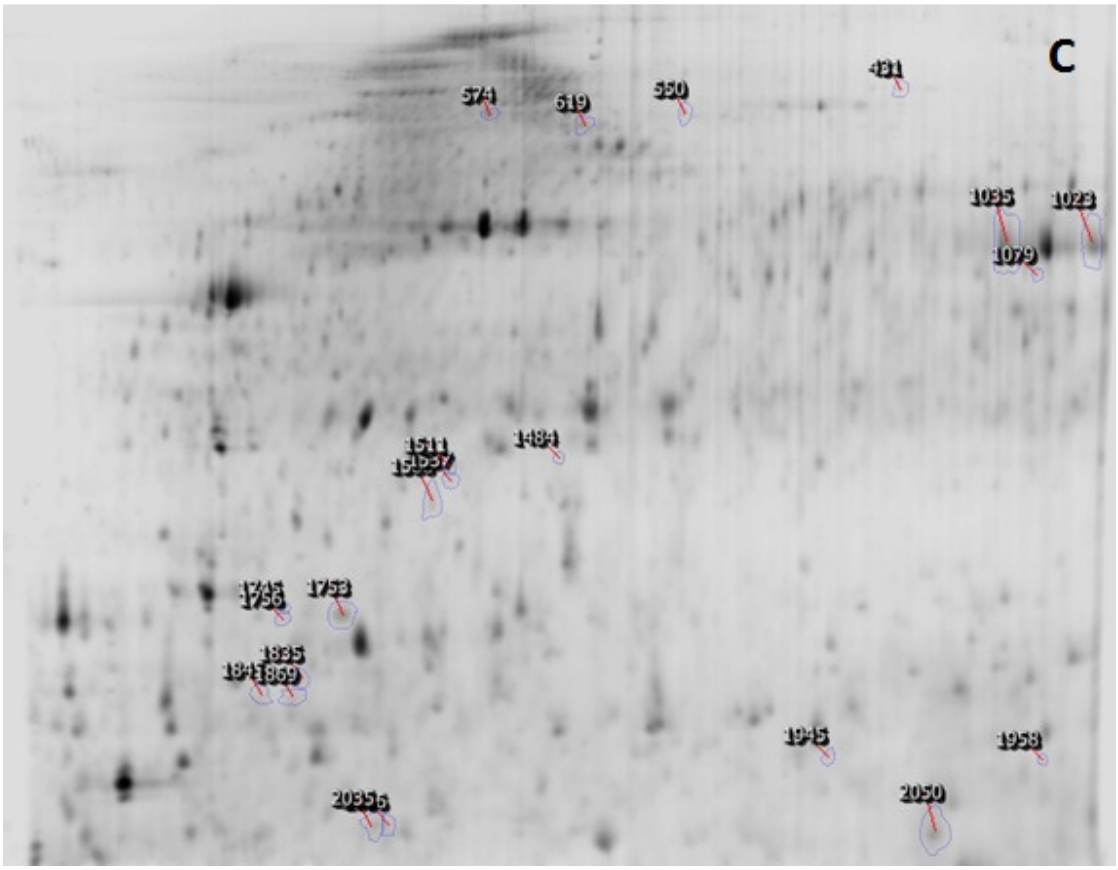

Figure 4.5. Minimal CyDye™ DIGE Fluor-stained 2D gel (pH 4-7 linear, 12.5 % Tris-HCl) from larval carcass of *S. aurata* at 27 DAH. Reference image representing protein spots whose expression was significantly affected by the dietary treatment (Student's t-test, $P < 0.05$). **A** – protein spots with fold-change from 1.2 to 1.5; **B** – most visible protein spots with fold-change from 1.5 to 2.0; **C** – protein spots with fold-change above 2.0.

Exploratory analysis of the normalised protein quantity using Principal Component Analysis showed that the larval carcass proteome was affected by dietary treatment, which elicited a clearly more systematic response by the proteome of larvae fed diet HPH than diet LPH, for which a higher dispersion was seen among replicates (Fig. 4.6.).

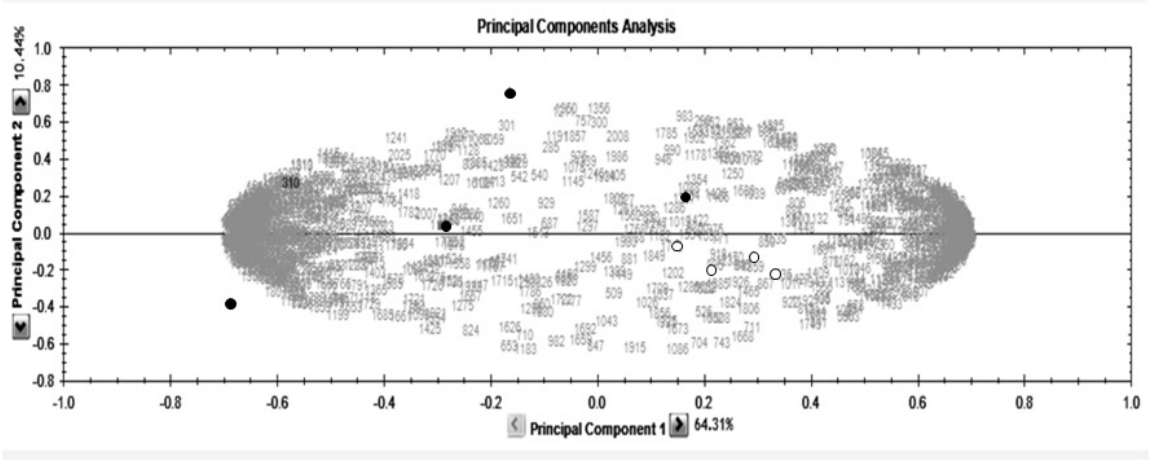

Figure 4.6. Principal component analysis biplot obtained from original data set of protein expression for each sample. Replicate gels (spot maps) from dietary treatment LPH are shown in black and from dietary treatment HPH in white.

A Student's t-test revealed 238 protein spots with significantly altered expression according to dietary inclusion level of PH, 142 spots (60 %) being under-expressed and 96 (40 %) over-expressed in HPH (fold-change > 1.2; $P < 0.05$). Of the protein spots whose expression was significantly affected by the different dietary inclusion levels and size distribution of FPH, 50 could be excised and subjected to in-gel tryptic digestion, 33 of which were reliably identified, corresponding to 19 proteins [skeletal alpha actin (10 spots); skeletal muscle myosin heavy chain (MyHC); fast skeletal muscle myosin light chain 1 (MyLC1); skeletal muscle myosin light chain 2 (MyLC2); fast skeletal muscle myosin light chain 3 (MyLC3); tropomyosin; intermediate filament proteins (type II keratin E3-like protein, type I keratin - 2 spots, and desmin-like protein); beta-enolase (2 spots); pyruvate kinase; glyceraldehyde-3-phosphate dehydrogenase; fructose-bisphosphate aldolase; mitochondrial ATP synthase subunits beta and D; muscle type creatine kinase 1 (3 spots); nucleoside diphosphate kinase, transferrin (2 spots); apolipoprotein A-1]. The remaining 17 protein spots could not be identified due to low protein abundance in the gels and also possibly because of lacking sequences in the databases searched (Table 4.3.).

Table 4.3. Protein spots identified by MALDI-ToF-ToF MS, using peptide fragment fingerprinting (PFF) in the option MS/MS Ion Search from the bioinformatics application Mascot. The PFF was made in the non redundant NCBI nr database for the Actinopterygii taxonomic level.

Spot #	p-value[a]	Fold-change	Protein identification	NCBI accession number	MW/pI T MW/pI E[b]	Coverage (%)	PM[c]	Best matched peptide sequence and E-value	Combined MOWSE score[d]
Higher abundance in LPH									
1033	0.049	1.8	Type II keratin E3-like protein	AAT44423	39/4.9 61.3/5.0	57	25	n.a.	(165, 7e-12)
1204	0.013	1.4	Type I keratin, cytoskeletal	AAI16530	50/4.9 49.5/4.4	20	16	n.a.	(81, 0.0019)
1241	0.001	1.4	Skeletal alpha actin	AAF22646.1	42/5.3 47.9/4.8	27	10	2(3) SYELPDGQVITIGNER 7e-05	127
1267	0.016	1.5	Type I keratin isoform 2	BAF56914	44/5.1 45.6/4.3	19	13	n.a.	(68, 0.0039)
1289	0.015	1.5	No protein match		44.4/5.1				
1321	0.026	1.5	Skeletal alpha actin	AAF22646.1	42/5.3 43.7/4.9	20	5	1(1) SYELPDGQVITIGNER 0.011	42
1328	0.034	1.6	Tropomyosin alpha	ADG29138	22/4.8 46.1/4.6	49	14	n.a.	(157, 4.4e-11)
1381	0.004	1.4	Skeletal alpha actin	AAF22646.1	42/5.3 40.1/5.1	20	11	2(3) AGFAGDDAPR 2e-07	185
1399	0.039	1.8	No protein match		38.6/5.0				
1477	0.020	1.4	Skeletal alpha actin	AAF22646.1	42/5.3 35.1/5.3	25	12	2(3) QEYDEAGPSIVHR 7.3e-08	184
1511	0.029	2.4	Skeletal alpha actin	AAF22646.1	42/5.3 33.1/5.1	35	15	2(3) AGFAGDDAPR 0.00037	122

Spot #	p-value[a]	Fold-change	Protein identification	NCBI accession number	MW/pIT MW/pIE[b]	Coverage (%)	PM[c]	Best matched peptide sequence and E-value	Combined MOWSE score[d]
1519	0.048	1.5	No protein match		32.7/5.0				
1537	0.012	2.6	Skeletal alpha actin	AAF22646.1	42/5.3 32.0/5.2	26	9	1(1) AGFAGDDAPR 0.041	89
1588	0.009	2.6	Skeletal alpha actin	AAF22646.1	42/5.3 29.9/5.1	20	10	1(2) AGFAGDDAPR 2.4e-05	100
1665	0.017	1.5	No protein match		25.3/4.7				
1748	0.030	1.4	Skeletal alpha actin	AAF22646.1	42/5.3 21.7/5.4	18	10	2(2) SYELPDGQVITIGNER 8.8e-08	178
1753	0.043	2.1	No protein match		21.4/4.9				
1823	0.032	1.8	No protein match		18.4/6.2				
1889	0.044	1.8	No protein match		16.3/4.7				
1948	0.025	1.9	No protein match		14.3/4.1				
1994	0.016	1.9	No protein match		13.3/5.4				
Higher abundance in HPH									
431	0.035	2.5	No protein match		96.3/6.4				
519	0.044	1.8	Transferrin	AEA41139	76/5.9 91.3/6.3	6	6	n.a.	(70, 0.015)
530	0.048	1.4	Transferrin	AEA41139	76/5.9 92.3/6.2	31	25	n.a.	(183, 1.1e-13)
619	0.002	2.0	Myosin heavy chain	ADG29145	67/5.4 86.0/5.5	26	20	1(2) EQDTSAHLER 0.0045	74
635	7.8e-04	1.7	No protein match		86.0/5.6				
801	0.006	1.8	Desmin-like	XP_003445343	54/5.3 75.6/5.5	32	18	n.a.	(103, 1.1e-05)
836	0.049	1.8	Beta-enolase	ADG29136	48/6.3 72.8/6.9	27	13	2(2) GNPTVEVDLWTAK 0.00027	101
839	0.043	1.8	Beta-enolase	ADG29136	48/6.3 72.8/6.8	28	12	2(2) AVDHVNKDIAPK 9.9e-05	100

Spot #	p-value[a]	Fold-change	Protein identification	NCBI accession number	MW/pIT MW/pIE[b]	Coverage (%)	PM[c]	Best matched peptide sequence and E-value	Combined MOWSE score[d]
856	0.019	1.7	ATP synthase subunit beta,mitochondrial	NP_001019600	55/5.1 72.1/4.9	36	17	2(3) DQEGQDVLLFIDNIFR 0.00021	144
864	0.048	1.6	Pyruvate kinase	ADG29124	42/8.8 71.7/6.4	23	14	1(2) NTGIVCTIGPASR 0.00024	59
969	0.028	1.8	Skeletal alpha actin	AAF22646.1	42/5.3 65.4/5.5	54	23	5(5) SYELPDGQVITIGNER 5.4e-11	350
974	0.043	1.5	Skeletal alpha actin	AAF22646.1	42/5.3 65.4/5.6	32	11	1(3) SYELPDGQVITIGNER 2.5e-05	124
1023	0.040	2.6	Creatine kinase 1	ABU42561	43/6.2 61.7/7.0	28	11	2(2) PFGNTHNNFK 0.0016	99
1029	0.039	2.0	Creatine kinase 1	ABU42561	43/6.2 62.0/6.9	25	8	1(2) GTGGVDTASVGGVFDISNADR 0.0023	69
1035	0.039	2.1	Creatine kinase 1	ABU42561	43/6.2 61.7/6.7	32	14	2(2) GTGGVDTASVGGVFDISNADR 1.1e-07	152
1079	0.016	2.2	Fructose-bisphosphate aldolase C	BAD17911	36/5.6 57.2/6.8	20	12	n.a.	(72, 0.013)
1091	0.019	1.7	Glyceraldehyde 3-phosphate dehydrogenase	BAB62812	36/6.4 56.6/6.6	22	7	n.a.	(84, 0.00086)
1129	0.015	1.3	No protein match		53.6/6.0				
1416	0.002	1.4	Apolipoprotein A-I	O42175	30/5.2 37.8/5.1	43	19	2(2) QMYDQAQTVDTDALR 4e-11	241
1468	0.025	1.3	Myosin light chain 1	P82159	20/4.5 34.7/4.6	36	7	2(2) AGFEDYVEGLR 2.8e-05	121

Spot #	p-value[a]	Fold-change	Protein identification	NCBI accession number	MW/pI T MW/pI E[b]	Coverage (%)	PM[c]	Best matched peptide sequence and E-value	Combined MOWSE score[d]
1589	0.006	1.3	Mitochondrial ATP synthase D subunit	ACO10059	19/6.6 33.6/6.4	23	8	1(1) AIDWVAFAER 0.0017	52
1712	0.018	1.8	No protein match		23.1/4.6				
1716	0.029	1.3	No protein match		23.2/5.3				
1727	0.004	1.5	No protein match		22.6/6.7				
1742	0.018	2.0	No protein match		22.0/6.3				
1755	0.032	1.6	Myosin light chain 3	AAD54228	17/4.4 21.2/4.1	50	9	n.a.	(82, 0.0014)
1779	0.028	1.6	Nucleoside diphosphate kinase	ACF75416	17/6.4 20.4/6.6	19	5	1(1) TFIAIKPDGVQR 0.046	36
1879	0.001	1.3	No protein match		16.4/6.0				
1951	0.008	1.5	Myosin light chain 2	AAD54229	19/4.6 14.4/4.8	48	12	1(2) EAFTIIDQNR 0.048	37

[a] p-value obtained from comparative analysis (Student's t-test) of protein spot expression between groups using Progenesis Same-Spots software (Nonlinear Dynamics, Newcastle upon Tyne, UK);

[b] theoretical (T) isoelectrical points (pI) and molecular weights (MW) based on the best result's sequence; experimental (E) isoelectric points (pI) and molecular weights (MW) estimated from the position of the spots in the gels; MW is given in kilo Daltons (kDa);

[c] number of peptides matched (E-value < 0.05), "n.a." when PFF gave no significant results;

[d] a non-probabilistic protein score, derived from the ions score (the Mowse score and corresponding E-value from peptide mass fingerprinting (PMF) is given when PFF gave no significant result).

The identified proteins with higher abundance in dietary treatment LPH were all cytoskeletal, that is, actin isoforms, skeletal muscle tropomyosin and keratins. A few actin isoforms were also over-expressed in dietary treatment HPH, as was an intermediate filament protein, desmin-like protein. Other cytoskeletal proteins with higher abundance in the carcass proteome of larvae fed diet HPH were myosin proteins from fast skeletal muscle. Proteins from the glycogenesis/gluconeogenesis pathway and other energy related metabolism proteins such as mitochondrial ATP synthase subunits and creatine kinase had an increased abundance in larvae fed the diet with higher inclusion level of larger peptides (diet HPH), as had the proteins nucleoside diphosphate kinase (NDPK), transferrin and apolipoprotein A-I precursor (Apo A-I) (Table 4.3.).

4.5. Discussion

The beneficial effect of dietary inclusions of some protein hydrolysates on fish larval development is generally recognised and taken into consideration for the design and manufacture of most commercially available microdiets for marine fish larvae (Holt et al., 2011; Gisbert et al., 2012). However, there is still a lack of knowledge on how different factors may play a role in influencing larval development, such as protein source, method of hydrolysis, dietary inclusion level, MW profile of the peptide fractions, leaching from diets, absorption rates in the GI tract, metabolic handling of absorbed AA, among others. With this basis, this study aimed to analyse how increasing the dietary inclusion of the larger peptide fraction from the same FPH influenced larval growth performance of the commercially important gilthead seabream.

So as to enable our analysis, it was necessary to co-feed the experimental microencapsulated inert diets with rotifers. This is because the successful rearing of *S. aurata* larvae solely on inert diets is still to be achieved and the manipulation of the PH fraction of live-feed remains extremely technically challenging (Nordgreen et al., 2008; Conceição et al., 2010). The alginate microdiets produced according to the patent ES P200201435 were chosen because these have been shown to satisfy conditions required for feed microparticles to be used during early larval stages of marine fish; that is, structural stability after rehydration, good buoyancy, appropriate size, palatability to allow a good acceptance and rapid ingestion by larvae, and also, an easier breaking down in the

immature larval gut. This procedure also has the advantage of using more environmentally friendly reagents at a lower cost and allows for a more balanced formulation because no high proportion of pure protein is required for microencapsulation (Yúfera *et al*., 2005, and references therein).

Using the co-feeding protocol with microdiet formulation above mentioned, the growth and survival of seabream observed were comparable to those reported for similar studies (Fernández-Díaz and Yúfera, 1997; Yúfera et al., 1999; Robin and Vincent, 2003; Robin and Peron, 2004; Yúfera et al., 2005; Seiliez et al., 2006; Chapter 3 of this thesis). Larvae fed the diet with a 20 % inclusion level of PH containing proportionally less oligo- and polypeptides (15 % of dietary ingredients, DM basis) achieved a superior weight by the end of the trial. This diet had a similar MW profile to the FPH, with same inclusion level, used in the diet High (H) of a previous study in white seabream larvae (de Vareilles et al., 2012 - Chapter 2) for which best growth performance was also reported. Enhanced growth was also recorded for gilthead seabream larvae in a previous experiment, when reared on a diet with 20 % inclusion level of a commercially available FPH, CPSP-90 (Sopropêche, France) (diet CPSP; Chapter 3 of this thesis). However, in that study (Chapter 3), the larvae from dietary treatment CPSP had a significantly lower survival rate. It was suggested that this might have been caused by a relative excess of small MW peptides and FAA. These are directly available for absorption by enterocytes and therefore may lead to a transient imbalance among AA inside the organism, with resulting detrimental effects. The extent of the detrimental effects likely depends on both the quality and quantity of dietary protein (see Wu, 2009 for review). In terms of quality, though a different PH was used in these two studies, both used fish as their protein source and their AA profile is similar, so it is unlikely that this be a reason for the negative effects observed in one experiment and not in the other. The dietary MW profile of PH is also comparable, with 7.6 % vs. 5 % FAA and di- and tripeptides, and 12.4 % vs. 15 % oligo- and polypeptides, in diet CPSP from the previous study (Chapter 3 of this thesis) vs. diet LPH in the present study, respectively. However, the structure and/or composition of peptides and their proportion among each other might have differed and this can play a role on the metabolic effects of the dietary PH (e.g. Carvalho et al., 2004; Savoie et al., 2006; Dabrowski et al., 2010; Gisbert et al., 2012). Moreover, the total dietary crude protein level was higher in the previous study (Chapter 3

of this thesis) and coupled to the PH composition and MW profile, could thus have led to a protein overloading which would not have occurred in the present study.

With diet HPH, we sought to increase the dietary inclusion level of digestible protein without increasing the amount of small MW PH (< 500 Da), on the basis that as previously mentioned, increasing protein digestibility of inert feed may improve larval performance, yet negative effects have been attributed to metabolic overloading by excess FAA and short peptides. However, a lower growth performance was obtained with this dietary treatment (35 % inclusion level of PH, DM basis) compared to the group receiving a 20 % PH inclusion level with proportionally less oligo- and polypeptides, diet LPH. It is unlikely that this is the result of different total dietary content of digestible protein in the two diets. Even though the final crude protein content obtained in diet HPH was slightly lower from the formulated value (Table 4.1.), when re-estimating the dietary content of digestible protein a similar final level of digestible protein between diets is observed (38 % in diet LPH and 37 % in diet HPH; estimation based on data by Morais et al., 2004a; Tonheim et al., 2005; Dias et al., 2010).

Assuming the diets had a comparable dietary level of digestible protein, the different growth results obtained should be interpreted in relation to the dietary peptide profile. Thus, increasing the fraction of oligo- and polypeptides from 15 % to 30 % of total dietary contents led to an inferior larval growth performance. The surplus of larger MW PH (> 500 Da) in diet HPH could have led to an AA overload of the larval digestive capacities such that protein synthesis for growth was impaired, despite containing the same amount of small peptides and FAA (< 500 Da). Though smaller MW molecules are more easily absorbed, oligo- and polypeptides can be broken down into di-, tripeptides and AA in the intestine. Not only can small peptides, in turn, be hydrolysed into FAA for which a wide variety of brush border membrane transporters exist, but there is a specific transporter (PepT1) in the enterocytes that specifically transports di- and tripeptides, which has been found in several fish species, including larval stages (Verri et al., 2003; Amberg et al., 2008; Terova et al., 2009; Ostaszewska et al., 2010; Rønnestad et al., 2010).

In fish larvae, it has been frequently observed that when the absorbed dietary AA do not match the profile needed for protein synthesis, they will be deaminated and used in energy

production, gluconeogenesis or lipogenesis, the preferential pathway being through oxidation via the tricarboxylic acid cycle (see Aragão et al., 2007; Conceição et al., 2011 and references therein). However, the nitrogen excretion and protein utilisation trials in this experiment do not enable to further elucidate if the lower growth observed for larvae fed diet HPH was related to an altered AA metabolism, with the exception that larvae fed this diet showed increased voracity compared to those from treatment LPH. That is, the fact that more larvae from group HPH compared to group LPH consumed *Artemia* nauplii after one hour's feeding possibly indicates that these were trying to compensate for dietary deficiencies. Gilthead seabream larvae showed a considerable capacity to digest *Artemia* proteins, with absorption, retention and catabolism values within the range of those usually reported for marine fish larvae in these types of studies (Conceição et al., 1998; 2007a; Morais et al., 2004b; Engrola et al., 2009b; 2010; Chapter 3 of this thesis). Indeed, the *Artemia* labelling trial allows the comparison of protein utilisation at a very high potential, comparatively to using inert feed, since *Artemia* is known to be a protein source with high digestibility in fish larvae (Rønnestad and Conceição, 2005). This possibly explains why there were no significant differences between larvae fed diet HPH and LPH in terms of protein utilisation; there might have been different metabolic responses when these were fed the alginate microdiets, but both were able to cope in the same way when fed *Artemia*. In the same line of thought, the lack of difference in mass-specific ammonia excretion between larvae of different dietary treament at the same age could be explained by their fasted state. Comparing the effect of an imbalanced dietary AA profile with a balanced profile on the development of white seabream larvae, Saavedra et al. (2009b) also report a lack of significant differences in nitrogen excretion between dietary treatments when larvae were fasted, despite there being higher excretion in the imbalanced treatment when the trial was performed on fed larvae. These authors hypothesise that when fasted, other nutrients such as lipids might be used in order to spare protein.

The comparative 2D-DIGE analysis of the larval carcass proteome highlighted the alteration in the protein expression profile related to different dietary PH inclusion level and peptide profiles. Interestingly, diet HPH seems to have elicited a more systematic response on protein expression than diet LPH; that is, for proteins whose expression was affected by dietary treatment, the expression pattern was more similar between replicates

from treatment HPH than from treatment LPH, where a higher dispersion of protein expression pattern was observed.

The identified proteins were involved in several cellular processes, the majority being cytoskeletal proteins, implicated in the maintenance of cell architecture, cell motility, proliferation, differentiation and organelle transport. Alpha actin, a major constituent of muscle tissue, composed a third of protein spots identified and practically all identified spots with increased abundance in larvae fed diet LPH. However, it is difficult to conclude further on the expression of alpha actin as there exist multiple isoforms and/or post-translational modifications whose occurrence seems to be regulated by numerous and complex mechanisms. Myosin light chain isoforms have also been shown to be closely regulated, particularly during fish development (Silva et al., 2010a and others therein). However, the fact that all isoforms identified, together with a myosin heavy chain, were over-expressed in larvae from dietary treatment HPH which had a comparatively inferior growth, could be pointing to increased protein degradation in the fast skeletal muscle, in turn reflected in increased MyLC solubility, as mentioned by Kjærgård and Jessen (2003) in a study on cod skeletal muscle proteome.

The increased abundance of muscle creatine kinase, mitochondrial ATP synthase subunits, NDPK and proteins belonging to the glycolytic/gluconeogenic pathways in larvae fed diet HPH may reflect a more important energy allocation to processes other than muscle tissue accretion, since these fish showed a depressed growth. As above mentioned, dietary AA imbalances can increase protein catabolism in fish, redirecting AA from protein accretion to energy production, gluconeogenesis or lipogenesis. If we assume that the surplus of PH in diet HPH impaired normal dietary nitrogen utilisation, then larvae from this group could have suffered from enhanced protein catabolism to meet basal energy requirements and to maintain AA homeostasis. With the current results it is not possible to ascertain that there was enhanced gluconeogenesis in larvae fed diet HPH since pyruvate kinase is specific only to the glycolytic pathway and gluconeogenesis occurs mainly in the liver and brain, whose proteome were not analysed. However, the higher abundance of Apo A-I could be indicating enhanced lipogenesis, as this protein is the main protein constituent of high density lipoproteins (HDL), which are known to play a role in reverse cholesterol transport

from tissues to the liver and in lipid metabolism. Finally, it is possible that these larvae showed a higher feeding activity in order to try to compensate for lower availability of dietary AA.

The current experiment was prolonged to 35 DAH for further skeletal development analysis, a period during which both experimental diets were replaced with a control diet whose PH fraction was composed of a 10 % dietary inclusion level of CPSP-90 (Diet CONT; Table 3.1. of Chapter 3 of this thesis). Though the skeletal development of seabream larvae is not within the scope of the present study, it was interesting to note that the larvae from dietary treatment HPH benefited from the switch of diets in terms of growth (Figure 4.1.), whereas those from dietary treatment LPH were not or maybe even negatively affected by the change. This provides further evidence that diet HPH was not suitable for seabream larvae. Supplying the larvae with a diet with a lower PH inclusion level and different MW profile of peptides seems to have led to compensatory growth in these larvae, showing the high adaptability of these organisms.

4.6. Final considerations

Accepting that progress is still to be made in terms of producing artificial microfeed and in the optimisation of weaning protocols, the alginate microencapsulated feed used in this experiment with a 20 % dietary inclusion level of a FPH composed of 25 % FAA, di- and tripeptides and 75 % peptides with MW between 500 and 30000 Da, was suitable for growth of gilthead seabream larvae. Increasing the PH dietary inclusion level to 35 % by adding the FPH fraction of MW between 500 and 30000 Da did not improve growth performance. The inferior growth performance and enhanced expression of proteins related to sarcomeric protein degradation and energy metabolism possibly show increased protein catabolism induced by this diet, which had a clear effect on protein expression in larval carcass. Albeit technically challenging, assessing feed intake and analysing the proteome of other larval tissues would greatly contribute to further elucidating how the dietary inclusion level and MW profile of PH act on larval development. Specifically, analysis the liver proteome, given the uniform structure and the key metabolic role of this organ, seems highly relevant to assess the effects of PH or alternative nitrogen sources on central metabolism, in order to understand what are the adaptive (or maladaptive) processes

involved (e.g. Hamza et al., 2010). Furthermore, application of comparative intestine proteomics, while challenging (due to issues such as the large amounts of membrane-bound proteins present, along with tissue complexity and the presence of gut microflora), can provide essential insight into factors underlying maturation of the digestive system and how dietary PH interact with these. For example, feed intake and *in vivo* apparent digestibility of the microdiets can be measured in larvae by marking the microdiets with fluorescent pigments or beads (Morris et al., 1990; Blair, 2005; Hansen et al., 2009) or inert metal oxides (Johnson et al., 2008). And using a non-gel-based proteomics approach such as reverse phase nanoflow liquid chromatography (LC), coupled to a high resolution mass spectrometer such as an ESI-LTQ-Orbitrap could be a good approach for analysing the larval intestine, as has recently been achieved by Nogueira et al. (2012) who analysed the larval midgut and its content of the cowpea weavil (*Callosobruchus maculatus*).

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Chapter 5. Size distribution of dietary protein hydrolysates affects skeletal muscle sarcoplasmic proteome and growth of juvenile zebrafish (*Danio rerio*)

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5.1. Abstract

Four experimental diets were fed to zebrafish (*Danio rerio*) to examine the effect of size distribution of dietary protein hydrolysates (PH) on growth of juvenile fish. The feeds were made to be isoproteic, isolipidic and isoenergetic, varying only in the fish protein hydrolysate composition. All diets contained 50 % crude protein, 25 % provided as plant-based protein and the other 25 % provided either as all fish meal (FM diet) or as fish meal with approximately 30% of the fish meal substituted by fish hydrolysate (FH diet) or by fractions of the size-fractionated hydrolysate, namely retenate after ultrafiltration of fish hydrolysate (UF diet) or retenate after nanofiltration of fish hydrolysate (NF diet). The diets were tested in triplicate in groups of 15 fish. The trial lasted from 33 to 47 days post-fertilisation (dpf), after which growth effects were evaluated by fork length measurement and a comparative proteomic analysis (2D-DIGE and MALDI-ToF-ToF MS) of the sarcoplasmic fraction of the trunk epaxial skeletal muscle.

The inclusion of a moderate level of PH with balanced distribution of its peptide size fractions did not improve growth of juveniles, nor induce remarkable changes in the sarcoplasmic proteome of the trunk epaxial muscle, in comparison with an intact protein-based diet. However, a diet rich in short peptides and FAA affected growth negatively, eliciting a clear response in the skeletal muscle proteome. The growth results are in agreement with other studies on teleosts, and did not suggest inferior handling of hydrolysates in this agastric species. The proteome response indicates a dietary effect on cellular phosphotransfer networks and a perturbed energy status, possibly indicating a reduced energetic capacity of the skeletal muscle and/or increased muscle degradation.

5.2. Introduction

It has been suggested that protein digestion and amino acids (AA) assimilation could set a limit on growth rate in the early life stages of fish. Protein is usually the main component of fish feed and fish rely on proteases for their digestion. Many studies have shown that the replacement of a fraction of protein content by partially hydrolysed (pre-digested) proteins could enhance performances of fish larval stages, since proteins are absorbed mainly as

smaller peptides or single AA (Rønnestad et al., 2007 and references therein). A moderate inclusion level of protein hydrolysate has been shown to benefit growth, development and/or survival of fish larvae in which the digestive system is actively developing (Cahu and Zambonino-Infante, 1995; Carvalho et al., 1997; Zambonino-Infante et al., 1997, Day et al., 1997; Tonheim et al., 2005; Nankervis and Southgate, 2009, Savoie et al., 2011), whilst having little or no effect on juvenile growth (Day et al., 1997; Gomes da Silva and Oliva-Teles, 1998, Oliva-Teles et al., 1999, Cahu and Zambonino-Infante, 2001; Savoie et al., 2011). However, there have also been positive results on growth and immune system reported for juveniles and adults (Espe et al., 1999; Refstie et al., 2004; Liang et al., 2006; Ostaszewska et al., 2010). From these studies, it seems that the optimum proportion of peptide and free AA (FAA) in respect to a major protein source for different fish species and different life stages needs to be addressed.

In fish, the white musculature may account for more than half of the body mass and is quantitatively the most important site for protein accretion (Carter and Houlihan, 2001), making this a particularly interesting tissue to study the growth effects induced by dietary nitrogen. However, the large number of membrane-associated proteins, the exceptionally high molecular mass of many muscle components, extensive posttranslational modifications in various muscle proteins and their organisation in highly complex supramolecular structures make it extremely difficult to carry out conventional biochemical studies of potential changes in protein clusters during physiological adaptations or pathological processes. In this respect, proteomics and subcellular proteomics attempt to isolate, separate and identify the entire protein constellation of a given muscle tissue or fibre population (Ohlendieck, 2011).

Indeed, in order to optimise formulated fish feeds, the application of novel tools in molecular biology will be necessary to detect various aspects of metabolism and influences on physiological responses to dietary treatments. Cutting edge technologies from "*omics*" approaches offer a comprehensive method to study biochemical systems by expanding the level of investigation from single biomolecules to a wide range of molecules present in a cell or tissue at once, in terms of their presence and relative abundance, without any *a priori* knowledge (Alves et al., 2010). Compared to transcriptomics, proteomics provides

not only information at a mechanistic level but can also capture changes in protein activity measured as post-translational modifications (PTMs). In fact the transcriptome does not account for the posttranscriptional and post-translational regulation of protein expression. The proteome can thus provide relevant information of an organism's physiological state not revealed by a transcriptomic approach (Rodrigues et al., 2012). Comparative proteomics via electrophoretic separation of proteins (2-DE) and identification by mass spectrometry (MS) of proteins whose expression is affected by the condition studied (e.g. dietary treatment), is an essentially hypothesis-generating approach. The protein species discovered through this approach should then be taken as a starting point for in-depth biochemical, cell biological and physiological characterisation of the tissue (or cell/sub-cell population) as affected by e.g. the dietary treatment.

One of the challenges of comparative proteomics is the ability to separate and quantify all the components of highly complex mixtures of proteins, despite the fact that the concentration of their components can span several orders of magnitude. Inevitably, the more highly expressed proteins, which are usually structural or homeostatic in nature, mask those with lower expression, often more biologically relevant proteins, including those involved in regulatory or signalling pathways. Fractionation procedures for tissue samples have been used for some time to address this situation (e.g. Vitorino et al., 2006; and references therein). Fractionation can simplify analysis by reducing the number of proteins in a given extract and improving the dynamic range by allowing larger loads per protein. Additionally, interpreting the observed changes in protein abundance may be eased by isolating a sub-population of the whole tissue proteome. Recent reports on proteomic analysis of skeletal muscle emphasise that fractionation methods, namely separating the myofibrillar and sarcoplasmic fractions, help to detect and identify more lowly expressed proteins (Kjæregård et al., 2004; 2006; Vitorino et al., 2006; Hamelin et al., 2007; Silva et al., 2010b). Separation appears useful for better detecting the low-expressed proteins in sarcoplasm, because myofibrillar proteins comprise more than 60 % of whole protein in skeletal muscle (Sato et al., 2009).

Another challenge faced by using a comparative proteomics approach for nutritional studies in aquaculture is that the interpretation of proteomic data requires availability of

information on genomic DNA and expressed mRNAs, but this information is still lacking in most of the 310 cultured species (FAO, 2011). Full genomes are still only available for some model species, such as zebrafish, stickleback, medaka, coelacanth, fugu and Tetraodon, although more focus is being given lately in studying the genomes of commercial species, such as tilapia, cod and salmon (Wenne et al., 2007; Crollius et al., 2005). However, for the present time, proteomic studies on aquaculture species still face some challenges at the level of protein identification (Rodrigues et al., 2012).

In the last decade, several studies have highlighted the possibility of considering zebrafish as a model species for nutritional and growth studies (Goolish et al., 1999; Meinelt et al., 2000; Carvalho et al., 2006; De-Santis and Jerry, 2007; Johnston et al., 2009; Siccardi et al., 2009; Palstra et al., 2010; Gómez-Requeni et al., 2010; 2011; Enyu and Shu-Chien 2011; Ulloa et al., 2011). Zebrafish possess the most developed genomic program compared to that of any other aquacultured fish, they are easy to maintain and breed, have short generation time and produce a large number of offspring. By using zebrafish as a model, a large number of dietary factors could be evaluated in far less time and at lower cost, and their effect studied more readily at a molecular level than in aquacultured fish.

With all of the above in mind, it was decided to use comparative proteomics (2-D DIGE and MS) on the sarcoplasmic fraction of the skeletal muscle of juvenile zebrafish fed inert feeds that differed in the inclusion of fish protein hydrolysates (FPH) and their peptide size distribution profile. For this, the fractionation method described by Kjærsgård and Jessen (2004) and validated by Silva et al. (2009b) was used on the trunk epaxial white muscle of juvenile zebrafish. The ultimate aim was to determine the effect (if any) of the dietary inclusion level and peptide size distribution profile of FPH on growth of juveniles of the agastric zebrafish and increase our understanding of the possible metabolic and regulatory pathways affected in skeletal muscle.

5.3. Materials and Methods

5.3.1. Fish rearing and sampling

Zebrafish (*Danio rerio*) were obtained and reared at the facilities of the Department of Biology of the University of Bergen (Norway). Water temperature was kept constant at 28

± 0.5 ºC, mean values for pH and water conductivity were 7.20 and 830 µS, respectively, and the diurnal light : dark cycle was set at 13 : 11 hours. According to standard procedures at the facilities, fish were fed *ad libitum* with a commercial diet (JBL Novo Tom Artemia, JBL GmbH & Co. KG, Neuhofen, Germany) until 15 days post-fertilisation (dpf). From 12 dpf, fish were co-fed with *Artemia* nauplii (Ocean Nutrition; Salt Creek Salt Lake City, UT, USA) and then solely *Artemia* until 25 dpf. Fish were then weaned by co-feeding onto the experimental diets for about a week. Feeding was performed four times and twice daily on week days and weekends, respectively. At 33 dpf fish were distributed into 12 rectangular plastic tanks (5 L) in groups of 15 fish each. Four experimental diets were tested, in triplicate. The growth trial lasted for 2 weeks, selected to occur during the exponential growth phase of juvenile zebrafish and before fish reached sexual maturation, based on a previous study at the same facilities (Gómez-Requeni et al., 2010).

At the beginning (33 dpf) and end of the growth trial (47 dpf), and following overnight fasting, fish were moderately anesthetised (3-aminobenzoic acid ethyl ester, MS-222; 50 µg mL^{-1}) for fork length measurement using a digital camera and the UTHSCSA Image-Tool software (University of Texas, Health Science Center, San Antonio, TX, USA). At 47 dpf, fish were then sacrificed by an overdose of MS-222 followed by pithing. The skeletal muscle tissue from the trunk epaxial region was excised on ice, briefly rinsed in Milli-Q grade water, snap frozen in liquid nitrogen and then stored at -80 ºC pending protein extraction.

5.3.2. Experimental diets and chemical analyses

Four agglomerated diets were produced at Nofima AS (Bergen, Norway) to be isoproteic, isolipidic and isoenergetic, and designed to vary only in the fish-source protein composition (Table 5.1.).

Table 5.1. Ingredients and chemical composition of experimental diets FM, FH, UF and NF.

Ingredients (%)	**FM**	**FH**	**UF**	**NF**
Full fish meal	30.50	20.00	24.20	25.10
Salmon byproduct hydrolysate (FH)		8.85		
Ultrafiltration retenate of FH			5.00	
Nanofiltration retenate of FH				7.50
Fish oil	11.20	12.00	11.70	11.90
Corn gluten	17.60	17.60	17.60	17.60
Wheat gluten	6.60	6.60	6.60	6.60
Wheat gluten hydrolysate	11.00	11.00	11.00	11.00
Wheat starch	15.84	16.72	16.63	13.85
Inositol	0.03	0.03	0.03	0.03
Betafin	0.40	0.40	0.40	0.40
Vitamin mix	2.00	2.00	2.00	2.00
Mineral mix without P	0.60	0.60	0.60	0.60
KH_2PO_4 (22.5 %)	1.15	1.10	1.14	1.09
NaH_2PO_4 (22.5 %)	1.15	1.10	1.14	1.09
Proximate composition (%)				
Protein	50.1	50.1	50.1	49.4
Lipid	15.0	15.0	15.0	15.3
Carbohydrate	22.1	22.8	22.8	20.3
Ash	7.9	7.0	7.1	11.4
Dry matter	95.5	95.4	95.4	95.7
Energy (MJ/kg)	21.7	21.8	21.8	21.3

On a dry weight basis, all diets were calculated to contain approximately 20 % carbohydrate and 15 % lipid. All contained approximately 50 % crude protein, 25 % provided as plant-based protein and the other 25 % provided either as all fish meal (25 % fish meal; FM diet) or as fish meal with approximately 30% of the fish meal substituted by fish hydrolysate (FH diet) or by fractions of the size-fractionated hydrolysate, namely retenate after ultrafiltration of fish hydrolysate (UF diet) or retenate after nanofiltration of fish hydrolysate (NF diet). Fish hydrolysate material contained only small or undetectable fractions of the largest peptides (10 – 20 kDa). The whole fish hydrolysate (included in diet

FH) contained the highest amount of all peptide fractions except the medium-sized peptides (1 – 5 kDa) (Fig. 5.1.). The retenate fish hydrolysate after ultra-filtration (included in diet UF) contained the least amount of FAA, and approximately 90 % of the protein evenly distributed between short and long-chained peptides. The fish hydrolysate retenate after nano-filtration (included in diet NF) contained the highest amount of the second smallest peptide fractions (100–1000 Da) and the lowest percentage of large polypeptides (5 – 20 kDa).

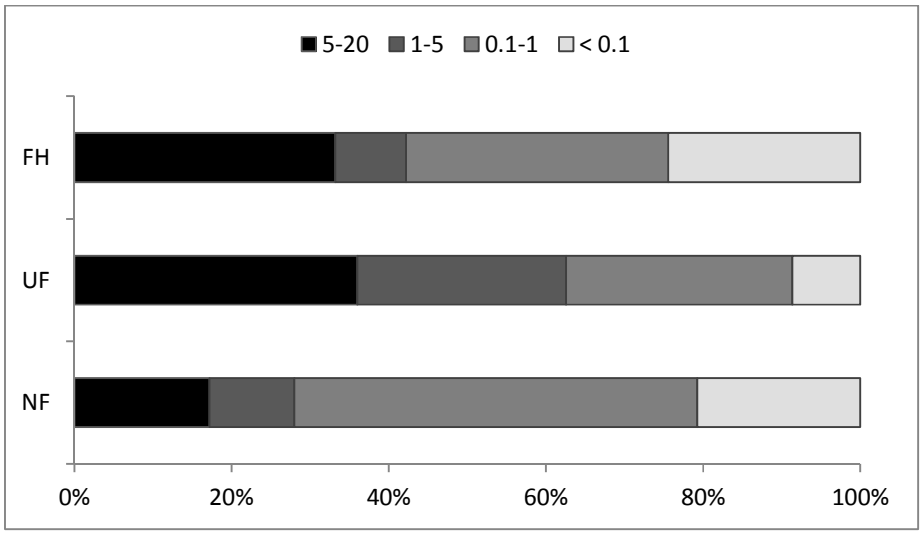

Figure 5.1. Peptide molecular weight distribution of hydrolysed fish meal fractions incorporated into the experimental feeds. "FH" – salmon byproduct hydrolysate; "UF" – retenate after ultrafiltration of fish hydrolysate; "NF" – retenate after nanofiltration of fish hydrolysate. All fish hydrolysate material contained undetectable fractions of the largest peptides (10 – 20 kDa; < 1.0 % of protein).

Crude protein (N×6.25) was determined by the Kjeldahl method (ISO5983-1997), moisture (ISO 6496-1999) and ash (ISO 5984-2002) gravimetrically after drying for 4 h at 105 °C and after combustion for 16 h at 550 °C, respectively. Gross energy was determined in a Parr adiabatic bomb calorimeter. All analyses were carried out in duplicates and if differences between parallels exceeded standardised values, new duplicate analyses were performed according to accredited procedures. Size distribution of peptides were analysed

by HPLC size exclusion chromatography using a TSK G2000 column and detection at 220 nm, as previously described in Aksnes et al. (2006).

5.3.3. Protein extraction and fluorescent labelling

One sarcoplasmic extract per tank were prepared from 70 mg wet weight of pooled muscle tissue homogenised by sonication in 350 µL of a mild extraction buffer (Tris-HCl 50 mM pH 7.4, EDTA 1mM, DTT 10 mM and a small amount of protease inhibitor cocktail). Mechanical homogenisation was performed in cycles of short sonication on ice to prevent sample heating, after which a 20 min centrifugation step was performed (11200 ×g, 4ºC), retaining the supernatant which contained mostly sarcoplasmic proteins. This method was adapted from Kjærsgård and Jessen (2004) and its applicability for comparative purposes has recently been explored and validated (Silva et al., 2010b). All protein extracts were then cleared from salts and contaminants using a standard TCA/acetone-based 2D electrophoresis sample precipitation kit (ReadyPrep™ 2D Cleanup Kit, Bio-Rad), resuspended in a standard DIGE lysis buffer (urea 7M, thiourea 2M, CHAPS 4%, Tris 30mM, pH 8.5) and quantified using a standard Bradford colorimetric method (Quick Start™ Bradford Protein Assay, Bio-Rad). For DIGE labelling, 37.5 µg of protein were labeled with 300 pmol of fluorescent amine reactive cyanine dyes freshly dissolved in anhydrous dimethyl formamide following the manufacturer's recommended protocols (CyDye™ DIGE fluor, minimal labeling kit, GE Healthcare). Labelling was performed on ice for 30 min in the dark and quenched with 1 mM lysine for 10 min on ice. Cy3 and Cy5 were used to label samples, 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. To normalise for Cy3 and Cy5 label differences, a fourth sample per dietary treatment was created by pooling an equal amount of protein from each extraction of that treatment. This sample gives no information on biological variation but helps to improve the estimate of the true central location of the distribution.

5.3.4. Two-dimensional gel electrophoresis

For gel electrophoretic separation, 37.5 µg of muscle protein of one replicate from each dietary treatment plus 37.5 µg of internal standard were diluted in Rehydratation Buffer

(Bio-Rad) to a final volume of 200 µl and loaded overnight on to an 11 cm Immobiline™ Drystrip pH 4-7 (Bio-Rad), by passive rehydration. This pH gradient was used because it was previously established that this range produced high resolution of skeletal muscle proteins and is the most highly populated p*I* range. In the first dimension, proteins were isoelectric focused using an Ettan™IPGphor™ 3 isoelectric focusing unit (GE Healthcare), at 20 ºC for a total of 29000 Vh at maximum 50 µA per strip. After the first dimension, strips were equilibrated using standard Bio-Rad reduction/alkylation buffers (30 min each step), transferred onto 13.3 x 8.7 cm 12 % Bis-Tris Criterion™ XT Precast gels (Bio-Rad) and run at 180 V using a MOPS running buffer (MOPS 50mM, Tris 50mM, EDTA 1mM, SDS 0.1%, pH 7.7).

5.3.5. Image and data analyses

After electrophoresis, gels were immediately scanned with a Typhoon Trio™ Variable Mode Imager (GE Healthcare) using a resolution of 100 µm and PMT value set to ensure that maximum pixel intensity was below saturation. Image analysis was performed using the Differential In-gel Analysis (DIA) and Biological Variation Analysis (BVA) modules of DeCyder v.7 software (GE Healthcare). A reference gel was automatically selected by the software using the default settings and based on an internal standard image. To be considered valid, any given spot had to be present in more than 2 replicates per dietary treatment. Spot detection, background subtraction, warping, matching, and normalisation were all set at the default settings of the software. Where possible, unmatched spots were edited on each multiplex group based on a 3D view of the spot, normalisation was restored, and the reference gels were updated. Protein spots considered for identification showed significant (One-way ANOVA, p-value < 0.05) differences in average normalised volume of 1.2-fold or more, between treatments. In addition, exploratory multivariate analysis by principal component analysis (PCA), using the in-built function, with mean centering and standard deviation scaling on protein, as well as clustering of the gels based on relative abundance of protein spots and according to correlation-based metrics, was performed as a complement to the One-way ANOVA, using the Extended Data Analysis (EDA) module of DeCyder software v.7 (GE Healthcare).

5.3.6. In gel trypsin digestion, MALDI-ToF-ToF MS and database searching

To identify the differentially expressed proteins, preparative gels were run prior to spot excision following the same protocol as described above except that labelling was not performed, each gel was loaded with either 300 or 500 μg proteins from mixed samples and Colloidal Coomassie Blue G250 staining (Neuhoff et al., 1988) was used.

Proteins of interest were excised and subjected to in-gel trypsin digestion. Briefly, the spots were successively washed with washing solution [NH_4HCO_3 25 mM in H2O/acetonitrile (ACN) 50 % v/v]. After reduction and alkylation (using DTT and iodoacetamide, respectively), protein spots were digested overnight with trypsin (Trypsin Gold, MS grade, Promega, 5 ng/μl in NH_4HCO_3 40 mM) and the resulting peptides extracted with trifluoroacetic acid (TFA) 0.1 %. After a final micropurification step, the peptides were then co-crystallized with a saturated matrix solution (α-cyano-4-hydrozy-cinnamic acid, 12 mg/ml, in 60 % ACN/0.2 % TFA) onto the target plate and analysed with an Ultraflex II MALDI-TOF-TOF mass spectrometer (Bruker-Daltonics).

Spectra were obtained in positive reflector mode and externally calibrated using the Peptide Calibration Standard II (Bruker Daltonics), while the internal calibration was performed with the trypsin autolytic products. These were analysed and converted to MS and MS/MS peak lists using FlexAnalysis 3.0 and Bio-Tools 3.0 (Bruker-Daltonics), removing peaks known to be common MS contaminants (human keratin and trypsin autolysis products). The obtained peak lists were then used as input to MASCOT MS/MS Ion searches of the *Danio rerio* subset of the NCBInr database, extended to the Chordata protein database when no reliable identification was possible in the *D. rerio* database, using the Matrix Science webserver (http://www.matrixscience.com/). These searches were performed assuming the formation of single charged peptides, carbamidomethylation of cysteine residues, possible oxidation of methionine residues and up to 1 missed cleavage. Mass tolerance was 70 ppm for MS data and 0.5 Da for MS/MS data. A protein spot was considered reliably identified when at least one MS/MS spectrum could be associated to a specific peptide in the database with high certainty (E-value << 0.05).

5.4. Results

5.4.1. Growth

During the two-weeks feeding trial, juvenile zebrafish readily accepted the experimental diets and maintained normal behaviour independent of dietary treatment. Survival was high and similar among treatments (Table 5.2.). At the end of the trial, the fish fed diet NF were smaller than those from the other treatments, with both final fork length and growth rate significantly lower than that of fish fed the diet with no protein hydrolysates (diet FM) (One-way ANOVA followed by post hoc Tukey's test, P-value < 0.05). Fish fed diet UF tended to show the second best growth rate (statistically non significant), after those fed the intact protein-based diet (FM).

Table 5.2. Growth and survival of *D. rerio* juveniles fed experimental feed differing in fish protein hydrolysate fraction. Values are means ± standard deviation (n = 3). Mean values for a selected row not sharing a common letter are significantly different (One-way ANOVA and post-hoc Tukey's test, P < 0.05).

	Treatment			
	FM	**FH**	**UF**	**NF**
FL (mm)				
33-35 DPF	17.04 ± 0.53	17.35 ± 0.39	16.77 ± 0.37	17.01 ± 0.50
47-49 DPF	23.96 ± 0.64^{a}	23.56 ± 0.98^{ab}	23.17 ± 0.76^{ab}	22.48 ± 0.80^{b}
Growth rate (mm day^{-1})	0.49 ± 0.03^{a}	0.44 ± 0.06^{ab}	0.46 ± 0.05^{ab}	0.39 ± 0.02^{b}
Survival (%)	88.89 ± 7.70	91.11 ± 3.85	91.11 ± 3.85	91.11 ± 7.70

5.4.2. Muscle sarcoplasmic proteome response

Analysis of the 24 spot maps obtained from 2D-DIGE of the trunk epaxial muscle sarcoplasmic proteome of juvenile zebrafish enabled the detection and quantification of 351 spots across all gels, with molecular weight between 10 and 200 kDa and isoelectric point between pH 4 and 7. Sixty of these protein spots were considered as differentially expressed between dietary treatments (fold-change ≥ 1.2; One-way ANOVA, p-value < 0.05). Data visualisation through principal component analysis (PCA) of this dataset

showed that, of the sarcoplasmic proteins whose expression was affected by dietary treatment, the response of those of zebrafish fed diet NF was clearly distinguished from other treatments (Fig. 5.2.). On the other hand, protein expression in dietary treatment "UF" most resembled that of "FM" and both were similar to "FH", though this latter showed the most dispersion in protein expression between replicates.

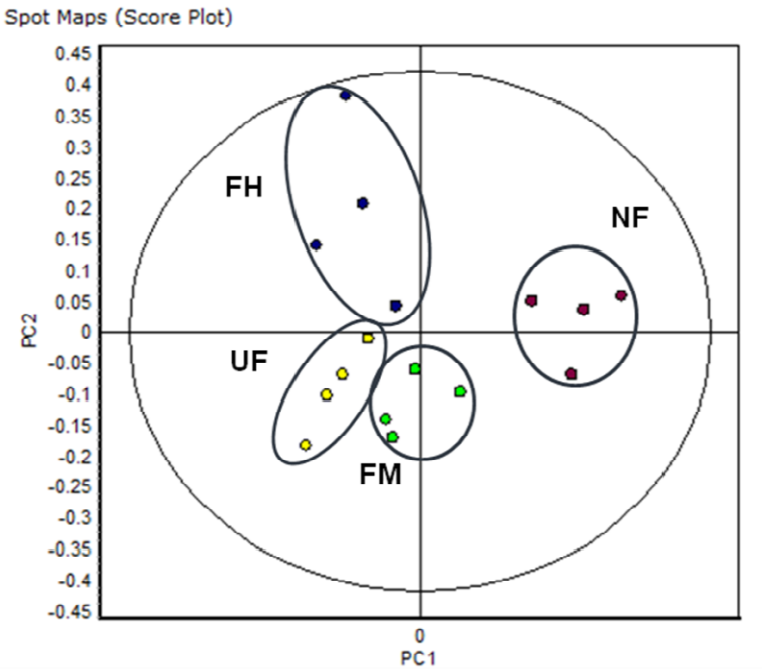

Figure 5.2. Principal component analysis (PCA) on differentially expressed proteins (One-way ANOVA, p-value < 0.05; fold-change ≥ 1.2), using the DeCyder 2-D software V.7.0 in-built function, with mean centering and standard deviation scaling on protein. PC1 explains 58.2 % of the variance and PC2 explains 20.4 %.

From the 27 regulated spots that could be excised from the gels (Fig. 5.3.), 18 were reliably identified by mass spectrometry (Table 5.3.); their expression pattern across treatments is shown in Table 5.4.. Proteins identified belong mainly to energy metabolism and nucleotide metabolism [mitochondrial ATP synthase, beta subunit – F1-ATPase/ATP5B (3 spots), adenylate kinase isoenzyme 1 – AK1 (4 spots), muscle creatine kinase – CK (5 spots), nucleoside diphosphate kinase – NDPK (3 spots)], lipid metabolism [apolipoprotein A1 precursor – Apo AI (2 spots)] and cellular oxidoreductase activity (peroxiredoxin 2 – Prx2).

In general, AK1, CK and NDPK were less abundant in dietary treatment "NF" compared with other groups, with the exception of a "modified" CK (spots 164, 166 and 167) and NDPK (spot 325) with higher molecular weight, whose expression pattern was opposite to their "non-modified" form. ATP5B and Apo AI showed similar expression patterns, being more abundant in the skeletal muscle of fish fed diet NF, and also in that of fish fed the intact protein-based diet (diet FM) relative to dietary treatments "FH" and "UF". Finally, Prx2 showed an inferior abundance in the muscle of fish fed diet NF compared with that of fish fed the other diets.

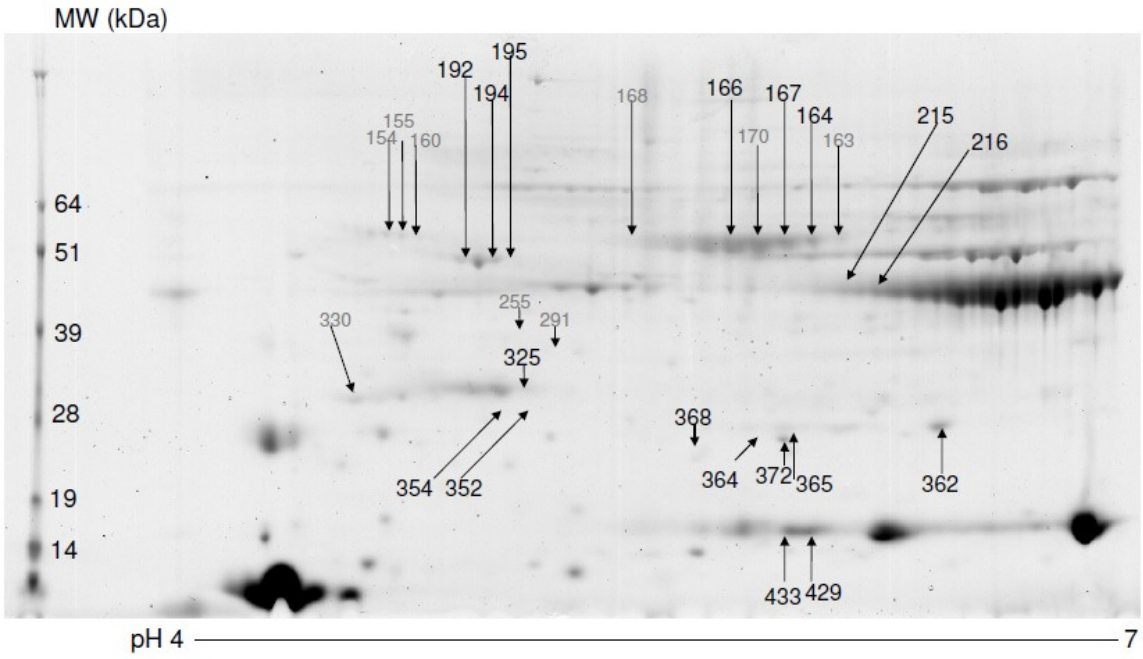

Figure 5.3. Protein map of two-dimensional gel electrophoresis of low-salt soluble proteins (sarcoplasmic fraction) of *D. rerio* trunk skeletal muscle, prepared by linear immobilised pH gradient (pH 4-7) in the first dimension and 12 % BisTris gel electrophoresis for the second dimension, stained with CBB G-250. Labelled spots indicate reliably identified proteins with significantly altered expression in response to dietary treatment. Spots in grey could not be reliably identified.

Table 5.3. MALDI-ToF-ToF MS identification of protein spots with significantly altered abundance between the sarcoplasmic fraction of trunk epaxial muscle from juvenile zebrafish fed the experimental diets (FM, FH, UF and NF). Peptide fragment fingerprinting (PFF) in the option MS/MS Ion Search from the bioinformatics application Mascot was made in the non redundant NCBI nr database for the *Danio rerio* taxonomic level. Caption a) theoretical (T) isoelectrical point (pI) and molecular weight (MW) based on the best result's sequence; experimental (E) pI and MW estimated from the position of the spots in the gel; MW is given in kDa; b) sequence coverage (%); c) number of peptides matched (E-value << 0.05); d) Combined MOWSE score (significant when above 31, $p < 0.05$).

Spot #	Protein identification	NCBI accession #	pI/MW T pI/MW E [a]	SC [b]	PM [c]	Best matched peptide sequence; E-value	Score [d]
164	muscle creatine kinase a	NP_571007	6.32/42.79 6.38/53.79	16	1	TFLVWVNEEDHLR 1.26E-07	64
166	muscle creatine kinase a	NP_571007	6.32/42.796.21 /53.41	12	1	TFLVWVNEEDHLR 1.26E-06	92
167	muscle creatine kinase a	NP_571007	6.32/42.79 6.35/53.49	22	1	TFLVWVNEEDHLR 1.00E-07	70
192	ATP synthase subunit beta,mitochondrial	NP_001019600	5.25/55.08 4.92/50.58	49	4	TIAMDGTEGLVR 7.94E-05	88
194	ATP synthase subunit beta,mitochondrial	NP_001019600	5.25/55.08 5.02/50.58	55	1	IPVGPETLGR 2.00E-05	47
195	ATP synthase subunit beta,mitochondrial	NP_001019600	5.25/55.08 5.07/50.58	42	2	IPVGPETLGR 3.98E-08	74
215	muscle creatine kinase b	NP_001099153	6.75/42.85 6.52/44.69	26	3	ELFDPVISDR 7.94E-08	381
216	muscle creatine kinase b	NP_001099153	6.75/42.85 6.61/44.69	19	2	TFLVWVNEEDHLR 6.31E-09	198
325	nucleoside diphosphate kinase b	NP_571002	6.75/17.23 5.10/31.67	50	2	TFIAVKPDGVQR 2.51E-06	96
352	Apolipoprotein A-I precursor	NP_571203	5.06/30.24 5.09/30.24	74	1	IAPHTQDLQTR 1.00E-08	80

Spot #	Protein identification	NCBI accession #	pI/MW T pI/MW E [a]	SC [b]	PM [c]	Best matched peptide sequence; E-value	Score [d]
354	Apolipoprotein A-I precursor	NP_571203	5.06/30.24 5.03/30.23	80	2	LEPVFQEYSALNR 1.00E-09	149
362	adenylate kinase isoenzyme 1	NP_001003993	7.68/21.49 6.61/24.23	19	1	ATEPVIAFYEQR 3.16E-09	122
364	adenylate kinase isoenzyme 1	NP_001003993	7.68/21.49 5.76/24.58	53	1	ATEPVIAFYEQR 2.51E-07	66
365	adenylate kinase isoenzyme 1	NP_001003993	7.68/21.49 5.89/24.33	44	2	GYLIDGYPR 3.98E-07	124
368	adenylate kinase isoenzyme 1	NP_001003993	7.68/21.49 5.62/24.58	19	1	ATEPVIAFYEQR 1.12E-06	96
372	peroxiredoxin 2	NP_001002468	5.93/21.84 5.87/24.14	22	1	QGGLGSMNIPLVADLTQSIS R 2.52E-04	70
429	nucleoside diphosphate kinase b	NP_571002	6.75/17.23 6.37/17.10	54	2	NLIHGSDSEK 1.58E-07	106
433	nucleoside diphosphate kinase b	NP_571002	6.75/17.23 6.27/16.50	67	2	TFIAVKPDGVQR 1.26E-11	168

Table 5.4. Fold change in abundance of proteins identified by MS according to the diet. Data were analysed by One-way ANOVA, followed by Tukey's HSD multiple comparison test. *P*: One-way ANOVA p-value. "-" indicates an underexpression of the protein in the first treatment relative to the second (and no symbol indicates overexpression). "." indicates non significance of fold-change (Tukey's HSD p-value > 0.05). "AK1" – adenylate kinase isoenzyme 1; "Apo AI" – apolipoprotein AI precursor; "ATP5B" – mitochondrial ATP synthase, beta subunit; "CK" – creatine kinase, muscle type; "NDK" – nucleoside diphosphate kinase; "Prx2" – peroxiredoxin 2.

Protein	Spot #	*P*	FM-FH	FM-UF	FM-NF	FH-UF	FH-NF	UF-NF
AK1	362	0.043	.	.	1.13	.	1.14	1.22
AK1	364	0.005	.	.	1.21	.	1.23	1.27
AK1	365	0.007	.	.	1.16	.	1.28	1.32
AK1	368	0.046	.	.	1.18	.	1.20	1.22
Apo AI	352	0.029	1.17	1.16	.	.	-1.26	-1.24
Apo AI	354	0.040	1.17	.	.	.	-1.32	-1.20
ATP5B	192	0.031	1.14	1.20	-1.14	.	-1.15	-1.20
ATP5B	194	0.001	1.30	1.24	-1.33	.	-1.72	-1.64
ATP5B	195	0.000	1.30	1.36	-1.90	.	-2.48	-2.58
CK	164	0.001	.	.	-1.26	.	-1.37	-1.35
CK	166	0.003	.	.	-1.35	.	-1.53	-1.33
CK	167	0.006	.	.	-1.25	.	-1.27	-1.25
CK	215	0.029	.	.	1.16	.	1.22	1.19
CK	216	0.006	.	.	1.14	.	1.18	1.20
NDK	325	0.039	.	.	-1.18	.	-1.27	-1.31
NDK	429	0.046	1.22	.	1.18	-1.16	.	1.12
NDK	433	0.049	1.19	.	1.20	-1.16	.	1.17
Prx2	372	0.040	.	.	1.15	.	1.14	1.20

5.5. Discussion

Despite the extensive use of zebrafish as a model organism for scientific research in a wide diversity of fields, there lacks information on nutritional requirements and diet optimisation for this species (Kaushik et al., 2011; Penglase et al., 2012). Still, the feeding protocol and experimental feed used in the present study allowed for an adequate growth and survival of zebrafish. The observed growth was comparable to a reference growth curve recently provided by Gómez-Requeni et al. (2010) for zebrafish reared at the same facilities on *Artemia* nauplii; and also to a more recent study performed by Kaushik et al. (2011) in which zebrafish were successfully reared solely on an inert dry feed from first-feeding.

In general, the dietary inclusion of hydrolysed fish protein (FPH) did not lead to a better growth performance of juvenile zebrafish compared to the intact protein-based diet (diet

FM). A number of studies report little or no effects of dietary FPH inclusion on growth and/or nitrogen utilisation in juveniles of several fish (common sole, Day et al. 1997; seabass, Gomes da Silva and Oliva-Teles, 1998; turbot, Oliva-Teles et al., 1999; Cahu and Zambonino-Infante, 2001; Atlantic salmon, Hevrøy et al., 2005; spotted wolfish, Savoie et al., 2011). However, to our knowledge, there are no equivalent studies in juveniles of stomachless fish in the available literature. Though not directly comparable, the results of Kwasek et al. (2010) are in accordance with those obtained presently, in that a 50 % dipeptide:intact protein diet contributed to better growth in juvenile Koi carp compared to either a pure dipeptide- or a FAA-based diet, but was no different from the intact protein-based diet.

In addition, it was seen that zebrafish receiving a diet without inclusion of FPH (diet FM) grew significantly better than those whose diet included the FPH richest in small peptides and FAA (< 1000 Da) and poorest in large polypeptides (5 – 20 kDa) (diet NF). This is in accordance with several studies on the utilisation of FAA-based diets by another cyprinid, the common carp, which observed an inferior utilisation of AA in terms of growth, when compared to equivalent protein-based diets (Murai, 1982; Murai et al., 1983; Kaushik and Dabrowski, 1983). In these studies, together with similar experiments using FAA or synthetic dipeptides as the sole protein source for both gastric and agastric teleosts, it was reported that in response to the relative flood of dietary AA these were excessively deaminated and/or excreted intact via gills and in urine, thus becoming inferior in terms of supporting growth (common carp, Murai et al., 1984; sturgeon, Ng et al., 1996; Atlantic cod, Berge et al., 1994; rainbow trout, Dabrowski et al., 2003; midas, Dabrowski et al., 2007; Koi carp, Kwasek et al., 2010).

In their review article on the effects of protein-, peptide- and FAA-based diets in fish nutrition, Dabrowski et al. (2010) mention that the lack of a stomach can negatively affect the digestion of certain types of protein but it is still unclear how this might affect the utilisation of dipeptides, stressing that the effects of dipeptide-based diets on absorption/metabolism of AA should not be equated with the response following feeding of FPH-based diets, which usually contain a mix of proteins, peptides and AA. In fact, as previously pointed out by Carvalho et al. (1997; 2004), when delivering dietary FPH to

fish, attention should be paid to the overall balance of the dietary peptide profile and not each molecular size fraction individually. This is because protein digestion products are transported from the intestinal lumen into the enterocytes both in the form of FAA, by a large variety of brush border membrane AA transporters, as in the form of di- and tripeptides, by the brush border solute carrier 15 (SLC15) membrane proteins, such as peptide transporter 1 (PEPT1) (Verri et al., 2003; 2011; Amberg et al., 2008; Terova et al., 2009; Ostaszewska et al., 2010; Rønnestad et al., 2010). In zebrafish, PEPT1 is a low affinity/high-capacity system and this transporter has been found to be highly expressed in the proximal intestine as early as 4 dpf, thus preceding functional maturation of the gut, first-feeding and complete yolk sack resorption (Verri et al., 2003). Additionally, there is evidence for the direct absorption of certain soluble intact proteins and polypeptides via pinocytosis and intracellular digestion in the digestive tract of fish (Govoni et al., 1986; McLean et al., 1990). Thus, though diet FH in our experiment had actually more FAA than diet NF, the larger fraction of short peptides (0.1-1 kDa) and low content in long polypeptides (5-20 kDa) in diet NF compared with the other diets possibly contributed to the lower growth of zebrafish fed this diet, as a result of the high dietary AA influx (see below). In the same line of thought, perhaps fish fed diet UF, in which approximately 90 % of the hydrolysed protein was evenly distributed between short and long-chained peptides (and the amount of FAA was smallest), tended to show the best growth rate of the FPH treatments and no significant differences with those fed the intact protein-based diet (diet FM). Additionally, protein solubility (supposedly higher in the FPH feeds compared with diet FM) apparently did not improve protein digestion and nitrogen utilisation for growth in these agastric juveniles, at least for the level used in these feeds. When comparing feeds with high protein solubility (by using soluble casein) with feeds containing similar or even lower levels of water-soluble nitrogen but in which the solubility was mainly conferred by hydrolysed protein, Carvalho et al. (2004) also reported a strong effect of the dietary peptide profile on carp larval development, independent of that of solubility.

Carvalho et al. (2004) suggested that a dietary excess of di- and tripeptides was linked to reduced early life-stage performance either due to saturation of the peptide transport mechanisms and/or to the rapid hydrolysis of low-molecular weight peptides, that produced an excessive AA load, that in turn saturated the AA intestinal transport mechanisms. In

addition, several AA share the same transporters, which can cause the transport of a particular AA to be competitively inhibited by the presence of other AA (Rønnestad et al., 2007; Narawene, 2011). Based on their own results with common carp larvae fed dipeptide-based diets, and on a study by Vabulas and Hartl (2005) on AA deprivation in mammalian cells, Zhang et al. (2006) suggest there is increasing evidence that providing an FAA flood in the form of FAA- or small peptide-rich diets upsets natural degradation (proteasome)-renewal balance in tissues and results in rapid AA depletion followed by impairment of protein synthesis. Again, whilst studying the nutrient absorption and growth in Atlantic salmon adults, Espe et al. (1999) observed improved growth in fish fed diets with up to 15 % inclusion level of FPH, relative to no or higher inclusion levels, but no change in protein synthesis in the skeletal muscle. These authors hypothesised that the better growth observed was due to lower protein degradation.

The comparative analysis of the zebrafish skeletal muscle proteome demonstrated that there was an effect of dietary treatment on sarcoplasmic protein expression, such that proteins from the fish receiving the nanofiltrate retenate of FPH (diet NF) showed a different response pattern from those belonging to the other dietary treatments; the proteome of fish fed diets FM and UF being the most similar, and that of fish fed diet FH having the most dispersion in response among replicates. So it seems that the skeletal muscle sarcoplasmic proteome reflected the different growth performances observed between dietary treatments, which in turn responded to the dietary peptide profile.

A significant portion of identified spots represented three proteins involved in nucleotide metabolism and phosphotransfer networks: creatine kinase (CK), adenylate kinase (AK), nucleoside diphosphate kinase (NDPK). Along with some glycolytic enzymes and other enzymes involved in guanine nucleotide phosphotransfer, CK, AK and NDPK ensure the rapid dissipation of ATP/ADP gradients across cells, effectively facilitating the spatial transmission of high-energy phosphoryl groups from ATP-producing to ATP-consuming sites. Thus it is not surprising that these enzymes are very abundant in the cytosol of tissues with high-energy demand such as skeletal muscle. Additionally, being key enzymes in the synthesis, equilibration and regulation of nucleotides, they are implicitly involved in essential cellular processes including DNA replication, gene expression, ion channel gating

and receptor or protein kinase-mediated signal transduction, thus often associated with protein synthesis, growth and development. Their inferior abundance in the skeletal muscle of zebrafish fed diet NF compared to other dietary treatments could therefore indicate reduced energy capacity and altered protein turn-over resulting in depressed growth. For instance, in rainbow trout, a down-regulation of CK was reported in atrophying skeletal muscle (Salem et al., 2010). On the other hand, increased expression of this enzyme and NDPK was associated with higher growth rates in rainbow trout fingerlings fed nucleotide-supplemented diets (Keyvanshokooh and Tahmasebi-Kohyani, 2012) and with body mass and length of yellow perch (Reddish et al., 2008). Alterations in AK, NDPK and CK expression profile have been associated with an abnormal regulation of nucleotide ratios in aged muscle fibres (O'Connell and Ohlendieck, 2009).

Interestingly, it was seen that two "modified" forms of CK and NDPK (protein spots identified as these enzymes but with an experimental (in-gel) molecular weight above the theoretical one) showed an opposite trend in expression from their "normal" form, that is, were more abundant in the skeletal muscle of fish fed diet NF relative to other treatments. It has frequently been observed that a deficiency in CK will lead to increased expression of AK and vice-versa, and similarly between AK and NDPK (e.g Ge et al., 2003; Keyvanshokooh and Tahmasebi-Kohyani, 2012; Silva et al., 2012). The often reported alterations in phosphotransfer CK-, AK-, guanine nucleotide- and glycolytic enzyme-catalysed activities may provide mechanisms for energetic plasticity critical in compensating for cellular energetic deficits (Dzeja et al., 2011). Could the "modified" forms of CK and NDPK with opposite expression pattern to their "normal" forms be playing a compensatory role in the skeletal muscle so as to maintain energy homeostasis and meet energy demand, along with the higher expression of ATP5B? In order to answer, further elucidation of these two enzymes' identity is necessary (see below).

Alternatively, given the "modified" NDPK protein spot showed twice the theoretical molecular weight of NDPK, it might represent NDPK dimers in the muscle of fish from the dietary treatment NF, the presence of which would indicate strong oxidative stress (Song et al., 2000). Indeed, the phosphotransfer network enzymes reported help to ensure the proper functioning of the respiratory chain, which could otherwise generate elevated levels of

superoxide and ROS (reactive oxygen species) (Wallimann et al., 2011). A higher abundace of ATP5B seen for this treatment might also be indicative of cellular oxidative stress, as was previously reported in a study on liver preoteome of Senegalese sole exposed to repeated handling stress, where an increased abundance of ATP5A1 was suggested to constitute an adaptation towards the maintenance of a lower charge gradient across the mitochondrial membrane, in an effort to prevent ROS formation (Cordeiro et al., 2012).

In accordance with this study, many proteins identified in skeletal muscle are represented by a large number of distinct two-dimensional spots in analytical gels (O'Connell and Ohlendieck, 2009). This points to the existence of multiple sub-species for each enzyme and explains why theoretical pI-values and/or molecular weight do not always perfectly agree with actual pI-values and/or molecular weight following 2-DE. One of the great advantages of proteomic technologies is that they can differentiate between differently charged or sized sub-species of individual proteins (Mullen and Ohlendieck, 2011). As advised by Forné et al. (2010), ambiguities inherent to common workflows used in proteomics for protein identification, such as the actual length of the sequence identified or the profile of any eventual post-translational modification(s), features intrinsically related to protein function, should be elucidated via other parallel, confirmatory approaches for consistent protein identification. Particularly for CK and NDPK, for which the DIGE analysis revealed differential effects of dietary treatment on different sub-species, it would be interesting to evaluate the exact molecular fate with, for example, antibody-based techniques such as Western blotting, immunofluorescence and/or immunohistochemistry, so as to confirm which sub-species was indeed up/down regulated. Additionally, further analysis of the identified protein spots using a phosphoproteomics approach would provide insight into the phosphorylation status of each sub-species. This in turn would greatly help elucidate the changes occurring in the skeletal muscle of zebrafish fed diet NF. This is because phosphorylation is a modification that regulates protein function, subcellular localization, complex formation and degradation of proteins. For example, ATP5B has numerous phosphorylation sites, and its phosphorylation status affects whether it participates in ATP production or hydrolysis (Højlund et al., 2003); thus a phosphoproteomics approach would help elucidate if the increased abundance of this protein was a sign of increased ATP production, or quite the contrary.

Finally, one of the aims of the study was to target the sarcoplasmic proteins using a fractionation technique that would help unveil the less abundant, but perhaps more relevant proteins directly involved in skeletal muscle growth, such as muscle growth regulators. However, either their response is not detectable in our study, or more likely, their presence is still masked by more abundant proteins such as the glycolytic, phosphotransfer network and other intermediate metabolism enzymes. Therefore, focusing on a cell specific population or organelle/sub-cellular compartments (nuclei, mitochondria) could dramatically decrease the proteins involved in unspecific responses and would increase the probability to unveil more proteins of interest.

5.6. Concluding remarks

The peptide profile of dietary FPH affected growth and skeletal muscle proteome of zebrafish juveniles. The inclusion of a moderate level of FPH with balanced distribution of its peptide size fractions did not improve growth of juveniles, nor induce striking changes in the sarcoplasmic proteome of the trunk epaxial muscle, in comparison with an intact protein-based diet. However, a diet rich in short peptides and FAA actually affected growth negatively, eliciting a clear response in the skeletal muscle proteome. The growth results are in agreement with other studies on teleosts, and did not suggest inferior handling of hydrolysates in this agastric species. The proteome response indicates a dietary effect on cellular phosphotransfer networks and a perturbed energy status, possibly indicating a reduced energetic capacity of the skeletal muscle and/or increased muscle degradation/stunted muscle growth. Given the multiple roles played by the main proteins identified, further studies on subspecies identification, cellular location and phosphorylation status would help understand the response to diet NF. Analysing the myofibrillar proteome could also bring valuable information concerning muscle degradation and altered protein turnover.

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Chapter 6. Dietary lysine imbalance affects muscle proteome in zebrafish (*Danio rerio*): a comparative 2D-DIGE study

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6.1. Abstract

Lysine (Lys) is an indispensable amino acid (AA) and generally the first limiting AA in vegetable protein sources in fish feeds. Inadequate dietary Lys availability may limit protein synthesis, accretion and growth of fish. This experiment aimed to further elucidate the role of Lys imbalance on growth by examining the myotomal muscle proteome of juvenile zebrafish (Danio rerio). Quadruplicate groups of 8 fish were fed either a low-Lys [Lys(−), 1.34 gkg−1], medium/ control (Lys, 2.47 gkg−1) or high-Lys [Lys(+), 4.63 gkg−1] diet. Fish growth was monitored from 33 to 49 days postfertilization (dpf) and trunk myotomal muscle proteome of Lys(−) and Lys(+) treatments were screened by 2D-DIGE and MALDI ToF tandem mass spectrometry. Growth rate was negatively affected by diet Lys(−). Out of 527±11 (mean± S.E.M.) protein spots detected (□10–150 kDa and 4–7 pI value), 30 were over-expressed and 22 under-expressed in Lys(−) fish (|fold-change|>1.2, p value <0.05). Higher myosin light chains abundance and other myofibrillar proteins in Lys(−) fish pointed to increased sarcomeric degradation, indicating a higher protein turnover for supplying basal energy-saving metabolism rather than growth and muscle protein accretion. The Lys deficiency also possibly induced a higher feeding activity, reflected in the over-expression of beta enolase and mitochondrial ATP synthase. Contrarily, in the faster growing fish [Lys(+)], over-expression of apolipoprotein A-I, F-actin capping protein and Pdlim7 point to increased energy storage as fat and enhanced muscle growth, particularly by mosaic hyperplasia. Thus using an exploratory approach, this study pinpoints interesting candidates for further elucidating the role of dietary Lys on growth of juvenile fish.

6.2. Introduction

The global increase in aquaculture production is expected to exceed the supply of fish-derived ingredients used in aquafeeds, making it imperative to find alternative sources, such as plant feedstuffs, to reduce production costs and increase sustainability (Gatlin et al. 2007). To make diets of similar nutritional quality to those containing more fish meal, it is necessary to use supplemental amino acids (AA) to ensure that nutritional needs are met, particularly as plant-proteins often show deficiencies in some indispensable AA (IAA).

Lysine (Lys) requirement in fish nutrition has received considerable attention in recent years as it is considered to be the first limiting AA in fish, as in higher non-ruminant vertebrates, and the level of Lys in plant-protein meals is often low compared to fish meal (Wilson 2002; Li et al. 2009). In fish, it has been demonstrated that Lys supplementation may enhance protein synthesis and deposition, and reduce nitrogen losses (e.g. Cheng et al., 2003; Conceição et al., 2003; Abimorad et al., 2009; Rathore et al., 2010). However, the metabolic pathways by which dietary Lys influences growth performance require further investigation.

In a previous study, the metabolic effects of different dietary Lys levels were examined by two-dimensional (2D)-proteomics in juvenile zebrafish (*Danio rerio*). This comparative proteomic analysis of whole-body zebrafish showed 45 protein spots differentially expressed across the 3 dietary treatments: Lys(−), made to be low in Lys; Control, formulated to respect Lys requirements in zebrafish; and Lys(+), with Lys supplemented above the estimated requirements of zebrafish. Lys deficiency was accompanied by a down-regulation of muscle proteins and up-regulation of proteins associated to fasting, energy deficit, growth arrest and apoptosis. Additionally, it was found that excess Lys was accompanied by an upregulation of proteins related to glycolysis, steroidogenesis and sexual maturation (Gómez-Requeni et al., 2011). A mass spectrometry-based proteomics approach was chosen because this is an unbiased and technology-driven approach for the comprehensive cataloguing of protein complements and represents an ideal analytical tool for the highthroughput discovery of protein alterations under specific conditions (Hochstrasser et al. 2002). The generation of data sets on protein expression levels makes proteomics a preeminent hypothesis-generating approach in modern biology (Ohlendieck 2011).

In this study, it was decided to apply 2D-fluorescense difference gel electrophoresis (DIGE), a highly discriminating technique, on the trunk myotomal muscle proteome of the juvenile zebrafish, so as to further explore possible pathways by which the dietary Lys levels influenced growth in our previous study (Gómez-Requeni et al., 2011). Growth or an increasing body size is directly linked to an increase in myotomal muscle, which can comprise from 40 to over 60 % of the body mass in teleosts (Zimmerman and Lowery

1999). Also, the myotomal muscle has an important ancillary metabolic function, its sarcomeric proteins constituting a reservoir of AA, being mobilised as an energy source by other tissues or for new protein synthesis such as in gonad generation (Johnston et al., 2011). In fish aquaculture, muscle is fundamentally important since it generally represents the final commercialised product.

The zebrafish provides an excellent biological model and has recently been suggested as a valid model for other aquaculture species (Dahm and Geisler, 2006; Gómez-Requeni et al., 2010) and more specifically in nutrigenomics, as well as muscle development research (e.g. Jury et al., 2008; Patterson et al., 2008; Johnston et al., 2009; Gómez-Requeni et al., 2011; Enyu and Shu-Chien, 2011).

Thus the aim of this study was to explore the effects of dietary Lys imbalances on the protein expression of myotomal muscle of juvenile zebrafish. To address this issue, the growth of zebrafish populations was monitored for approximately 2 weeks (Gómez-Requeni et al., 2011) and the myotomal muscle proteome profile was screened by means of 2D-DIGE and mass spectrometry (MS).

6.3. Materials and Methods

6.3.1. Animals, experimental conditions and sampling procedure

Zebrafish were reared at the facilities of the High Technology Center (Bergen, Norway). Ninety-six fish of 33 days post-fertilisation (dpf) were distributed into 12 plastic tanks (5 L) in groups of 8 fish each. Photoperiod was 13:11 h of light/dark and water temperature was kept constant at 28°C.

Three experimental isoproteic, isolipidic and isoenergetic agglomerated diets were prepared at Nofima AS in Bergen, Norway, containing different levels of Lys whereas the remaining AA were kept at similar levels. The experimental diets' composition and AA profiles are shown in Tables 6.1. and 6.2., respectively. The diets were equally low in fishmeal in order to obtain low total Lys levels from the basal dietary raw materials and contained equal amounts of different plant protein raw materials, fish oil and feed additives. In addition, the three diets contained 142 gkg−1 diet of the corresponding crystalline AA mix: a full mix

for the control diet (diet Control), a mix without Lys [diet Lys(−)] and a mix with Lys added in excess [diet Lys(+)]. The full AA mix was prepared considering the data obtained on zebrafish whole body AA content in a previous study (Gómez- Requeni et al., 2010), based on the "ideal protein concept" (ARC, 1981; Boisen et al., 2000) and the Lys requirement of common carp (NRC 1993). The experimental diets were offered to visual satiation to quadruplicate groups of fish (33–49 dpf) four times daily during week days and twice per day during the weekends.

Table 6.1. Ingredients and chemical composition of the three experimental diets. (previously published in Gómez-Requeni et al. 2011)

Ingredient (%)	**Control**	**Lys(-)**	**Lys(+)**
Fish meal	20.60	20.60	20.60
Corn gluten 176/07	13.25	13.25	13.25
Wheat gluten 225/07	13.25	13.25	13.25
Wheat starch 143/07	19.86	19.87	19.83
Fish oil [1]	11.80	11.80	11.80
Vitamin mix [2]	2.00	2.00	2.00
Mineral mix [3]	0.60	0.60	0.60
Betafine (Choline chloride)[4]	0.40	0.40	0.40
Inositol [5]	0.03	0.03	0.03
KH_2PO_4 (22.5 %)	1.28	1.28	1.28
NaH_2PO_4 (22.5 %)	1.28	1.28	1.28
AA mix full[6]	14.22	0	0
AA mix Lys-[7]	0	14.21	0
AA mix Lys+[8]	0	0	14.25
Taurine	1.43	1.43	1.43
Proximate composition (%)			
Dry matter	90.70	92.20	91.90
Crude Protein	45.40	45.60	45.80
Crude Fat	14.40	15.30	14.70
Ash	5.00	5.00	4.90

[a] NorSeaOil O1/07, Norsildmel, Norway
[b] Provided per kg of feed: vitamin D3, 3,000 I.E.; vitamin E, 160 mg; thiamin, 20 mg; riboflavin, 30 mg; pyridoxine–HCl, 25 mg; vitamin C, 200 mg; calcium pantothenate, 60 mg; biotin. 1 mg; folic acid, 10 mg; niacin, 200 mg; vitamin B12, 0.05 mg; menadion bisulphite, 20 mg

[c] Provided per kg of feed: magnesium, 56 mg; potassium, 450 mg; zinc, 90 mg; iron, 56 mg; manganese, 11 mg; copper, 5.6 mg
[d] Betafin BCR, Finnsugar Bioproducts, Finland
[e] Danisco Animal Nutrition, Finland
[f] AA mix full provided in g/100 g: Asn, 9.12; Gln, 13.30; Ser, 5.34; Gly, 7.67; His, 2.23; Arg, 6.79; Thr, 4.37; Ala, 6.50; Pro, 4.46; Tyr, 2.43; Val, 4.95; Met, 3.20; Ile, 4.46; Leu, 7.57; Phe, 4.08; Lys, 9.72; Cys, 1.65; Trp, 2.17
[g]AA mix Lys− provided in g/100 g: Asn, 10.12; Gln, 14.76; Ser, 5.92; Gly, 8.51; His, 2.48; Arg, 7.54; Thr, 4.85; Ala, 7.22; Pro, 4.95; Tyr, 2.69; Val, 5.49; Met, 3.55; Ile, 4.95; Leu, 8.40; Phe, 4.52; Cys, 1.83; Trp, 2.20
[h]AA mix Lys+ provided in g/100 g: Asn, 6.99; Gln, 10.19; Ser, 4.09; Gly, 5.88; His, 1.71; Arg, 5.21; Thr, 4.40; Ala, 4.98; Pro, 3.42; Tyr, 1.86; Val, 3.79; Met, 2.45; Ile, 3.42; Leu, 5.80; Phe, 3.12; Lys, 29.26; Cys, 1.26; Trp, 2.15

Table 6.2. Amino acid composition of the three experimental diets (previously published in Gómez-Requeni et al., 2011)

Amino acid (g.kg^{-1} diet)	**Control**	**Lys(-)**	**Lys(+)**
Asn	3.39	3.29	3.05
Gln	9.26	8.87	8.70
Hyp	0.12	0.12	0.12
Ser	2.28	2.18	2.09
Gly	2.6	2.50	2.28
His	0.97	0.98	0.89
Arg	2.56	2.54	2.24
Thr	1.66	1.56	1.64
Ala	2.74	2.74	2.45
Pro	3.18	3.22	3.08
Tyr	1.48	1.22	1.24
Val	2.13	2.17	2.02
Met	1.25	1.26	1.18
Ile	1.95	2.01	1.91
Leu	4.11	4.24	3.94
Phe	2.14	2.24	2.04
Lys	**2.47**	**1.34**	**4.63**
Cys	0.76	0.76	0.71
Trp	0.52	0.61	0.59

At 49 dpf, after overnight fasting, fish were killed by pithing after moderate anaesthesia (3-aminobenzoic acid ethyl ester, MS-222; 50 μg mL−1) and pictures were taken with a digital camera of every fish for fork length measurements, using the UTHSCSA Image-Tool

software (University of Texas, Health Science Center, San Antonio, TX, USA). Skeletal muscle tissue from the trunk epaxial region of 4 randomly selected fish from each tank (16 per dietary treatment) was excised on ice and immediately stored at −80°C pending protein extraction. Comparative 2D-DIGE analysis of the trunk myotomal muscle was performed only between the two most extreme conditions [Lys(−) and Lys(+)].

6.3.2. Feed composition analysis

Crude proximate composition and total AA analyses were performed by the Analytical Laboratory of Nofima AS in Bergen, Norway, as previously described (Gómez-Requeni et al., 2011). Briefly, crude protein (N×6.25) was determined by the Kjeldahl method (ISO 5983-1997), moisture (ISO 6496-1999) and ash (ISO 5984-2002) were determined gravimetrically, and total lipid was determined according to Bligh and Dyer (1959). For total AA profile determination, samples were hydrolysed in 6M HCl for 22 h at 110°C and analysed by HPLC using a fluorescence technique for detection (Cohen and Michaud, 1993).

6.3.3. Protein extraction, purification and fluorescent labelling

After pooling all muscle tissue from zebrafish belonging to the same tank, proteins were extracted in 1:7.5 w/v lysis buffer (7M urea, 2 M thiourea, 4 % CHAPS, 30 mM Tris) with 1 % v/v protease inhibitor cocktail (Sigma-Aldrich), by 6×5 s sonication on ice followed by centrifugation at 18,000×g twice for 5 min at 4°C. Non-protein contaminants were removed from the protein extracts using the ReadyPrep 2-D Cleanup Kit (Bio-Rad). Precipitated proteins were resuspended in the lysis buffer. The pH of the protein extract was adjusted on ice to 8.5 by addition of the appropriate volume of dilute NaOH. All protein quantifications were performed using Quick StartTM Bradford Protein Assay (Bio-Rad). For DIGE minimal labelling, 50 μg of muscle protein was labelled with 400 pmol of fluorescent amine reactive cyanine dyes freshly dissolved in anhydrous dimethyl formamide following the manufacturer's recommended protocol (GE Healthcare, Uppsala). Labelling was performed on ice for 30 min in the dark and quenched with 1 mM Lys for 10 min. Two replicates per dietary treatment were labelledwithCy3 and twowith Cy5 to normalise for

label differences while an internal control consisting of equal quantities of protein from all replicates was labelled with Cy2.

6.3.4. Two-dimensional gel electrophoresis

For gel electrophoretic separation, 50 µg of muscle protein of one replicate from each dietary treatment plus 50 µg of internal standard were diluted in Rehydratation Buffer (Bio-

Rad) to a final volume of 200 µL and loaded overnight on to an 11 cm Immobiline™ Drystrip pH 4–7 (GE Healthcare), by passive rehydration. In the first dimension, proteins were isoelectric focused using an Ettan™IPGphor™ 3 isoelectric focusing unit (GE Healthcare), at 20°C for a total of 23,000 Vh at maximum 50 µA per strip. After the first dimension, strips were equilibrated (cysteine sulfhydryl groups reduced then carbamidomethylated) using standard Bio-Rad reducing (2 % w/v DTT) and alkylating (2.8 % w/v iodoacetamide) buffers (6M urea, 20 % v/v glycerol, 2 % w/v SDS and 0.5 M Tris–HCl pH 8.8), 30 min each step, at room temperature.

The strips were transferred on to 13.3×8.7 cm 12 % Bis–Tris Criterion™ XT Precast Gels (Bio-Rad) and proteins separated in a second dimension by SDS-PAGE, using a MOPS-based running buffer (50 mM MOPS, 50 mM Tris, 0.1 w/v SDS, 1 mM EDTA). The electrophoresis proceeded at 180 V in two Criterion Cell (Bio-Rad) linked to the same PowerPac™ Universal power supply (Bio-Rad).

6.3.5. Image and data analyses

After electrophoresis, gels were immediately scanned with a Typhoon Trio™ Variable Mode Imager (GE Healthcare, Uppsala) using a resolution of 100 µm and PMT value set to ensure that maximum pixel intensity was below saturation. Image analysis was performed using the Differential In-gel Analysis (DIA) and Biological Variation Analysis (BVA) modules of DeCyder v.7 software (GE Healthcare). A reference gel was automatically selected by the software using the default settings and based on an internal standard image. To be considered valid, any given spot had to be present in more than 2 replicates per dietary treatment. Spot detection, background subtraction, warping, matching, and normalisation were all set at the default settings of the software. Where possible,

unmatched spots were edited on each multiplex group based on a 3D view of the spot, normalisation was restored, and the reference gels were updated. Protein spots considered for identification showed significant (Student's t test for independent samples, p value<0.05) differences in average normalised volume of 1.2-fold or more, between treatments. In addition, exploratory multivariate analysis by principal component analysis (PCA), using the in-built function, with mean centring and standard deviation scaling on protein, as well as clustering of the gels based on relative abundance of protein spots and according to correlation-based metrics, was performed as a complement to the Student's t test, using the Extended Data Analysis (EDA) module of DeCyder software v.7 (GE Healthcare).

6.3.6. Protein identification

To identify the differentially expressed proteins, preparative gels were run prior to spot excision following the same protocol as described above except that fluorescent labelling was not performed, each gel was loaded with either 300 or 500 µg proteins from mixed samples and Colloidal Coomassie Blue G250 staining (Neuhoff et al., 1988) was used. Proteins of interest were excised and subjected to in-gel trypsin digestion. Briefly, the spots were successively washed with washing solution [25 mM NH_4HCO_3 in 50 % v/v H_2O/acetonitrile (ACN)]. The proteins were submitted to a reduction and alkylation step using 10 mM DTT at 56°C for 45 min, followed by 55 mM iodoacetamide in the dark at room temperature for 45 min. Finally, gel pieces were thoroughly washed as before and dried in a SpeedVac Concentrator 5301 (Eppendorf, Germany). The dried gel spots were rehydrated by addition of digestion buffer (40 mM NH_4HCO_3 and 5 ng/µl of trypsin (Trypsin Gold, MS grade, Promega) during 30 min incubation on ice and digestion was performed overnight at 37°C. Following tryptic digestion, the supernatant was collected and 1 % trifluoroacetic acid (TFA) was added to the gel plugs, followed by 60 % ACN/0.1 % TFA in order to extract the peptides more efficiently. Both these extracts were combined with the first supernatant and these samples were dried again.

The tryptic peptides were desalted and further concentrated using small disks of C18 3 M Empore Disks (Varian A.B., Solna) placed into 10 µl pipet tips and were eluted with saturated matrix solution (α-cyano-4-hydrozy-cinnamic acid (12 mg/ml, Bruker Daltonics,

Bremen) in 60 % ACN/0.2 % TFA) onto the target plate. Matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS; Autoflex and Ultraflex, Bruker Daltonics) and MALDI with MS/MS (Ultraflex with a LIFT module, Bruker Daltonics) was used for mass analyses of the peptide mixtures. The spectra were externally calibrated using Peptide Calibration Standard II (Bruker Daltonics, Bremen), while the internal calibration was performed with the trypsin autolytic products. FlexAnalysis 3.0 (Bruker Daltonics, Bremen) was used to create the peak list and Bio-Tools 3.0 (Bruker Daltonics, Bremen) was used for interpretation of MS and MS/MS spectra, and proteins were identified by peptide mass fingerprinting (PMF) via the database search program MASCOT (http://www.matrixscience.com), using the *Danio rerio* NCBInr protein database, extended to the Chordata NCBInr protein database when no reliable identification was possible in the D. rerio database. MS/MS analysis and repeated MASCOT database searches of a minimum of two precursor ions recognised in the PMF search were performed to confirm the PMF-based protein identification, whenever possible. It was assumed that the peptides were monoisotopic, cysteine residues were carbamidomethylated and methionine residues possibly oxidised. The fingerprinting method allowed for a maximum of one missed tryptic cleavage per protein. The maximum deviation permitted in matching the parent ion/fragment ion mass values was 70 ppm/0.8 Da, respectively. Following the database searches, the MOWSE score, number of peptide matches, sequence coverage, molecular weight and pI value were used to evaluate the results.

6.4. Results

During the 16 days feeding trial, juvenile zebrafish (*D. rerio*) readily accepted the experimental diets and maintained normal behaviour independent of dietary treatment, with no mortality recorded. The average initial and final fork length were not significantly different among treatments (one-way ANOVA followed by post hoc Tukey's test, p value < 0.05). However, the growth rates (expressed as mm day^{-1}) were significantly lower in fish fed with diet Lys(–) (0.23±0.01 mm day^{-1}) compared to the control group (0.27±0.01 mm day^{-1}) and the Lys(+) group (0.29±0.01 mm day^{-1}), which had the highest growth rate (Gómez-Requeni et al., 2011).

Comparative 2D-DIGE analysis of the trunk myotomal muscle, using the BVA module of DeCyder software (GE Healthcare) applied to the resulting gel images, each one representing a biological quadruplicate of each experimental condition, enabled the detection and quantification of 527±11 (mean±S.E.M.) protein spots across all spot maps, covering a molecular mass range of ca. 10 to 150 kDa and pI values between 4 and 7 (Fig. 6.1.).

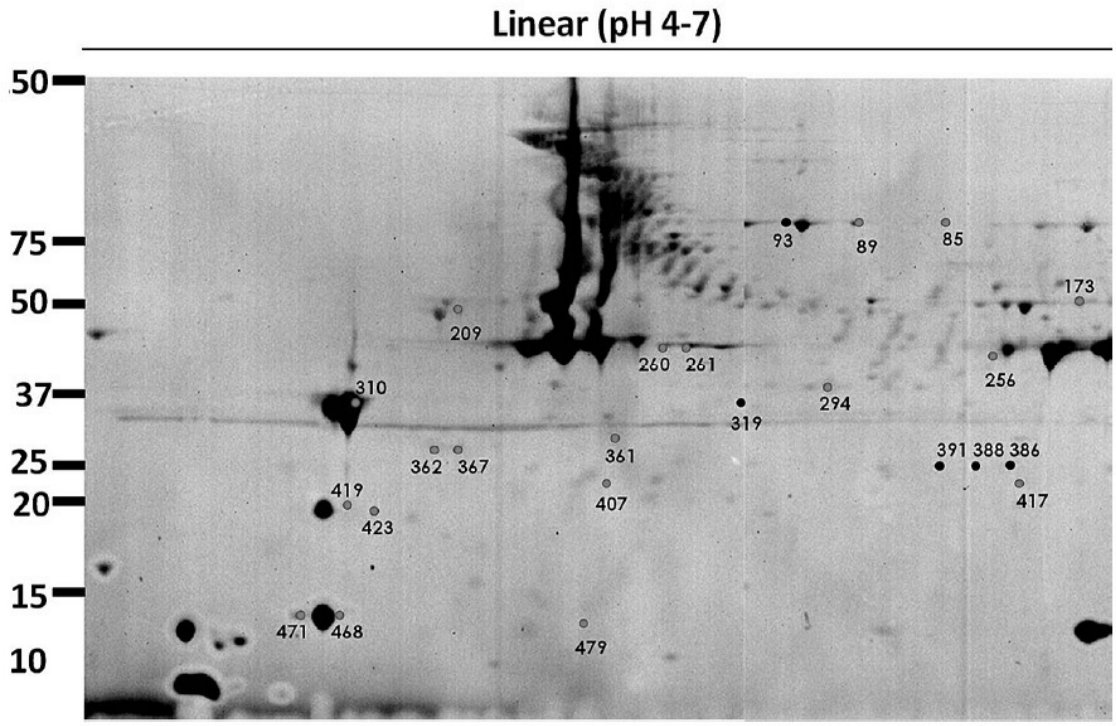

Figure 6.1. 2-D PAGE image of 300 µg skeletal muscle protein (whole extraction) from a mix of zebrafish fed lysine deficient and lysine enriched experimental diets, performed on 11 cm ImmobilineTM Drystrip pH 4–7 (GE Healthcare) and 13.3×8.7 cm 12 % Bis–Tris Criterion™ XT Precast Gels (Bio-Rad), and stained with Colloidal Coomassie Blue, G-250. Numbered spots represent significantly differentially expressed protein spots ($p<0.05$, Student's t test; $|\text{fold-change}|>1.2$) between treatments, selected for sequencing. Light grey circles are positively identified spots. Black circles are unidentified protein spots.

A PCA resulted in the separation of samples according to dietary treatment, with 40 % of all variation explained along the first principal component (PC1; Fig. 6.2.(a)). Univariate analysis of the normalised protein quantity using a Student's t test revealed 52 protein spots

with significantly altered expression due to dietary Lys content, 30 spots being under-expressed and the remaining over-expressed in Lys(+) (|fold-change|>1.2; $p<0.05$). Application of PCA to this refined set of variables reduced spurious variations, PC1 then accounting for 62 % of the total variance (Fig. 6.2.(b)).

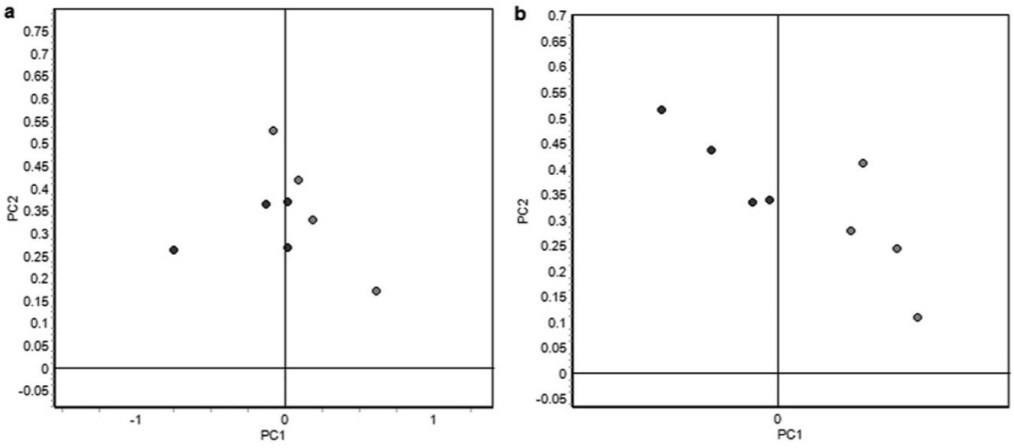

Figure 6.2. Principal component analysis biplot, with mean centring and standard deviation scaling on protein, obtained (a) from original data set and (b) after removal of spurious variables. Samples from juvenile zebrafish fed the Lys(+) or the Lys(−) diet are labelled in light grey and black, respectively. (a) The first principal component (PC1) accounted for 40 % of the total variance and PC2 for 31 %. (b) PC1 accounted for 62 % of the total variance and PC2 for 21 %.

A hierarchical cluster analysis (by the complete linkage method and using a distance metric based on Pearson's correlation) confirmed the information provided by the PCA projections, showing that spot maps from the same treatment were more similar, according to the chosen criterion (Fig. 6.3.), thus indicating that Lys content in the diet of juvenile zebrafish affected trunk myotomal muscle protein expression.

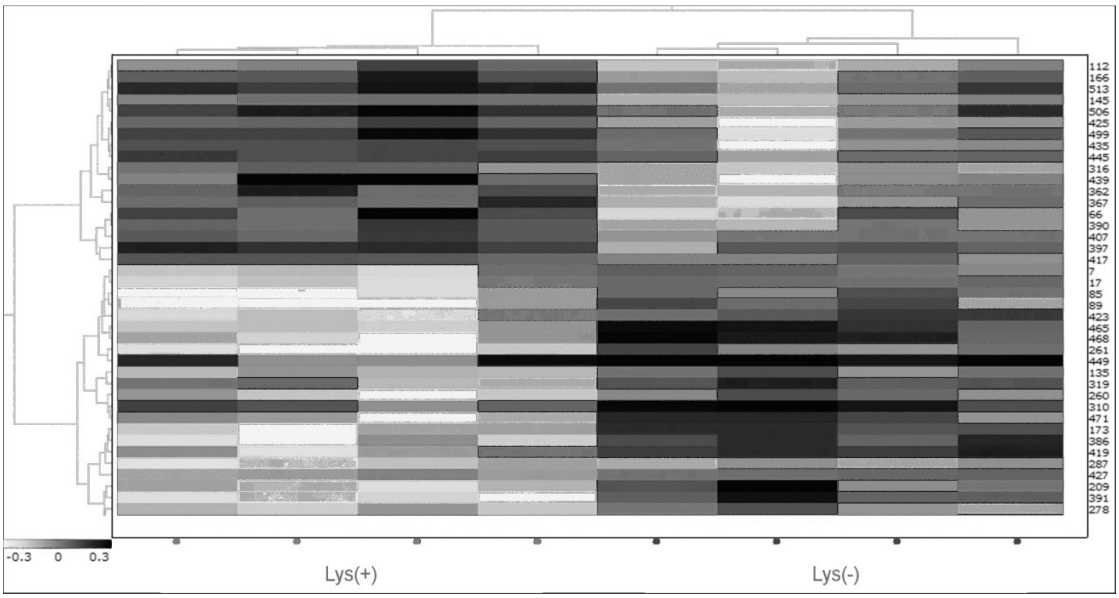

Figure 6.3. Heat map showing relative abundance of identified proteins for all samples. Spots were grouped using Euclidian distance by agglomerative hierarchical clustering (complete linkage method). Only spots present in more than 80 % of spot maps and with p value lower than 0.05 (Student's t test) were included. Light shades indicate a lower than average expression of protein spots and dark shades indicate a higher than average expression. Samples from Lys(+) treatment are labelled in light grey and from Lys(−) treatment in black. Numbers refer to spot IDs.

Of the protein spots whose expression was significantly affected by dietary Lys content, 24 could be excised and subjected to in-gel tryptic digestion, 18 of which were successfully identified, corresponding to 11 proteins (Table 6.3.). Two additional protein spots (spots 93 and 423) could be reliably identified, though their MASCOT result was not significant. The remaining 4 protein spots could not be identified (Fig. 6.1.), mainly due to a low abundance in the gels or possibly an inadequate protein digestion.

Table 6.3. Protein spots with significantly altered abundance between muscle from Lys(−) and Lys(+) zebrafish.

Spot #	Protein identification	Accession #[a]	Fold change[b]	S.C. (M.P.M.)[c]	pI/MW(kDa)		Score (PMF)[d]	P.M.[e]	Best P.M. p-value[f]
					Observed	Theoretical			
Proteins increased in abundance in fish fed Lys(-) diet									
85	myosin-binding protein H-like	gi|153945848	1.77	20 (11)	6.35/79.50	5.39/57.57	(84)	n.a.	n.a.
89	myosin-binding protein H-like	gi|153945848	1.96	30 (17)	6.20/75.69	5.39/57.57	82	3	KPGNFDGGVYSCR 7.94E-07
260	actin, alpha 1b, skeletal muscle	gi|70778800	1.55	35 (10)	5.66/41.95	5.29/42.20	(79)	n.a.	n.a.
261	actin, alpha, cardiac muscle 1b	gi|28277651	1.90	40 (11)	5.72/41.95	5.23/42.3	63	2	QEYDEAGPSIVHR 3.16E07
294	fast skeletal myosin heavy chain 4	gi|33088009	1.44	34 (12)	6.15/34.46	5.70/40.88	42	1	DAQLHLDDAVR 6.31E-05
310	tropomyosin alpha-1 chain	gi|18859505	1.57	62 (26)	4.78/32.01	4.70/32.76	68	2	KLVIVEGELER 3.16E-06
419	fast skeletal myosin alkali light chain 1	gi|41053385	1.72	60 (9)	4.72/19.57	4.63/20.98	238	4	ATYDDYVEGLR 5.01E-08
423	fast skeletal myosin alkali light chain 1	gi|41053385	1.74	29 (6)	4.80/19.10	4.63/20.98	28	1	EAFLLFDR 3.98E-03
468	myosin, light polypeptide 2, skeletal muscle	gi|18859049	2.26	95 (24)	4.72/11.68	4.68/18.97	259	3	NICYVITHGEEKEE 2.00E-12
471	myosin, light polypeptide 2, skeletal muscle	gi|18859049	1.73	85 (9)	4.60/11.68	4.68/18.97	326	4	NICYVITHGEEKEE 2.00E-12
173	beta-enolase	gi|47551317	1.95	43 (14)	6.84/52.34	6.25/47.84	(99)	n.a.	n.a.

Spot #	Protein identification	Accession #[a]	Fold change[b]	S.C. (M.P.M.)[c]	pI/kDa		Score (PMF)[d]	P.M.[e]	Best P.M. p-value[f]
					Observed	Theoretical			
209	mitochondrial ATP synthase beta subunit-like	gi|66773080	1.90	48 (27)	5.08/48.62	5.25/55.08	194	3	IPVGPETLGR 5.01E-07
Proteins decreased in abundance in fish fed Lys(-) diet									
256	fast skeletal myosin heavy polypeptide 1	gi|8698685	1.49	38 (22)	6.55/38.97	5.52/48.69	128	2	DAQLHLDDAVR 2.00E-10
367	actin, alpha, cardiac muscle 1b	gi|28277651	1.46	25 (7)	5.04/26.95	5.23/42.30	119	2	SYELPDGQVITIGN ER 1.58E-11
407	actin, alpha 1b, skeletal muscle	gi|70778800	1.22	28 (10)	5.46/22.69	5.29/42.20	46	1	GYSFVTTAER 2.00E-05
479	actin, alpha 1b, skeletal muscle	gi|70778800	2.00	32 (13)	5.38/11.12	5.29/42.20	59	1	AGFAGDDAPR 1.26E-06
361	F-actin-capping protein subunit beta	gi|41053959	1.37	31 (9)	5.51/27.62	5.70/30.95	80	2	KLEVEANNAFDQY R 2.00E-07
362	apolipoprotein A-I precursor	gi|18858281	1.43	64 (27)	5.00/27.62	5.06/30.24	32	1	IAPHTQDLQTR 6.31E-04
417	Pdlim7 protein	gi|45709024	1.20	50 (12)	6.62/21.60	6.97/23.51	77	3	LEGPACFIPNDR 2.51E-06

[a] NCBInr RefSeq accession number.

[b] Fold-change of protein abundance between treatments.

[c] Percentage of sequence coverage and number of mass peaks matched to sequence.

[d] Ion score obtained in MSMS ion search (significant when above 31, $P < 0.05$). When no reliable MSMS data available, the protein score from PMF is given (significant when above 59, $P < 0.05$).

[e] Number of significant peptide matches in MSMS ion search (ion score > 31; E-value < 0.05).

[f] p-value was calculated as $10^{-0.1 \times \text{score}}$, where "score" is the ion score of the best matched peptide.

Sixteen spots matched proteins involved in the cytoskeletal network and the contractile apparatus of skeletal muscle, 11 of which were more abundant in Lys(−) fish [myosin-binding protein H-like (MyBP-H, spots 85, 89 and 93), fast skeletal myosin heavy chain (myhc4, spot 294), fast skeletal myosin light chains (myl1, spots 419 and 423; mylz2, spots 468 and 471), tropomyosin alpha 1 chain (tpma, spot 310) and alpha actin (spots 260 and 261)] and 5 of which were less abundant [fast skeletal muscle myosin heavy chain (myhz1, spot 256), F-actin-capping protein subunit beta (capzb, spot 361) and alpha actin fragments (spots 367, 407 and 479)] (Table 6.3.; Fig.s 6.1. and 6.3.). Myl1 from *D. rerio* matched the peptide fingerprint for spot 423, although the scores were not significant (Table 3.). However, this is likely to be a correct identification because it is consistent with a nearby spot (spot 419; Fig. 6.1.) matching best to this protein, with which spot 423 shows many mass peaks and matched peptides in common, the mass difference between these being lower than the mass accuracy of the instrument. An equal similarity was found between spot 93 and spots 85 and 89, though no conclusive results could be obtained from the PMF search using the database search program MASCOT. This spot [p*I* = 6; MW = 75.69 kDa; 1.64 times more expressed in Lys(−)] is probably also MyBP-H, given that it is in a "spot train" (Fig. 6.1.) and has many tryptic peptides in common with spots 85 and 89, the difference between the same value mass peaks of these spots being lower than the mass accuracy of the instrument.

In addition to structural proteins, significant increases in abundance in the skeletal muscle of Lys(−) zebrafish were also found for proteins involved in energy metabolism [betaenolase (glycolytic pathway; spot 173) and mitochondrial ATP synthase beta subunit-like (oxidative phosphorylation; spot 209)] (Fig.s 6.1. and 3; Table 3).

Finally, apolipoprotein A-I precursor (Apo A-I, lipid transport; spot 362) and a protein belonging to the PDZLIM protein family, Pdlim7 (spot 417) whose members are reported to act as signal mediators in various cellular processes such as migration, signal transduction and differentiation, showed an increased abundance in the trunk myotomal muscle of juvenile zebrafish fed the Lys enriched diet, Lys(+).

6.5. Discussion

Feeding juvenile zebrafish a diet with a Lys content below the estimated requirements of the fish, Lys(−), was accompanied by a depressed growth rate, whereas an excess of Lys above dietary requirements, Lys(+), did not have any apparent deleterious effect relative to the control group but did not lead to a significantly improved fish growth. This underlines the importance of meeting the dietary Lys requirements of animals for proper growth and development, as previously discussed in detail in Gómez-Requeni et al. (2011). So as to further explore the possible pathways of actuation of Lys on growth, it was sought to take a closer look at the proteome of myotomal muscle. This tissue typically composes 40–75% of teleost total mass and it is known that an increasing body size in fish is directly linked to the growth of myotomal muscle (Zimmerman and Lowery, 1999).

The wide and dynamic expression range of proteins within the skeletal muscle makes it impossible to separate and detect all protein species with currently available techniques (Ohlendieck, 2011). Therefore, the presence of certain classes of proteins such as integral proteins, components with an extreme p*I* value, certain post-translational modifications or very high molecular mass will be underestimated. Provided one accepts this, the highly sensitive DIGE technique can be considered appropriate for the study of soluble proteins in the molecular range of 10–200 kDa (Doran et al. 2009). The broad distribution of skeletal muscle protein spots represented in the expression profiles of this study agreed with published results from international databanks (e.g., Bosworth et al. 2005; Lu et al., 2010; Veiseth-Kent et al., 2010) and the effect of dietary Lys content was notable on protein expression.

Several myosin chains were identified among the differentially expressed myofibrillar proteins from the muscle extracts. Skeletal muscle myosin is composed of two heavy chains (MyHC), two essential light chains, and two regulatory light chains. The relative content of MyHC and myosin light chains (MyLC) in muscle determines its contractile properties, including ATPase activity, maximum shortening speed and force-velocity relationship. In teleosts, numerous distinct isoforms of the myosin chains are known, their expression being dependent on muscle fibre type (slow/red, fast/white or intermediate; Rowlerson et al., 1985), location in the body and developmental stage (Silva et al., 2010a; Chu et al., 2011),

and influenced by environmental, particularly temperature (Johnston and Temple, 2002), nutritional (Hevrøy et al., 2006; Campos et al., 2010) and hormonal factors (Moutou et al., 2001), with a great variety among taxa. In contrast to other vertebrates, slow and fast muscle fibres of teleosts form physically discrete layers with slow fibres residing subcutaneously around the horizontal septum and fast fibres constituting the majority of the myotomal musculature (van Raamsdonk et al., 1982). Thus it is not surprising that all myosin chain isoforms identified in this study belonged to the fast skeletal muscle. In Lys(+) fish, the abundance of myhc4, mylz2 and myl1 was decreased, whilst myhz1 had a higher abundance in the gels of these juveniles with the highest growth rate. One possible hypothesis for this observation could be linked to a different growth pattern between these two groups.

Myosin chain isoform expression has been shown to also be associated with fish muscle growth pattern, that is, the unique combination of hypertrophy (expansion of previously formed fibres) and mosaic hyperplasia (input of new fibres on the surface of developed muscle fibres) by which fish skeletal muscle grows throughout their life. For example, Ennion et al. (1995) mention a novel MyHC isoform in carp, FG2, expressed transiently during the differentiation of satellite cells into muscle fibres. They thus suggest a role of this specific MyHC in muscle cell recruitment characteristic of hyperplasic growth. In European sea bass (*Dicentrarchus labrax*), early temperature increase influenced positively larval and early postlarval muscle growth dynamics, as well as postlarval activation of the satellite cells population, as assessed by the different mATPase activities which in turn reflect different myosin isoforms expression (López-Albors et al., 2003). In another study on how embryonic temperature can affect muscle growth pattern in later stages, MyHC genes were down-regulated in zebrafish characterised by a hypertrophic phenotype with no more or little fibre recruitment occurring; specifically, one transcript of MyHC1 (Myhz1(2)) was highly expressed in nascent muscle fibres but not large diameter fibres. That is, it was up-regulated in the smaller fish with growth still marked by mosaic hyperplasia (Johnston et al., 2009). More recently, in transgenic zebrafish characterised by a muscle-specific over-expression of the growth hormone receptor (GHR) gene and showing increased mosaic hyperplasic growth of white skeletal muscle, a down-regulation of mylz2, myhc4 and skeletal alpha actin was observed (Figueiredo et al., 2011). Mosaic

hyperplasia has been shown to be responsible for an approximately fivefold increase in fast fibre number in zebrafish (Johnston et al., 2009) and will normally contribute to attaining a large adult size. It could be that the fish fed the Lys(+) diet were characterised by a growth pattern dominated by mosaic hyperplasia whereas those fed the low-Lys diet were either in a less advanced stage of white skeletal muscle development or had a paused growth due to possible energy/indispensable AA (IAA) saving mechanisms. It has been seen that changes in dietary nutrients are able to induce changes in the relative contribution to muscle growth by hyperplasia and hypertrophy. For instance, in juvenile blackspot seabream (*Pagellus bogaraveo*), high-protein diets favoured muscle hyperplasia (Silva et al., 2009) and in European sea bass larvae medium to high dietary vitamin D content favoured hypertrophic growth. This high dietary vitamin D level also stimulated mosaic hyperplasia of white skeletal muscle fibres, showing that the larvae fed the medium to high vitamin D diets were more advanced in muscle development at 44 DPH than those fed the low concentration (Alami-Durante et al., 2011).

However, this hypothesis should be treated with caution as for one, the MyHC isoforms identified in this study are in fact both products of the myosin heavy polypeptide 1.1 (myhz1.1) gene. Further genomic research is necessary to confirm the exact nature of the different isoforms expressed, particularly as it is not clear whether the MyHC isoforms expressed in this study exist as transcribed and translated products or represent proteolytic fragments of the original isoforms. For another, alpha actin, myhc4 and mylz2 codify for main structural muscle proteins and are thus associated to hypertrophy by Figueiredo et al. (2011). In their study, the transgenic zebrafish with enhanced hyperplasic growth showed no size difference relative to the non-transgenic fish at sampling time. It is therefore realistic to suppose the overall expression of these "hypertrophic" genes was comparatively lower in the transgenic fish. However, in the present study, the Lys(−) fish were smaller, therefore the overall expression of these structural muscle proteins might not have been higher than in the Lys(+) fish. This is because these latter fish would be expected to have larger fibres as well as mosaic hyperplasia, like the seabass larvae fed the high vitamin D content in the study of Alami-Durante et al. (2011). It would be interesting and indeed crucial to perform a histological characterization of the muscle tissue to further explore this hypothesis.

Another hypothesis for the increased abundance of MyLCs in the gels from Lys(−) zebrafish is that the white skeletal muscle was suffering from degradation. Indeed, in a study on cod skeletal muscle proteome, Kjærsgård and Jessen (2003) mention that an increase in MyLCs could be indicative of myosin degradation that leads to an increase in solubility of the MyLCs. This could explain their increased abundance observed in the Lys(−) gels. When investigating the effect of Lys supplementation on plasma protein expression of broiler chicks, Corzo et al. (2005) noticed than an individual dietary AA deficiency does not necessarily translate into decreasing protein synthesis proportionate to body weight, but rather significant changes may be occurring within the types of proteins undergoing anabolism. Fish swiftly use proteins as oxidative substrates and proteins have traditionally been considered to be the usual gluconeogenic precursors during starvation in fish. In a situation of unfavourable feeding condition, switching on ATP-generating catabolic pathways whilst inhibiting ATP-consuming biosynthetic pathways such as protein synthesis is a promising means to limit energy expenditures (Salem et al., 2010). The Lys deficient diet probably represented an unbalanced dietary AA composition, which has been shown to lead to poor protein retention and, subsequently, a higher portion of protein being directed towards catabolic processes (Gómez-Requeni et al., 2003). It could be that this dietary deficiency induced a higher degradation of sarcomeric proteins to provide the Lys required for more vital compounds. For example, when a diet rich in soybean meal and low indispensable to dispensable amino acid (IAA/DAA) ratio was ingested by rainbow trout (*Oncorhynchus mykiss*), the decreased fish somatic growth and protein accretion was accompanied by higher nitrogen excretion rates and lower protein retention, linked to enhanced protein degradation (Alami-Durante et al., 2010). To confirm this hypothesis, it would be necessary to analyse parameters allowing identification of a link with protein turnover.

The disassembly of the myofibrillar apparatus associated with increased catabolism of this protein fraction of the myotomal muscle in Lys(−) zebrafish could also explain the over-expression of MyBP -H. This protein is a thick filament component mainly found in fast skeletal muscle and is suspected to have a role in the assembly of the myofibrillar apparatus (Jagoe et al. 2002). Therefore the disassembly could result in its higher solubility.

Muscle-specific beta-enolase, which catalyses the ninth step of glycolysis, and the beta subunit of mitochondrial ATP synthase, a component of the respiratory chain, were both over-expressed in the fish fed the Lys(−) diet. Given that these zebrafish showed a depressed growth rate, this over-expression may reflect a more important allocation of energy to processes other than muscle tissue accretion. Possibly, these fish showed a higher feeding activity in order to try to compensate for the Lys deficiency of the diet. The over-expression of alpha tropomyosin in these fish could also point to a higher activity since in concert with troponins, tropomyosin plays an important role in regulating the interaction of myosin with the actin filament for muscle contraction (Winder and Ayscough, 2005).

The under-expression of the apolipoprotein A-I (Apo AI) precursor in the muscle of Lys(−) treatment supports the increased energy demand scenario. Apo A-I is the main protein constituent of high density lipoproteins (HDL), which are known to play a role in reverse cholesterol transport from tissues to the liver and in lipid metabolism. This protein's expression decreased in the muscle of stressed or chronically immune-stimulated fish (Johansen et al., 2006; Veiseth-Kent et al., 2010), suggesting that cholesterol was not being transported normally and supporting the idea of increased energy demand and decreased need for energy storage in these fish. A more extensive analysis of energy and fatty acid metabolism during Lys deficiency in fish is necessary to further examine these ideas.

The over-expression of Apo A-I in Lys(+) fish could also indicate that these larger zebrafish are close to begin allocating energy for reproduction, as discussed previously (Gómez-Requeni et al., 2011). Additionally, this over-expression might agree with the increased growth rate observed in these zebrafish. Indeed, Ferrari et al. (1990) reported that the expression of the gene encoding Apo A-I was tightly linked to the expression of a muscle-differentiated phenotype and specific to multinucleated myotubes in cultured chicken myogenic cells. They hypothesised that Apo A-I might function in maintaining water-insoluble lipids dissolved in the cells and interstitial fluid for the formation of myotubes, which involves remodelling of membrane components and *de novo* synthesis of sarcoplasmic reticulum.

Interestingly, the F-actin capping protein beta subunit (Capzb), showed an increased abundance in the zebrafish with the highest growth rate [Lys(+)]. Capzb binds in a calcium-

independent manner to the fast growing ends of actin filaments thereby blocking the exchange of actin subunits at these ends. It has been reported that cell movement increased in cells with increased capping protein and decreased in cells with decreased capping (Hug et al., 1995). These authors hypothesised that the function of this protein is to keep actin filaments short so that the meshwork is isotropic and pliable. It was seen that an inhibition of this capping protein's ability to bind actin dramatically altered the actin organisation in muscle cells undergoing myofibrillogenesis.

Finally, the protein Pdlim7 or Enigma was found to be over-expressed in the Lys(+) fish. This protein also alters actin dynamics when localised to actin filaments (Krcmery et al., 2010, references therein) though much remains to be elucidated on its possible roles via cytoskeletal remodelling. Pre-existing knowledge agrees with its increased expression in zebrafish fed the Lys-enriched diet showing an enhanced growth rate and particularly, to a situation of increased cell proliferation and new fibre recruitment characteristic of mosaic hyperplasia. Indeed, Pdlim7 was recently considered to play a role in muscle development and regeneration in a study of cattle skeletal muscle (Moreno-Sánchez et al., 2010). Moreover, recent work in zebrafish revealed that Pdlim7 is required for proper skeletal muscle development and maintenance (Camarata et al., 2010; Krcmery et al., 2010). Pdlim7 has been associated with insulin-like growth factor receptor and insulin receptor signalling (Wu et al., 1996; Durick et al., 1998) and cell proliferation (Jung et al., 2010) and could thus possibly play a role in the signaling pathways controlling protein synthesis and gene transcription in skeletal muscle. These directly influence muscle growth processes, that is, protein synthesis and hypertrophic growth or activation of myogenic regulatory factors responsible for myoblast proliferation (e.g. Figueiredo et al., 2011) and are known to be directly affected by dietary AA supply (Rehfeldt et al., 2010; Johnston et al., 2011, references therein).

6.6. Final considerations

This study presents the effects of a dietary Lys imbalance on the growth of zebrafish reared under best practice laboratory protocols. Having previously characterised the whole body proteome profile of zebrafish at the end of the growth period to obtain knowledge about the metabolic consequences of an essential AA dietary imbalance (Gómez-Requeni et al.,

2011), this study allowed a deeper insight into how a dietary Lys level affects the white skeletal trunk muscle of juvenile zebrafish.

A decrease in growth rate in fish fed with a Lys deficient diet was found when compared to fish fed with diets with a theoretically non-deficient Lys profile. The analysis of the muscle proteome generally agreed with main conclusions drawn from the whole-body proteome (Gómez-Requeni et al., 2011). Specifically, the decreased growth rate observed in the Lys(−) pointed to an enhanced catabolism of the myofibrillar apparatus induced by the dietary AA deficiency or imbalance. This deficiency could in turn have induced a higher feeding activity in these fish for compensatory reasons, and perturbed normal transport of cholesterol and energy storage.

On the other hand, the higher growth rate observed in fish fed an enriched-Lys diet seems to reflect an active hyperplasic growth and enhanced skeletal muscle development. This would make Lys one of the factors that might regulate the process of hyperplasia in fish skeletal muscle and thus be of particular importance to optimise muscle growth in aquaculture.

The usefulness of screening molecular approaches such as 2D-proteomics, as a hypothesis-generating approach in modern biology, is highlighted to further establish the consequences of dietary shifts on the regulation and plasticity of metabolic pathways. Particularly, the role of Pdlim7 in nutritionally regulated muscle growth should be investigated.

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Chapter 7. General Discussion

7.1. Effects of dietary fish protein hydrolysates (FPH) on growth and nitrogen utilisation

The results from the research performed in the framework of this PhD study corroborate with the general premise that a partial substitution of intact dietary protein by its hydrolysed products can improve performance in fish larval stages but not juveniles because the larval digestive tract capacity to process dietary protein is limited by its proteolytic capacity but not its absorptive capacity. Indeed, a moderate dietary inclusion level of FPH improved growth in white seabream (*Diplodus sargus*) larvae (Chapter 2) and gilthead seabream (*Sparus aurata*) larvae (Chapter 3 and 4) but did not result in growth improvement of juvenile zebrafish, even when compared to juveniles fed an intact protein-based diet (Chapter 5). To our best knowledge, this is the first time this type of study is performed with white seabream, a promising, relatively new species in Mediterranean aquaculture. Our results show that the dietary inclusion level of FPH for these larvae could be up to 20 % (DM basis), composed of 25 % di- and tripeptides and free amino acids (FAA) and 75 % larger oligo- and polypeptides, though the more common use of a 10 % dietary inclusion level of the commercially available FPH CPSP-90 also gave positive growth results. These inclusion levels were also the most appropriate in terms of survival, nitrogen utilisation and growth for gilthead seabream larvae (Chapter 3, diet CONT – 10 % CPSP-90; Chapter 4, diet LPH – 20% FPH with similar peptide size distribution profile as in diet High, Chapter 2).

Additionally, observations from previous studies (e.g. Cahu et al., 1999, Kolkovski and Tandler, 2000, Carvalho et al., 2004, Zhang et al., 2006) were further confirmed concerning the detrimental effects on growth, survival and general nitrogen utilisation that a dietary excess of short peptides and FAA can have on the organism, both in larvae (Chapters 2 and 3) and in zebrafish juveniles (Chapter 5). This is generally believed to be due to the fact that elevated luminal peptide and FAA concentrations can not only saturate the intestinal transporters mechanisms, but also the large pulse of incoming FAA in circulation (post-absorption) may overload the metabolic handling systems, leading to increased AA

oxidation and reduced dietary protein retention and anabolic efficiency (e.g. Cahu et al., 1999; Tonheim et al., 2005; Zhang et al., 2006; Aragão et al., 2007).

Furthermore, results obtained from this research re-emphasize the importance of considering multiple factors involved when attempting to compare across studies and make claims regarding the efficacy of dietary FPH. Particularly, their origin, their concentration in the feed and the molecular structure of their peptidic chains. For instance, in Chapter 3, survival of *S. aurata* larvae fed a feed containing a 20 % inclusion level of CPSP-90 was detrimentally affected whereas a 20 % inclusion level of another FPH with a similar peptide size distribution profile and similar total AA profile led to improved growth and unaffected survival in both white seabream and gilthead seabream larvae (Chapters 2 (diet High) and 4 (diet LPH). The generally higher total crude protein content of the feeds in Chapter 3 compared to the other two studies possibly exacerbated the negative effects caused by a dietary nitrogen overloading. Also, in Chapters 4 and 5, though diet HPH did not differ with diet LPH concerning the short peptides (MW < 500 Da) content (Chapter 4) and diet NF in Chapter 5 was not the richest in FAA among dietary treatments, growth of fish fed these feeds (diets HPH and NF) was negatively affected. These feeds contained more oligo- and polypetides (diet HPH) or short peptides (MW < 1000 Da; diet NF) than the other dietary treatments being compared and this stresses the importance of considering the overall balance of the dietary peptide profile and not each molecular size fraction individually. This is mainly because protein digestion products are transported from the intestinal lumen into the enterocytes not only in the form of FAA, by a large variety of brush border membrane AA transporters, as in the form of di- and tripeptides, by the brush border solute carrier 15 (SLC15) membrane proteins, such as peptide transporter 1 (PEPT1) (Verri et al., 2003; 2011; Amberg et al., 2008; Terova et al., 2009; Ostaszewska et al., 2010; Rønnestad et al., 2010). Additionally, there is also evidence for the direct absorption of certain soluble intact proteins and polypeptides by pinocytosis in the digestive tract of fish (Govoni et al., 1986; McLean et al., 1990).

So in Chapter 4, increasing the inclusion level of the larger size-fractions of hydrolysed protein under the assumption that this would increase nitrogen bioavailability in the larval feed without providing an immediate elevated influx of AA into the system, did not result

in improved growth but quite the contrary; this was further put in evidence by the compensatory growth observed in these larvae when they stopped receiving diet HPH. This is an interesting result for future consideration when formulating larval feed.

Finally, as well said in Gisbert et al. (2012), the evaluation of an experimental feed with modified formulation in terms of potential growth enhancement has many constrains when working with marine fish larvae, since growth ultimately depends on several variables not only related to the characteristics of the microfeed, but also with the larval feeding behavior and digestive capacities (Aragão et al., 2007); these aspects are especially relevant in the case of dietary proteins, peptides, and amino acids (Kvåle et al., 2007), and the final response can be restricted by the deficiency in other nutrients (Aragão et al., 2007; Yúfera et al., 2011). To cite but a few examples, nutrient leaching during feeding is a large problem with regard to being able to supply fish larvae with water-soluble nutrients such as protein hydrolysates, vitamins and minerals (López-Alvarado et al., 1994; Yúfera et al., 2002; Kvåle et al., 2006); some PH and FAA are known to play a role as attractants (Kolkovski et al., 1997; Cahu and Zambonino-Infante, 2001; Koven et al., 2001), whilst on the contrary, some methods of preparation of PH can result in bitter tasting peptides (Hevrøy et al., 2005); the dietary ratio of digestible protein to digestible energy has been shown to influence how PH are metabolized (Aguirre et al., 1995), making it particularly important to control the dietary composition in all ingredients when testing different FPH contents.

Although we assume that leaching of soluble protein, peptides and FAA into the experimental tanks was minimal in our studies because larvae and juvenile zebrafish ingested the feed almost immediately (personal observation), we cannot totally reject the hypothesis that leaching occurred whilst preparing the larval feeds in Chapter 4, because though these were prepared in the same way as feeds in Chapter 3, their final crude protein content was slightly lower than the formulated one. Controlling for leaching of soluble nutrients is currently one of the main bottlenecks in microfeed production and studies on nutritional requirements of larvae are strongly dependent on the resolution of this problem.

Concerning the role of dietary PH in the palatability of the inert feeds, it is bound to affect ingestion rate by larvae, and this in turn is known to affect protein retention (Govoni et al.,

1986; Øie et al., 1997; Kvåle et al., 2007; Rønnestad et al., 2007). Therefore having a method to measure intake and ingestion rate of the experimental feed by larvae would greatly contribute to further understand the results obtained in our experiments with seabream larvae and similar studies. Furthermore, a method that enables measuring feed intake and ingestion rate *in vivo*, such as that recently developed by Hansen et al. (2009) using incorporation of fluorescent microspheres into the microfeed, could also provide information on apparent protein digestibility without having recourse to expensive and hazardous radiolabelling of inert feeds; but it would still need to be coupled to a method such as the *Artemia* radiolabelling performed in our studies because it would not provide information on metabolism and nutrient utilisation (Conceição et al., 2007b).

Another interesting aspect to mention is that dietary FPH have been shown to affect the immune system and health status of fish, not only by the production of bioactive peptides with immune-stimulating and antibacterial properties during the hydrolysing procedure, but also because such substrates as PH can boost bacterial proliferation in the gut (Bøgwald et al., 1996; Gildberg et al., 1996; Carvalho et al., 2004; Daoud et al., 2005; Liang et al., 2006; Kotzamanis et al., 2007; Hermannsdóttir et al., 2009). In larval stages, mortality can be high due to opportunistic pathogens, and the early weaning of larvae with compound feeds may cause bacterial growth, especially that of *Vibrio* species (Gatesoupe, 1997). Severe and moderate mortalities caused by vibriosis outbreaks have been reported in sea bream and sea bass larvae (Grisez et al., 1997). This could possibly explain the higher mortality observed in larvae fed the 20 % dietary inclusion level of CPSP in Chapter 3 and therefore, as also suggested by Kotzamanis et al. (2007), it will be particularly important to monitor the intestinal microbiota of larvae in future studies with dietary protein hydrolysates.

7.2. Comparative proteomics as a tool to further explore how dietary FPH affect growth and nitrogen utilisation

One of the main aims of the research done in the framework of this PhD was to work with two-dimensional electrophoresis and matrix-assisted laser desorption/ionisation tandem time-of-flight mass spectrometry (2-DE and MALDI-ToF-ToF MS; gel-based proteomics) as an exploratory approach to gain a better understanding of the metabolic adjustments and

the molecular mechanisms affected by dietary nitrogen sources, particularly those involved in muscle growth and development in seabream larvae and zebrafish juveniles. To our best knowledge, this work provides the largest whole-body proteome map for teleost larvae identified to date, and the first for white seabream (*Diplodus sargus*) larvae (Chapter 2). Furthermore, the use of this novel approach enabled to visualise a response pattern to dietary treatment that was not detectable by growth and survival analysis solely (Chapters 2, 3 and 5).

Notably, in Chapter 3, the increased abundance in myosin chains from the skeletal muscle of larvae fed a 10 % dietary inclusion level of CPSP-90 agreed with the elevated dietary nitrogen retention and growth potential reported for these larvae despite the fact that they were generally smaller than those fed a 20 % CPSP-90 inclusion level at the end of the trial. In Chapter 5, the main growth difference was observed between juvenile zebrafish fed an intact protein-based feed (diet FM) and those whose feed was richest in short peptides and FAA (diet NF), with not such a clear distinction among the other two experimental feeds containing FPH with varying peptide size distribution profiles (diets UF and FH). However, the proteomic analysis of the skeletal muscle sarcoplasmic fraction revealed that each dietary treatment elicited a specific response; the feed with the most balanced peptide size distribution profile (diet UF) being closest to the intact protein-based feed (diet FM) in terms of effects on the sarcoplasmic proteome, and the feed richest in short peptides and FAA (diet NF) eliciting a distinct response from all other dietary treatments. This further demonstrated how the peptide size distribution profile of the dietary PH affects muscle protein metabolism.

Lastly, in Chapter 2, the principal growth difference was observed between larvae fed the same inclusion level of the same FPH that differed only in peptide size distribution profile (diets High and Low). However, the main difference in protein expression profile lay between larvae fed a 20 % inclusion level of FPH, independent of peptide protein profile (diets High and Low) and a 10 % dietary inclusion level of the commercially available FPH, CPSP-90 (Sopropêche, France). This once more evinces the importance of considering not only the dietary inclusion level of PH, but their origin and the molecular structure of the constituent peptides when attempting to evaluate the efficacy of dietary

FPH. Hence in the following chapters, only the inclusion level of the same FPH (Chapter 3), then different peptide size distribution profiles of the same FPH (Chapters 4 and 5) were compared. Also, although the identification of some developmental stage-related proteins (e.g. myosin light chain isoforms, type II keratins) corroborated with the growth patterns observed in larvae of white seabream (Chapter 2) and the increased abundance of proteins belonging to the energetic metabolism, along with glutamine synthase and antiquitin, pointed to increased AA catabolism in larvae fed the diet richest in short peptides and FAA (diet Low, Chapter 2), further interpretation of results was encumbered by the complexity of analysing the proteome of an entire organism. This is the reason why in following chapters, with the aim of focusing on skeletal muscle tissue, only the carcass was analysed in studies with larvae (Chapters 3 and 4) and then, by using juvenile zebrafish, it was possible to narrow down to just trunk epaxial skeletal muscle, fractionated (Chapter 5) or whole (Chapter 6). The recent improvement of the 2-DE method, 2D-DIGE (fluorescence difference gel electrophoresis), was used in these following chapters because it is a highly sensitive staining technique that gives the possibility to run several samples on a single gel and also significantly improves handling of gel-to-gel variability through the use of a common reference channel across all gels (Unlü et al., 1997; Minden et al. 2009).

Not only did the proteomics approach allow visualising a response pattern to dietary treatment not detectable just by growth and survival analysis, but in general, the proteins whose expression was found to be affected by dietary treatment either complemented growth and nitrogen utilisation results or helped to further interpret these. As for instance in Chapter 3, where the increased abundance of myosin chains in the carcass proteome of *S. aurata* larvae fed a 10 % dietary inclusion level of CPSP-90 corroborated with the elevated nitrogen retention observed for these larvae, pointing to enhanced growth potential in these larvae at time of sampling, despite their final average length and weight being generally smaller than that of larvae fed a 20 % inclusion level of CPSP-90. Or in Chapter 4, in which the enhanced expression of proteins related to sarcomeric degradation and energy metabolism in the carcass of *S. aurata* larvae fed with a higher dietary inclusion of large FPH (diet HPH) pointed to increased protein catabolism and agreed with the lower growth rate observed for these larvae, despite their nitrogen excretion when fasted and their capacity to digest live prey proteins having been unaffected by dietary treatment at the

developmental stage studied. Also in Chapter 5, the sarcoplasmic proteome indicated a reduced energetic capacity of the skeletal muscle in juvenile zebrafish with the lowest growth rates and fed the feed richest in short peptides and FAA. Finally in Chapter 6, expression of sarcomeric proteins and components of the energetic metabolism pointed to enhanced catabolism of the myofibrillar apparatus and possibly higher feeding activity in zebrafish juveniles receiving a feed deficient in the indispensable AA, lysine, thus corroborating the lower growth rate reported for this group compared with fish receiving a diet theoretically non-deficient in lysine. Moreover, data for protein expression in this latter group supported the enhanced growth and development through skeletal muscle hyperplasia.

Some brief considerations on the muscle proteomics approach used in this thesis should be mentioned here. We focused on the skeletal muscle because it accounts for over half of fishes' body mass, usually the majority of absorbed nutrients are invested in accreting muscle tissue (Mommsen, 2001) and it is characterised by the highest growth rate efficiency of any organ (Houlihan et al., 1995), thus making it an obvious candidate for studying fish growth. However, the wide and dynamic expression range of proteins within the skeletal muscle make it impossible to separate and detect all protein species with currently available biochemical techniques (Ohlendieck, 2011). Using a 2-DE approach, the presence of certain classes of proteins such as integral proteins, components with an extreme p*I* value, certain post-translational modifications or very high molecular weight will be underestimated. However, provided one accepts this, the highly sensitive DIGE technique can be considered appropriate for the study of soluble proteins in the molecular range of 10–200 kDa (Doran et al., 2009). Be that as it may, one still has to deal with the abundant myofibrillar proteins, which comprise more than 60 % of whole protein in skeletal muscle (Sato et al. 2009) and will mask the presence of proteins with lower expression, often more biologically relevant proteins, including those involved in regulatory or signalling pathways (Jarrold et al. 2005). Hence the reason for isolating the sarcoplasmic fraction for proteome analysis, as was done in Chapter 5. This approach enabled us to detect that the skeletal muscle phosphotransfer networks might be involved in this tissue's response to a dietary excess of short peptides and FAA in juvenile zebrafish, and looking back, this might have happened also in Chapter 4, from the expression of

proteins belonging to the phosphotransfer network in carcass of *S. aurata* larvae fed diet HPH. Though this, together with the depressed growth rate observed in juveniles, additionally pointed to a possible muscle degradation/stunted muscle growth scenario, no further conclusion could be made in this sense due to lack of information on the myofibrillar proteins. In a parallel study (Chapter 6), this time testing for growth effects of dietary content in an AA, lysine, known to affect muscle growth in possibly all life stages and many animals, we analysed the skeletal muscle proteome (whole). The results indicated that dietary lysine content might actually be involved in growth pattern regulation of zebrafish juvenile trunk epaxial white muscle and that a dietary lysine deficiency can lead to muscle degradation. However, once more, it is difficult to be sure if the change of abundance of myosin chains and other components of the myofibrillar apparatus in the polyacrylamide gels reflected a change of expression in these proteins or a change in their solubility (and thus degradation of the myofibrillar apparatus). So from these two approaches, it becomes clear that, although both the sarcoplasmic fraction and the total proteome can provide important information *per se*, interpretation of the results in a reliable way would be improved by coupling these approaches in one same study.

Finally, using a proteomics approach led to the discovery of some potentially interesting candidate proteins or metabolic and/or regulatory pathways that warrant further investigation. Namely, apolipoprotein A-IV, recently reported to play a role in appetite and satiety regulation in rodent models (Simon et al., 2011) and expressed in white seabream larvae fed diet High (Chapter 2), for which the highest growth rate was reported. Also, the expression of many sub-species of proteins belonging to the phosphotransfer network in skeletal muscle of juvenile zebrafish fed diet NF (Chapter 5), for which the lowest growth rate was reported, and possible effects of dietary FPH on phosphotransfer networks and/or nucleotide metabolism in skeletal muscle of fish. And last but not least, the protein Enigma/Pdlim7 and its possibly role in muscle growth by hyperplasia, together with the potential role of dietary lysine in the regulation of white skeletal muscle growth pattern in juvenile zebrafish (Chapter 6).

7.3. Brief considerations on the applicability of zebrafish (*Danio rerio*) as a model for dietary nitrogen utilisation and growth

Zebrafish (*Danio rerio*) is a reputed model organism that has been extensively used in fields such as developmental biology, disease and immunity, drug discovery, toxicology and physiology. More recently, zebrafish have also served as a model for nutrition and growth studies in aquacultured fish (e.g. Dahm and Geisler, 2006; De-Santis and Jerry 2007; Drew et al., 2008; Jury et al., 2008; Gómez-Requeni et al., 2010; 2011; Enyu and Shu-Chien, 2011; Figueiredo et al., 2011; Ulloa et al., 2011). The fact that they possess the most developed genomic program compared to that of any aquacultured fish to date, are easy to maintain and breed, have short generation time and produce a large number of offspring, make this species a candidate for evaluating a large number of dietary factors in far less time and at lower cost; and the effect of these dietary factors can be more easily studied at a molecular level in zebrafish than in other currently aquacultured fish. Indeed, it was much easier to reliably identify differentially expressed proteins in our trials with zebrafish juveniles than with seabream larvae. This is because protein identifications by mass spectrometry depend not only on the quality of spectra, but also on the quality of the sequence database used. Thus, by using zebrafish, we were able to pinpoint some proteins and molecular pathways worth further investigation, such as the effect of a dietary excess of short peptides and FAA on the phosphotransfer networks and/or nucleotide metabolism in white skeletal muscle of juvenile fish, or the possible role of lysine on the regulation of growth pattern in the trunk epaxial white muscle of juvenile fish.

However, since teleosts represent a large taxonomic group with a large diversity of physiological, morphological and ecological adaptations, it is very important to take the unique physiological characteristics of each model species selected for study into consideration. Thus, though zebrafish has recently been validated as a good model for muscle growth in aquaculture, by also showing an indeterminate growth pattern more typical for larger fish (Patterson et al., 2008; Johnston et al., 2009), perhaps for analyzing the dietary effects of FPH on growth of seabream, a marine species that reaches a larger size and for which there is substantial coverage on genome/EST information, such as the Atlantic salmon (*Salmo salar*), might provide a better model. Additionally, given current development in DNA (and RNA) sequencing technology (Mukhopadhyay, 2009), it is expected that the coverage of genome/EST information on commercially relevant species

will be greatly expanded, certainly improving the quality of MS-based peptide/protein identification methods (Rodrigues et al., 2012).

Additionally, zebrafish, like other cyprinids, are functionally stomachless, as are many marine teleost larvae. However, any further comparison is not recommended since the developmental stage and intestinal tract maturation in each case is most probably entirely different. The maturation of the intestinal tract, together with other developmental and metamorphic processes are actively occuring in most marine teleost altricial larvae whereas juvenile zebrafish are already presumably fully developed in terms of gut maturation and capacity to digest and utilise protein from their natural diet (which, being omnivores and generalists, consists in a wide variety of food items). Additionally, further research on the distribution of digestive enzymes, their activities, and functional roles in zebrafish is necessary. Nevertheless, given the particularities of early development and ease with which they can be reared or maintained and with which we can intervene at the cellular or molecular levels within the whole organism, zebrafish remains a model of choice for basic research (Kaushik et al., 2011).

Interestingly, though it was not possible to work with the larval stages of zebrafish in this research due to an unsuccessful earlier weaning onto our experimental feeds, this has very recently been achieved by Kaushik et al. (2011), who reared zebrafish from first-feeding using an inert feed. This, together with progress on establishing a standard formula diet for this species (Penglase et al. 2012), opens a great potential for the application of zebrafish as a model for nutritional studies in larvae.

Chapter 8. Conclusion and Future Perspectives

8.1. Conclusions

The purpose of this research was to further understand how the inclusion of a more digestible form of protein (protein hydrolysates) into the feeds for fish early-life stages affects nitrogen utilisation, by employing a comparative gel-based proteomics approach and focusing more particularly on skeletal muscle growth. Additionally, this approach and focus were further applied for studying the effects of dietary lysine content on juvenile growth, because this amino acid (AA) is indispensable and often the most limiting in alternative, plant-based, protein sources used in fish feeds.

It was seen that a moderate dietary inclusion level (10 – 20 %, DM basis) of fish protein hydrolysate (FPH) into the larval feed of both white and gilthead seabream can improve larval growth, provided the dietary total crude protein level and content of di- and tripeptides and free AA (FAA) are considered and kept below a threshold level. On the other hand, in the agastric juvenile zebrafish, increasing the solubility and digestibility of dietary protein by providing a 30 % inclusion level (DM basis) of FPH with balanced peptide size distribution profile does not lead to beneficial effects in terms of growth when compared to an intact protein-based feed.

Independent of developmental stage, environmental conditions and species (marine, temperate-water seabream larvae or fresh-water, tropical zebrafish juveniles), providing a dietary excess of short peptides and FAA had detrimental effects on growth and/or survival and general nitrogen utilisation, reflected principally by altered muscle protein turnover with enhanced catabolism. This effect was also induced by a dietary lysine deficiency in juvenile zebrafish, thus corroborating the general observation that dietary AA imbalances in fish lead to reduced anabolic efficiency.

Furthermore, it was seen that a special attention must be given to the molecular structure of the constituent peptides of the dietary FPH and their overall dietary concentration. This was particularly evinced in Chapter 4, where increasing the inclusion level of the larger size-

fractions of hydrolysed protein under the assumption that this would increase nitrogen bioavailability in the larval feed without providing an immediate elevated influx of AA into the system, did not result in improved growth but quite the contrary.

Using comparative proteomics technologies (2-DE/2D-DIGE and MALDI-ToF-ToF MS) was a useful approach since the proteins whose expression was found to be affected by dietary treatment either complemented growth and nitrogen utilisation results or helped to further interpret these. Moreover, the use of this novel approach enabled to visualise a response pattern to dietary treatment that was not detectable by growth and survival analysis solely and that warrant future research. Additionally, the suggestion of specific candidates for further targeted studies underlines the usefulness of this approach for these studies. However, the principal take-home message from this research and inherent to the dynamism and sensitivity of the proteome towards environmental signals and subtle changes in physiological state, is that further studies will greatly benefit from reducing sample complexity (tissue heterogeneity) and obtaining and using as much contextual information as possible (including both classical and/or –omics observations) to improve interpretation of the proteomics results.

8.2. Future Perspectives

Understanding how dietary nitrogen affects growth and development of the early life-stages of fish is of great interest for aquaculture, and for biological sciences in general. Although the research performed within the framework of this PhD contributed to this end, it was faced with many challenges, which are mainly inherent to current technological limitations when working with marine teleost larvae, microfeeds, FPH and 2-DE proteomics. Future research in this field will depend on improved larval rearing protocols (e.g. optimal water quality control, optimized temperature and photoperiod conditions, species-tailored weaning and feeding strategies) and development of methods for working with these small sized-organisms that are fragile to manipulation, such as improvement of protocols to assess feed intake, feed digestibility, mortality rate, etc. Additionally, technological advances in microfeed production, given the limitations in manipulating the nutritional

composition of live feed, will surely constitute a break-through for future nutritional studies in larvae; namely tackling constraints in developing digestible, well accepted microfeeds with a well defined nutritional composition at the time of ingestion. More specifically, dietary FPH represent a challenge in itself due to their complexity, namely due to the difficulty in controlling their peptidic composition. This in turn influences a multitude of factors because of differences in AA absorption rates among FAA, di- and tripeptides and protein dietary sources. This results in different metabolic handling of the AA and their availability for protein synthesis. Also, the possible effects of peptides on gene expression and other bioactive properties of specific peptides must not be forgotten (knowing that theoretically there are 8400 different di- and tripeptides, through all possible combinations of the 20 AA). And also, the fact that FPH can serve directly as substrates for the gut microflora, some having a direct bioactive effect in the gut epithelial layer and after absorption. Future research on this subject will undoubtedly benefit from technological advances in controlled hydrolysis and screening methods for characterising the peptidic composition and AA profile of the dietary FPH. For example, protein hydrolysates produced by enzymatic treatment (together with temperature, pH and time manipulation) theoretically contain well defined peptide profiles and should be further explored. Additionally, important contributions shall come from the study of AA transporters in larval ontogeny (besides the already more studied enzymatic development and maturation of the digestive system) and from the recent interest in PepT1 and future systematic and continued use of various peptide substrates to study nutritional requirements of fish and effects on growth and immunological state.

Finally, the proteomics approach is useful since it provides an un-targeted, discovery-orientated approach, thus providing an unbiased holistic measure of an organism's response to an external stimulus. From the techniques applied and results obtained with this research, and due to the dynamic and complex nature of the proteome, we conclude that future research will benefit from approaches that help to determine the exact location in the muscle tissue of the proteins whose expression was affected by dietary treatment and also that characterise the sub-species identified, namely in terms of post-translational modifications (PTMs). This is because the location and modified state, such as

phosphorylation status, are determinant factors of protein function. Additionally, it is crucial to obtain as much contextual information as possible, including both classical observations, such as the nitrogen excretion and tracer studies used in this research, intestinal enzyme expression for gut maturation, or histological analyses for studying muscle growth patterns, and/or –omics observations to support the proteomics observations. Analysing the proteome of other organs, as for example the liver and gut, would further contribute to understanding the general picture. To resolve the issue of the presence of proteins involved in signalling and regulatory pathways being masked by highly abundant myofibrillar proteins and those belonging to the intermediate metabolism in skeletal muscle, possible additional sub-cellular fractionation (e.g. nuclear, mitochondrial, cytosolic) could be applied. This is currently complicated for studies where the biological material is limited, such as when working with tiny fish larvae.

However, as technology continuously develops, new approaches have a good potential to becoming invaluable tools for this type of research. Namely MS imaging, which involves the direct digestion of histological samples fixed to a suitable support, followed by direct MS/MS. To cite Silva et al. (2013), this is the equivalent of using immunocytochemistry methods, but in a nonspecific way, providing information on a key variable: location. Along with the development of computational methods, MS imaging will provide useful information on proteome distributions over any tissue, distinguish between sub-populations of cells with different proteome profiles and pinpoint exactly where proteome changes occur. This will undoubtedly greatly increase our understanding of biological processes which are intrinsically morphological and relevant for aquaculture research, such as larval development or muscle growth.

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