UNIVERSIDADE DO ALGARVE

Using proteomic technologies to understand the impact of stress and nutritional factors on fish metabolism, welfare and quality

Tomé Pereira de Azevedo Santos Silva

Tese

Doutoramento em Ciências da Vida, do Mar, da Terra e do Ambiente

Ramo de Ciências Biológicas

(Especialidade em Biologia Molecular)

Trabalho efetuado sob a orientação de:

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

Doutor Jorge Proença Dias, Centro de Ciências do Mar do Algarve (CCMAR), Faro, Portugal.

Doutor Flemming Jessen, DTU Fødevareinstituttet (DTU Food), Kgs. Lyngby, Dinamarca.

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Declaração de autoria do trabalho

Declaro ser o autor 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

As the scale of aquacultural activities increases, we increasingly face challenges, not only in terms of sustainability, but also regarding issues like animal welfare and product quality. Within these research fields, proteomics (along with other -omics) is establishing itself as an invaluable tool for an untargeted assessment of the impact of exogenous stimuli on fish metabolism.

This dissertation describes work developed in this area, where proteomic technologies were used to understand the impact of stress and nutritional factors on fish metabolism, welfare and quality. Gilthead seabream was chosen as the main biological model, due to its high importance in the Portuguese aquaculture sector, with Senegalese sole as secondary model. These studies were focused on both skeletal muscle and liver tissue.

Results demonstrated that a reproducible proteomic analysis of the sarcoplasmic fraction of gilthead seabream muscle is feasible, with pre-slaughter stress inducing a clear hastening of the transition between the pre-rigor and post-rigor profiles. Comparatively, glycerol supplementation (as a tool to modulate muscle glycogen reserves) was shown to have a more subtle impact on the sarcoplasmic proteome of gilthead seabream, with results generally suggesting adaptive effects associated with this dietary factor. Hepatic proteome analyses revealed a high sensitivity towards both stress and dietary factors, with stress factors again displaying a broader impact on protein expression. In these experiments, the proteomic responses to sources of stress displayed specificities that depend on both the biological model and the type/duration of stressor, despite some degree of overlap in terms of the affected pathways.

Concluding, despite the apparent resilience of gilthead seabream quality attributes in regards to nutritional and/or stress factors, proteome analysis revealed that these factors have an impact on both muscle and liver metabolism, being likely to affect post-mortem muscle degradation dynamics. The suggestion of specific candidates for further targeted studies underlines the usefulness of proteomics in this context.

Keywords: aquaculture, gilthead seabream, proteomics, stress, quality, nutrition

Resumo

A aquacultura, apesar de se tratar de uma atividade humana que já conta com milénios de existência, entrou numa fase de crescimento surpreendente a partir da década de 60 do século passado, sendo que atualmente ultrapassa já o sector pesqueiro como principal fornecedor de produtos alimentares derivados de organismos aquáticos. Por outro lado, embora este crescimento prodigioso tenha acarretado mais-valias no que respeita ao custo e acessibilidade destes produtos alimentares (de elevada qualidade nutricional) às populações em geral, cada vez mais nos deparamos com diversos desafios, não só ao nível da sustentabilidade a longo-prazo destas atividades, como ao nível de questões relacionadas com o bem-estar animal e com a qualidade (nutricional e organolética) do produto final. Neste contexto, a proteómica (assim como a genómica, transcriptómica e metabolómica) tem-se estabelecido nos últimos anos como uma valiosa ferramenta na avaliação holística do impacto de fatores extrínsecos sobre os processos celulares dos peixes.

O trabalho descrito nesta dissertação foca-se precisamente na aplicação de ferramentas proteómicas para o estudo do impacto do *stress* (a curto e a longo-prazo) no metabolismo dos peixes e processos de decomposição *post-mortem* (e, consequentemente, o seu efeito a nível do bem-estar dos peixes e da qualidade do produto final), assim como a interação de fatores nutricionais com estes fatores de *stress*. Para o efeito, procurou-se efetuar análises proteómicas ao nível de dois tecidos: por um lado, o músculo esquelético (i.e. o filete), dado que constitui a principal parte comestível do peixe; por outro, o fígado, que é o principal órgão responsável pelo controlo central dos processos metabólicos. Estes estudos foram realizados utilizando a dourada (*Sparus aurata*) como principal modelo animal, dada a sua importância no sector nacional de aquacultura. Para efeitos de comparação, foi ainda efetuado um estudo em linguado (*Solea senegalensis*), visto se tratar também de uma espécie relevante na aquacultura nacional e apresentar particular sensibilidade a fatores de *stress*, quando em cativeiro.

No decorrer deste trabalho, foram realizados vários ensaios focados no tecido muscular da dourada, particularmente no que respeita a fatores imediatamente *pre-mortem* e o seu impacto nos processos de decomposição e, consequentemente, nas propriedades organoléticas do produto final. Os resultados obtidos confirmam a possibilidade de isolar a fração sarcoplasmática do proteoma muscular da dourada de forma reprodutível, dado que a sua aplicação aparentemente não aumenta a quantidade de ruído técnico inerente à análise do

proteoma (Capítulo 2). A aplicação desta metodologia no estudo do *stress* pré-abate demonstrou um forte efeito deste tipo de *stress* pré-mortem no proteoma sarcoplasmático, claramente acelerando a transição entre um perfil pré-rigor e um perfil pós-rigor (Capítulo 3). Para além disso, apesar do impacto de fatores de *stress* sobre o proteoma muscular da dourada ser muito maior e mais abrangente do que o de fatores nutricionais, também se tornou claro que estes últimos (particularmente, o caso da suplementação alimentar com glicerol) podem induzir um efeito positivo ao nível das reservas de energia pré-mortem, podendo portanto constituir um fator mitigante no impacto dos efeitos de fatores de *stress* (Capítulo 4).

Em relação aos ensaios focados na resposta proteómica do fígado face a fatores nutricionais e de *stress*, os resultados geralmente demonstram uma maior sensibilidade (comparado ao tecido muscular) face a estímulos externos, com os fatores de *stress* uma vez mais induzindo um maior impacto no proteoma comparativamente aos fatores nutricionais. Comparando os dois modelos animais utilizados (dourada e linguado), verificou-se que, apesar das semelhanças entre os dois modelos quando sujeitos a fatores de *stress* crónico em termos de vias metabólicas afetadas, existe uma fraca sobreposição em termos de proteínas especificamente afetadas, sugerindo a existência de idiossincrasias inerentes a cada espécie no que toca à sua resposta a fatores de *stress* (Capítulos 5 e 6). De facto, mesmo a aplicação de diferentes fatores de *stress* no mesmo modelo (dourada), em situações experimentais idênticas, demonstra a existência de uma dependência entre o tipo/duração do fator de *stress* aplicado e a resposta proteómica induzida, a nível hepático (Capítulo 5). Finalmente, conseguiu-se demonstrar que os dados obtidos por espectroscopia de infravermelho transmissiva do fígado de dourada fornecem importante informação que é tanto consistente como complementar à informação fornecida por metodologias proteómicas, providenciando um contributo significativo para o estudo do impacto do *stress* térmico sazonal no metabolismo da dourada, assim como para a formulação de dietas especiais fortificadas que providenciem um efeito positivo demonstrável no sentido de mitigar o impacto deste fator de *stress* (Capítulo 7).

Concluindo, este trabalho demonstrou que tanto o *stress* crónico como o *stress* pré-abate induzem efeitos significativos a nível do proteoma muscular e hepático de peixes como a dourada e o linguado. É importante notar também que, em algumas das experiências, se demonstrou o potencial de fatores nutricionais na mitigação de alguns dos efeitos do *stress* induzido (tanto a nível hepático como a nível muscular). Para além disso, apesar da observação de claros efeitos induzidos por fatores nutricionais e de *stress* sobre o proteoma muscular, poucos efeitos foram notados em termos de impacto nas propriedades organoléticas

do produto final (nomeadamente, textura, aroma e aspeto visual) que, apesar de positivo (dado ilustrar a robustez dos traços fenotípicos e propriedades organoléticas da dourada, face a fatores extrínsecos), torna difícil a utilização da dourada como modelo ótimo no estudo das relações entre o bem-estar animal e possíveis efeitos indesejados a nível da qualidade do produto final. Apesar disto, é inegável que estes fatores apresentam um impacto não-negligenciável sobre o bem-estar e o metabolismo dos peixes, facto atestado pelos resultados descritos nesta dissertação. Exemplos específicos de candidatos de estudo interessantes em futuros trabalhos nesta área incluem a proteína DJ-1, a proteína inibidora da Raf cinase (RKIP), a fosfohistidina fosfatase (PHP), entre outras proteínas regulatórias cuja expressão se demonstrou ser afetada por fatores nutricionais e de *stress*. Tudo isto demonstra a utilidade da proteómica no contexto da aquacultura, particularmente na área do bem-estar animal, como uma ferramenta sensível na deteção de sinais de *stress* fisiológico/celular e desvios metabólicos, mesmo quando estes não são aparentes mediante o recurso a técnicas clássicas.

Palavras-chave: aquacultura, dourada, proteómica, *stress*, qualidade, nutrição

Table of Contents

Acknowledgments		3
Summary		5
Resumo		7
Table of Contents		11
Objectives		13
Chapter 1.	Introduction – Proteomics and Aquaculture	15
1.1	Current issues in aquaculture	17
1.2	The concept and importance of fish welfare	19
1.3	The concept and importance of fish quality	22
1.4	Proteomic (and other –omic) technologies	29
1.5	Analytical approaches for an integrative biology	38
Chapter 2.	On the reproducibility of a fractionation procedure for fish muscle proteomics	45
Chapter 3.	Effects of pre-slaughter stress levels on the postmortem sarcoplasmic proteomic profile of gilthead seabream muscle	57
Chapter 4.	Dietary tools to modulate glycogen storage in gilthead seabream muscle: glycerol supplementation	81
Chapter 5.	Characterization of the impact of crowding and repeated handling stress on gilthead seabream liver proteome	113
Chapter 6.	Characterization of the impact of repeated handling stress on Senegalese sole liver proteome	135
Chapter 7.	Integrated proteomic and metabolic profiling of gilthead seabream liver to track interactions between dietary factors and seasonal temperature variations	161
Conclusions and Future Perspectives		195
References		199

Objectives.

The main purpose of my PhD study was to explore and study the impact of short and long-term stress on both fish muscle proteome, to assess their effect on post-mortem muscle/meat conversion and decomposition processes, as well as downstream quality attributes, and hepatic proteome, to assess their effect on pre-mortem fish welfare and metabolic status, using gilthead seabream as main model and Senegalese sole as secondary model. Besides this, focus was given on assessing possible interactions between dietary factors and the biological impact of these stressors. Finally, it was also important to use this untargeted information for, on one hand, the discovery of novel indicators of fish welfare and metabolic/nutritional status, and on the other hand, the assessment of the validity of already-established indicators.

Chapter 1.

Introduction – Aquaculture and Proteomics.

Part of this chapter has been previously published in:

Pedro M. Rodrigues, Tomé S. Silva, Jorge Dias, Flemming Jessen. **Proteomics in aquaculture: Applications and trends.** Journal of Proteomics **2012**, 75 (14), 4325-4345. (doi:10.1016/j.jprot.2012.03.042)

1.1. Current issues in aquaculture

Aquaculture is defined as the farming of aquatic organisms, covering both freshwater and marine fish, along with crustaceans, molluscs and algae. Although this practice has been known to man for millennia, its growth has increased considerably since the 1960's, while the productivity of fisheries has reached a *plateau* in the early 1990's. Currently, aquaculture has already surpassed fisheries in terms of production volume and this trend is unlikely to reverse. In fact, the growth rate in aquaculture production already surpasses the rate of human population growth, which indicates a trend towards an increasingly higher reliance on aquaculture to provide fish products (and other food products derived from aquatic organisms)[1]. In the particular case of Portugal, similarly to other countries in southern Europe, aquaculture of marine species (like gilthead seabream, white seabream, European sea-bass, clams, oysters and flat fish species) is particularly strong, although there is also culture of freshwater species (like eels and salmonids)[2].

While there are obvious positive aspects to this incredible recent growth in aquaculture production (e.g.: reducing dependence/impact on fishery resources; making high nutritional value food products more affordable, improving human nutrition), there are also negative aspects and challenges that concern both farmers, researchers and stakeholders. Looking in broad terms to the type of concerns and research published, we can generally cluster them according to a few subjects (Figure 1.1). These will increasingly become essential topics of research, as the dimension of aquacultural activities increase (along with their importance in human nutrition), in order to overcome the challenges, mitigate the negative aspects and guarantee their long-term (economical, social, environmental, ecological) sustainability.

Some of the research is focused on issues related to "reproduction", mainly at the level of maintaining viable broodstock in captivity, genetic studies, sperm cryopreservation and issues related to sexual maturation, among other subjects (e.g. [3-10]). The issue of sexual maturation can also be seen as part of the "development" cluster, which approaches essential matters like early morphogenesis, metabolic programming, larval digestive ontogeny and the onset of morphological deformations/malformations (e.g. [11-15]). This last topic leads us to another important cluster of research, which deals with "health" issues, such as the study of captivity-specific syndromes, as well as issues related to the prophylaxis and theurapeutics of viral, bacterial and parasitic infections (e.g. [16-25]).

Another field of research concerns the "biotechnology"-related applications of marine organisms, such as their use in bioreactors (e.g. for biofuel production) or for bioremediation purposes (e.g. [26-37]). In this more empirical sense, there is also plenty of research related to the specificities of "production systems", particularly on the development of high-performance intensive culture systems (with water recirculation) and on the optimization of logistic/management practices, but also on the exploration of alternative, more extensive and/or integrated (multi-trophic) aquaculture systems (e.g. [38-49]). These last are also a matter of study in the field of "sustainability", where a big emphasis is put on studying the impact of aquacultural activities on the environment and wild ecosystems (e.g. [50-54]). Also within this subject is the matter of sustainable feeds, which often involves the (at least partial) replacement of traditional marine-based ingredients for plant-based ingredients in production of feeds for aquaculture (e.g. [55-59]).

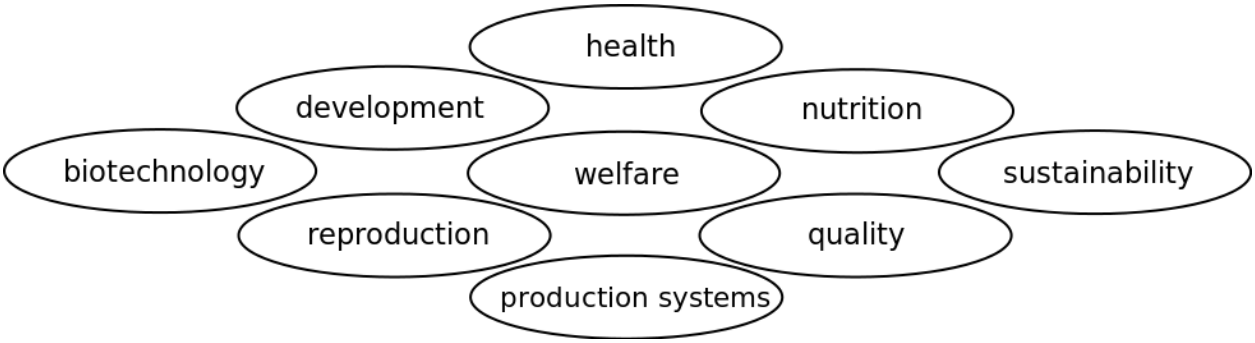

Figure 1.1 – Main (often overlapping) clusters of research within aquaculture.

In the field of "nutrition", there is a wide range of studies, not only on issues related to sustainability and the use of new ingredient sources, but also on the determination of fish nutritional requirements (e.g. [60-63]) , development of optimized feeds for specific developmental stages (e.g. [64-67]), as well as the exploration of the effect of a wide range of supplements and micronutrients (e.g. immune boosters, attractants/phagostimulants, pigments, antioxidants). Finally, there are also the fields of fish "welfare" and "quality", which are further discussed in the succeeding sections, being the main topics approached in this dissertation.

1.2. The concept and importance of fish welfare

Animal welfare is a complex concept that is anything but straightforward to define and measure. In this context, there is not a single commonly-agreed framework and the whole concept within aquaculture has evolved over time, from strictly performance-oriented measures to the current, more feelings-based approaches[68-75]. Here, a commonly cited definition of animal welfare puts it in terms of "freedom from hunger and thirst, discomfort, pain, injury, disease, fear and distress, and the freedom to express normal behaviour"[68]. From this definition, it is quite evident that no easy and single all-encompassing measure of welfare is available.

Aquacultured organisms (and fish in particular) are often subjected to stressful situations, some of which unavoidable, that might compromise their welfare. While, until recently, the most general accepted measure of welfare has been physical health and performance, where a variety of physiological and biochemical measures have been used, more importance is increasingly being given to the observation and characterization of behavioural alterations, along with the discovery and development of novel molecular indicators of metabolic status. An overview of the factors which often impair fish welfare, along with the most used indicators of compromised fish welfare can be seen in Figure 1.2.

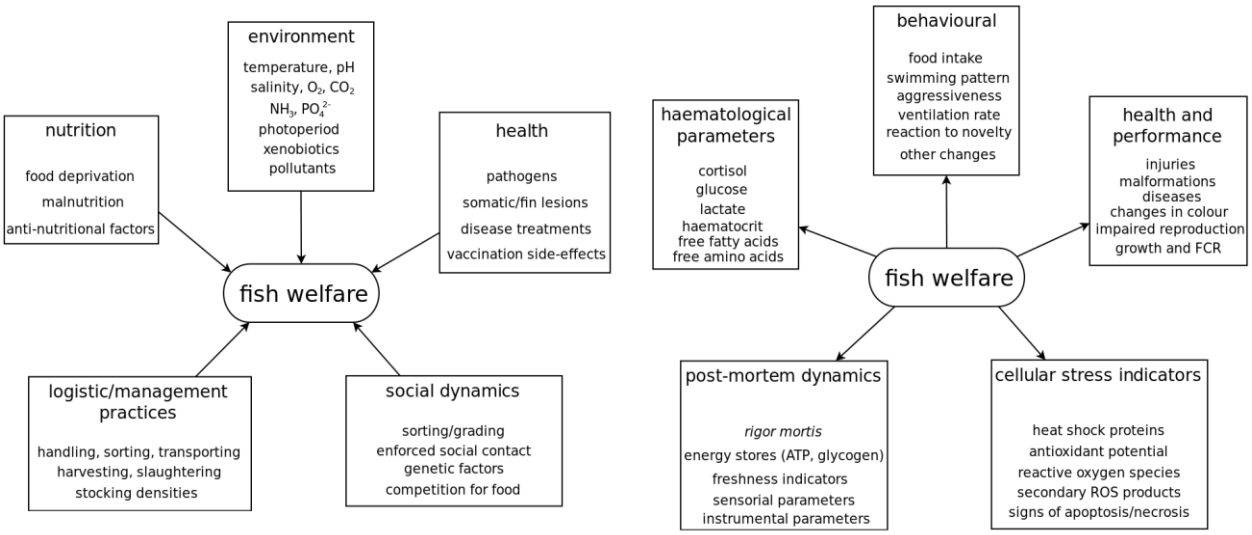

Figure 1.2 – Diagrams showing the main factors affecting fish welfare (on the left), along with the commonly used indicators of impaired fish welfare (on the right).

When fish are subjected to stressors or situations that otherwise compromise their welfare, a series of neurological and endocrine changes occur (dubbed the "primary stress response"), which involve the release of epinephrine and cortisol to the bloodstream. These changes elicit secondary behavioural and metabolic adaptations which improve the short-term capacity of fish to cope with (or otherwise avoid) challenging situations. On the other hand, chronic exposure to sources of stress with consequent repeated activation of the primary stress response has a deleterious effect on fish physiology and health, eventually leading to effects such as anorexia, poor growth performance, immunodepression and impaired reproductive function[76-78]. Also, it is important to remember that aquacultured fish, unlike wild fish, are subjected to unavoidable physical constraints which often undermine the effectiveness of their natural behavioural responses to stress. This obviously implies that aquacultured fish have a reduced capacity to cope with the cumulative effect of stressors through their own means, which underlines the necessity to minimize the occurrence of avoidable sources of stress and/or find ways of mitigating their effect.

The importance of fish welfare in aquaculture is derived not only from pragmatic reasons (i.e. preventing poor fish performance, health and quality), but also legitimate ethical concerns[79], as studies increasingly suggest that fish sense pain similarly to mammals, recollect aversive experiences and are capable of displaying clear preferences[80-87]. This suggests that, despite the particularly underdeveloped neocortex of fish (compared to mammals) and their reduced self-awareness and cognitive abilities, fish can certainly sense noxious stimuli and probably experience, to some level, both pain and anticipatory aversion (i.e. fear).

Currently, the scientific view on the biological adaptability within a changing environment has shifted from the classic 'homeostatic' model, where organisms are seen as continuously striving towards a static (conceptually optimal) state, to an 'allostatic' model, that more accurately accounts for the adaptive and dynamic nature of biological systems[88-89]. This model assumes that understimulation (due to lack of signals and challenges from the environment) can be as detrimental to fish welfare and development as overstimulation. Also, it distinguishes adaptive changes ('eustress') from maladaptive changes ('distress'), where the latter usually occur when the intensity or duration of the challenges overcome the organism's coping capacities. An overview of the relationship between stressors and biological responses within the context of finfish aquaculture can be seen in Figure 1.3.

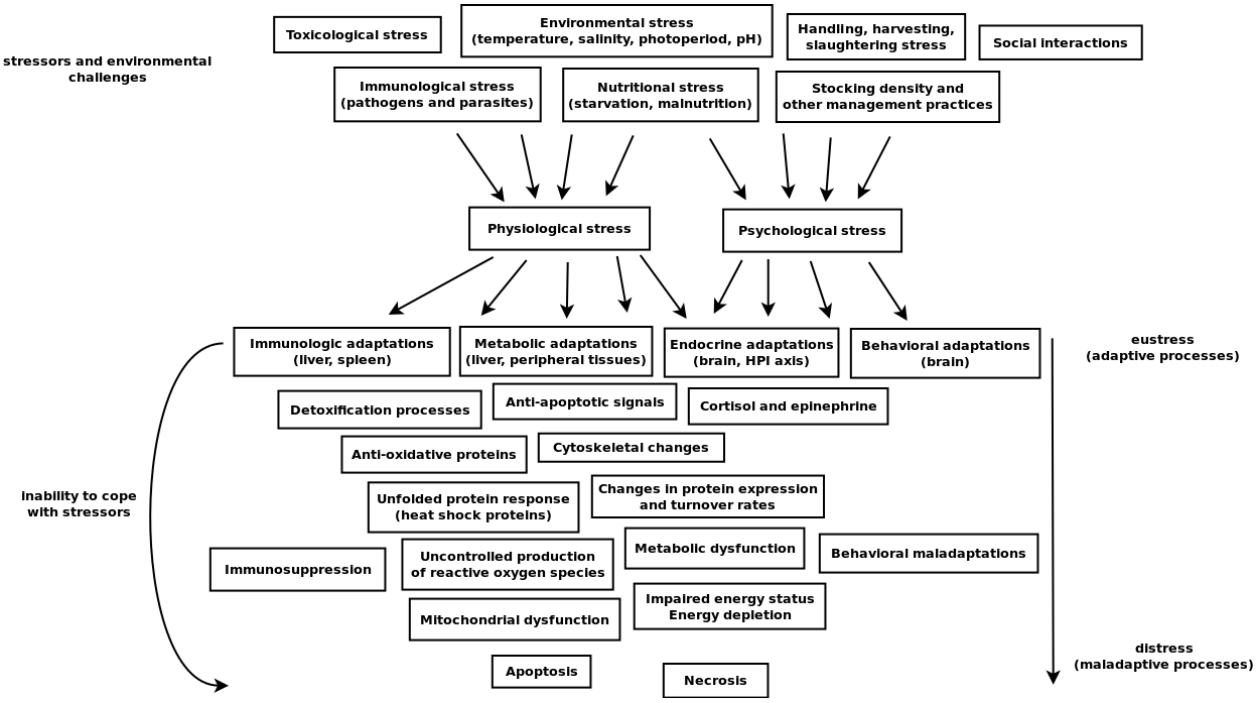

Figure 1.3 – Global overview of the relationship between stressors and physiological/psychological/cellular stress, within an "allostatic" framework.

As already mentioned, the assessment of situations of impaired fish welfare are often based on (besides the immediately obvious secondary and tertiary effects of the response to stress) the measurements of the plasma levels of metabolites associated with stress response, namely cortisol, glucose and lactate. Despite the usefulness of cortisol measurements in the detection of hypothalamic-pituitary-interrenal axis activation, it is clear that this information is not sufficient by itself to assess fish welfare, for several reasons: first, the estimated absolute cortisol levels in plasma should be biased, as blood sampling itself constitutes a source of stress; second, although most fish display a common pattern of increased cortisol levels while exposed to a source of stress, followed by a return to basal levels within hours when the stressor is removed, the basal cortisol level itself is very context-dependent (being affected by factors such as feeding, season, maturation, photoperiod and unknown stressors); and third, both the basal levels of cortisol and the degree of cortisol induction during stressful events are very much species-dependent, displaying a high degree of inter-individual variation (which is somewhat explained through the "bold *vs.* shy" paradigm) and, again, a high degree of context-dependence (the magnitude of cortisol induction depends on factors such as the type and duration of the stressor, as well as whether the source of stress is predictable and/or avoidable)[76, 88, 90-95]. The same type of problems also applies to the plasma levels of glucose

and lactate, being impossible to define reliable reference or threshold values for "stress *vs.* welfare", again, very much due to the allostatic nature of biological organisms. This underlines the importance of context when interpreting these haematological parameters and the general need for integrated multidisciplinary approaches towards fish welfare that not only take into account all the different known indicators of well-being, but also explore eventual unexpected or yet unknown signs of impaired fish welfare (through the use of, e.g., high-throughput "omics" technologies). In fact, proteomics is increasingly being used as a method to obtain unbiased information regarding the impact of stressors on both plasma and organs/tissues, with a significant focus being given to the liver (due to its central role in most key metabolic processes), but with studies also being performed on skeletal muscle, brain, osmorregulatory and immune-related organs and tissues[96].

1.3. The concept and importance of fish quality

Fish quality is an important aspect of aquaculture production and research that pertains to the value-determining attributes of fish products and consumers' expectations regarding these attributes. It usually refers specifically to the assessment of the organoleptic properties of fish products (both through sensory and instrumental approaches), as well as to the study of the impact of different factors on these organoleptic properties. This concept and field of study, when taken in its wider definition, also encompasses issues related to food safety (e.g. [50, 52, 97-101]), nutritional value (e.g. [102]), food certification/traceability (e.g. [103]) and management of post-harvest product stability and shelf-life (e.g. [104-110]), as all these aspects are also highly determining to the products' ultimate quality and value, from the consumers' point-of-view[111-112].

Although, in general terms, consumers tend to expect organoleptic attributes of cultured fish to be as close as possible to wild fish, the typical (i.e. in the wild) values of the different attributes (along with their respective weight in the determination of fish quality) are very much species-dependent (and sometimes even population-dependent). Furthermore, it is important to take into account that consumers' expectations are inherently subjective and even prone to change over time, which implies that it might not always be possible to define universally optimal organoleptic attributes, even within a particular species.

Given that the edible part of fish is usually the fillet, which is mostly composed by skeletal muscle (along with variable amounts of connective and adipose tissue), factors such as muscle cellularity, composition and macrostructure are highly determining of the products' ultimate quality. Within this context, the assessment of fish meat quality appears to be more challenging than for land animals, as fish myosystem types are more diverse and their idiosyncrasies less well characterized[113]. In addition to the fillets, the properties of skin, eyes, gills and mucus can affect the product's desirability, and fish quality measurements also take these into account.

Regarding the specific organoleptic properties that are relevant to the definition of fish quality, the main focus has been given to concepts such as freshness, flavour, taste, aroma, texture and visual appearance. "Freshness" is a special type of attribute that encompasses the other attributes, reflecting the apparent elapsed time since slaughter. Similarly, the concept of "flavour" should also be seen as a meta-attribute (resulting of the interaction between "taste", "aroma" and "texture", along with temperature and chemesthetic factors). The other attributes ("taste", "aroma", "texture" and "visual appearance") can be seen as distinct aspects, thought not necessarily independent, as there are common underlying factors to these distinct organoleptic properties.

The concept of "taste" refers to the sensory information mediated through the gustative receptors of taste buds, which can be classified according to five known types (with distinct activation triggers): salty (alkali cations), sour (protons), sweet (saccharides and analogs), umami (L-glutamate, IMP, GMP) and bitter (hundreds of different substances)[114-115]. As expected, the subjective experience of taste can be mostly associated to the different free concentrations of gustative triggers present in the edible parts of fish, although there are also (human) genetic factors that might be determining to the ultimate perception of taste (particularly in the case of sweet and bitterness)[116-118].

Regarding "aroma", it basically concerns the olfactory perception elicited by volatile substances that are released both before and during mastication[119]. Like with "taste", it results from a form of chemoreception, but mediated by olfactory receptor cells located inside the nasal cavity, along the olfactory epithelium. On the other hand, olfactory information is much richer than gustative information, as there is a wider range of different olfactory receptors than gustative receptors. Another difference between the two systems is that gustation requires direct physical contact of the reception organ (i.e. tongue) with the subject, unlike olfaction. The specific olfactory triggers in fish products include alcohols, aldehydes, ketones, esters, sulfides, mercaptans and amines. In the case of marine teleosts, the aroma of fresh fish

is relatively simple and mostly composed of alcohols, aldehydes and ketones, which result from the activity of endogenous lipoxygenases on polyunsaturated fatty acids[120-123]. As time post-mortem elapses and spoilage begins, other types of more pungent volatiles start being produced mostly as a result of further oxidative processes and bacterial metabolism, namely dimethylamine, trimethylamine, sulfides, mercaptans, among others[110, 124-126].

Regardless of these differences, both types of chemoreceptive perception within the context of fish products are obviously highly dependent on body composition, but also post-mortem degradative processes, bacterial proliferation, lipid oxidation or any other processes that affect the composition of volatiles, fatty acids, small ions, nucleotides, free amino acids, saccharides and other substances. Even structural factors (i.e. "texture") can have an influence on how taste and aroma develop, by modulating the release of these compounds before and during mastication, which is why it is often appropriate to consider a "flavour" meta-attribute that results from and encompasses all these different inter-dependent aspects.

Another very important quality-defining attribute in fish products is "texture", which is related to the mechanical perception of food in the mouth and therefore, in this particular case, to the structural and physical properties of the edible parts of fish (mostly skeletal muscle, but also connective and adipose tissue). Descriptions of the textural properties of fish meat usually employ subjective organoleptic terms, such as firmness, chewiness, tenderness, juiciness and pastiness, that seem to be correlated to objective structural characteristics of meat, both in terms of initial apparent hardness and resilience in response to deformation forces, as in terms of secondary properties which only become apparent throughout the mastication process (e.g. springiness, cohesiveness, adhesiveness)[127]. In the specific case of fish, as already stated, there is a wide variability between species in terms of myotome organization and specific structural properties, although they generally display softer texture than land animals, due to their relatively sparse extracellular collagen matrix. Another general trend is that cultured fish also display generally softer texture than wild fish, which is seen as a negative attribute. In part, this is usually attributed to the fact that cultured fish are often constrained to a more sedentary lifestyle, which affects the development of their muscular system[113]. As such, the study of muscle physical properties and their relation to the subjective perception of texture, in the context of aquaculture, is given high importance.

Finally, "visual appearance" is also an essential criterion in the assessment of fish quality, both in terms of a fish's external appearance (regarding, e.g., body shape, eye attributes, gill coloration and the presence of species-specific marks) as in terms of fillet colour[128]. Although appearance might seem like an attribute of lesser importance (in the sense

that it generally doesn't affect much the flavour perception of fish products), it constitutes one of the main criteria consumers use to gauge product freshness and significant deviations from consumers' expectations (e.g. in terms of body shape or flesh coloration) can prevent the marketability of fish, even if they display no spoilage or other compromised organoleptic traits. For some species (e.g. salmon), it might be important to supplement their diets with specific crustacean or algae-derived pigments (in the case of salmonids, astaxanthin and other carotenoid pigments) which are usually present in their diet (in the wild), not only due to eventual nutritional/heath-related issues, but to enable the development of expected visual traits (e.g. meat coloration or specific external marks) that depend on the dietary availability of these pigments[129-132].

Given the dependence of all these organoleptic traits on the physical and chemical properties of fish products, it should not be surprising that fish quality traits can be affected by a wide range of different factors, in a heavily species-dependent way. These factors can be classified according to whether they affect the pre-mortem or intrinsic attributes of fish (which would reflect in measured quality parameters immediately post-mortem) and/or the post-mortem temporal evolution of these traits (which would reflect on the rate of spoilage and deterioration of freshness-related attributes, in terms of quality traits measured several hours/days post-mortem). In the first case, we have factors related to nutrition, health, development and environment (e.g. temperature, photoperiod, salinity, pH, water depth, O_2 levels, CO_2 levels, stocking density), which have been shown, in particular cases, to have a direct impact on fillet composition and physical properties and, therefore, on downstream quality attributes[113, 133-135]. In the second case, it is important to take into account that, when fish are slaughtered, peripheral tissues (like skeletal muscle) begin to experience energy depletion and anoxia, as the circulatory system stops to provide oxygen and fuel. This leads to a cascade of tightly-related processes and events which are characteristic of muscle-meat conversion and meat degradation (Figure 1.4) and that can ultimately lead to changes in quality criteria, depending on how intensive and extensive these processes are[128, 136]. At this level, several pre- and post-mortem factors are known to interact and influence the development of these processes: on one hand, as explained in the preceding section, we have factors related to fish nutrition and welfare (such as pre-mortem energetic/metabolic/oxidative state and the type of harvesting/slaughtering methods used)[136-141] and, on the other hand, we have factors which are related to the type of post-processing and conditioning used to manage post-mortem spoilage processes[142-143]. It is relevant to note that, while these post-mortem conditioning processes generally tend to slow down spoilage (both in terms of autolytical

processes, as in terms of microbiological proliferation) and increase shelf-life, they often also entail changes in terms of organoleptic properties (particularly in the case of salting, smoking, freezing, drying or freeze-drying), which obviously have to be taken into account.

Regarding the assessment of fish quality itself, there are several possible complementary approaches, though the use of trained sensory panels is highly important (and generally seen as the "golden standard"), regardless of other instrumental methods applied, since the latter generally fail to capture the whole range of information provided by human sensorial organs. Furthermore, the subjective perception of organoleptic traits (as it relates to consumers) is more readily aprehended through a sensory analysis performed by trained individuals than by instruments, as humans instinctively generate an integrated perception (e.g. flavour) from very differing type of stimuli (e.g. chemical, thermal, mechanical), which is something that purely instrument-based approaches might have difficulties with.

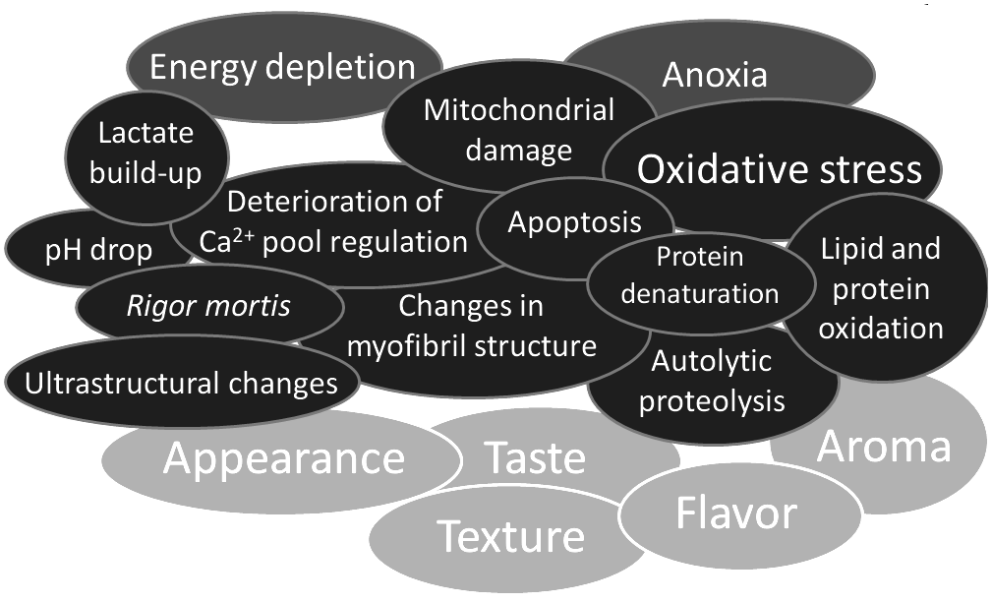

Figure 1.4 – Diagram showing the different endogenous processes that occur in skeletal muscle post-slaughter, due to the onset of energy depletion and anoxia, which can ultimately affect the different organoleptic properties of fish products.

Given the difficulty of quantitatively assessing something which is inherently subjective, methods have been devised in an attempt to standardize species-specific quality evaluations, like the QIM (Quality Index Method) schemes[144-150]. Other approaches, as already mentioned, rely on instrumental information to obtain objective measurements which can then be interpreted using both human knowledge-based and/or machine learning-based approaches. A specific example would be the use of data fusion methods on multi-

instrumental datasets to achieve an objective index which possesses properties consistent with the QIM indicator[151-153].

Looking at the specific instrumentation that can be used to assess quality-related parameters in meat products (Figure 1.5), including fish products, it becomes clear there is a wide range of complementary approaches possible to evaluate their physical and chemical properties (and, therefore, associated organoleptic attributes). Regarding physical properties, they are classically assessed using Texture Profile Analysis or similar approaches that consist in measuring deformation behaviour as a function of (repeated) applied pressure, in an attempt to directly replicate the dynamics of mastication[154]. Besides directly mechanical methods, other ways of assessing structural and physical properties are possible, from microwave imaging[155-157] to impedance[158-160] and calorimetric[109, 161] measurements. There is also a great wealth of techniques (fluorescence anisotropy, circular dichroism and birefringence measurements) that exploit the fact that muscle tissue is optically active, due to its anisotropic nature, and the fact that some metabolites (like tryptophan) can act as intrinsic fluorophores, providing essential structural information[159]. Unsurprisingly, many of the methods adopted by chemometrics/analytical chemistry are also used in the context of food quality assessment (e.g. mass spectrometry, nuclear magnetic ressonance and FTIR spectroscopy-based methods), since these are the most straightforward and comprehensive ways of obtaining thorough information on tissue and volatile chemical composition[162-167]. Apart from these, other indirect methods of assessing tissue composition can also be used (e.g. ultrasonic, X-ray and microwave imaging), as changes in tissue composition often entail predictable changes in measurable physical properties as well[159]. Characterization of volatile composition is also increasingly being studied through the use of specialized arrays of sensors (generically dubbed "electronic noses")[152, 168-170]. Besides all of these, several types of biochemical and microbiological assays are also employed, particularly in the assessment of fish freshness and safety.

In recent years, it became increasingly clear that the use of state-of-the-art "omics" technologies (e.g. genomics, transcriptomics, metabolomics, proteomics) can provide useful information on the impact of pre-mortem factors (e.g. genetic, nutrition, welfare, development) on both the intrinsic (pre-mortem) properties of skeletal muscle and its post-mortem degradation dynamics[171-176]. Also, there are signs that it might be possible to directly correlate quality traits with particular genes, transcripts or proteins[177], providing essential clues for a better understanding of the emergence of macroscopic organoleptic features from

microscopic physico-chemical properties. The next section therefore explores their relevance in aquaculture research, particularly in the study of issues related to fish welfare and quality.

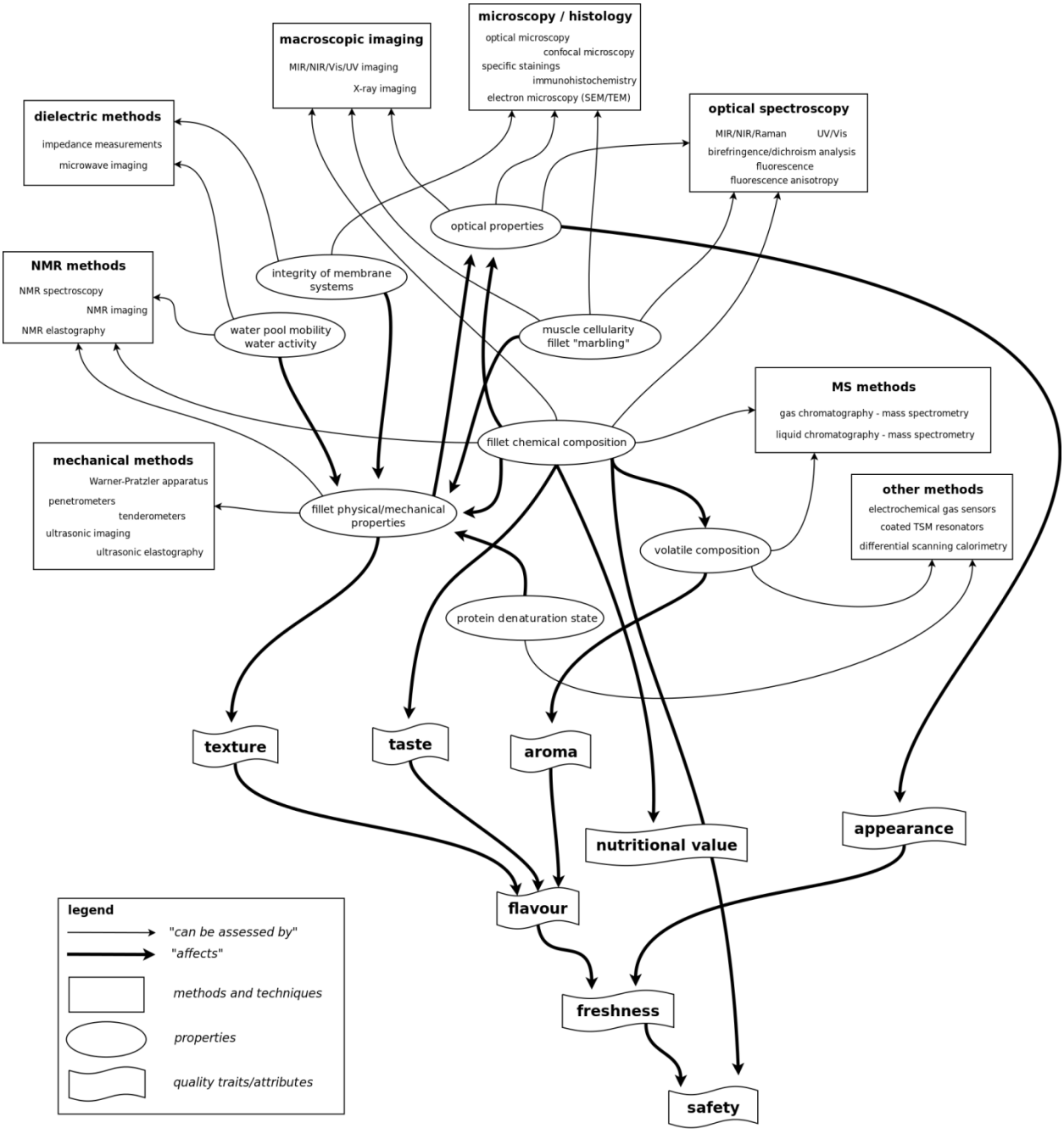

Figure 1.5 – Diagram detailing the different kinds of methods and techniques used in the context of fish quality, explicitly detailing the type of properties normally assessed and their relation with ultimate quality traits. Microbiological assays, also used in the context of food safety, were omitted.

1.4. Proteomic (and other –omic) technologies

Modern biological sciences are increasingly adopting state-of-the-art high-throughput instrumentation which allows for thorough characterization of broad classes of molecules, enabling approaches towards knowledge discovery which are less hypothesis-driven and more data-driven. These approaches are commonly known as "omic" or "omics" approaches, with the best known examples being metabolomics, genomics, transcriptomics and proteomics (where the object of study of each are the complete characterization of metabolites, genes, RNA transcripts and proteins in living organisms, respectively). They rely on a wide array of technologies that allow the separation and characterization of all these different classes of biomolecules.

Metabolomics (or "metabonomics") usually focuses on small molecules, requiring analytical methods which are compatible with complex mixtures of very distinct substances (in terms of chemical behaviour). Most approaches in these field are based on either MS (mass spectrometry), (^{1}H) NMR (nuclear magnetic ressonance) or IR (infrared) spectroscopy, all of these classically used in the field of chemometrics/analytical chemistry[178]. Particularly in the case of MS, detection methods are often coupled to separation methods like capillary electrophoresis, gas or liquid chromatography. While some authors make the distinction between "metabolomics" (to refer to more targeted, usually MS-based approaches) and "metabonomics" (to refer to more holistic, usually NMR or IR spectroscopy-based approaches), the object of study is, in any case, either the metabolome or a specific subset of the metabolome (e.g. lipidome, glycome). The overlap between metabolomic techniques and many of the techniques used in the context of fish quality (particularly in the assessment of taste and aroma) should not be surprising, as organoleptic properties often depend on the concentration of small metabolites[179].

Genomics and transcriptomics are both concerned with nucleic acids (DNA and RNA, respectively), so most techniques are common between the two fields, as it is trivial to convert RNA to the (much more stable) corresponding cDNA. These fields have mostly started with the use of Sanger sequencing, cDNA microarrays and assorted technology, but these are increasingly being replaced by cheaper, higher-throughput, next-generation genome sequencing platforms (based on, e.g., pyrosequencing, DNA nanoballs, SOLiD sequencing and nanopore technologies)[180-185].

Proteomics is concerned with proteins and peptides in particular, which are a broader group of molecules than nucleic acids (in terms of possible chemical behaviour). It usually relies on electrophoretic and/or chromatographic methods for separations, while using mass spectrometry for thorough characterization of peptides (or digested proteins). These methods often rely on genomic and transcriptomic data to infer protein identity[186].

Looking at living organisms from a systems point of view, we can see that the information provided by these different "omics" is complementary (Figure 1.6). Most (if not all) phenotypic traits can be modeled, in a first-order approximation, as an interaction between proteome and metabolome, as these contain the fractions of biomolecules with direct structural function (and therefore responsible for most macroscopic features). We know, from chemical kinetics, that (again, as first-order approximation) metabolic fluxes can be modeled as systems of differential equations, which depend on both the current metabolomic state and the current proteomic state. For larger time-scales it is important to take into account that the proteome is not exactly static, and higher-order terms, like the transcriptome and protein turnover processes, become important to accurately model metabolic fluxes and the emergence of macroscopic features. This is further complicated by the fact that both metabolome and proteome affect transcriptomic activity by feedback mechanisms (e.g. ligand-regulated transcriptional factors). Despite the complexity of the metabolome-proteome-transcriptome relations, a useful car analogy can be established: if the metabolome represents a car's position, the proteome would represent the car's instant velocity and the transcriptome would represent the car's acceleration. This simple analogy, of course, breaks down once we take into account other things like protein turnover regulation processes, alternative splicing, post-translational modifications, ligand-(in)dependent transcription factors, non-enzymatic reactions, protein signaling cascades and the influence of environmental factors on both genome, transcriptome, proteome and metabolome. These imply the acceleration (transcriptome) in the metabolic phase space of a cell also depends on both velocity (proteome) and position (metabolome). In a way, more like a self-driving car.

So, in order to truly understand "how the car drives itself" (i.e. the underlying chemical dynamics of living systems), it is increasingly clear that a thorough and quantitative understanding of genome, transcriptome, proteome and metabolome (as well as how environmental factors affect these) is required. One of the challenges for "omics" approaches in the near future will be precisely to improve instrumentation and methodologies in order to transition from current "relative quantitation" methods (mostly of a comparative nature) to

"absolute quantification" methods, enabling the development and validation of quantitative *in silico* modeling approaches and high-confidence phenotype prediction methods[187-188].

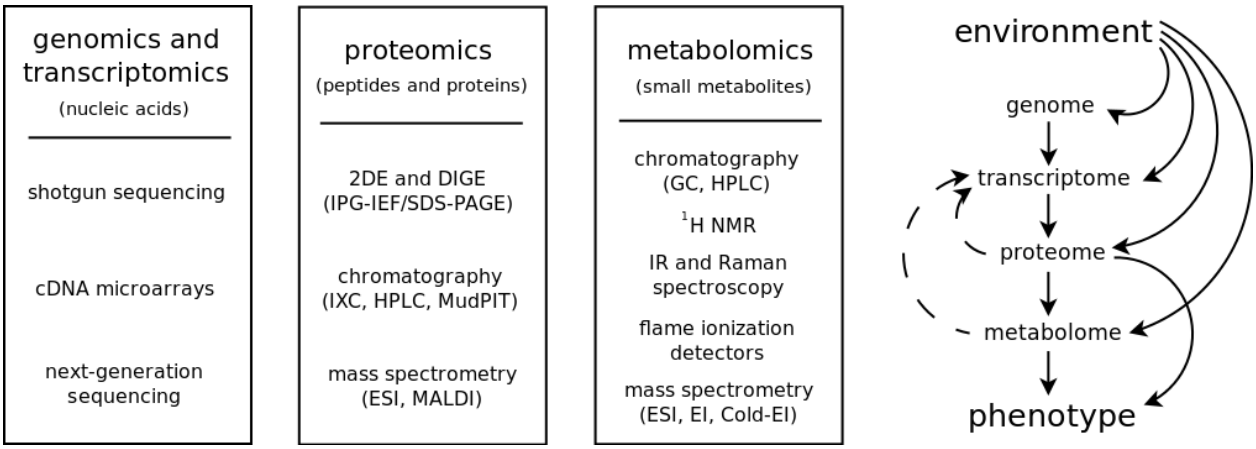

Figure 1.6 – Diagram showing the major technologies underlying the main "omics" (on the left), along with a scheme depicting the information flows from genotype to phenotype (on the right).

This dissertation focuses mostly on gel-based comparative proteomics and attempts to provide some examples demonstrating the usefulness of these technologies in aquaculture and fish science, particularly regarding fish nutrition, welfare and quality issues. The use of proteomics to study teleosts and invertebrates is a booming field which has already shown many results in several areas, as described by recent reviews[96, 189-190]. The importance of proteomics in this context is, as stated above, linked with the role of proteins as catalysts and direct effectors of most biological functions: knowing how a stimulus affects the proteome allows one to infer how and which cellular functions and metabolic processes are directly affected by that stimulus.

In technical terms, most proteomic workflows (both gel-based and gel-free) include a common set of steps: sample preparation; peptide/protein separation and quantification; peptide/protein identification and characterization. This is schematically depicted in Figure 1.7.

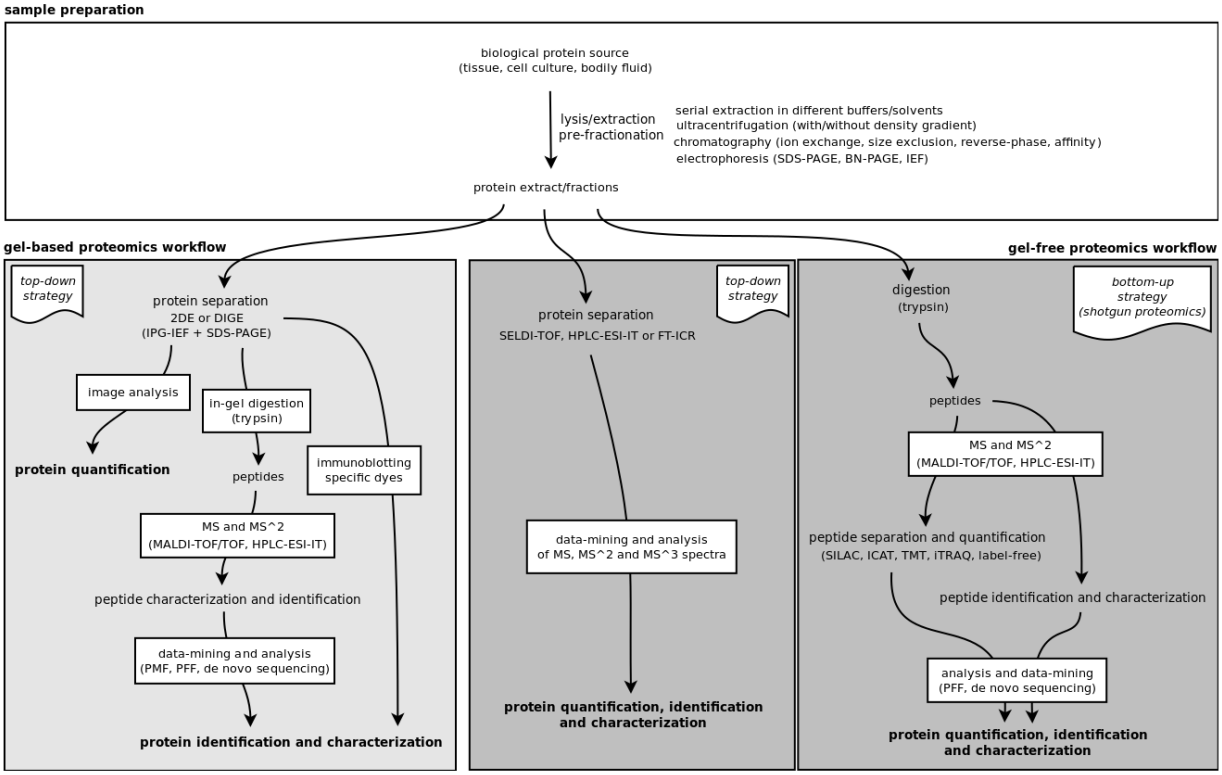

Figure 1.7 – Schematic depiction of the typical gel-based and gel-free proteomic workflows.

1.4.1. Gel-based proteomics

Sample preparation

The first step is usually protein extraction, since most analytical techniques used in proteomics require prior solubilization of proteins in an appropriate solvent (aqueous buffers, organic solvents). This makes sample preparation a critical step in the analytical workflow[186, 191]. The purpose of this step is to attempt thorough solubilization of all (or a specific subset of) the proteins present in a given bodily fluid, organ, tissue or cell extract. Commonly used aqueous extraction buffers often contain (besides buffering agents) detergents, chaotropes, reducing agents and protease inhibitors, ensuring that enzymatic activity is halted during extraction and that intra and inter-molecular interactions between proteins are minimized, preventing aggregation. Although standard extraction buffer formulations work reasonably well for a broad range of samples (eukaryotic cell cultures, bodily fluids and soft tissues of animals), some types of organisms (algae, bacteria) or protein sub-populations (membrane proteins, proteins embedded in a calcified matrix) often require different types of extraction buffers for optimal results[191].

To improve protein separation and identification, several procedures can be considered. Sample fractionation is often a choice for sample pre-processing prior to proteome analysis, simplifying protein extracts and/or improving the dynamic range of a protein mixture. Fractionation methods are usually based on chromatography, electrophoresis, differential solubility and/or (ultra) centrifugation and the most popular are usually aimed at isolation of organelles and sub-cellular compartments (nuclei, mitochondria), enrichment in particular sub-populations of proteins (phosphorylated, glycosylated, secreted, membranar) and depletion of highly abundant proteins (like albumin and immunoglobulins in blood plasma, or actin/myosin/tropomyosin in muscle).

Protein separation and quantification

The two most important analytical techniques in proteomics are two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS). Due to the central role that these two methods have in proteomic studies, most experiments are broadly classified as either "gel-based proteomics" or "gel-free proteomics", depending on whether 2-DE is used for protein separation and quantification or not.

Currently, 2-DE is still, by far, the most common strategy for protein separation and quantification, enabling the separation, detection and quantification of hundreds (and sometimes thousands) of different proteins from a single extract, taking advantage of the fact that the isoelectric point of a protein is mostly uncorrelated to its molecular weight. On the other hand, issues like gel-to-gel variation, limited linear dynamic range, limited throughput, and protein co-migration still pose challenges to gel-based proteomics[186]. Although most software geared toward the analysis of 2-DE gels, like Progenesis SameSpots (Nonlinear Dynamics), PDQuest (Bio-Rad Laboratories) and DeCyder (GE Healthcare), try to overcome some of these issues with a simplified, semi-automated analysis workflow, they still cannot achieve totally unambiguous results without significant human intervention and visual inspection.

Nevertheless, there are also some advantages of gel-based approaches: first of all, they are generally less expensive than purely MS-based approaches. Besides that, information on small post-translational modifications (PMTs) and highly-homologous isoforms can often be obtained directly, since these tend to shift the isoelectric point of a protein without extensively changing its molecular weight.

Classically, detection and quantification methods for 2-DE are usually based on Coomassie Brilliant Blue (CBB) or silver staining, which enable estimation of protein

quantity by scanning 2-DE gels in the visible range[192]. Recently, the development of multiplex 2-DE (dubbed "difference gel electrophoresis" or DIGE), which instead involves tagging the protein samples with different fluorophores prior to 2-DE, not only allows several samples to be run on a single gel, but also significantly improves gel-to-gel variability, by providing a common reference channel across all gels of an experiment[193]. This recent improvement of the 2-DE method has been increasing in popularity in aquaculture-related proteome studies with successful results in several species[194-198].

Protein identification and characterization

Although immunostaining methods are a valid choice for protein identification after 2-DE, the overwhelming majority of gel-based proteomic studies rely on digestion of detected proteins and analysis of the resulting peptides by MS for their identification and characterization, since it does not rely on having specific antibodies against every single protein to be identified. Given the accuracy of current MS instruments and the low number of proteins usually present on a single 2-DE spot, the results obtained can be quite reliable. Instruments currently employed for this purpose include ESI-Ion Trap, MALDI-TOF/TOF and ESI-QTOF mass spectrometers to a lesser extent. Identification of proteins can be assessed either directly through its peptide mass fingerprint (PMF), for the case of organisms with fully sequenced genome, or by analysis of the fragmentation spectra of such peptides (PFF, peptide fragment fingerprinting or even *de novo* sequencing) obtained through tandem MS.

1.4.2. Gel-free proteomics

Due to the above-mentioned issues associated with 2-DE and the continuous improvement and cost reduction of MS-based methods, gel-free strategies are becoming increasingly popular, since they generally allow higher analytical throughput and deeper proteome coverage than gel-based methods. In a way, 2-DE can be seen as an elaborate pre-fractionation step that precedes MS analysis in a typical proteomic workflow and, as such, some researchers prefer to omit this step and apply different strategies which scale much better.

In contrast to the usual "top-down" approach of gel-based proteomic studies, where proteins are separated and quantified before being digested and identified, most gel-free studies employ a "bottom-up" approach. Here, proteins are digested from the start and

analyses (separation, quantification, characterization) are done at the peptide level. Since digestion of a complex protein mixture dramatically increases its complexity, gel-free methods are usually combined with fractionation methods to reduce the number of different peptides entering the mass spectrometer at any given moment, maximizing the total number of distinct peptides detected over the course of a sample run. By far, the most common pre-fractionation methods used in gel-free proteomics are based on liquid-phase chromatography procedures, which can readily be coupled to ESI-based mass spectrometers. Even multidimensional chromatographic separations are commonly used, as in the case of MudPIT, where peptides are separated by charge (SCX-HPLC) and hydrophobicity (RP-HPLC) prior to MS analysis. Despite the fact that label-free methods exist, most gel-free workflows rely on stable isotope labeling for peptide quantification, either by metabolic incorporation of radioactive amino acids in proteins (SILAC) or by post-extraction chemical modification (ICAT, TMT, iTRAQ). With stable isotope labeling, several samples can be analyzed in parallel on the same MS run (with identical peptides from distinct samples separated by a small mass shift) and relative abundance can be estimated from the ratio of peak areas of identical peptides. At the end of a “bottom-up” analysis, information on protein abundance/identity is inferred from the obtained information on peptide abundance/identity, using computational tools. This is, in itself, a challenging part of “bottom-up” approach and still a matter of research in the field of mass spectrometry.

Although a direct “top-down” approach is intrinsically more challenging, due to the difficulty of ionizing whole proteins, the higher resolution of MS instruments using FT-ICR or Orbitrap technologies make “top-down” approaches increasingly practical, beyond the more common “bottom-up” approaches.

1.4.3. Identifying proteins from peptides

Most proteomic studies (both gel-based and gel-free) attempt to identify proteins by looking at peptides, as large proteins still constitute a challenge for MS-based methods. The classical method used (dubbed "peptide mass fingerprinting" or PMF) is based on *in silico* digestion of genomic/EST sequences following the pattern of a predictable endonuclease (usually trypsin) to obtain a list of peptide masses (or "mass fingerprint") for each database entry. Identification is performed by comparing experimentally-obtained MS mass lists with those generated *in silico*, and choosing significantly similar matches. Of course, this only works reliably when working with a model species (like zebrafish), for which there is full

genome data, and for simple digests (like 2DE spot digests). On the other hand, by using tandem MS instruments, one obtains not only a peptide mass list, but also information on the fragmentation mass spectra of those peptides, providing a fingerprint for each peptide that directly reflects its sequence. The most common strategy here ("peptide fragment fingerprinting" or PFF) also involves starting with a genomic/EST database and performing *in silico* digestion with a standard endonuclease. Then, for each peptide, the masses of all likely fragments are deduced from prior models. Identification of peptides is then performed by comparison of experimentally obtained MS/MS spectra against all possible fragmentation spectra in the database. Since a fragmentation spectrum (unlike mass) is usually very specific for a certain peptide sequence, identification of proteins can often be attained from a single high-quality peptide match. Nevertheless, unless one works with species for which there is a great wealth of genomic/EST data available, this strategy only works for highly-conserved peptides.

It is therefore important to underline that protein identifications depend not only on the quality of spectra, but also on the quality of the sequence database used. Generally, a good approach is to start by searching genomic databases (since they tend to be smaller and therefore fast to query) and then EST databases. The results obtained are often different, since genomic databases reflect genomic DNA sequences while EST databases reflect mRNA sequences (which are closer to the actually translated amino acid sequences). Observations on the frequency of alternative splicing events in teleosts suggests that this difference is particularly important for species with a compact genome (like *Takifugu rubripes*, for which ~43% of mapped genes seem to be affected by alternative splicing) and less so for species with a high level of gene duplication (like zebrafish, for which only ~17% of mapped genes seem to be affected by alternative splicing)[199]. Also, given current developments in DNA sequencing technology[180], 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.

1.4.4. Challenges and new technologies

Looking at the results of proteomic experiments, we observe that there are several issues that prevent perfect consistency of results across experiments on a single topic, despite the fact that there is usually significant overlap in terms of affected pathways. Some of these

differences can be attributed to technical limitations, like the influence of sample preparation methods, the "user" factor, the difficulty of ionizing certain peptides, as well as other instrumental and analytical limitations. Most of these can hopefully be overcome by increased automation, improvements in instrument quality and a decrease in user intervention throughout the workflow. On the other hand, the dynamic and complex nature of the proteome provides several intrinsic challenges during interpretation of proteomic results. This can be seen, for example, in experiments coupling transcriptome and proteome observations, which often show a weak correlation between the two profiles (when sampled at the same time)[200-201]. This dynamism and the sensitivity of the proteome towards environmental signals and subtle changes in physiological state justifies the necessity of obtaining and using as much contextual information as possible (including both classical and/or –omics observations) to support proteomic observations and having into account the importance of time and locality on the proteome.

The fact that most proteins display post-translational modifications (PTMs) further adds to the proteomes' complexities and is increasingly seen as essential aspect of proteome research, given the biologically important roles some of these PTMs are known to have. Although gel-based approaches can use either specific dyes (like Pro-Q Diamond, for detection of phosphorylated proteins) or immunoblotting for detection of PTMs, the real workhorse of PTM analysis is mass spectrometry[202-204]. Specific detection methods are often combined with protein/peptide enrichment steps, to target for some specifically modified proteins (like affinity chromatography with lectins, to enrich for glycoproteins, or with TiO_2, to enrich for phosphopeptides).

As technology continuously develops, the future of proteome research is still uncertain, but there are already novel approaches which have good potential to become invaluable tools in aquaculture research (and marine biology in general). One of them is MS imaging. This technology involves the direct digestion of histological samples fixed to a suitable support, followed by direct MS/MS analysis (for example, by applying a matrix solution on the sample and performing MALDI-MS all over the surface, point by point), which is the equivalent of using immunocytochemistry methods, but in a non-specific way, providing proteomic information on a key variable: location[205-207]. This is very important because, unless cell sorting or microdissection techniques are applied prior to proteomic analysis of a given tissue, the profiles obtained are very likely to represent an "average profile" of a heterogeneous population of cells, since most organs and tissues are heterogeneous. Most importantly, this heterogeneity is often location-dependent, which

implies that MS imaging can provide a type of deep morpho-proteomic information that would not be accessible otherwise. It is interesting to note that MS imaging does not necessarily require protein identification: using computational methods (dimensionality reduction methods, tree classifiers, neural networks and similar machine learning tools) it is possible to map all the MS and MS/MS information obtained for each point in space as a pixel, where color information is defined so that it reflects the similarity relations between proteomes. This means that MS imaging can provide useful information on proteome distributions over any tissue (regardless of source), distinguish between sub-populations of cells with different proteome profiles and pinpoint exactly where proteome changes occur. The size of matrix crystals are a limiting factor preventing increased resolution in MALDI imaging, so there is still a lot of research on alternative matrices or ionization methods for MS imaging, like SIMS and DESI[208-210]. New breakthroughs in this field will certainly revolutionize proteomics and greatly increase our understanding of biological processes which are intrinsically morphological in nature and relevant for aquaculture research (like larval development or muscle growth).

Another interesting technology is the development of protein array/protein chip approaches[211]. These allow for very high throughput at the expense of high specificity, which currently makes the development of such tools relatively expensive and challenging, particularly for non-model or non sequenced organisms. Nevertheless, as these types of technologies develop, the potential for very large scale studies regarding major issues in aquaculture suddenly becomes feasible, opening the doors to new, more in-depth approaches for comparative proteome studies.

1.5. Analytical approaches for an integrative biology

While the technical aspects of proteomics, as seen in the previous section, are an active field of study of a great importance, the analytical treatment of proteomic data is also a big concern for many scientists, as classical statistical tools can be somewhat lacking in their ability to extract all potential information from high-dimensional datasets such as the ones produced through both gel-based and gel-free proteomic approaches[212-278]. Although this section is mostly centered on gel-based proteomic methodologies, many of these points also apply (*mutatis mutandis*) to other "small n, high p" datasets[279], such as the ones generated by other –omics, and even more classical situations involving co-measurement of several parameters.

1.5.1. Experimental design

Although not specific to proteomics, issues of experimental design should be a main priority, as poor or incorrect decisions at this point can easily compromise a whole experiment. Some of the publications within the field of proteome data analysis already cover most of the main issues regarding experimental design in this context[245, 247, 249, 251-252, 280].

The first issue concerns the number and type of replicates to use. While the standard seems to be a minimum of 4-6 replicates per experimental group, the optimal number of replicates can only be determined through a power analysis (i.e. *a posteriori*). This is important in terms of resource management, particularly in the cases where a high number of replicates is feasible, since it might make more sense in those cases to perform two experiments with less replicates rather than a single big experiment. Regarding the type of replication employed, a distinction should be done between "biological replication" and "technical replication". While it is important to understand the magnitude and structure of the noise introduced throughout the technical steps, the general consensus is that biological replication is more important than technical replication, since the technical variability has no biological meaning (unlike biological variability), which implies that technical replication should never be performed at the expense of biological replication. Another consensus is that one should also try to avoid, as much as logistically possible, pooling biological samples, to prevent losing information on relevant information on biological variability.

1.5.2. Image analysis

After running a gel-based experiment, gels are digitalized using an appropriate scanner (depending on the type of staining used) and some form of image analysis is performed, in order to obtain information on how the different gel features vary between gels. While there is some research on 2DE gel analysis methods that work directly at the pixel level (e.g. [218, 227, 262]), practically all known 2DE gel analysis software consider "spots" as the basic feature and attempt to summarize all the image information by providing measures of "spot abundance" for each spot, across all gels. This is usually done by matching each spot in each gel to the equivalent spot in all other gels and then integrating the volume (area × intensity) of each spot either explicitly or by modeling each spot as a smooth curve (usually a bivariate Gaussian function) and integrating the fitted curve instead. Although this may seem trivial, in practice,

2DE gels often display characteristics (e.g. uneven background, fused spots) which present challenges in terms of image segmentation (i.e. in terms of defining the boundaries of each spot), even if we assume near-perfect gel matching and spot detection.

In terms of the specific software used, the overwhelming majority of gel-based proteomic studies use some form of proprietary software, which are usually classified either as taking a "classical 2DE analysis approach" or a "4th generation 2DE analysis approach", with the difference being that the "classical" 2DE software packages usually perform spot detection before matching, while newer software packages usually perform image matching/warping before spot detection. A deeper view on the issues of image analysis is outside the scope of this thesis, and unnecessary given the existence of good reviews on the subject (e.g. [221, 227, 231, 253, 263, 266, 281]), but it is important to note that some variability in results (i.e. introduction of technical noise) can be attributed to this step. It is expected that improved, more automated, image analysis pipelines will help to mitigate these problems, in the near future.

1.5.3. Pre-processing

Regardless of the software used, the end result of 2DE image analysis can be seen as a table or a matrix, where each row represents a sample/gel, while each column represents a variable/spot. Before a statistical analysis can be performed, several types of pre-processing algorithms are applied to correct for some of the technical issues.

The first issue concerns the possible existence of missing values. Particularly in the case of software packages that use a "classical 2DE analysis approach" (i.e. where spot detection is performed before matching), it is very common to have wide variations in the number of spots detected per gel (often more than 10%, mostly due to gel-gel technical variability), which implies the existence of "empty cells" (or "missing values"). This problem can be solved in several ways[232-234, 249, 252, 282]. First, it is important to take into account that these missing values can sometimes simply be a symptom of a reduction in protein abundance beyond the threshold of detection (either of the staining or of the peak detection algorithm), in which case it would make sense to replace the missing value either by a zero or by some estimate of the local background intensity. On the other hand, more often than not, these missing values actually result from inconsistent spot detection results and/or incorrect spot matching (as explained above, due to technical variability). These situations can be sometimes (but not always) mitigated by time-consuming manual editing of each spot. A common

strategy here involves removal of columns (i.e. spots) which contain missing values. Another option (that should be performed after removal of spots with a high proportion of missing values) involves the use of imputation methods to estimate the missing values, which seems to have a good performance in the cases where missing values are few (always less than 25%, but preferably less than 5% of the total number of cells) and randomly distributed. Care should be taken, nonetheless, if using these imputation methods for anything other than exploratory data visualization (due to the "no such thing as a free lunch" principle).

Another issue relates to the need for "data normalization" before analysis, to correct for such things as variations in protein load per gel, staining variations (both in terms of spot intensity as in terms of background intensity), scanning variations and/or distinct fluorophore efficiencies (in the case of DIGE approaches). The options here are many, but the simplest solution involves expressing each spot abundance as a ratio between that value and the sum of the abundances of the spots in the same gel (i.e. the total spot volume for each gel). Though this solution should mostly solve biases related to variations in protein load, there are often nonlinearities (e.g. high-intensity, possibly saturated spots) which compromise the validity of "total spot volume"-based normalization methods. A possible way of addressing this point would be to remove the highest-intensity spots from the calculation of total spot volume or simply use a smaller set of "housekeeping proteins" (which can be chosen through a preliminary analysis) as reference. Other (less often used) normalization methods include LOESS regression-based and quantile-based normalization. More recently, the use of normalization methods inspired by the ones used in transcriptomics (e.g. SameSpots' normalization method and the VSN method[283]) have become more prevalent, mostly due to the fact that these truncate differentially-expressed spots from the regressions (implicitly restricting normalization to be performed based on "housekeeping proteins"), while explicitly modelling both multiplicative (e.g. differences in protein load/spot staining) and additive biases (e.g. background staining).

Finally, there's the issue of data transformation, which attempts to address the positive correlation that exists between a spot abundance and that spot variance. This can be attributed to the fact that high-abundance spots tend to have larger areas and, therefore, volume calculations for high-abundance spots are affected by a larger amount of noise (since the errors of single pixels add up), which is a problem since (for example) ANOVA-based analyses usually assume not only that spot abundances are normally distributed and independent, but also that variances are homogeneous (i.e. homoscedasticity). The classical way of solving this issue is through a log-transformation, which usually has a strong variance

stabilization effect on highly-abundant spots. On the other hand, log-transformation cannot handle negative values and is rather unstable for values near zero, which is probably why other transformations (like arsinh and cubic root) seem to provide better results[249].There are also suggestions that more generalized transformations should be used (like the Box-Cox transform or other types of power transforms), with the specific transform function being chosen through (e.g. maximum-likelihood) fitting.

1.5.4. Data visualization/exploratory data analysis

The first step after applying the appropriate normalization and transformation methods should be to look at the data itself, as it constitutes an important diagnostic step in the assessment of the quality of data (and of the underlying experiment). Also, it helps to avoid incorrectly taking statistical artifacts as meaningful biological signal, such as (e.g.) in pathological cases where the Yule-Simpson effect applies.

The problem with proteomic dataset (and other –omics datasets) is that the common tools for exploratory data analysis (e.g. boxplots, scatter plots) are inadequate for visualization of points in a p-dimensional space (where p is often on the order of hundreds or thousands of variables/spots). On the other hand, the intrinsic dimensionality of the subspace implied by the samples (minus noise) is usually quite low, particularly with simple experimental setups (e.g. one homogeneous group is compared against another homogeneous group), which justifies the use of (unsupervised) multivariate dimensionality reduction approaches that attempt to preserve most of the high-dimensional information through either projections (e.g. principal component analysis, PCA) or embeddings (e.g. multidimensional scaling, MDS) in low-dimension spaces (usually 2 or 3). Several papers within the field of proteomic data analysis explore, discuss and review some of these methods[222, 242, 249, 252, 255-256, 262-263, 272-273, 281-282].

Projection methods (of which PCA is the most well-known example, but also include supervised extensions such as partial least squares regression and correspondence analysis) usually formulate the problem as a linear algebra one, often under the form of a simple singular value decomposition problem, for which there are several known efficient algorithms. One important issue with these methods regards the scaling of variables, which is highly advisable in the case of significant deviations from homoscedasticity (see [272] for a deeper discussion on possible scaling methods for PCA within the context of gel proteomics).

Embedding methods (of which MDS is the most well-known example) take a different (two-step) approach: first, a dissimilarity matrix is calculated, using a chosen metric (or non-metric) measure of dissimilarity from the high-dimensional data. This dissimilarity matrix contains a type of distance information between every two samples/gels (or variables/spots), which can then be reconstructed in a low-dimensional space (usually 2D or 3D) in a way that some measure of reconstruction error is minimized (which is the underlying idea of multidimensional scaling). It is important to stress the importance of the chosen dissimilarity measure: these can include obvious measures such as Euclidean, Mahalanobis or Minkowski distances (turning it somewhat into a generalization of PCA), but also other type of dissimilarity measures that are more powerful than Euclidean distance-based distances (e.g. cosine distance, distances based on Pearson correlation, Kendall correlation). There is also a class of (more advanced) methods that are sensitive to local structure, such as the graph-based isomap method, maximum variance unfolding or self-organizing maps. The problem with some of these relates to the fact that they require dense sampling of the phase space (i.e. high number of replication) for an accurate "manifold learning".

Finally, it is worth to separately talk about a popular and useful subset of embedding methods generally dubbed "clustering methods". These also involve the estimation of a dissimilarity matrix from a dataset, which is then used to (either through agglomerative or partitional methods) to separate either gels or spots according to how similar they are. The usual way of representing this, in the case of hierarchical clustering, is through dendograms, which provide a clear and easy to interpret embedding that attempts to reproduce the dissimilarity structure of the dataset. Also in this case, it is important to consider not only the type of dissimilarity measure used, but also the underlying criteria for clustering.

1.5.5. Feature selection

The classical approach for feature selection (i.e. deciding which spots are relevant) involves the use of univariate methods on each feature separately, to test for differences between experimental groups in terms of some measure of central tendency (usually the "mean"). For this effect, ANOVA-based methods are usually employed, although they generally require low deviations from normality and homoscedasticity. Another often used option is the application of non-parametric tests, such as the Wilcoxon-Mann-Whitney test, which displays a higher robustness in the presence of outliers, besides having softer requirements (distributions should be similar, but not necessarily normal or homoscedastic).

Another class of possible non-parametric approaches includes the use of resampling/bootstrapping methods to explicitly estimate the underlying distribution of a given test or pivotal quantity (e.g. Cohen's d) under the null hypothesis.

It is important to take into account that, when performing univariate hypothesis testing over hundreds (or thousands) of variables, one has to explicitly correct for this, in order to effectively control the frequency of false positives (Type I errors). Several types of "multiple testing correction" methods are known, some of which exploit knowledge on the distribution of p-values and the correlation between the different tests to achieve a better performance over the classic Bonferroni correction. In the context of proteomics, several publications cover this issue (e.g. [249, 252]).

Besides the classical (i.e. univariate) approaches for feature/variable selection, it is increasingly clear that (also unsupervised, but particularly supervised) multivariate approaches, including regression (e.g. partial least squares regression/projection to latent structures, PLS-discriminant analysis, PLS with jack-knifing procedure, PLS with CovProc), classification (e.g. decision trees, support vector machines, linear discriminant analysis, soft independent modelling by class analogy), blind signal separation (e.g. independent component analysis) and even machine learning, manifold learning and dimensionality reduction (e.g. artificial neural networks, self-organizing maps, multidimensional scaling, isomap, maximum variance unfolding) methods, are essential tools that complement univariate approaches in the context of both data visualization and feature selection, also providing important insights on variable correlation that are otherwise impossible to obtain through univariate approaches[222, 242, 249, 252, 255-256, 262-263, 272-273, 281-282]. These can be particularly powerful when applied to experiments involving co-measurement of several types of information (i.e. "data fusion") and/or multifactorial experimental designs.

1.5.6. Importance of bioinformatic tools and databases

As discussed in section 1.4.3 of this dissertation, the quality of genomic and transcriptomic databases is essential for correct proteomic identifications. Besides these databases, other type of bioinformatic databases (related to interactomics[284]) and tools that automatically datamine literature repositories for information on protein-protein or protein-ligand interactions are increasingly seen as a powerful aid in the (often difficult) interpretation of proteomic (and other –omic) data or even in the assessment of the use of any specific protein as an indicator or biomarker[285].

Chapter 2.

On the reproducibility of a fractionation procedure for fish muscle proteomics.

A previous version of this chapter has been published as:

Tomé S. Silva, Odete D. Cordeiro, Flemming Jessen, Jorge Dias, Pedro M. Rodrigues. **On the reproducibility of a fractionation procedure for fish muscle proteomics.** American Biotechnology Laboratory **2010**, 28 (4), 8-13.

Abstract

Fractionation procedures are often used in comparative proteomics for the depletion of highly abundant proteins, raising the concentration of low abundance proteins above the detection threshold. We aimed to verify if the noise and inter-batch bias introduced by an additional fractionation step in a 2DE workflow hampered the detection of the time-dependent response of gilthead seabream to pre-slaughter stress. Multivariate analysis of the resulting datasets by multidimensional scaling indicates that the biological responses have a greater magnitude than the noise introduced by the fractionation and that inter-batch bias is no larger than the bias of performing different IEF/SDS-PAGE runs.

2.1. Introduction

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. Sub-cellular fractionation procedures for muscle tissue have been used for some time in both gel and MS-based proteomics to address this situation[286-287], simplifying 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. Specific examples of the use of these techniques for proteomic analysis include organelle and nucleus isolation[288-292], enrichment of extracts for secretory[293] or membranar proteins[294] and albumin/immunoglobulin depletion of serum samples[295-296]. On the other hand, since its use implies a greater number of sample processing steps than doing the whole extract, it is expected that some noise may be introduced by a fractionation procedure. It seems therefore important for comparative proteomic experiments to simultaneously ensure that no inter-batch biases are introduced and that intra-batch noise is minimized, so that the benefits of performing this step outweigh its disadvantages.

This study aims to assess if the noise introduced by a specific muscle fractionation procedure previously described by Kjærsgård and Jessen for the analysis of rainbow trout muscle[297-298] is significant by comparing it to the technical noise introduced by the different steps of the whole procedure, using the gilthead seabream (*Sparus aurata*), a marine teleost species, as model. It was also important to verify the presence of any relevant inter-batch bias that could hamper the detection of the biological responses aimed to be measured (response to pre-slaughter stress), in order to determine the validity of this fractionation method for large-scale comparative muscle proteome analysis.

2.2. Materials and methods

A preliminary assessment of the effect of the fractionation method described by Kjærsgård and Jessen[297-298] was performed by applying it twice to a single muscle sample, in order to obtain two myofibrilar fractions (containing high amounts of myosin, tropomyosin

and actin) and two sarcoplasmic fractions. A whole extract of this sample was also obtained by using a standard 2DE extraction buffer (urea 7 M, thiourea 2 M, 2 % (w/v) CHAPS, DTT 50 mM, EDTA 1 mM, 1 % (v/v) IPG 3-10 ampholytes and a protease inhibitor cocktail). All protein extracts were cleared from salts and contaminants using a standard TCA/acetone-based 2DE sample precipitation kit and a small-format gel was run for these three extracts (myofibrilar fraction, sarcoplasmic fraction, whole extract) using a standard 2DE protocol (7 cm pH 4-7 strips and 4-12% Bis-Tris gels stained with colloidal CBB). After this initial step, small-format gels were also used to separate in triplicate the sarcoplasmic fractions of the two fractionations for an initial evaluation of batch-to-batch variation, having run all gels in the same IEF/SDS-PAGE batch. Image analysis was performed using PDQuest™ 2DE gel analysis software which performs semi-automatic detection, alignment, background subtraction, quantification and normalization of protein spots. Further data analysis was performed using the R statistical computing environment[299].

For the experiment itself, two groups of five gilthead seabreams (*Sparus aurata*) each were subjected to distinct levels of pre-slaughter stress (profound anesthesia vs. net crowding for 30 mins) and sacrificed by the administration of a lethal dose of AQUI-S™ anesthetic, in order to assess its effects on flesh quality. Three samples of the dorsal muscle were collected from each individual at distinct time points (post-slaughter at 0h, pre-*rigor mortis* at 6h and post-*rigor mortis* at 48h) for a total of thirty samples. These samples were homogenized, fractionated and freed from contaminants as described earlier. The extraction/fractionation procedure was performed in five batches to prevent intra-batch variability (due to the impossibility of carrying out some of its steps in parallel for all samples). The sarcoplasmic fraction of each sample was then separated by 2DE using a standard large-format gel protocol (three batches of IEF, using 24 cm pH 4-7 strips and five batches of SDS-PAGE using 12,5% Tris-Glycine gels stained with colloidal CBB). Sample distribution among batches was designed to prevent variable confounding, by balancing and randomization to avoid the least possible artificial correlation between sample source, pre-slaughter stress, time, extraction/fractionation batch, IEF batch and SDS-PAGE batch. Each of the thirty 2DE gels obtained was digitalized and analyzed using SameSpots™ 2DE gel analysis software which performs semi-automatic detection, alignment, background subtraction and quantification of protein spots, besides being analysed with PDQuest™ 2DE gel analysis software. Further data analysis was performed using the R statistical computing environment[299].

2.3. Results

The initial small-format gel trial showed an improvement in gel quality and sensitivity threshold for a number of proteins when comparing the whole extract map with the sarcoplasmic map (Figure 2.1). This can be attributed both to the depletion of high abundance spots in the acidic region and removal of a smeared protein train in the high molecular range (which can be seen in both the whole extract and myofibrilar fraction gels). For this reason, reproducibility analysis of the fractionation procedure was performed by focusing on the sarcoplasmic fraction.

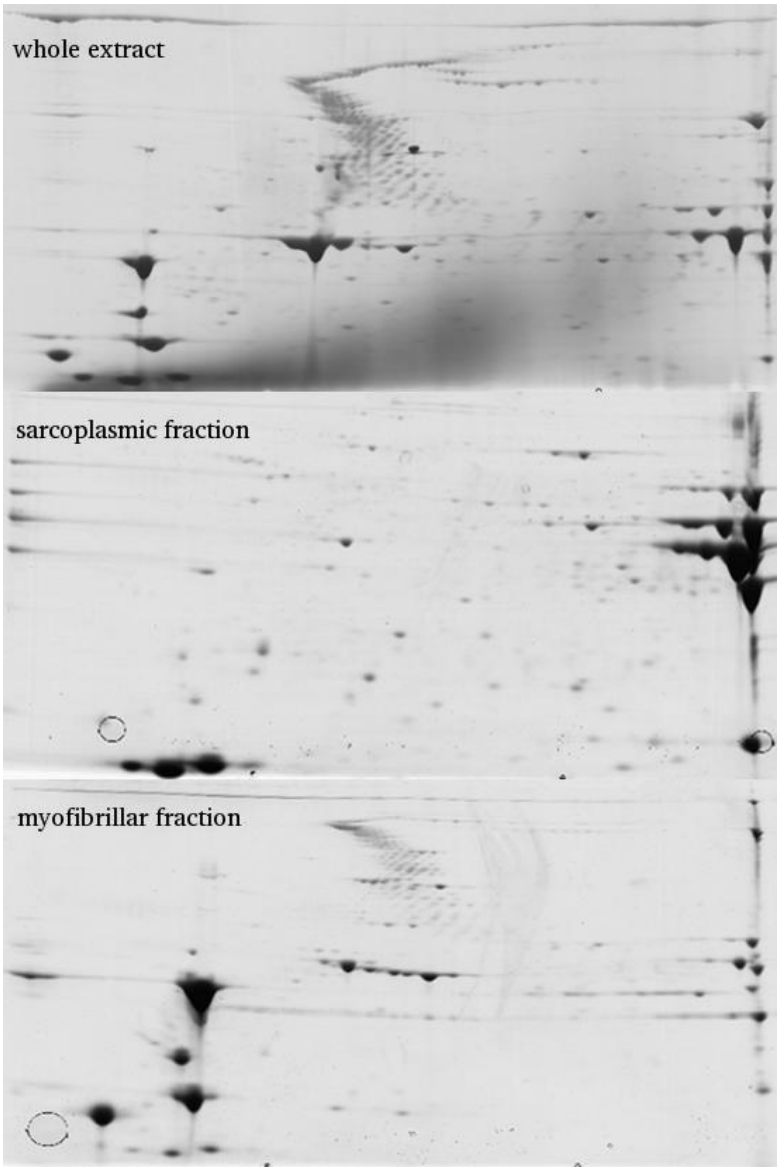

Figure 2.1 – Protein maps in small-format gels for gilthead seabream whole muscle extract; sarcoplasmic fraction and myofibrilar fraction.

Using the 141-spot dataset resulting from the small-format gel trial after VSN transformation (for normalization and variance stabilization[283, 300-301]), dissimilarity measures were calculated between every pair of gels: 1-|τ|, where τ is Kendall tau correlation coefficient; 1-|ρ|, where ρ is the Pearson correlation coefficient; and the Euclidian distance between gels. Visualization of the dissimilarity matrices obtained was performed using classical metric multidimensional scaling (MDS, Figure 2.2). Stability of the MDS plots was assessed by repeating (n = 10000) the multidimensional scaling for truncated copies of the original data matrix (obtained by randomly removing 1/3 of the variables) and superimposing the results on the original plot using the Procrustes algorithm (as implemented in the "vegan" R package[302]).

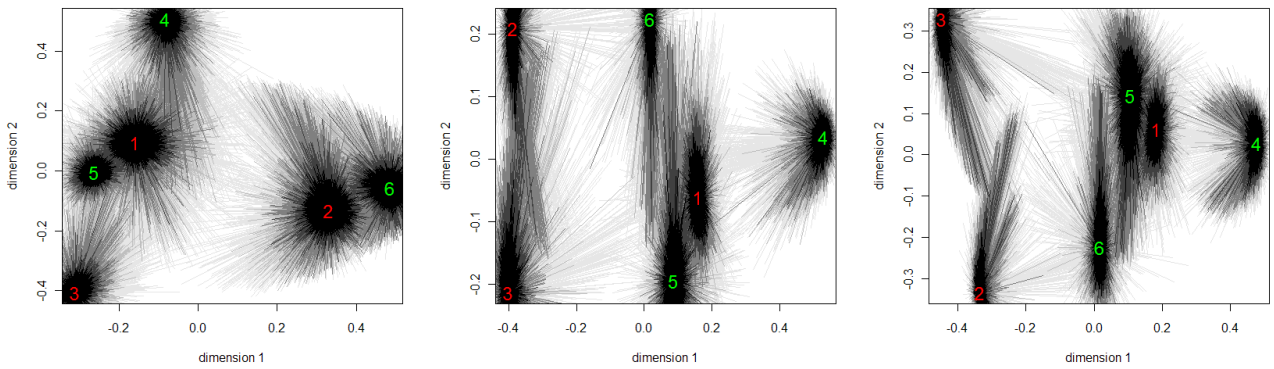

Figure 2.2 – Plot of a small set of gels of the same muscle sample in a 2D space. Each segment represents an MDS calculation using the average distance obtained from k truncated datasets (where goes from k = 1, for light gray, up to k = 20, for black segments). The position of each data point was obtained based on the average distance across all 10000 truncated datasets. One sample of muscle was extracted and fractionated in two batches (red and green). The left image was calculated using 1-|τ| as dissimilarity measure, the center image was calculated using 1-|ρ| as dissimilarity measure and the right image was calculated using the Euclidian distance between gels.

A similar analysis was applied to the 311-spot SameSpots dataset resulting from the main experiment itself, except for the addition of an arsinh transformation ($\log_e(x+(x^2+1)^{0.5})$) for variance stabilization (Figure 2.3), as well as to the 145-spot PDQuest dataset obtained from the same set of gels after VSN transformation (Figure 2.4).

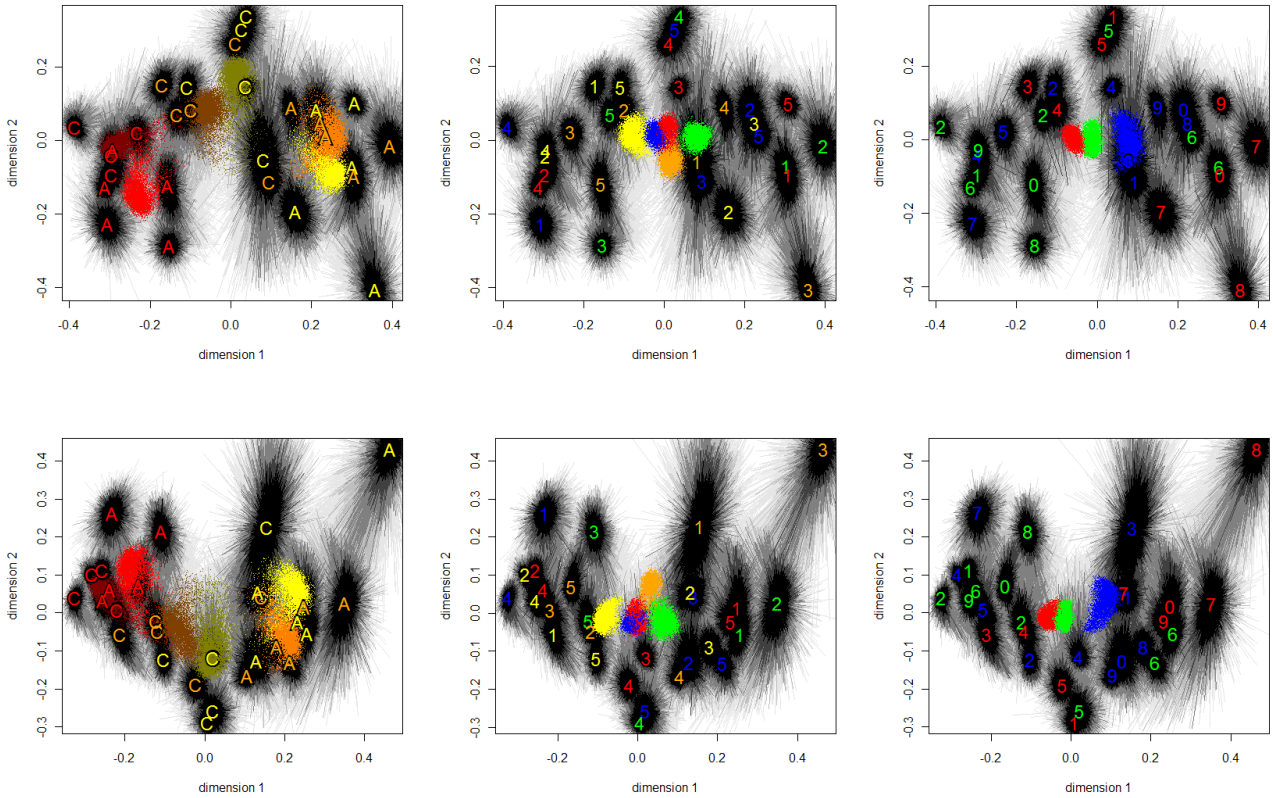

Figure 2.3 – Plot of the gels analysed with SameSpots in a 2D space by multidimensional scaling. Each segment represents an MDS calculation using the average distance obtained from k truncated datasets (where goes from k = 1, for light gray, up to k = 20, for black segments). The position of each data point was obtained based on the average distance across all 10000 truncated datasets. The first column represents the treatment (net Crowding / Anesthesia) and the sampling time (yellow [0 h], orange [6 hours], red [48 h]); the second column represents the SDS-PAGE batch (numbers) and the extraction batch (color-coded); the last column represents the fish ID number and IEF batch (color-coded). Each pixel of the colored blobs represents the centroid of the groups according to the color-coded variables (extraction batch, IEF batch) calculated for each of the truncated datasets, except for the first column, where the centroids were calculated for all 6 possible time/treatment groups (darker color for points corresponding to the "net crowding" treatment). The top images were calculated using 1-|τ| as dissimilarity measure and the bottom images were calculated using the Euclidian distance between gels.

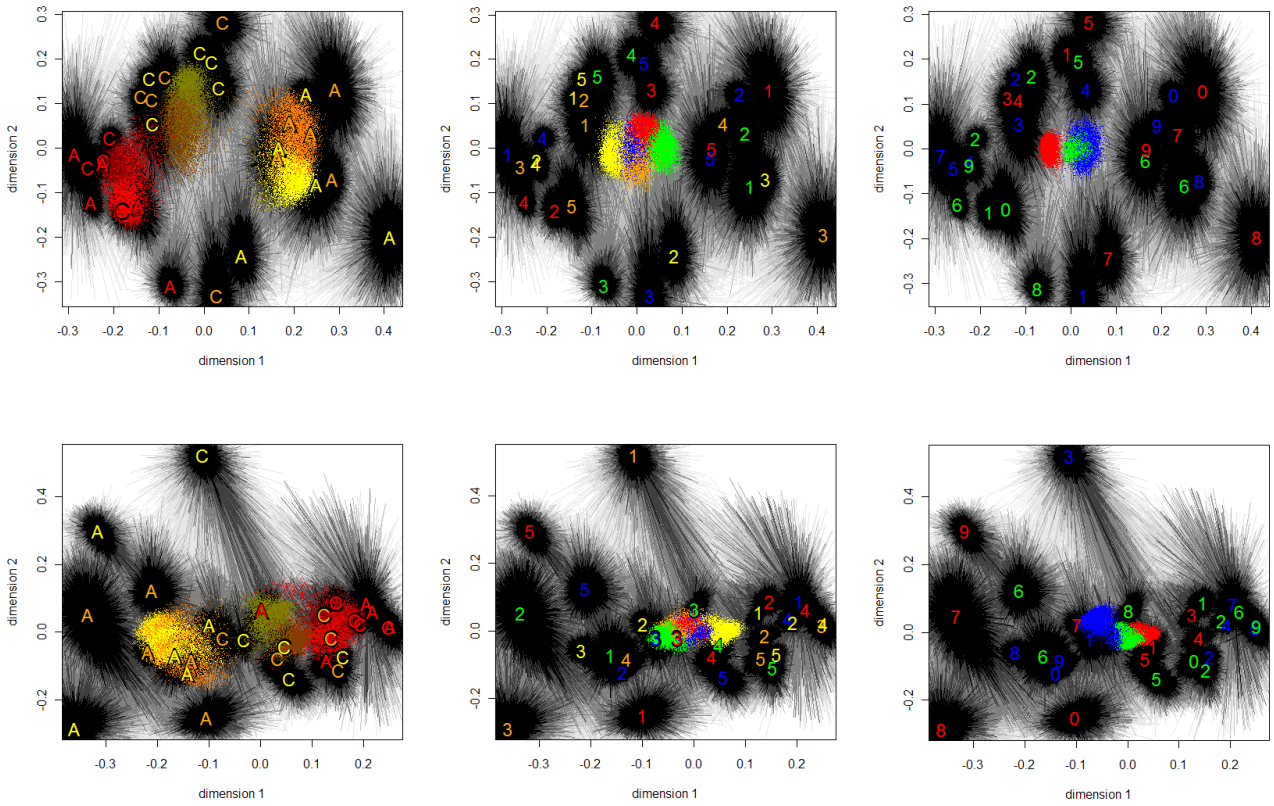

Figure 2.4 – Plot of the gels analysed with PDQuest in a 2D space by multidimensional scaling. Each segment represents an MDS calculation using the average distance obtained from k truncated datasets (where goes from k = 1, for light gray, up to k = 20, for black segments). The position of each data point was obtained based on the average distance across all 10000 truncated datasets. The first column represents the treatment (net Crowding / Anesthesia) and the sampling time (yellow [0 h], orange [6 hours], red [48 h]); the second column represents the SDS-PAGE batch (numbers) and the extraction batch (color-coded); the last column represents the fish ID number and IEF batch (color-coded). Each pixel of the colored blobs represents the centroid of the groups according to the color-coded variables (extraction batch, IEF batch) calculated for each of the truncated datasets, except for the first column, where the centroids were calculated for all 6 possible time/treatment groups (darker color for points corresponding to the "net crowding" treatment). The top images were calculated using 1-|τ| as dissimilarity measure and the bottom images were calculated using the Euclidian distance between gels.

2.4. Discussion

Multidimensional scaling plots using different dissimilarity measures for the first trial gels (Figure 2.2) shows no significant difference between two distinct fractionations of a sample run on two gels and a single fractionation of a sample run on two different gels.

Interestingly enough, there is a transition in structure when the dissimilarity measure based on the Kendall tau is used, resulting in a plot with more isotropic, stable distortions; this stresses the relevance that different metrics can have on the resulting embedding. Regardless, according to all three measures there is no consistent bias among gels from the same extraction/fractionation batches that enables a distinction between the two groups (i.e. the two nearest neighbors of each gel are predominantly gels from the other fractionation batch).

In Figure 2.3, multidimensional scaling reveals that the dataset obtained with SameSpots for the main experiment displays significant differences between the two levels of pre-slaughter stress (especially on the first two sampling points); a significant difference can also be seen between all points pre-*rigor mortis* and the points post-*rigor mortis*, although these early points from the net crowding treatment seem to have a profile more similar to the late sampling points. This is consistent with an accelerated onset of *rigor mortis* in the stressed fish, which was observed during this experiment. In contrast, there is no apparent structure that can be seen as a result of the different extraction batches, as the centroids are clustered together in the center and their mutual distances have similar magnitudes to their dispersions. This is similar to what happens with the IEF grouping centroids and unlike what happens to the time/treatment centroids. As Figure 2.4 illustrates, a similar trend is obtained when the gel images are analysed with PDQuest, with the experimentally relevant variables (pre-slaughter stress, time) having a greater impact in the dataset's structure than the technical variables (including fractionation batch).

The dendogram (which is the most common way of displaying dissimilarity matrices) does not seem as informative as the MDS plots to describe the dissimilarity structure between gels due to the fact that it imposes an hierarchical structure on the dataset regardless of the implied structure, which can obscure some interesting spatial relations between the data points. Nevertheless, as observed in Figure 2.5, no clustering can be seen as a result of the extraction batch, as gels seem to be grouped roughly in three groups according to the biological variables in a consistent way to what was observed by MDS (grouped as anesthesia t0+anesthesia t6, net crowding t0+net crowding t6, anesthesia t48+net crowding t48; with some overlap between anesthesia t6/net crowding t0 and anesthesia t48/net crowding t6).

Unsupervised clustering and low-dimensional embedding visualization methods applied to the datasets obtained during this study using two distinct gel systems and two distinct image analysis software systems suggest that the use of this fractionation procedure introduces a relatively low amount of noise (compared to the baseline technical noise) and allows for reduced inter-batch bias, due to the fact that samples fractionated in different

batches displayed the same level of variation as two samples fractionated in the same batch. In addition, when attempting to cluster the samples using several different dissimilarity metrics, no grouping made a clear distinction between fractionation batches. This indicates that this fractionation method can be useful for some comparative proteomic studies involving muscle tissue, especially in cases where assessing the quantity of low abundance soluble proteins is important or it is essential to pinpoint the observed proteomic changes to a specific location in the cell.

Chapter 3.

Effects of pre-slaughter stress levels on the postmortem sarcoplasmic proteomic profile of gilthead seabream muscle.

This chapter has been previously published as:

Tomé S. Silva, Odete D. Cordeiro, Elisabete D. Matos, Tune Wulff, Jorge P. Dias, Flemming Jessen, Pedro M. Rodrigues. **Effects of pre-slaughter stress levels on the postmortem sarcoplasmic proteomic profile of gilthead seabream muscle.** Journal of Agriculture and Food Chemistry **2012**, 60 (37), 9443-9453. (doi:10.1021/jf301766e)

Abstract

Fish welfare is an important concern in aquaculture, not only due to the ethical implications, but also for productivity and quality-related reasons. The purpose of this study was to track soluble proteome expression in postmortem gilthead seabream muscle and to observe how pre-slaughter stress affects these postmortem processes.

For the experiment, two groups of gilthead seabream (n=5) were subjected to distinct levels of pre-slaughter stress, with three muscle samples being taken from each fish. Proteins were extracted from the muscle samples, fractionated and separated by 2DE. Protein identification was performed by MALDI-TOF-TOF MS.

Analysis of the results indicates changes on several cellular pathways, with some of these changes being attributable to oxidative and proteolytic activity on sarcoplasmic proteins, together with leaking of myofibrillar proteins. These processes appear to have been hastened by pre-slaughter stress, confirming that it induces clear postmortem changes in the muscle proteome of gilthead seabream.

3.1. Introduction

The gilthead seabream (*Sparus aurata*) is an important marine aquaculture species that is commercially reared in Southern Europe. The most important criteria regarding aquaculture fish like the gilthead seabream are health value, food safety and quality (specifically, optimal organoleptic characteristics such as texture, aroma and taste). Fish welfare is also an important factor, not only due to the ethical implications, but also because there are indications that fish welfare generally promotes improved health status, higher productivities and higher product quality criteria[68].

Fish muscle organoleptic traits are generally multifactorial in nature: they depend on factors such as muscle cellularity, mobility of water pools, composition (fatty acid profile, salts, collagen, amino acids), myofibrillar structural integrity and oxidative/proteolytic status. Besides that, the relative weight of these different factors on the final product quality is different between fish species, making it impossible to directly extrapolate information obtained for a single species across all.

Although some knowledge about the impact of postmortem processes on the flesh quality of gilthead seabream is available[109, 303-305], it is also important to study how the pre-slaughter stress that aquaculture gilthead seabream is often exposed to may interact with such postmortem processes and possibly impair fish muscle quality.

Due to the complex and multigenic nature of flesh quality traits, the use of a proteomic approach which enables the simultaneous detection of hundreds of proteins is highly beneficial, by providing a global vision on the protein content of tissues in specific physiological states. For this study, we chose to analyze specifically the sarcoplasmic fraction of gilthead seabream muscle in an attempt to maximize the number of visualized proteins (by depletion of the highly-abundant myofibrillar proteins), particularly those associated with metabolic and regulatory processes.

In parallel with the proteomic analysis, several other physiological and biochemical parameters were measured[306], which contribute to the reconstruction of a plausible model of gilthead seabream muscle degradation.

3.2. Materials and Methods

3.2.1. Fish rearing

Two groups of 32 gilthead seabream with a mean initial body weight of 473 ± 108 g were reared in 1000 L circular plastic tanks at CCMAR facilities and fed a commercial diet, once a day, until apparent satiation, reaching a final body weight of 536 ± 96 g. The seabream originated from a commercial fish farm and had been reared at CCMAR facilities during the previous 12 months. These were subjected to natural photoperiod and sea water from Ria Formosa (37°00′ N, 07°56′ W) was supplied by a flow-through system (mean rearing density: 17 kg/m^3, mean temperature: 23.1 ± 1.2 °C, salinity: 37.4 ± 0.5‰) with artificial aeration (dissolved oxygen: 88 ± 11%). Prior to harvesting, fish were starved for 48 h.

3.2.2. Pre-slaughter harvesting stress and sampling

For the experiment itself, two groups of five gilthead seabream (*Sparus aurata*) each were subjected to distinct levels of pre-slaughter stress (profound anesthesia *versus* net crowding for 20 min), and sacrificed by the administration of a lethal dose (270 ppm) of a water dispersible isoeugenol-based anesthetic (AQUI-S™ New Zealand Ltd., Lower Hutt, New Zealand, an anesthetic approved in several non-EU countries, with zero withdrawal period for use in fish for human consumption) in order to isolate the effects of pre-slaughter stress on flesh quality.

For the profound anesthesia condition, the water level in the tank was slowly lowered, without disturbing the fish, and AQUI-S was added to the tank (previously diluted in 1 L of salt water), for a final concentration of 60 ppm of AQUI-S. Once lethargic, fish were immediately slaughtered. For the net crowding condition, fish were confined to an extremely high density (approximately 140 kg/m^3) inside a cylindrical net, and the water level was slightly lowered until the fish became agitated. Fish were kept in this condition for approximately 20 min prior to slaughter. Three samples of the dorsal muscle were collected from each individual at distinct time points (post-slaughter at 0 hr, pre-*rigor mortis* at 6 hr, and post-*rigor mortis* at 48 hr) for a total of 30 samples.

In parallel with the proteomic analysis, several other parameters were measured for the same fish, namely pH, lipid oxidation levels (using a thiobarbituric acid assay), collagen content (by hydroxyproline assay), sulphated glycosaminoglycan content, instrumental texture parameters and rigor index temporal profile. A separate analysis of the non-proteomic data has

already been published[306].

3.2.3. Protein extraction and fractionation

Sarcoplasmic extracts for each of the 30 muscle samples were prepared from 200 mg of tissue, which was homogenized (Ultra Turrax T25 Basic, IKA Labortechnik) in 1.5 mL of a mild extraction buffer (Tris-HCl 50 mM pH 7.4, dithiothreitol (DTT) 10 mM, EDTA 1 mM and a small amount of a protease inhibitor cocktail) three times for 30 s with a 30 s pause between cycles to prevent sample heating. After mechanical homogenization, a 20 minutes centrifugation step was performed (11200 ×g, 4ºC), retaining the supernatant which contained mostly sarcoplasmic proteins. Extracts were kept on ice or at 4 ºC during the whole extraction/fractionation procedure. This procedure was performed in five batches to prevent intrabatch variability (due to the impossibility of carrying out some of its steps in parallel for all samples). This method was adapted from Kjaersgard et al.[307] and its applicability for comparative purposes has recently been explored[308]. All protein extracts were then cleared from salts and contaminants using a standard TCA/acetone-based 2DE sample precipitation kit (ReadyPrep 2-D Cleanup Kit, Bio-Rad) and quantified using a standard Bradford colorimetric method.

3.2.4. Two-dimensional gel electrophoresis (IPG-IEF/SDS-PAGE)

600 μg of protein from each sarcoplasmic extract was loaded on to 24 cm IPG ReadyStrip pH 4–7 strips (Bio-Rad) by passively re-swelling them overnight in 450 μL of total volume (using a standard IPG-IEF buffer for sample dilution, as appropriate). Proteins were separated in the first dimension by isoelectric focusing using an Ettan IPGphor (Amersham), at 20 °C, for a total of about 43 kV.hours. Strips were then equilibrated using standard Bio-Rad reduction/alkylation buffers (15 min each step), loaded on to manually cast large-format 12,5% Tris-HCl SDS-PAGE gels and run at 50 mA/gel (after a short initial period at 10 mA/gel), using a standard Tris-glycine-SDS running buffer. After electrophoresis, gels were stained with EZBlue™ colloidal CBB stain (Sigma), according to a manufacturer's instructions. Due to the impossibility of running the whole 2DE procedure in parallel for all samples, three batches of IEF and five batches of SDS-PAGE were performed. Sample distribution among batches was chosen to prevent variable confounding by balancing and randomization (avoiding introducing any artificial correlation between sample source, pre-slaughter stress level, time of sampling, extraction/fractionation batch, IEF batch, and SDS-PAGE batch).

3.2.5. Gel scanning and image analysis

After staining, each of the 30 gels was digitized using a GS-800 calibrated densitometer (Bio-Rad) at a 42.3 μm/pixel resolution and analyzed using Progenesis SameSpots^{TM} 2DE gel analysis software (Nonlinear Dynamics, Newcastle upon Tyne, U.K.) — which performs semiautomatic detection, alignment, background subtraction, and quantification of protein spots. The software automatically assigns a unique numeric identifier to each spot. Normalization of background-subtracted spot volumes across gels is done by estimating a per-gel gain factor calculated from a recursive median of the log-transformed spot abundance values, which makes it robust to spot volume distributions with abnormal skewness and/or kurtosis. As an alternative to SameSpots' native normalization algorithm, we also employed the VSN ("variance stabilization and normalization") method, which applies an inverse hyperbolic sine transformation after removing additive and multiplicative biases estimated with least trimmed sum of squares (LTS) regression.

Experimental spot pI values were estimated from the spots' horizontal positions on the 2DE gel, and linearly interpolating between the two pH extremes (4 and 7). Molecular weights were estimated by running a separate gel with an appropriate molecular weight marker to obtain a calibration curve for the estimation of a spot's log(Mw) based on its vertical position.

3.2.6. Data visualization and feature selection

Datasets exported from SameSpots were then analyzed using the R statistical computing environment[299]. Data visualization was performed using principal component analysis (PCA) and metric multidimensional scaling (MDS) taking $1-|\tau|$ as dissimilarity measure, where τ is Kendall's rank correlation between gels. For variable selection, partial least squares regression (PLS2) was used, as implemented in The Unscrambler software (Camo Software, Oslo, Norway). 44 distinct PLS models were obtained by: choosing different normalization methods (both SameSpots' native algorithm and the VSN method); optional pre-filtering of probably irrelevant spots (using the Mann-Whitney univariate test for comparison between groups, and taking a p-value > 0.05 threshold as indicative of low relevancy); choosing different scaling methods (autoscaling, group scaling and mean-centering by subject followed by group scaling); using 3 different Y-matrices (where the *time* factor is expressed in 3 different ways). These models were obtained by progressive removal of features using jack-knife validation, in a way that the explained validated Y-variance and

the validated correlation between PLS components and Y-matrix variables were maximized. The values obtained for these parameters were generally around 70-80% and 0.7-0.9, respectively. Protein spots which contributed to explain the X-matrix variation according to the Y-matrix factors (*treatment* and *time*) in more than 10 PLS models were considered for manual excision from the 2DE gels and subsequent identification by MS.

3.2.7. Protein identification by MALDI-TOF-TOF

After reduction and alkylation (using DTT and iodoacetamide, respectively), protein spots were digested with trypsin and the reaction stopped with 0.1% trifluoroacetic acid. After a concentration/desalting step, the peptides were co-crystallized with the matrix (DHB) on a MALDI plate and analyzed with an Ultraflex II MALDI-TOF-TOF mass spectrometer (Bruker-Daltonics). Spectra were obtained in positive reflector mode and externally calibrated using a tryptic digest of β-lactoglobulin. These were analysed and converted to MS and MS/MS peak lists using FlexAnalysis 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 Actinopterygii subset of the NCBInr 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). Whenever no significant hits were obtained with the NCBInr database, searches were repeated against the Actinopterygii subset of the Vertebrates_EST database. Identity of non-annotated transcripts was assessed using blastp (http://blast.ncbi.nlm.nih.gov/) against the NCBInr database. For each identified spot, we also attempted to determine the specific isoform (whenever isoform-specific peptides could be identified from the MS/MS data).

3.3. Results

Analysis of the 30 gel images enabled the detection and quantification of 313 spots across all gels. A representative example can be seen in Figure 3.1. Preliminary visualization of the 30 × 313 matrix using PCA (Figure 3.2) suggests the samples can be roughly divided in three clusters: pre-rigor unstressed fish (*cluster 1*); pre-rigor stressed fish (*cluster 2*); post-rigor stressed and unstressed fish (*cluster 3*). This implies the observed differences between treatments are greater pre-rigor than post-rigor. Also, cluster 3 seems more similar to cluster 2 than to cluster 1, which suggests that, in general, the application of pre-slaughter stress seems to have sped up the transition between the pre-rigor proteomic profile and the post-rigor proteomic profile. This is consistent with our observations of both a quicker onset of rigor in fish subjected to pre-slaughter stress and a general reduction in the differences between the unstressed and stressed fish groups over time for other parameters (like measured muscle pH)[306].

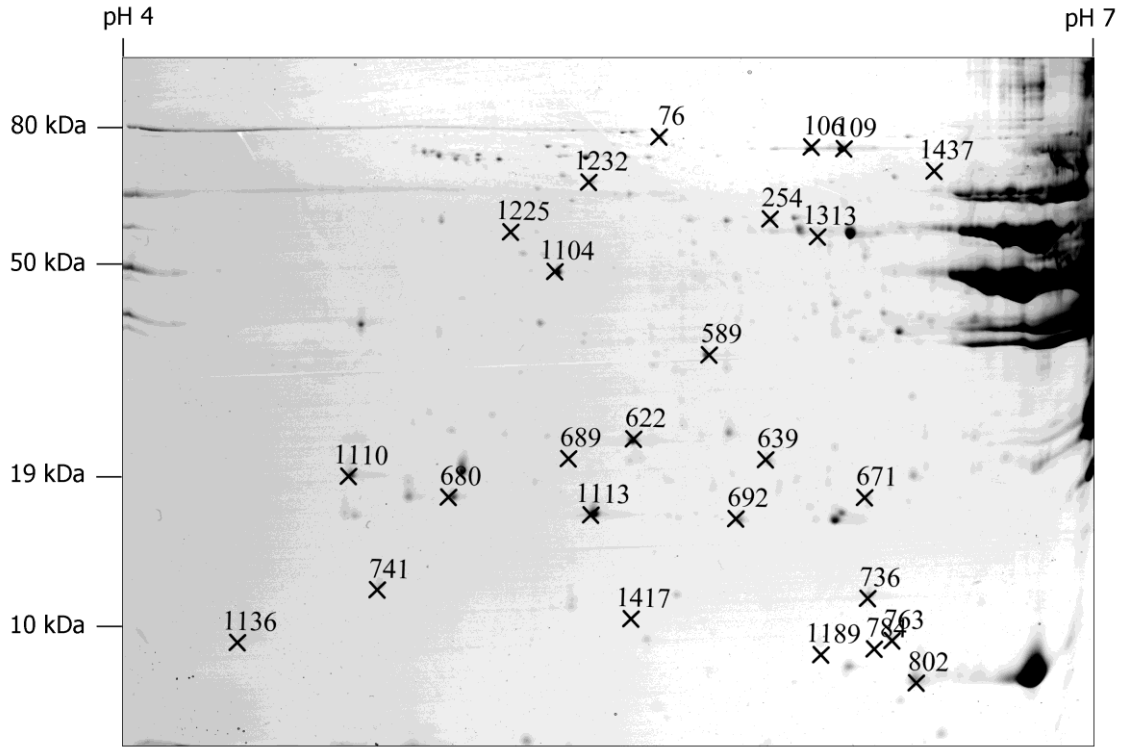

Figure 3.1 – Representative high-resolution and contrast-enhanced example of obtained 2DE gels, denoting the position of reliably identified protein spots.

As it can be seen in Figure I (Supporting information), most protein spots were not included in any of the 44 obtained PLS models, with only 79 of the 313 being included in

more than 10 models. Visualization with PCA suggests this reduced 79-spot dataset contains a lower amount of irrelevant information, while simultaneously preserving most of the biological information (see Figure 3.2). Particularly interesting is the fact that true dimensionality of the dataset is only clear in this second PCA analysis, where the first two components can be directly associated with the two experimentally-implied factors ("harvesting stress condition" and "time elapsed since slaughter").

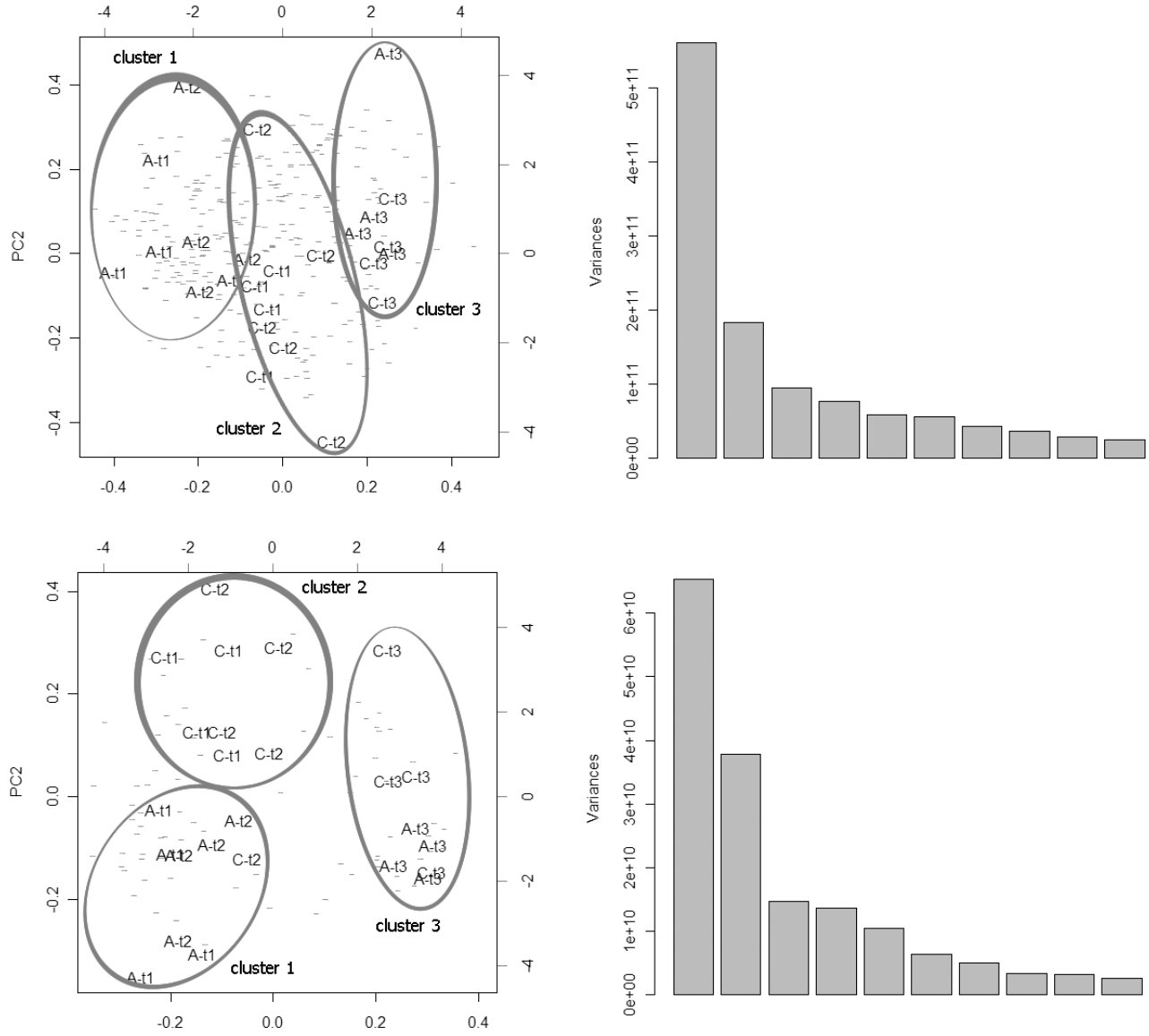

Figure 3.2 – Principal component analysis before (above, 313 variables) and after variable selection (below, 79 variables). On the left, biplots showing the similarities between all samples (as measured by the Euclidian distance, after mean-centering and auto-scaling). Samples are labeled with "A" (for "anesthesia") or "C" (for "crowded") and the corresponding sampling time ("t1", "t2" or "t3", meaning t = 0 h, 6 h and 48 h, respectively). On the right, explained variance for each principal component.

These observations were confirmed by using a different similarity metric (based on Kendall's correlation) and using metric multidimensional scaling to plot the results in 2D (Supporting information, Figure II).

Another interesting observation when comparing several measured physiological parameters with the results obtained by the proteomic analysis, was that two of the protein spots showed a significant degree of correlation with the measured muscle pH ($\rho = 0.80$ and 0.89, respectively; or $\tau = 0.74$ and 0.82, respectively, if Kendall's correlation measure is used), as it can be seen in Figure IV (Supporting information). Repeated simulations with randomly permuted versions of the original dataset seem to indicate that values of τ above 0.46 (or below -0.46) only occur by chance with a probability below 0.0001. Besides protein spots selected by PLS regression, we also sought to identify these two protein spots displaying correlation to measured muscle pH.

All non-overlapping spots deemed relevant which displayed acceptable resolution and intensity were excised from the gels, digested with trypsin and subjected to MS analysis. Of these, we were able to reliably identify 25 of them (see Figure 3.1 and Table 3.1). It should be noted that, for some of the identified spots, observed deviations between estimated and theoretical molecular weights suggest that they are very likely to constitute proteolytic fragments of the respective full-length proteins. A quick overview of the results for these spots can be seen in Table 3.2 and the heatmap of Figure 3.3. More detailed time-dependent protein abundance profiles for these proteins can also be seen in Figure VI (Supporting information).

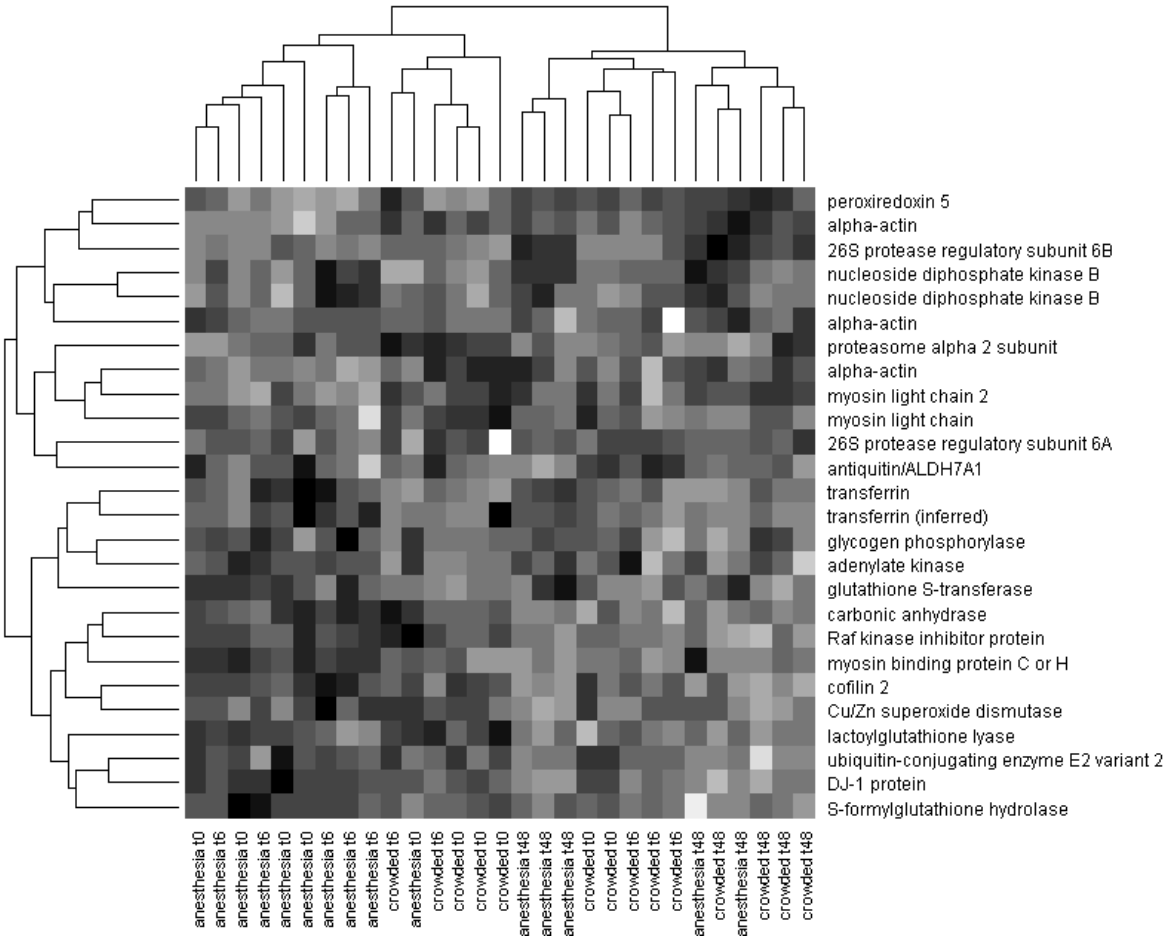

Figure 3.3 – Heatmap showing relative abundance of each sequenced spot across all samples, along with simultaneous clustering of spots and gels (using Euclidian distance as dissimilarity measure and the "complete linkage" criterion for agglomerative hierarchical clustering) for this reduced dataset. Dark colors indicate lower-than-average expression while light colors indicate higher-than-average expression.

Table 3.1 – List of reliably sequenced proteins, along with relevant MS-related information. Theoretical Mw and pI values were estimated from the protein's composition (based on available sequence data). Only peptides with E-value below 0.05 (discarding oxidized versions and other duplicates) were counted for the calculation of the number of matched peptides per protein, estimated coverage and combined Mowse score. Entries marked with an asterisk (*) indicate a possibility that the identified spot constitutes a proteolytic fragment, while entries with a double-asterisk (**) indicate that it is very likely that the identified spot constitutes a proteolytic fragment.

spot ID	protein identification	accession #	species	exp. Mw/pI	theo. Mw/pI	coverage (%)	# peptide matches	best peptide match: sequence, E-value	combined Mowse score
76	myosin binding protein C or H	AAI46704.1	*Danio rerio*	81 kDa / 5.4	58 kDa / 5.4	4%	3	TGDWFTVLEHYHR, 9.2e-07	139
106	transferrin	CAL92187.1	*Chaenocephalus aceratus*	76 kDa / 5.8	76 kDa / 6.4	2%	1	ASAEEQYYGYAGAFR, 0.00026	75
109	transferrin (inferred)	----------	----------	76 kDa / 5.9	----------	----------	----------	----------	----------
254	antiquitin/ALDH7A1	AAX54912.1	*Acanthopagrus schlegelii*	57 kDa / 5.7	56 kDa / 5.9	7%	5	AISFVGSNSAGEYIYER, 4.7e-09	231
589	S-formylglutathione hydrolase	ACO09141.1	*Osmerus mordax*	32 kDa / 5.5	32 kDa / 6.0	2%	1	KIPVVFR, 0.0039	47
622	carbonic anhydrase**	AAQ89896.1	*Oreochromis mossambicus*	23 kDa / 5.4	29 kDa / 7.7	5%	1	YPAELHLVHWNTK, 5.6e-07	104
639	glutathione S-transferase**	AAQ56182.1	*Sparus aurata*	21 kDa / 5.7	35 kDa / 8.5	22%	5	LIPDSPAEQALTYQR, 3.5e-06	427
671	adenylate kinase 1-2*	ACM41863.1	*Epinephelus coioides*	18 kDa / 5.9	21 kDa / 7.6	31%	6	KVDSELPVDEVFGQVSK, 1.8e-08	388
680	lactoylglutathione lyase*	ACO09023.1	*Osmerus mordax*	17 kDa / 4.9	20 kDa / 5.2	12%	4	GFGHIGIAVPDVYEACK, 6.2e-07	143
689	proteasome alpha 2 subunit*	ACI66157.1	*Salmo salar*	21 kDa / 5.2	26 kDa / 6.0	8%	1	LVQIEYALAAVAAGAPSVGIK, 7e-08	104
692	dj-1 protein*	CAG07041.1	*Tetraodon nigroviridis*	16 kDa / 5.6	20 kDa / 6.1	24%	3	AGIAVTVAGLTGKEPVQCSR, 6.4e-10	204
736	cofilin 2 (muscle)**	NP_991263.1	*Danio rerio*	12 kDa / 6.0	19 kDa / 7.6	18%	2	KEDLVFIFWAPEGAPLK, 4e-08	223
741	myosin light chain 2**	BAA95125.1	*Thunnus thynnus*	12 kDa / 4.7	19 kDa / 4.7	64%	8	EFLEELLTTQCDR, 2.1e-07	705
763	nucleoside diphosphate kinase B**	AAG13336.1	*Gillichthys mirabilis*	10 kDa / 6.0	17 kDa / 6.8	19%	2	TFIAVKPDGVQR, 0.0066	133
784	Cu/Zn superoxide dismutase	CAI79044.1	*Sparus aurata*	9 kDa / 5.9	7 kDa / 5.4	28%	1	MLTLSGPLSIIGR, 7.1e-07	138
802	nucleoside diphosphate kinase B**	ADG29125.1	*Epinephelus coioides*	9 kDa / 6.1	17 kDa / 6.8	40%	4	GDLCINIGR, 0.0023	159
1104	alpha-actin	AAA29847.1	*Styela clava*	45 kDa / 5.1	42 kDa / 5.2	20%	6	SYELPDGQVITIGNER, 1.3e-07	486
1110	myosin light chain	BAA12731.1	*Cyprinus carpio*	19 kDa / 4.6	21 kDa / 4.7	13%	3	VAYNQIADIMR, 4.7e-06	128
1113	phosphatidylethanolamine binding protein**	AAB06983.1	*Mus musculus*	16 kDa / 5.2	21 kDa / 5.2	18%	2	YNLGAPVAGTCYQAEWDDYVPK, 2.5e-06	172
1136	beta-actin**	AAH45846.1	*Danio rerio*	9 kDa / 4.3	42 kDa / 5.3	2%	1	IWHHTFYNELR, 9.6e-05	80
1189	ubiquitin-conjugating enzyme E2 variant 2**	NP_998680.1	*Danio rerio*	9 kDa / 5.9	16 kDa / 7.8	8%	2	VECGSRYPEAAPTVR, 0.0085	93
1225	26S protease regulatory subunit 6B	AAH55215.1	*Danio rerio*	52 kDa / 5.0	47 kDa / 5.0	15%	4	ENAPAIIFIDEIDAIATK, 0.00013	303
1232	glycogen phosphorylase**	AJ719440.1	*Gallus gallus*	65 kDa / 5.2	97 kDa / 6.0	1%	1	IIDGWQVEEADDWLR, 2.6e-05	86
1313	26S protease regulatory subunit 6A	AAB24840.1	*Homo sapiens*	53 kDa / 5.8	49 kDa / 5.1	12%	4	DSYLILETLPTEYDSR, 5.4e-08	322
1417	peroxiredoxin 5**	ADI78068.1	*Sparus aurata*	11 kDa / 5.3	28 kDa / 8.9	7%	1	AVDLLLDSDQIVQVLGNKR, 4.7e-10	129
1437	skeletal alpha-actin	CAA24598.1	*Sparus aurata*	71 kDa / 6.1	42 kDa / 5.5	7%	2	SYELPDGQVITIGNER, 4.8e-08	168

Table 3.2 – Differential expression trends and univariate statistics for each identified protein. Direction and magnitude of effect size (Cohen's d) for the stress factor (at each time point) and for the time factor (separately for anesthesia and crowded groups). Effect sizes over one standard deviation are marked in bold. Asterisk (*) next to a number denotes statistical significance of the observed effect (assessed by Mann-Whitney-Wilcoxon's U-test, $p < 0.05$). Protein names marked with an asterisk (*) indicate a possibility that the identified spot constitutes a proteolytic fragment, while entries with a double-asterisk (**) indicate that it is very likely that the identified spot constitutes a proteolytic fragment.

		harvesting stress effect			time effect					
		anesthesia → crowding			T0 → T6		T0 → T48		T6 →T48	
spot #	protein identification	T0	T6	T48	A	C	A	C	A	C
76	myosin binding protein C or H	**2,62***	**2,84***	-0,42	0,16	**1,87**	**1,87**	-0,24	-0,27	-0,45
106	transferrin	0,93	**2,07***	0,89	-0,36	0,77	0,25	-0,12	0,58	0,53
109	transferrin (inferred)	0,28	**2,63***	**1,12**	-0,72	**1,26***	0,29	0,72	0,69	**1,01**
254	antiquitin/ALDH7A1	0,34	**-1,28***	**-1,03**	0,88	0,13	**1,31***	-0,92	0,76	0,03
589	S-formylglutathione hydrolase	0,33	**1,93***	0,54	-0,32	**1,33***	**1,09***	**1,38***	0,89	**1,03***
622	carbonic anhydrase**	**1,42***	**1,02**	0,77	0,33	**2,21***	**3,11***	0,20	0,78	0,85*
639	glutathione S-transferase**	**1,97***	**1,73***	**1,15***	0,31	0,16	0,40	0,10	0,74	0,77
671	adenylate kinase 1-2*	**2,31***	**1,51**	**1,42***	-0,25	0,66	0,38	0,73	0,63	**1,11**
680	lactoylglutathione lyase*	0,83	-0,55	**1,47***	**1,53***	0,30	**3,38***	-0,26	**2,17***	0,93
689	proteasome alpha 2 subunit*	0,56	0,59	0,50	0,24	**2,06***	**2,00***	0,11	0,74	0,76
692	dj-1 protein*	**1,28**	**2,88***	0,75	0,84	**6,01***	**5,09***	**2,35***	0,98*	**1,21***
736	cofilin 2 (muscle)**	-0,02	**1,86***	0,68	-0,60	**5,12***	**6,27***	**1,35***	0,88*	0,99*
741	myosin light chain 2**	**-1,55***	-0,86	0,27	**1,88***	**-3,02***	-0,93	**1,06**	-0,35	0,56
763	nucleoside diphosphate kinase B**	-0,10	**1,54***	0,95	**-2,22***	0,45	**-2,01***	**-1,23***	0,74	0,43
784	Cu/Zn superoxide dismutase	-0,20	0,84	0,64	-0,95	**2,64***	**1,43***	0,02	0,98	0,98*
802	nucleoside diphosphate kinase B**	-0,86	**1,50***	**1,18***	**-3,13***	-0,78	**-4,07***	-0,34	0,61	0,52

3.4. Discussion

During the course of this study, we were able to observe time-variable postmortem changes in the sarcoplasmic proteome of gilthead seabream modulated by pre-slaughter stress. Identified proteins included cytoskeletal proteins, as well as proteins related to the redox homeostasis, proteasome/ubiquitin pathway, detoxification, energy homeostasis and signal transduction. We sought to interpret observed changes in light of current literature. A schematic overview of the possible relationships between pre-slaughter stress and these cellular processes can be seen in Figure 3.4.

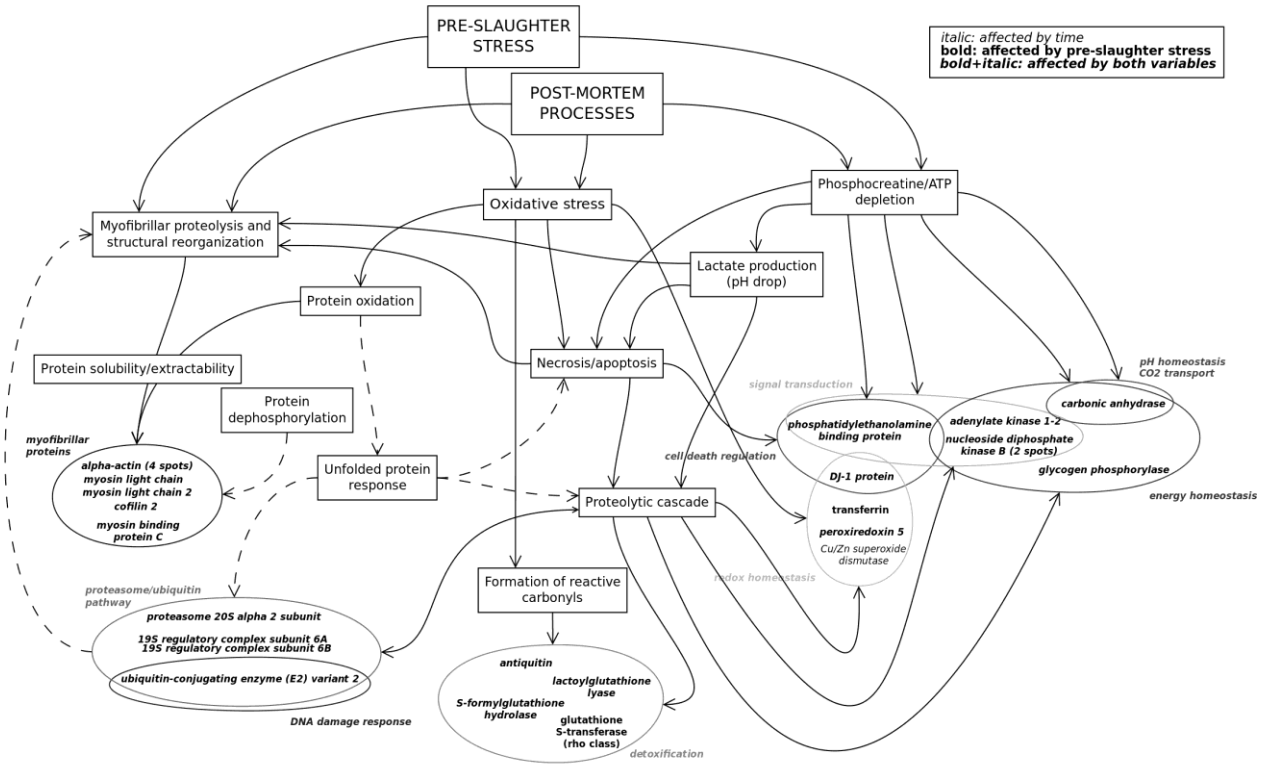

Figure 3.4 – Schematic overview of the putative interactions between induced pre-slaughter stress and known post-mortem processes in muscle.

3.4.1. Energy homeostasis and signal transduction

Efficient coupling of spatially segregated ATP-producing and ATP-consuming cellular processes requires the action of several enzymes belonging to the so called "phosphotransfer networks", such as creatine kinase (CK), adenylate kinase (AK), nucleoside diphosphate kinase (NDPK), carbonic anhydrase (CA) and some glycolytic enzymes, which help to rapidly dissipate ATP/ADP gradients across the cell, effectively facilitating the spatial

transmission of high-energy phosphoryl groups from ATP-producing to ATP-consuming sites. The high rate of energy production and consumption in active muscle suggests that phosphotransfer networks should have an essential role in the correct and efficient function of this tissue. This was evident from the 2DE gels of the sarcoplasmic fraction of muscle, where a high abundance of these proteins was observed (particularly CK).

During this study, a progressive increase in the abundance of an AK spot was noted in fish subjected to stress, although some of these seemed to have lower inductions (displaying an abundance temporal profile similar to control fish). On the other hand, given this spot's observed molecular weight, it is possible that it constitutes an ~18 KDa proteolytic fragment of the 21KDa full-length AK, which would imply a higher (but variable) degree of proteolysis in stressed fish, with non-stressed fish displaying a general lack of proteolytic activity towards AK. A possible explanation for the increase in proteolytic activity against AK might be that stress-induced expression of AK has simply increased availability of this protein, increasing the chances of proteolytic events involving AK. Another possibility is that changes in the ATP/ADP/AMP proportions due to induced pre-slaughter stress might affect steady-state concentrations of the different AK conformers, leading to a differential observed proteolytic activity towards AK depending on the pre-mortem energetic status. Finally, it also has to be considered that pre-slaughter stress might have simply sped up the cascade of proteolysis (i.e. proteolytic activation of proteases), resulting in generally higher levels of proteolysis towards all sarcoplasmic proteins.

Besides AK, we were also able to detect three other protein spots (two NDPK spots and one CA spot) which are almost certainly proteolytic fragments. In both cases, stressed fish displayed increased abundance of these spots, compared to control (in the case of CA, at all sampling times; in the case of NDPK, only on later sampling time-points). Also, a general trend of increased abundance over time could be observed for both spots. Explanations for the observed increase in the abundance of proteolytic fragments can be linked, as said above for AK, on several factors. At least in the particular case of NDPK, nucleotide binding has been shown to stabilize it[309], suggesting that stress-induced ATP depletion might directly affect NDPK's susceptibility to proteolysis. Interestingly, recent studies on sea bass showed that NDPK muscle abundance is likely to decrease postmortem, regardless of the fish storage temperature, due to proteolysis [310-311], which is consistent with what we observed.

Another relevant protein was identified as phosphatidylethanolamine binding protein (PEBP), which is also known as the Raf-1 kinase inhibitor protein (RKIP), an ubiquitous protein with a role in signal transduction (namely, as a suppressor of the mitogen-activated

protein kinase [MAPK] pathway)[312]. Studies have shown RKIP prevents phosphorylation of MEK1/MAP2K1, NIK/MAP3K14 and TAK1/MAP3K7 through competitive inhibition, therefore suppressing signaling to downstream kinases and associated transcription factors (like AP-1 and NF-κB)[313]. In our experiment, increased levels of a RKIP proteolytic fragment in fish subjected to pre-slaughter stress was generally observed, as well as a positive correlation between elapsed time since slaughter and protein abundance in both treatments. It is possible that the phosphorylation state of RKIP might affect its susceptibility to proteasome-mediated proteolysis[314], which could be the underlying link relating pre-slaughter stress with increased RKIP proteolysis.

Another protein associated with energy homeostasis in muscle is glycogen phosphorylase, which is responsible for mobilization of glucose stored as glycogen, enabling a quick response to increased energy requirements. In this study, we observed higher abundance of a proteolytic fragment of glycogen phosphorylase in fish subjected to pre-slaughter stress, especially at 6 h after slaughter, although the effect is still visible 48 h after slaughter. This difference, again, points towards higher levels of proteolysis in fish stressed pre-slaughter, possibly associated to a higher degree of myofibrilar degeneration (as glycogen phosphorylase is known to tightly bind to myofibrillar proteins, which suggests its apparent abundance in sarcoplasmic extracts is likely to be affected by leakage and oxidation of myofibrillar proteins[315]).

3.4.2. Cellular response to redox stress

Peroxiredoxin 5 is a 3-Cys hypoxia-inducible mitochondrial isoform of peroxiredoxin which, when overexpressed, seems to induce a protective effect against oxidative stress-induced apoptosis. During our experiment, the postmortem abundance of a proteolytic fragment of this protein is progressively reduced with time. This would imply that peroxiredoxin 5 is particularly susceptible to early proteolysis, as high amounts of this fragment can be seen on the first sampling, with subsequent samplings showing a clear reduction in the abundance of this spot. Again, pre-slaughter stress appears to hasten this process, as stressed fish displayed consistently lower abundance levels of this proteolytic fragment at all time points, compared to unstressed fish.

Another important element in cellular redox homeostasis is the Cu/Zn superoxide dismutase: a protein which catalyzes the conversion of superoxide radicals into oxygen and hydrogen peroxide. Although no significant differences were found in stressed fish, the abundance of this protein was increased post-rigor in both treatments (compared to pre-rigor),

suggesting an increased expression (or limited turnover) of this protein. This could be a reflection of the importance of this protein in the cellular defense against the highly toxic superoxide radicals, in contrast with peroxiredoxin 5, which can be functionally complemented by the activity of other peroxidases (like catalase).

Iron is a metabolically essential metal which can act as pro-oxidant when in its free form, catalyzing the formation of hydroxyl and peroxide radicals from hydrogen peroxide. The pro-oxidant activity of iron in skeletal muscle is mainly controlled by transferrin and ferritin, which have the capacity to chelate this metal, maintaining it in the ferric form (Fe^{3+}). Two spots identified as transferrin displayed generally higher abundance in fish subjected to pre-slaughter stress, especially at 6 and 48h after slaughtering. This could be a sign of a higher degree of iron import into muscle (complexed with transferrin) or a lower degree of transferrin recycling in response to the applied stress. It's interesting to note that higher levels of transferrin have already been detected in the sarcoplasmic fraction of PSE (pale, soft, exudative) bovine meat[316], having been explained as a possible consequence of the effects of hypoxia on angiogenesis and tissue remodeling. Further insight into the interaction between pre-slaughter stress and iron homeostasis in muscle could be obtained if the levels of ferritin and total iron were also assessed in parallel.

Another protein related to cellular redox stress is the DJ-1 protein (also known as PARK7, due to its association with Parkinson's disease). The biological function of this protein is still not completely figured out, although it seems to have (among others) antioxidant and protein chaperone roles and, specifically in the skeletal muscle of mice, to be involved in the regulation of the Ca^{2+} pool[317]. During the course of our study, we observed an increase over time in the abundance of what appears to be a proteolytic fragment of this protein, as well as generally higher abundance in fish subjected to pre-slaughter stress. At least two experiments on postmortem muscle degradation in land animals also revealed an increase in the abundance of several DJ-1 protein spots over time[318-319]. Besides that, another study on the effects of pre-slaughter stress on the soluble muscle proteome of trout showed results consistent to ours: the application of pre-slaughter stress induced lower concentrations of the full-length protein in muscle, pointing towards a higher degree of proteolytic activity[320].

3.4.3. Detoxification processes

Antiquitin (also known as ALDH7A1) is a highly conserved member of the aldehyde dehydrogenase family which catalyzes conversion of L-2-aminoadipate-6-semialdehyde (an intermediate in lysine catabolism) into L-2-aminoadipate, using NAD^+ as cofactor. A recent

study with purified recombinant human ALDH7A1 suggests it is probably also related to osmotic regulation (due to observed betaine aldehyde dehydrogenase activity) and lipid peroxidation (due to observed dehydrogenase activity regarding some lipid peroxidation products)[321]. During our experiment, slightly decreased levels of ALDH7A1 were detected in fish subjected to pre-slaughter stress, specifically after 6h postmortem. This could be a sign of increased oxidation, proteolysis and/or aggregation of ALDH7A1 in fish subjected to pre-slaughter stress.

S-formylglutathione hydrolase (also known as esterase D) participates in formaldehyde detoxification by catalyzing hydrolysis of S-formylglutathione into formate and glutathione. Formaldehyde can be endogenously produced from methanol and methylamine, being also a side-product of methionine catabolism, histidine catabolism and oxidative demethylation of nucleic acids. During our experiment, we observed generally increased levels of S-formylglutathione hydrolase in fish subjected to pre-slaughter stress at early time-points, which suggests a direct cellular response to induced physiological stress. We also observed a small induction of this protein (regardless of stress state) after slaughter, which is consistent with observations on postmortem porcine muscle[322].

Furthermore, there were two other spots identified as proteolytic fragments of proteins also involved in glutathione metabolism: glutathione S-transferase and lactoylglutathione lyase. In the case of glutathione S-transferase, it is interesting to note that non-stressed fish display very stable low abundances for this proteolytic fragment, while stressed fish display relatively higher and more variable abundances. In the case of lactoylglutathione lyase, abundance increases over time, but pre-slaughter stressed fish display generally higher abundance levels. This seems to indicate that the breakdown of the detoxification systems is generally hastened by the action of pre-slaughter stress.

One last fact to consider when interpreting observations related to the detoxification pathways is that both groups of fish were slaughtered using a lethal dosage of isoeugenol. As suggested by Matos et al.[306], it is possible that isoeugenol might interact with oxidative stress and detoxification processes.

3.4.4. Proteasome/ubiquitin pathway

Although the caspase pathway seems to be the most important proteolytic system regarding fish muscle death and postmortem degradation, there are signs that other proteolytic systems are likely to be involved (namely, the calpain, cathepsin, matrix metalloprotease and proteasome systems) and that the relative importance of these systems on fish meat quality

may be species-dependent. During our experiment, we observed abundance variations in several spots related to the 26S proteasome system: two of them are components of the 19S regulatory complex, one is a fragment of a component of the 20S proteasome and the last one is a fragment of an ubiquitin-conjugating enzyme variant.

The abundance of two components (with ATPase activity) of the 19S regulatory complex, namely the 6A subunit (also known as S6', PSMC3, TBP1 or Rpt5) and the 6B subunit (also known as S6, PSMC4, TBP7 or Rpt3), was seen to change over time, depending on pre-slaughter stress levels. According to our observations, the abundance of the 6A subunit had a tendency to stabilize over time and there seemed to be lower amounts of this subunit in stressed fish. Since this subunit is involved in polyubiquitin recognition, this may imply that either the proteasome pathway has lower proteolytic activity in stressed fish or that it occurs via the ubiquitin-independent pathway (which acts preferentially during oxidative stress). Interestingly, the 6A subunit has been implicated in the regulation of HIF1α abundance (a transcription factor that regulates gene expression in response to hypoxia), mediated by its interaction with pVHL (an E3 enzyme). In fact, overexpression of the 6A subunit has been shown to promote HIF1α degradation and blocking its expression via siRNA has been shown to reduce HIF1α degradation by the proteasome [323]. This suggests that decreased levels of this subunit could constitute a regulatory mechanism to increase HIF1α half-life in response to the applied pre-slaughter stress. In the case of subunit 6B, the time profile seems to suggest a postmortem induction followed by a progressive reduction in abundance (i.e. a peak), which was delayed in control fish (compared to fish subjected to pre-slaughter stress). Still, given the low abundance of this protein at 48 h in both groups, the idea that the ubiquitin-independent pathway and/or other proteolytic pathways contribute more significantly for postmortem muscle degradation than the ubiquitin-dependent pathway seems plausible.

Regarding the proteolytic fragments observed, for the case of ubiquitin-conjugating enzyme E2 variant 2, an increase in the abundance of this fragment could be observed over time, with pre-slaughter stress inducing even higher levels. This effect is more pronounced in later time-points, again suggesting a higher degree of proteolysis in pre-slaughter stressed fish. In the case of the proteolytic fragment of 20S proteasome subunit alpha 2, it seems to increase over time for non-stressed fish, while, for stressed fish, it initially increases and then decreases in abundance, resulting in distinct abundance levels at 48h (compared to non-stressed). This should be a sign of proteolytic activity in stressed fish against this fragment, suggesting again generally higher levels of proteolytic activity in pre-slaughter stressed fish.

The results obtained therefore suggest either a shift from ubiquitin-dependent to ubiquitin-independent proteasome proteolytic activity or a general breakdown of the proteasome system, suggesting this system might be of secondary relevance regarding postmortem proteolytic events in gilthead seabream muscle. It is nevertheless possible that some of the observed changes reflect the role of the proteasome in the post-translational regulation (i.e. turnover) of transcription factors.

3.4.5. Myofibrillar proteins

After slaughtering, biochemical and physical changes that occur in fish muscle have a significant impact in the oxidation, solubility, phosphorylation and proteolytic state of the structural/contractile proteins contained in the myofibrils. Analysis of the presence of these proteins in sarcoplasmic extracts has to also take into account the phenomenon of leakage that is associated to postmortem myofibril degeneration.

In this work, we observed changes in the abundance of several putative myofibrilar proteins in our sarcoplasmic extracts: actin (3 spots: apparently one full-length and two actin fragment spots), myosin light chain 1, myosin light chain 2, cofilin-2 and myosin binding protein (isoform C or H). The three actin spots showed a general reduction in abundance over time. These could be a reflection of proteolytic activity towards actin and/or reduced protein solubility due to denaturation/aggregation. For two of those spots (the ones with lower molecular weight), the group subjected to pre-slaughter stress displayed lower abundance, while the other spot (apparently full-length actin) showed no significant differences between groups. This general reduction in actin abundance over time (for both full-length actin and fragments) can be explained by denaturation and aggregation due to postmortem oxidative stress, which could reduce their extractability. If this assessment is correct, this seems to be an indirect confirmation of higher levels of oxidative stress in fish subjected to pre-slaughter stress.

The two myosin light chains identified during this experiment also displayed generally lower abundance in fish subjected to pre-slaughter stress. One of them (myosin light chain 1, a non-phosphorylatable isoform) appeared at a very high molecular weight, which suggests it could be some soluble aggregate or otherwise covalently modified form of the protein. Its abundance apparently increased over time, but no significant differences between groups could be noted at 48 h postmortem. The other myosin light chain spot observed (isoform 2, phosphorylatable) displayed an induction around 6 h postmortem and then its abundance levels fell again to low levels. If this specific spot corresponds to the phosphorylated isoform

of this protein, the observed abundance profile could just be a reflection of the postmortem myofibril contraction dynamics and energetic state.

Myosin binding protein (MyBP) is mainly localized in the C-zone of the thick filament, aiding in the assembly and maintenance of the myosin bundles which constitute the thick filaments. We have observed an ultimate high abundance of MyBP in both groups. In the group subjected to pre-slaughter stress, MyBP levels were already high immediately after slaughter, which suggests a contribution of pre-slaughter stress to hasten the process of postmortem myofibril disassembly, increasing MyBP extractability.

Interestingly, previous observations by Jessen et al., correlating 2DE with sensory analysis data, suggest actin, fast myosin light chain 2, myosin binding protein and phosphorylated cofilin-2 may have a relation with rainbow trout muscle texture. Western blot with anti-phospho-cofilin confirmed positive correlation of phosphorylated cofilin-2 abundance measured immediately after slaughter with juicy texture, suggesting cofilin-2 dephosphorylation is an important mechanism influencing juiciness in rainbow trout fish muscle (unpublished results). A study on pig muscle identified a cofilin-2 spot in the TES-soluble fraction of the muscle which displayed decreased abundance over time[322]. In our case, we have identified a cofilin-2 spot which displayed increased abundance over time, with a negligible pre-slaughter stress effect. This is consistent with the fact that no significant differences were noted in terms of instrumentally measured texture parameters between our control and stressed groups[306].

In general, when observing this group of proteins as a whole, although transient effects can be seen immediately after slaughter and at 6 h after slaughter, no significant differences can be seen at 48 h between the control and stressed groups.

3.4.6. Correlations between proteomic and physiological/biochemical observations

During this study, we decided to co-measure several physiological and biochemical parameters on the same set of gilthead seabream muscle samples, in parallel with the proteomic analysis. Besides providing us with a more complete view of the interaction of pre-slaughter stress with the postmortem cellular processes in the muscle tissue, it enabled us to look for correlations between proteomic and non-proteomic observations which could be useful in the discovery and validation of surrogate measurements for the estimation of proteomic data from (possibly higher-throughput and cheaper to obtain) non-proteomic data.

As mentioned in the Results section, the only relatively strong correlations observed were between measured pH values (shown in Figure III of the Supporting information) and

the abundance levels of two protein spots (see Figure IV of the Supporting information). So far, we could only identify one of the spots as being one of the alpha-actin spots, although the unidentified spot (referred to as "spot #1115") displays an even better correlation with measured muscle pH. It is interesting to note that none of these variables correlate well with elapsed time since slaughter, so it is unlikely that these observed correlations are a simple reflection of time, since the relation remains even if we calculate the partial correlations (with the influence of the time variable removed).

Regarding the underlying reasons to the observed correlation between measured muscle pH and an alpha-actin spot, it might be difficult to pinpoint them without more information. Besides differences in protein abundance, the observed change in spot abundance should be due to leakage of myofibrillar proteins, but may also involve post-translational modifications (e.g. oxidation state, phosphorylation) and/or changes in solubility/extractability.

Therefore, although any causal relationship between measured muscle pH and the abundance of such proteins, particularly myofibrillar ones, in the sarcoplasmic fraction of muscle should not be assumed without further data, it might be interesting to further explore this possibility, if any of these proteins are shown to be predictive of some specific physiological state of the muscle.

3.5. Final considerations

Regardless of the fine details of how the physiological response to pre-slaughter stress occurs in the muscle of gilthead seabream, there are clear modulations of the postmortem cellular processes, especially at the level of cytoskeletal/myofibrillar proteins, energy homeostasis processes, response to oxidative stress, signal transduction and cell cycle regulation, with most of these changes being attributable to myofibril degeneration, oxidative and proteolytic activity. Although some of the specific proteins identified have not yet been characterized as being specifically associated with postmortem processes in fish muscle, the overall results generally follow what is expected, given what is currently known about the cellular response to external sources of stress and energy depletion in muscle.

Despite the observed differences between the low stress and high stress groups (especially in the early time points) at the proteome level and in terms of measured muscle

pH, there were no observable differences between groups in terms of texture properties (namely, hardness and cohesiveness of cooked and raw fillets)[306]. This shows, on one hand, a relative robustness of gilthead seabream muscle properties to pre-slaughter stress and, on the other hand, the sensitivity of proteomic data in detecting a biological response to certain stimulus, even when no differences are apparent according to macroscopic quality criteria. These factors underline the relevance of proteomics in the context of animal farming, not only in the field of fish welfare and harm reduction, but also fish quality, given the observed hastening effect of pre-slaughter stress on post-mortem muscle degradation processes.

Supporting information available

This information is available in the CD-ROM version of this dissertation and online, at "http://dx.doi.org/10.1021/jf301766e".

Chapter 4.

Dietary tools to modulate glycogen storage in gilthead seabream muscle: glycerol supplementation.

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Abstract

The quality and shelf-life of fish meat products depend on the skeletal muscle's energetic state at slaughter, as meat decomposition processes can be exacerbated by energy depletion. In this study, we tested dietary glycerol as a way of replenishing muscle glycogen reserves of farmed gilthead seabream. Two diets were tested in duplicate (n=35/tank).

Results show 5% inclusion of crude glycerol in gilthead seabream diets induces increased muscle glycogen, ATP levels and firmness, with no deleterious effects in terms of growth, proximate composition, fatty acid profile, oxidative state and organoleptic properties (aroma and color). Proteomic analysis showed a low impact of glycerol-supplementation on muscle metabolism, with most changes probably reflecting increased stress coping capacity in glycerol-fed fish.

This suggests inclusion of crude glycerol in gilthead seabream diets (particularly in the finishing phase) seems like a viable strategy to increase glycogen deposition in muscle without negatively impacting fish welfare and quality.

4.1. Introduction

When fish are slaughtered, skeletal muscle and other peripheral tissues experience anoxia and energy depletion, since the circulatory system fails to provide an adequate supply of oxygen and fuel. As skeletal muscle becomes anaerobic and energy production efficiency drops, glycogen is quickly metabolized to replace ATP reserves and pH drops due to the onset of lactic fermentation[324-325].

This leads to a cascade of tightly-related processes and events (like Ca^{2+} leakage, oxidative stress, protein and lipid oxidation, protein denaturation, rigor onset, mitochondrial damage, breakdown of primary metabolism, proteolysis, rigor resolution, cell death processes) that characterize the post-mortem transition of fish muscle to fish meat and which, depending on how extensive and intensive they are, can ultimately lead to detrimental changes in the meat quality criteria[326].

The dynamics of these processes are affected by contextual factors (such as prior muscle energetic and oxidative status, temperature, pH) and therefore, it is important to take into account that certain management/logistic procedures can exacerbate (e.g. pre-slaughter stress) or delay (e.g. refrigeration) muscle decomposition and therefore affect the final product quality traits (like texture) and shelf-life[327-328]. Specifically for gilthead seabream, there are indications that increased pre-mortem starvation periods (and, therefore, impaired pre-mortem energetic states) contribute to reduced shelf-life[329].

Given this, delaying energy depletion in muscle through dietary manipulation (by increasing its pre-mortem glycogen reserves) could contribute to the preservation of the muscle's organoleptic traits (particularly texture). Similar types of strategies were already tried for cod[330] and pig[331-333], where they attempted to delay these post-mortem dynamics either by trying to slow down the glycolytic rate (with magnesium, sodium oxalate, ascorbate and quercetin) or through the use of muscle buffering substances such as citrate or histidine.

In this trial, we chose to study the effect of crude glycerol derived from biodiesel production in gilthead seabream muscle. There is evidence that different species of fish use glycerol in different ways[334-336]. In some cases, glycerol is not used itself as a gluconeogenic precursor, but still provides energy, sparing other gluconeogenic precursors (like lactate) and therefore indirectly contributing towards glucose production and glycogen replenishing, which seems to be the case for rainbow trout[337].

Regarding land-based animals, like cows[338], pigs[339-343] and chicken[344], there is a great wealth of studies regarding glycerol-inclusion in feeds and, at least for inclusion levels below 10%, it was often found to increase growth rates, increase feed intake, improve meat yield, increase ultimate muscle pH, replenish muscle glycogen reserves and improve water holding capacity. On the other hand, some of these studies have also shown that glycerol can have some impact on body composition, fatty acid profile and other organoleptic properties.

In fish and other marine organisms, although there are already some studies within this subject, knowledge on the dietary use of crude glycerol is still scarce and limited to a few species, namely Nile tilapia and channel catfish[345-346]. Furthermore, no information on the possible impact of dietary glycerol on organoleptic properties has been obtained in any of these works. For gilthead seabream specifically, the only relevant study we found relates to addition of pure glycerol to the rearing water of prelarvae and larvae, for the purpose of boosting hepatic glycogen reserves, in order to improve survival during early stages of development[347]. Results of this study confirmed dose-dependent hepatic glycogen deposition due to glycerol uptake.

Our study was therefore mainly focused on testing the putative ability of glycerol to help replenish skeletal muscle glycogen energy reserves, and therefore improve its pre-mortem energy status, in farmed gilthead seabream. We also sought to verify if this dietary supplementation induced any deleterious effects in terms of fish health and quality. For this, an interdisciplinary approach was undertaken, having obtained complementary information on physiological and quality status through both unspecific (proteome expression data) and specific means (biochemical, histological, instrumental and sensory organoleptic data), providing a holistic and unbiased view on the effects of crude glycerol on farmed gilthead seabream.

4.2. Materials and Methods

4.2.1. Experimental diets

Table 4.1 shows the formulations of the diets used in this trial. A control diet (CTRL), similar to commercial seabream feed, was formulated with practical ingredients to contain 50 % crude protein, 18 % crude fat and 20 $kJ.g^{-1}$ gross energy. Another diet was formulated, similar to the control diet, with 5 % crude glycerol, incorporated at the expense of a crude

source of pea starch. All diets were formulated to fulfil the known nutritional requirements of the species. Main ingredients were grinded (below 250 µm) in a micropulverizer hammer mill Hosokawa, model #1. Powder ingredients were then mixed accordingly to the target formulation in a Double-helix Mixer TGC, model 500L to attain a basal mixture. No oils were incorporated at this stage. All diets were manufactured by extrusion (pellet size 5.0 mm) by means of a pilot-scale twin-screw extruder CLEXTRAL BC45 (Clextral, France) with a screw diameter of 55.5 mm and temperature ranging 105-110 ºC. Upon extrusion, all batches of extruded feeds were dried in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 2 hours at 60 ºC. Following drying, pellets were allowed to cool at room temperature, and subsequently the oil fraction was added under vacuum coating conditions in a Pegasus vacuum mixer (PG-10VCLAB, DINNISEN, The Netherlands). Throughout the duration of the trial, experimental feeds were stored at room temperature. Samples of each diet were taken for proximate composition analysis (Table 4.1).

Table 4.1 – Ingredients and proximate composition of the experimental diets.

Ingredients (%)	CTRL	GLY
Fishmeal LT[1]	15.0	15.0
Fishmeal 65[2]	20.0	20.0
Soy protein concentrate[3]	5.0	5.0
Wheat gluten[4]	5.0	7.0
Corn gluten[5]	8.0	8.0
Soybean meal 48[6]	10.0	10.0
Wheat meal	12.0	10.0
Aquatex G2000[7]	10.0	5.0
Fish oil	13.0	13.0
Vit & Min Premix[8]	1.0	1.0
Binder (diatomaceous earth)[9]	1.0	1.0
Crude glycerol[10]	0.0	5.0
Proximate composition		
Dry matter (DM) (%)	94.6 ± 0.1	93.6 ± 0.1
Crude protein (% DM)	48.4 ± 0.2	47.8 ± 0.2
Lipid (% DM)	17.8 ± 0.2	18.1 ± 0.3
Ash (% DM)	6.7 ± 0.0	6.6 ± 0.0
Phosphorus (% DM)	1.11 ± 0.1	1.16 ± 0.0
Gross energy (kJ/g DM)	20.7 ± 0.2	20.6 ± 0.1

[1]Peruvian fishmeal LT: 71% crude protein (CP), 11% crude fat (CF), EXALMAR, Peru.
[2]Fair Average Quality (FAQ) fishmeal: 62% CP, 12%CF, COFACO, Portugal.
[3]Soycomil P: 65% CP, 0.7% CF, ADM, The Netherlands.
[4]VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France.
[5]GLUTALYS: 61% CP, 8% CF, ROQUETTE, France.
[6]Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL, Portugal.
[7]Dehulled grinded pea grits: 24% CP, 0.4% CF, SOTEXPRO, France.
[8]PVO40.01 SPAROS standard premix for marine fish, PREMIX Lda, Portugal.
[9]Kielseguhr: LIGRANA GmbH, Germany.
[10]Biodiesel derived crude glycerol: 82% glycerol, <0.03% methanol, IBEROL, Portugal.

4.2.2. Fish and rearing conditions

Four groups of 42 gilthead seabream with a mean initial body weight of 321 ± 45 g were reared in 1000 L tanks at CCMAR facilities and hand-fed twice a day until apparent satiation. Natural sea water was supplied (mean temperature 26 ± 2 ºC; mean salinity 36.6 ± 0.7 ‰), by a flow-through system with artificial aeration (mean dissolved oxygen 82 ± 20 %). The experimental diets were tested in duplicate over a period of 90 days. Prior to harvesting, fish were starved for 48 h (common practice in the industry).

4.2.3. Sampling

Fish from both treatments were slaughtered by immersion in ice-saltwater slurry until death (common practice in the industry). Fish were then stored with the ventral side upward, covered with plastic and flaked ice in insulated polystyrene boxes.

Fourteen fish from each treatment (seven from each dietary replicate) were weighed, scaled and filleted 24 h after death. One fillet from each of eight fish was separately packed and stored at 4 ºC until sensory assessment, carried out approximately 48 h after slaughter. The remaining fillets were either used for proximate composition and fatty acid profiles (n = 10), or were frozen at -20 ºC for biochemical assays. Additionally, from four fish of each treatment (right fillets), small (1.0 × 0.5 × 0.5 cm) white muscle samples were taken from the dorsal area, under the dorsal fin, and fixed in Carnoy's solution at 4 ºC (for 24 h, with occasional shaking) for muscle morphology by histology.

Twelve fish from each treatment (six from each dietary replicate) were used for muscle pH measurements (n = 6) and rigor mortis measurements (n = 6) at 0, 3, 6, 9, 24 and 48 h after death.

Samples for biochemical and proteomic analysis were obtained from 8 fish per treatment (four from each dietary replicate) at the time of death (T0) and six hours after death (T6), immediately snap frozen in liquid nitrogen and stored at -80 ºC prior to analysis.

Eight fish from each treatment (four from each dietary replicate) were kept for 48 h in a temperature controlled room (4 ºC). Afterwards, the fish were weighed, filleted, fork length, fillet yield, liver and visceral weight were recorded and samples for biochemical and proteomic analysis were snap frozen in liquid nitrogen and stored at -80º C (T48). Unless otherwise specified, all analyses were run on individual fish (n = 8), in triplicate.

Table 4.2 contains an overview of how the fish sampling and distribution between the different assays and techniques was performed.

Table 4.2 – Distribution of samples taken from each individual fish (per experimental treatment).

Fish ID	Left fillet	Right fillet
1-4	Sensory analysis Fatty acid analysis Proximate composition	Instrumental texture (raw)
5-8	Instrumental texture (cooked) Colour measurements	Sensory analisys Fatty acid analysis Proximate composition
9-14	Instrumental texture (cooked) Colour measurements	Instrumental texture (raw)
15-20	muscle pH	muscle pH
21-26	Rigor mortis	Rigor mortis
27-30	Histological characterization *Time 0:* - proteomic analysis - volatile compound analysis - lipid oxidation - muscle glycogen - muscle ATP - specific enzymes activity	*Time 6:* - lipid oxidation - muscle glycogen - muscle ATP - specific enzymes activity
31-34	*Time 6:* - lipid oxidation - muscle glycogen - muscle ATP - specific enzymes activity	*Time 0:* - proteomic analysis - volatile compound analysis - lipid oxidation - muscle glycogen - muscle ATP - specific enzymes activity
35-42	*Time 48:* - lipid oxidation - muscle glycogen - muscle ATP - specific enzymes activity	

4.2.4. Proximate composition

Ash (Method 942.05), moisture (Method 950.46), crude protein (Method 992.15) and fat (Method 991.36) contents were determined by the reference methods[348], using minced fish muscle, without skin. Four pools of two fish per treatment were used.

4.2.5. Fatty acid analysis

Fatty acid composition was determined through the analysis of methyl esters, as previously described[349], from homogenized and lyophilized skinless white muscle. Briefly, the analysis was executed using a Varian Star CP 3800 GC (Walnut Creek, CA), equipped with auto sampler and flame ionization detector. The separation of the different methyl esters was performed in a polyethylene glycol capillary column DB-WAX (Folsom, CA, USA) (30 m x 0.25 mm id, film thickness: 0.25 µm). Identification of fatty acid methyl esters was achieved by comparison of their retention times with those of mixed chromatographic standards (Sigma Aldrich). Peak areas were determined using the Varian software. Results were expressed as relative percentage of total fatty acids. Four pools of two fish per treatment were used.

4.2.6. Instrumental texture analysis

The fillets were kept at 8 – 11 ºC until the texture analyses (approximately 48 h after death). All the right fillets were used for raw texture measurement while the corresponding left fillets were cooked in a saturated steam oven (Rational Combi-Master CM6 Cross Kuchentechnik GmbH, Landsberg a. Lech) during 6 min at 100 ºC. The fillets were allowed to cool down at room temperature and then were kept at 8 – 11 ºC until the texture analysis (approximately two hours). Fillet texture was measured instrumentally using a Texture Analyser Model Instron 4301 (Instron Engineering, Canton, MA, USA) equipped with a load cell of 1 kN and a spherical plunger (12.5 mm diameter). Compression was applied on raw and cooked fillets. The plunger was pressed into the fillets at a constant speed of 2 $mm.s^{-1}$ and penetrated 6.0 and 3.0 mm into the raw and cooked fillets, respectively. This penetration depth was selected as the maximum distance which could be applied without breaking the muscle fibres and affecting the muscle structure. Measurements were performed on each fillet (n = 10 per treatment) in the position corresponding to maximum thickness. Thickness at this point displayed low variability between fillets (control: 16 ± 2 mm; glycerol: 17 ± 2 mm) and should not account for any observed texture differences between treatments.

4.2.7. Colour measurements

Instrumental colour measurements were performed with a tristimulus colorimeter (Macbeth Color-Eye 3000), in a 10 mm diameter measuring area. A 10° standard observer and a D65 illuminant were used. Before each measurement session the colorimeter was calibrated with a white porcelain plate. The coordinates L^*, a^* and b^* (CIELAB colour space)

were used as they relate to the human eye response to colour. The Chroma (C^*) of the fillets was calculated according to the formula[350]: $C^* = (a^{*2} + b^{*2})^{1/2}$. Colour was measured on the white dorsal muscle, on ten cooked fillets per treatment. At each location a value resulting from five consecutive measurements was obtained and the mean value of three locations was used.

4.2.8. Sensory analysis

Sensory evaluation was carried out in an acclimatized room equipped with individual booths. To reduce the variability within the fillets, the parts close to the head and the tail were removed. Each fillet was individually wrapped with perforated aluminium foil and cooked for 6 min at 100 ºC in a saturated steam oven (Rational Combi-Master CM6 Cross Kuchentechnik CmbH, Landsberg a. Lech, Germany). Cooked fillets were presented to the assessors sequentially in coded white dishes under normal white lighting. Eight fillets per treatment were assessed by a panel composed of eight trained assessors, male and female, non-smokers, with ages between 34 and 60 years. Each panellist evaluated one fillet per treatment, using a 12 cm unstructured line scale in which the left end of the scale corresponded to "none" or "absence" while the right end represented high intensity[351], to evaluate the intensity of the odour (typical odour) and colour (white/yellowish) of the fillets.

4.2.9. Volatile compound extraction and analysis

The volatile profiling of the raw fish fillets was evaluated using automated HS-SPME coupled to GC–MS, as previously described[352]. The analysis was performed on samples taken at the time of death, snap frozen in liquid nitrogen and kept at -80ºC prior to analysis. Two pools of four fish per treatment were used.

4.2.10. Histological characterization

After fixation in Carnoy's solution, the muscle samples were then routinely dehydrated in a graded ethanol series, cleared in xylol, and finally embedded in paraffin. Four 10 µm-thick section were cut per block, and then stained with haematoxylin-eosin for morphometric analysis, and with Periodic Acid Schiff (PAS) for glycogen content, before being coversliped.

The morphometric study was made using an interactive image analysis system (Olympus Cell*Family), working with a live-image captured by CCD-video camera (ColorView Soft Imaging System, Olympus) and a light microscope (BX51, Olympus,

Japan). Relative number (density) of white muscle fibres per unit area (μm^2), mean individual muscle fibre area and mean cross-sectional fibre diameter were estimated according to standard procedures[353].

Assessment of muscle glycogen content followed a semi-quantitative approach based solely in three degrees: 1 (low) – when muscle fibres in PAS sections present a weak coloration; 2 (mild) – PAS sections presented a mild coloration; 3 (intense) – PAS sections presented a strong coloration. Six fish per treatment were used for histological characterization.

4.2.11. Measurement of muscle pH

Muscle pH was measured (n = 6 per treatment) directly on the muscle using a pH meter with an insertion electrode (model pH Spear, Eutech Instruments), on 3 replicate locations in the dorsal area of the left fillet.

4.2.12. Rigor mortis

The *rigor* index was evaluated (n = 6 per treatment) as previously described[328]. Fish were handled carefully in order to prevent secondary effects on the development of the *rigor* state.

4.2.13. Lipid oxidation

Oxidation of lipids was determined (n = 8 per treatment) by measuring the thiobarbituric acid reactive substances (TBARS), as previously described[328].

4.2.14. Measurement of glycogen content

The amount of glycogen in the white muscle samples was determined (n = 8 per treatment) as previously described[354]. Glycogen content of the samples was expressed as µg per mg muscle (dry weight).

4.2.15. Measurement of ATP

Lyophilized muscle (around 10 mg) was suspended in 500 µL of a solution containing TCA (2 % w/v) and EDTA (2 mM) and the mixture was vortexed vigorously. Samples were then centrifuged (10000 *g*, 4 ºC, 40 min) and 500 µL of a buffer (pH 3.87) containing Tris-HCl (40 mM), magnesium chloride hexahydrate (20 mM) and EDTA (4 mM) were added to 100 µL of the supernatant. This extract was used to measure ATP content in the muscle (n = 8

per treatment) using the ATP determination kit (Biaffin GmbH & Co KG, Cat. #LBR-S010), and values were expressed as nmol ATP per g of muscle (dry weight).

4.2.16. Enzyme activity assays

The extraction of enzymes was performed as previously described[355]. Briefly, 2 g of muscle was homogenized twice for 30 s using an Ultra Turrax T25 Basic (IKA Labortechnik) in 6 ml of extraction buffer (50 mM Tris–HCl (pH 7.5), 10 mM β-mercaptoethanol, 1 mM EDTA). The supernatant was collected after centrifugation at 10000 g for 40 min at 4 ºC and frozen at -80 ºC prior to analysis.

Cathepsins B and H activity measurements were performed as previously described[355]. Cathepsin activities in the protein extracts were determined at 30 ºC in a 298 µl reaction (6 µl 5% CHAPS, 1 µl of 1.40 M β-mercaptoethanol, 16 µl of 5 % (w/v) Brij™ 35, 5 µl of synthetic fluorogenic substrate prepared in ultrapure water and 70 µl of 0.4 mM acetate/acetic acid (pH 4) buffer containing 10 mM β-mercaptoethanol and 1 mM EDTA). Z-arginine-arginine-7-amido-4-methylcoumarin hydrochloride (10 mM) and L-arginine-7-amido-4-methylcoumarin hydrochloride (5 mM) were used as the fluorogenic substrates for Cathepsin B and Cathepsin H, respectively. The reaction was initiated by adding 200 µl of protein extract. Collagenase-like activity measurement was done as previously described[356]. After thawing, the extracts were centrifuged (7840 g, 4º C for 10 min), and the enzymatic activity was measured against a synthetic fluorogenic substrate, N-succinyl-glycine-proline-leucine-glycine-proline-7-amido-4-ethylcoumarin (SGP). Concentration of SGP (dissolved in DMSO) was 0.0625 mM in 100 mM bis-Tris, 5 mM $CaCl_2$, pH 7.0. Samples were run in triplicate and a standard curve prepared with 7-amido-4-methylcoumarin (AMC) as well as a control with extraction buffer instead of enzyme extract were run in parallel. Increase in fluorescence was monitored using a fluorescence spectrophotometer (Synergy™ 4, BioTek, Winooski, USA), and the excitation (λ_{ex}) and emission (λ_{em}) wavelengths were set to 385 and 460 nm, respectively. One unit of enzyme activity was defined as the amount of enzyme that hydrolyses 1 nmol of substrate per minute, and the results were expressed as $U.g^{-1}$(protein).

Glycogen phosphorylase activity (a + b) was measured spectrophotometrically (340 nm) by following the release of G-1-P from glycogen, using a method previously described[357]. The reaction mixture contained 50 mM Tris (pH 7.6), 5 mM EDTA, 10 mM KH_2PO_4/Na_2HPO_4 (pH 7.6), 10 mM L-cystein, 0.3 mM NADP, 0.05 mM glucose-1,6-diphosphate, 2 mM AMP, 1.68 U phosphoglucomutase, 28 U glucose-6-phosphate dehydrogenase and 0.2 $mg.ml^{-1}$ glycogen. In a microplate, 200 µl of reaction mixture were

added to 25 µl of sample and the slope of increase in absorbance was determined. The slope of increase in absorbance was determined and activity results were expressed as $\Delta abs.mg^{-1}(protein).min^{-1}$.

Protein content in the enzyme extracts was determined using the DC Protein Assay (Bio-Rad, Cat. #500-0111), using bovine serum albumin as standard. Enzymatic assays were performed for 8 fish per treament.

4.2.17. Protein extraction and labeling for DIGE

Proteins from muscle samples were extracted and the sarcoplasmic fraction retained, following a method previously described[358]. All protein extracts were then cleared from salts and contaminants using a standard TCA/acetone-based 2DE sample precipitation kit (Bio-Rad), adjusted to pH 8.5 with NaOH and quantified using a standard Bradford colorimetric method (Bio-Rad). An internal standard was obtained by mixing equal amounts of protein from each sample (n = 6 per treatment) in a different container. Minimal CyDye labeling was performed on all samples (Cy3 and Cy5) and internal standard (Cy2) following the manufacturer's recommended procedures (GE Healthcare), ensuring proper dye balancing to prevent variable confounding.

4.2.18. Protein separation by two-dimensional gel electrophoresis

Each IPG strip (24 cm, pH 4-7, Bio-Rad) was rehydrated by passively re-swelling them overnight with a Cy3-labeled sample, a Cy5-labeled sample and the Cy2-labeled internal standard (50 µg of protein from each, for a total of 150 µg protein, pooled and diluted in standard IPG-IEF rehydration buffer to a final volume of 450 µL). Isoelectric focusing was performed using an Ettan IPGphor (GE Healthcare), at 20 ºC, for a total of about 70 kV.hours and with current below 75 µA per strip. Strips were then equilibrated using standard Bio-Rad 2DE reduction/alkylation buffers (15 min each step), loaded on to manually cast large-format 12.5% Tris-HCl SDS-PAGE gels and run at 50 mA/gel (after an initial 30 mins period at 10 mA/gel), using a standard Tris-glycine-SDS running buffer, until the bromophenol blue front reached the end of the gels.

4.2.19. Gel image acquisition and analysis

Labeled proteins in the gels were visualized using a Typhoon TRIO (GE Healthcare), using three different filters (520BP40, 580BP30 and 670BP30), at 100 µm resolution. Obtained images were analyzed using Progenesis SameSpots™ 2DE gel analysis software

(Nonlinear Dynamics) — which performs semiautomatic gel alignment, spot detection, background subtraction and abundance estimation. Student's t-test was then applied on resulting protein abundance data (expressed in relation to the internal standard), having considered proteins with a $P < 0.05$ as being likely to be affected by the dietary supplementation. These protein spots were then manually excised from preparative gels post-stained with colloidal CBB for digestion and identification.

4.2.20. Protein identification by MS analysis of peptides and database search

After reduction and alkylation (using DTT and iodoacetamide, respectively), protein spots were digested with trypsin and the resulting peptides extracted with acetonitrile and trifluoroacetic acid. After a final desalting step, the peptides were then co-crystallized with the matrix (DHB) on a MALDI plate and analyzed with a MALDI-TOF-TOF mass spectrometer. The obtained MS and MS/MS mass lists were then used as input to MASCOT MS/MS Ion searches of the Actinopterygii subset of the NCBInr 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. Whenever no significant hits were obtained with the NCBInr database, searches were repeated against the Actinopterygii subset of the Vertebrates_EST database. Identity of transcripts was assessed using blastp (http://blast.ncbi.nlm.nih.gov/) against the NCBInr database. For each identified spot, we also attempted to determine the specific isoform (whenever isoform-specific peptides could be identified from the MS/MS data).

4.2.21. Statistical analyses

All results are expressed as mean ± standard deviation. Unless otherwise specified, a two-way ANOVA was used to test the effects of time post-mortem and inclusion of glycerol in the diets, followed by a Tukey HSD test if appropriate, except for analysis performed at only one time-point, where results were compared using Student’s t-test. The comparison of histological degrees of muscle glycogen was performed using the Mann–Whitney test. Statistical significance was defined as $P < 0.05$. All tests were run with SPSS ver.17.0 (SPSS Inc., Chicago, USA).

4.3. Results

At the end of the feeding trial, seabream weight did not differ significantly between dietary treatments (Table 4.3). Similarly, no differences were found in terms of fish length, viscerosomatic and hepatosomatic indices and proximate composition of the fillets. However, seabream fed the GLY diet had a significantly higher fillet yield (34 ± 3 % vs. 31 ± 3 % for CTRL; skinless fillets, $P = 0.010$).

Table 4.3 – Weight, length, fillet yield, viscerosomatic index, hepatosomatic index and proximate composition of the fillets of gilthead seabream fed the experimental diets.

	CTRL	GLY
Weight (g)	623 ± 66	617 ± 61
Length (cm)	29 ± 1	30 ± 1
Fillet yield (%)	31 ± 3	34 ± 3*
Hepatosomatic Index (%)	1.5 ± 0.2	1.5 ± 0.2
Viscerosomatic Index (%)	5.4 ± 0.7	5.3 ± 0.9
Proximate composition (%)[a]		
Lipids	9 ± 3	9 ± 3
Protein	21 ± 1	22 ± 1
Moisture	69 ± 2	68 ± 3
Ash	1.4 ± 0.0	1.4 ± 0.0

Values are means ± standard deviation ($n = 16$). [a]$n = 4$ pools of 2 fish. Means with * are significantly different from the control ($P<0.05$).

Table 4.4 shows an overview of the results for the fatty acid profiles of seabream muscle from both diets. The individual fatty acids analysed did not differ between diets tested (results not shown), and there were no significant differences between major lipid classes.

The instrumental texture of cooked fillets was not significantly affected by the inclusion of glycerol in the diet. However, glycerol significantly increased the hardness of raw fillets (control: 2.5 ± 0.9 N *vs.* glycerol: 3.5 ± 1.6 N), which can be seen as a positive effect.

Table 4.4 – Summarized fatty acid profiles of seabream from the two dietary treatments, expressed as percentage of total fatty acids.

Fatty acids	CTRL	GLY
C20:4n-6 [AA]	0.7 ± 0.2	0.7 ± 0.1
C20:5n-3 [EPA]	5.2 ± 1.2	5.1 ± 0.5
C22:6n-3 [DHA]	9 ± 4	8 ± 1
Σ saturated	32 ± 4	31 ± 2
Σ monounsaturated	36 ± 2	37 ± 1
Σ polyunsaturated	31 ± 7	30 ± 2
Σ n-3	21 ± 6	20 ± 2
Σ n-6	8.5 ± 0.5	8.7 ± 0.5
Ratio Σ n-3/Σ n-6	2.4 ± 0.6	2.2 ± 0.1

Values are means ± standard deviations (n = 4 pools of 2 fish).

The microscopy analysis of the glycogen content of gilthead seabream muscle showed significant differences between treatments. Fish fed the glycerol diet showed a significantly higher glycogen deposition (grade 3) compared to the control (grade 2), as can be confirmed by the more intense coloration of muscle fibres (Figures 4.1A and 4.1B). Muscle cellularity parameters did not vary between dietary treatments. Although fish fed the glycerol diet had a tendency towards larger white muscle fibres compared to the control (80 ± 4 µm *vs.* 74 ± 6 µm for the CTRL), this difference was not significant ($P > 0.05$).

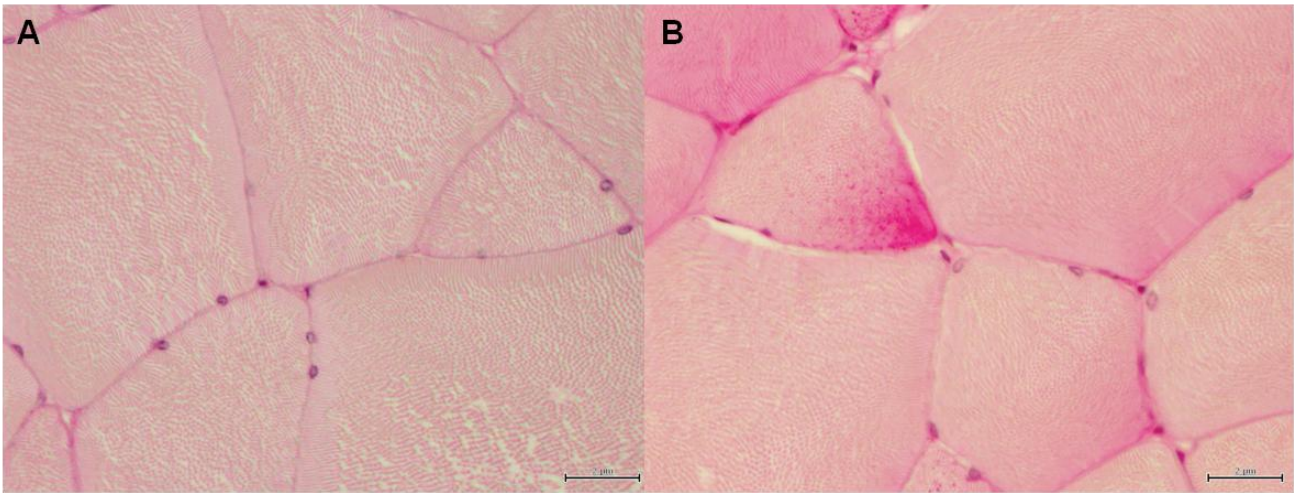

Figure 4.1 – Transversal sections of white muscle fibres of gilthead seabream fed A) the control and B) glycerol supplemented diet after staining with PAS (100x). Scale size is 2 µm.

Muscle colour was not affected by dietary treatments (Table 4.5). Likewise, there were no significant differences ($P > 0.05$) in volatiles analysed by GC-MS (Table 4.6). If we apply a relaxed significance threshold of $P < 0.1$, to ensure a more conservative assessment of the impact of glycerol on aroma, we observe that glycerol-fed fish display slightly higher values for 2-nonanone, (E)-2-nonenol, (E)-2-decen-1-ol and undecanal compared to control, while these display slightly higher values of total aromatic hydrocarbons compared to glycerol-fed fish. Nonetheless, sensory panellists did not find differences in colour and aroma of seabream fed either the CTRL or the GLY diet (Figure 4.2), which is consistent with the lack of major changes in terms of volatile composition.

Table 4.5 – Instrumental colour of cooked fillets from seabream fed the two experimental diets.

	CTRL	GLY
*L**	85 ± 2	86 ± 2
*a**	-2.1 ± 0.3	-2.2 ± 0.3
*b**	9 ± 2	8 ± 2
*C**	9 ± 2	9 ± 1

Values are means ± standard deviation (n=10).

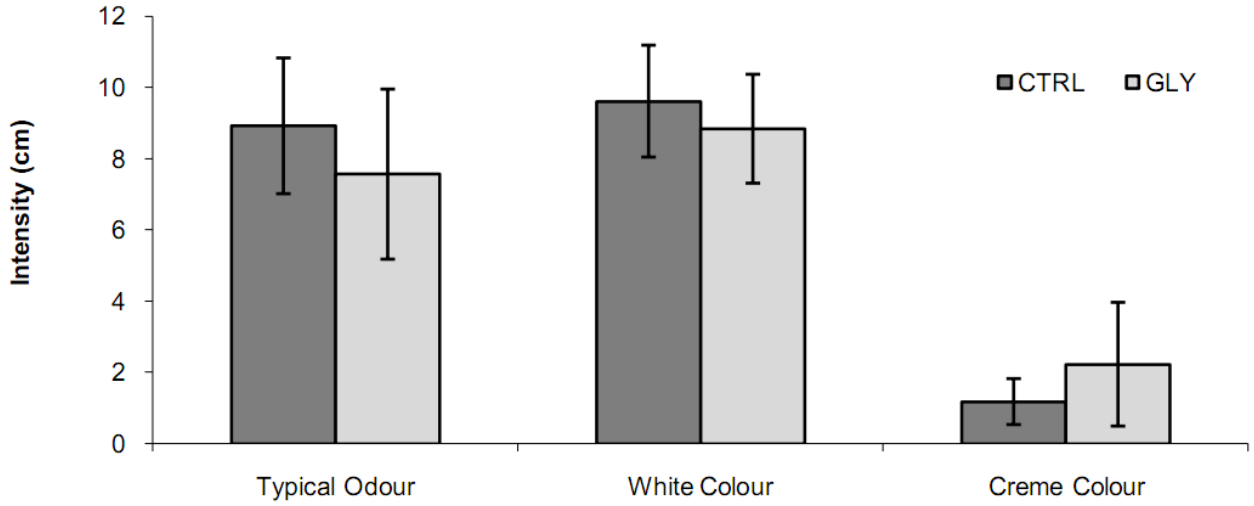

Figure 4.2 – Sensory scores of gilthead seabream fed the control and glycerol supplemented diet. Values are means ± standard deviation (*n* = 8).

Table 4.6 – Concentration of volatile compounds in gilthead seabream fed the two experimental diets.

Compounds (µg.g^{-1})	CTRL	GLY
3-Methyl-butanal	1.34 ± 0.3	1.89 ± 0.3
1-Penten-3-ol	566 ± 44	498 ± 146
Hexanal	43 ± 17	47 ± 9
Ethylbenzene	12 ± 9	7 ± 1
(Z)-4-Heptenal	1.2 ± 1.0	1.4 ± 0.8
Heptanal	1.6 ± 0.4	1.9 ± 0.5
1-Octen-3-ol	6 ± 2	7 ± 2
Octanal	2.4 ± 0.3	2.6 ± 0.5
Eucalyptol	0.04 ± 0.04	0.07 ± 0.02
(E)-2-Octenal	0.02 ± 0.03	0.35 ± 0.27
2-Nonanone	0.28 ± 0.06	0.51 ± 0.08
(E)-2-Nonenol	7.0 ± 1.7	12.4 ± 0.3
(E.Z)-2.6-Nonadienal	0.02 ± 0.03	0.87 ± 1.10
4-Ethyl-benzaldehyde	0.6 ± 0.1	0.6 ± 0.2
(E)-2-Decen-1-ol	0.5 ± 0.1	1.0 ± 0.2
Undecanal	7 ± 2	14 ± 4
Σ aldehydes	13.62	23.55
Σ alcohols	13.97	20.14
Σ aromatic hydrocarbons	11.55	6.68
Σ ketones	0.28	0.51

Values are means ± standard deviation (n = 2 pools of 4 fish each).

Muscle pH (Figure 4.3) decreased markedly throughout storage time. Seabream fed the GLY diet displayed higher muscle pH at the time of death (P = 0.005), but this difference was no longer present 3 h after death.

Rigor mortis showed a typical evolution, with the seabream attaining full rigor between 9 and 24 h post-mortem, and this pattern was similar for seabream fed any of the diets (results not shown).

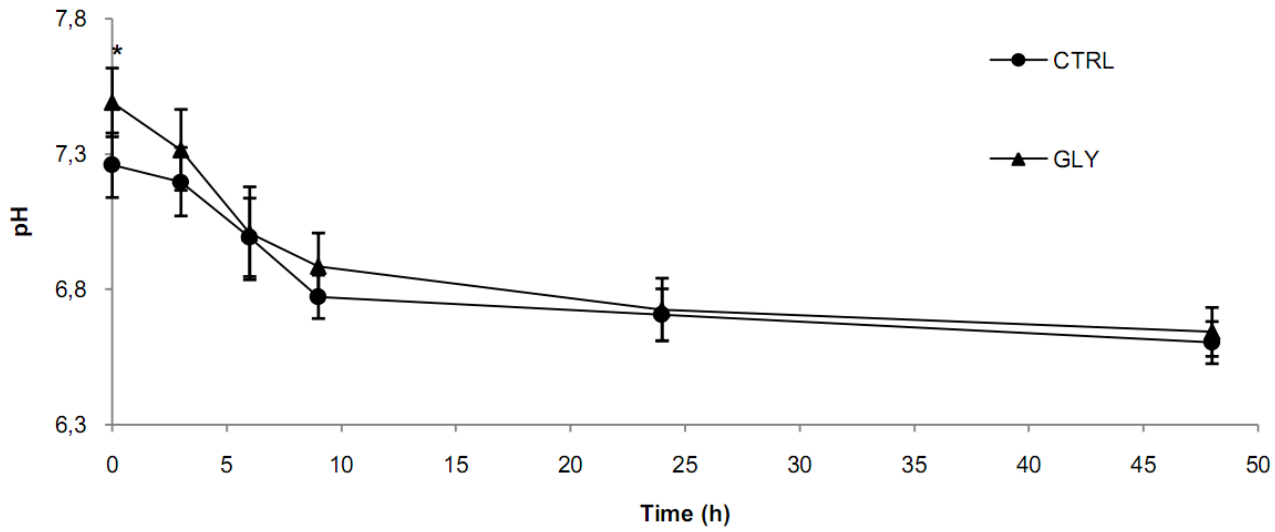

Figure 4.3 – Post-mortem muscle pH of gilthead seabream fed the control and glycerol supplemented diet, over 48 hours in ice storage. Values are means ± standard deviation (n = 6). Means with * are significantly different from the control (P<0.05).

Muscle thiobarbituric acid reactive substances ranged from 4.8 ± 0.4 to 6.4 ± 0.5 µg MDA.g^{-1} and were not affected by storage time nor dietary treatments (see Figure II of Supporting information). However, at 6 h post-mortem, glycerol fed seabream showed a slightly lower lipid oxidation, although not significantly different from the control (P = 0.057).

Figure 4.4A shows the muscle's glycogen content. Seabream fed the glycerol supplemented diet showed a higher muscle glycogen content at the time of death (P = 0.044), but this difference was no longer evident 6 h post-mortem. At 48 h glycogen levels in the muscle had decreased below the detection threshold (results not shown).

ATP content of the muscle decreased throughout storage time (Figure 4.4B). At the time of death, seabream fed GLY had a higher ATP content in the muscle ($P = 0.024$), but no differences were evident at 6 and 48 h post-mortem.

Cathepsin B activity increased throughout storage time, with average activity at 48 h post-mortem (23 ± 10 $U.g^{-1}$ protein) being significantly higher than activity at 0 and 6 h post-mortem (8 ± 2 and 12 ± 3 $U.g^{-1}$ protein, respectively, $P < 0.001$), but dietary treatments did not influence cathepsin B activity. Similarly, cathepsin H activity did not differ between dietary treatments, although the evolution throughout storage time showed a higher activity at 6 h post-mortem (375 ± 90 $U.g^{-1}$ protein) compared to 0 and 48 h post-mortem (298 ± 103 and 253 ± 71 $U.g^{-1}$ protein, respectively, $P = 0.002$). Collagenase activity was not significantly affected by either storage time or dietary treatments, and varied between 193 ± 70 and 282 ± 73 $U.g^{-1}$ (protein).

Glycogen phosphorylase activity (Figure 4.4C) was not significantly affected by storage time ($P = 0.178$). Supplementation with glycerol in the diets resulted in a higher glycogen phosphorylase activity ($P = 0.029$), specifically at 6 h post-mortem, when seabream fed CTRL diet showed a marked decrease in activity (0.056 ± 0.008 $\Delta abs.mg^{-1}$ (protein).min^{-1}) while GLY fed seabream glycogen phosphorylase activity remained similar in all time points (average 0.092 ± 0.026 $\Delta abs.mg^{-1}$ (protein).min^{-1}).

In terms of protein expression, 2D-DIGE analysis revealed a relatively low impact of the glycerol-containing diet on muscle proteome expression, with only 17 (out of 387 quantified proteins) displaying significant differences between treatments ($P < 0.05$). Of these 17 proteins, 9 were reliably identified using mass spectrometry (Table 4.7). Abundance estimation for these 17 proteins can be seen in Figure 4.5.

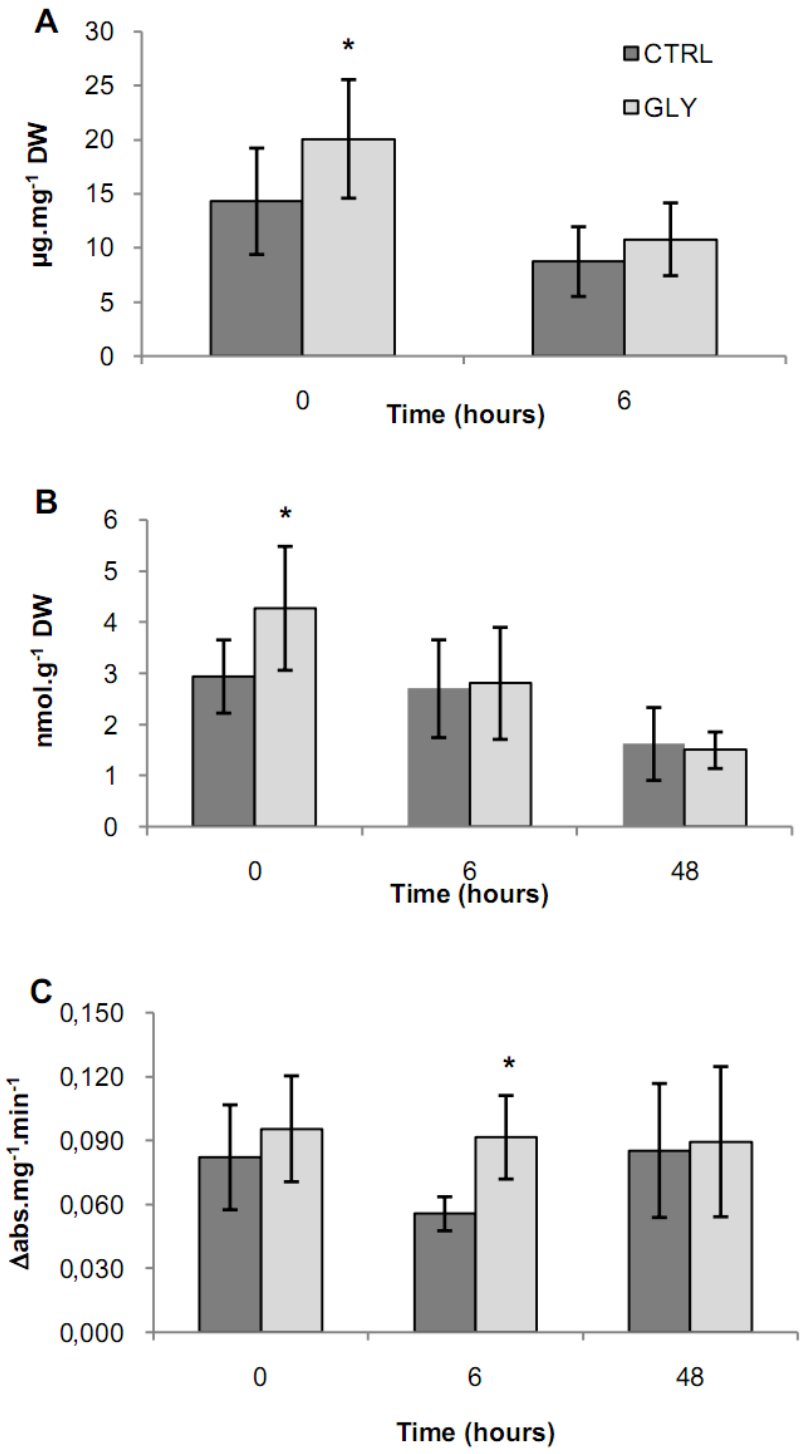

Figure 4.4 – Muscle glycogen (a) and ATP (b) content of gilthead seabream fed the control and glycerol supplemented diet. Muscle glycogen phosphorylase activity (c) of gilthead seabream fed the control and glycerol supplemented diet. Values are means ± standard deviation (n = 8). Means with * are significantly different from the control (P<0.05).

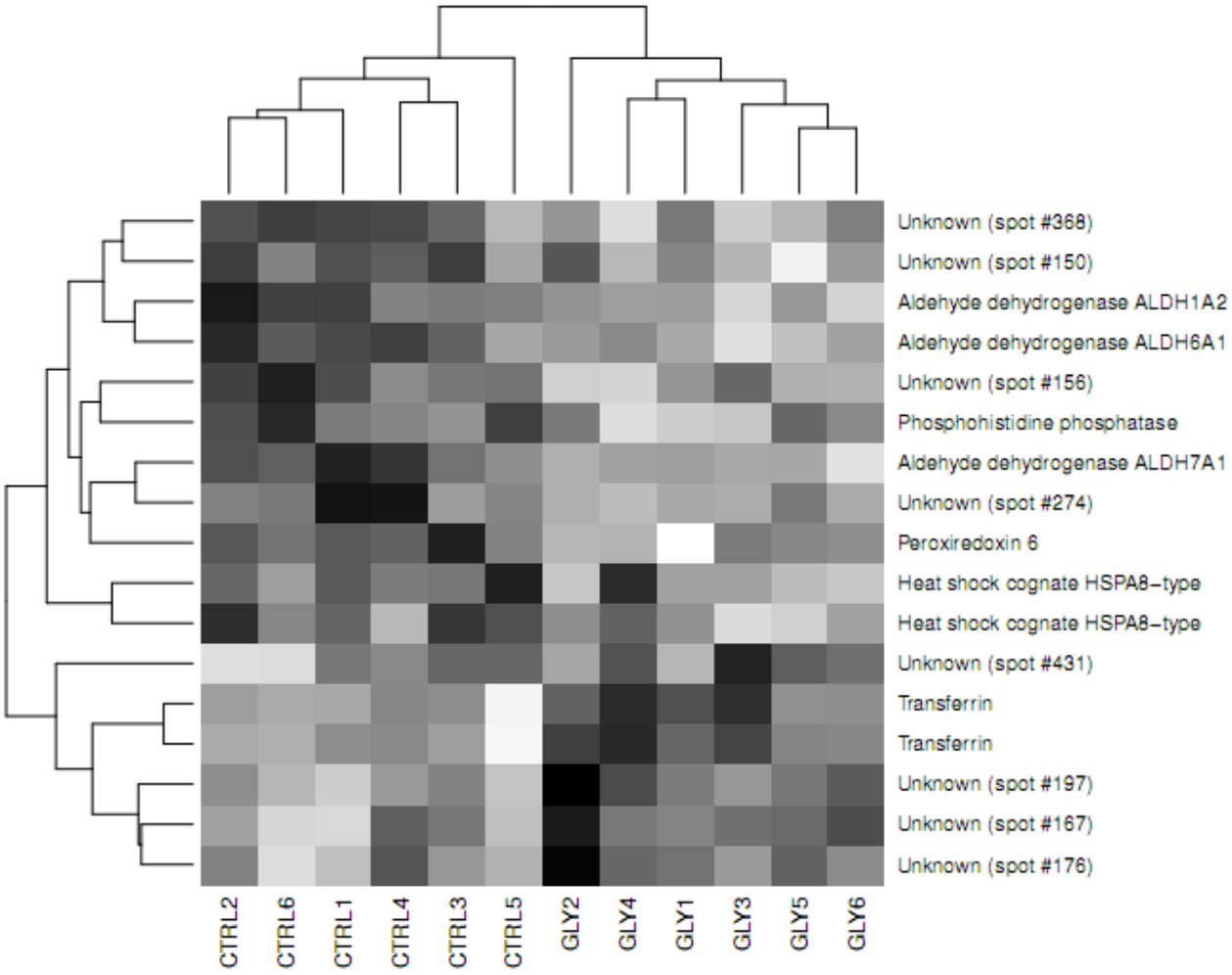

Figure 4.5 – Heat-map detailing protein expression levels across all muscle samples at time-point 0h, for all protein spots significantly different between control and glycerol-fed individuals (p-value < 0.05). Dark tones indicate "below average expression", while bright tones indicate "above average expression". Protein identity is present, whenever available.

Table 4.7 – Protein spots identified by mass spectrometry (using peptide fragment fingerprinting), along with relevant details. Experimental Mw and pI values were estimated from the spots' positions on the 2DE gel. Theoretical Mw and pI values were estimated using available sequence data). Only peptides with E-value below 0.05 (discarding oxidized versions and other duplicates) were counted for the calculation of the number of matched peptides per protein, estimated coverage and combined Mowse score.

Spot #	*Protein identity*	*Accession #*	*Exp. Mw/pI*	*Theo. Mw/pI*	*Coverage (%)*	*# matches*	*Best peptide match: sequence, E-value*	*Mowse score*
124	Transferrin	ACN80997.1	77 kDa / 6.0	74 kDa / 5.9	0*	0*	VPAHAVVTR, 0.08	39
129	Transferrin	AEA41139.1	76 kDa / 6.1	76 kDa / 5.9	3	2	ASSIEQYYGYAGAFR, 1.1e-05	103
188	Heat shock cognate HSPA8-type	CAA72216.1	61 kDa / 5.3	71 kDa / 5.2	3	2	ARFEELNADLFR, 0.0079	84
189	Heat shock cognate HSPA8-type	AAF71255.1	61 kDa / 5.3	72 kDa / 5.3	5	3	ARFEELNADLFR, 9.2e-05	169
202	Aldehyde dehydrogenase ALDH7A1	AAX54912.1	57 kDa / 5.9	56 kDa / 5.9	6	3	IYVEGVGEVQEYVDVCDYAVGLSR, 0.016	53
204	Aldehyde dehydrogenase ALDH6A1	FM154143	56 kDa / 6.0	55 kDa / 6.7	7	4	EWLPELVER, 0.00021	174
211	Aldehyde dehydrogenase ALDH1A2	FM152135	56 kDa / 5.9	57 kDa / 5.9	5	4	DIDKANYVSSGLR, 7.2e-11	222
421	Peroxiredoxin 6	ADI78069.1	28 kDa / 6.4	25 kDa / 6.3	14	2	LSILYPATTGR, 1.6e-06	86
532	Phosphohistidine phosphatase	FM145195	16 kDa / 6.3	16 kDa / 8.8	16	2	IPDVEIDPEGTFK, 6.9e-07	181

* despite the fact that no peptides were found with an E-value below 0.05 for this protein spot, there were two borderline peptide matches and, since the spot's position in the 2DE gel is consistent with a transferrin isoform, we assumed it was valid to exceptionally consider this identification as reliable.

4.4. Discussion

4.4.1. Effects on fish growth and performance

Results from this trial showed a generally beneficial effect regarding the inclusion of crude glycerol in gilthead seabream diets. Looking at measured zootechnical parameters, we observed good performance results for both diets, confirming that glycerol inclusion at 5 % does not seem to induce any immediately apparent deleterious effects in adult gilthead seabream. Specifically, proximate composition and hepatosomatic index values obtained generally suggest the fish are able to metabolically cope in an adequate way with the partial replacement of typical carbohydrates/starch for glycerol as an energy yielding substance. This is consistent with results obtained for channel catfish, where it was shown that inclusion of up to 10 % glycerol in diets induced no detrimental effects on typical zootechnical performance criteria[345]. In fact, unlike the results of this catfish study, our results showed no induced differences even in terms of body moisture content.

Interestingly, although no differences could be seen in terms of final weight, glycerol-fed fish displayed higher fillet yield, compared to control. A similar situation has already been described in broilers fed 2.5 % and 5 % glycerol diets, where a higher breast yield was obtained (compared to 0 % glycerol diet), although no differences in final weight could be measured[344]. This effect was attributed to higher protein deposition due to the sparing effect glycerol can have on glucogenic amino acids like alanine and glutamate. Our data from a separate study, in which we assessed the effect of dietary glycerol on the growth performance and metabolic nutrient utilization in juvenile gilthead seabream, confirms that a 5 % dietary glycerol level significantly improved dietary protein and fat retention (J. Dias, pers. comm.).

4.4.2. Effects on metabolism and pre-mortem muscle energy status

In terms of the putative ability of dietary glycerol to improve the pre-mortem energetic status of muscle, our results do seem to confirm it for gilthead seabream: both spectrophotometric and histological assays showed increased glycogen deposition in the muscle fibres of glycerol-fed fish, as well as increased levels of ATP (measured immediately post-mortem). Downstream indicators of the muscle energetic status (muscle pH and rigor index) show delayed (or at least unaffected) progression of their usual temporal profiles for glycerol-fed fish, which is consistent with the glycogen and ATP measurements. These signals of improved muscle energy were particularly apparent immediately post-mortem,

while after 6 h post-mortem little differences could be measured between the two experimental groups.

To contextualize these results, it is important to note that both control and experimental diet were formulated to ensure that all known nutritional requirements for adult gilthead seabream were adequately met, and that these were fed during a period of less demanding energetic requirements (finishing phase). Besides this, we attempted to replicate as accurately as possible standard aquaculture practices, namely enforcing a 48 h pre-slaughter starvation period, catching the fish with a net and slaughtering them using ice-water slurry. These practices unavoidably constitute challenges and therefore affect fish metabolism, in part due to the combined effect of glucagon, epinephrine and glucocorticoids (like cortisol), and also due to the induced hypoxia and energy depletion.

Metabolically, gilthead seabream is relatively flexible, having a high tolerance towards dietary carbohydrates, compared to other carnivores like rainbow trout[359]. Specifically, studies have shown that gilthead seabream does not display the same type of hyperglycaemia that rainbow trout does when fed carbohydrates, but only a postprandial transient peak (at least in part due to repression of hepatic gluconeogenesis)[360]. On the other hand, during fasting or feed-restriction periods, gilthead seabream liver displays both higher gluconeogenic rates and lower glucose catabolic capacity (due to decreased expression of glucokinase)[361]. Furthermore, several studies point out that cortisol and epinephrine are likely to have a cumulative effect with feed deprivation on hepatic and peripheral metabolism[362-363]. Finally, there are indications that, at least for gilthead seabream, 3-carbon compounds may have a higher importance than glucose in providing energy and carbon for short-term hepatic glycogen replenishment during feeding-fasting cycles[364], as well as during stressful situations associated with feed deprivation[363], possibly because triacylglycerides are an important secondary store of energy in gilthead seabream and their mobilization entails higher glycerol availability.

Regarding skeletal muscle metabolism, information on glycerol uptake and glycogenesis in gilthead seabream is scarce, but there are indications that, in rainbow trout, it can have (at least indirectly) a positive effect on glycogen deposition in muscle[337]. Our results, coupled to the knowledge that gilthead seabream is more adaptable to and tolerant towards carbohydrates than rainbow trout, clearly suggest glycerol does contribute significantly to increased glycogen deposition in muscle, either directly (possibly after being converted to glucose by hepatic gluconeogenesis) or indirectly (as it is the case for rainbow trout, through a sparing effect on lactate and other glucogenic metabolites). Besides this, we

were aware that dietary glycerol, particularly during low energy demand periods, could induce increased lipogenesis in liver, muscle or adipose tissue, as well as induce changes in the fatty acid profiles (i.e. increased saturation levels), as it was demonstrated for other animal models[339-340]. As mentioned before, none of these issues occurred during this experiment, as no differences or abnormalities could be noted in terms of HSI, VSI, fatty acid profile or whole body composition. These results again reinforce the notion that gilthead seabream has a relatively high metabolic adaptability to dietary carbohydrates (particularly glycerol) for a carnivorous teleost.

4.4.3. Effects on fish meat quality

When looking at the impact of glycerol on ultimate fish meat quality indicators, results were also encouraging, as dietary glycerol induced no deleterious effects in terms of aroma, colour, proteolytic potential and texture parameters (instrumental and morphometric).

In fact, increased hardness of raw fillets is seen as a very positive result in terms of quality and freshness measures for gilthead seabream meat[365]. Although none of the studies on the effect of glycerol on marine species explored the possible impact on their organoleptic properties, a few experiments in pigs support the idea that glycerol might increase muscle hardness/firmness, particularly when included in a finishing diet[341, 343]. This last study attempts to explain this through the effect glycerol supplementation seems to have in terms of fatty acid composition, namely a general increase in the degree of fatty acid saturation. This impact in fatty acid profile was not observed in the current experiment though, suggesting that the underlying factor in seabream pertains to a different cellular trait. A likely possibility is that the slight texture differences observed are attributed to proteins of the extracellular matrix (like collagen), which would explain why no differences could be observed for the cooked fillets (as discussed elsewhere[366]). It is therefore plausible that glycerol supplementation might have induced a sparing effect on glutamate (required for proline synthesis, a limiting factor in collagen production), which would explain the instrumental texture observations for raw and cooked fillets, despite no significant differences in terms of cellularity parameters (i.e. fibre diameters).

Another relevant factor in fish meat quality is aroma: a complex feature that arises from the release of a mixture of volatile components mostly resulting from bacterial metabolism, lipid oxidation, enzymatic reactions and other decomposition processes. According to[367], fresh saltwater fish give off a relatively low number of different volatiles, with 1-penten-3-ol as major component: this is generally consistent to what we observed. This

particular fact is also evident in other analyses of fresh gilthead seabream[368-370]. Lipid oxidation usually generates different kinds of volatile aldehydes, such as pentanal, hexanal, octanal and nonanal, but hexanal is often predominant. Aldehydes are particularly relevant volatiles since their presence is subjectively noticeable at comparatively low quantities. Of the four volatile components that could be considered slightly increased ($P < 0.1$) in glycerol-fed fish, two are pleasant-smelling alcohols (2-nonenol and 2-decenol), one is a pleasant-smelling ketone (2-nonanone) and one is a more pungent, although pleasant-smelling, aldehyde (undecanal). All of these are usually associated with organoleptic terms such as fresh, green, herbal, waxy, fruity, citric, sweet and fatty, suggesting these substances are not likely to be responsible for any fishy or otherwise foul odours. This was corroborated by the sensorial panel analysis, which found no differences between the two fish groups.

4.4.4. Effects on muscle sarcoplasmic proteome

The proteomic results mostly confirm the low impact of glycerol on muscle metabolism, as only a very reduced set of proteins (17 out of 387) were identified as being affected by the dietary treatment, even when taking a relatively relaxed threshold for statistical significance (Student's t-test, $P < 0.05$, with no multiple comparison correction). Looking at affected pathways, we observe mostly changes at the level of proteins involved in detoxification processes (ALDH1A2, ALDH6A1, ALDH7A1), response to oxidative stress (peroxiredoxin, HSPA8 proteins, transferrins) and signaling (phosphohistidine phosphatase), with most signs pointing towards higher expression of stress response proteins in glycerol-fed fish.

Regarding proteins associated with detoxification processes, specifically in the case of ALDH7A1 (known to be involved in the detoxification of lipid peroxidation products), this enhanced expression seems to suggest that increased lipid peroxidation was occurring in glycerol-fed fish. On the other hand, this observation is inconsistent with the fact that we observed lower levels of TBARS in these fish. This could be an indication that increased expression of ALDH isoforms should probably be interpreted not as a sign of increased lipid oxidation, but as a sign of increasing muscle capacity to process and neutralize lipid peroxidation products.

Looking at proteins involved in the response to oxidative stress, we found that, in comparison to control fish, glycerol-fed fish showed increased expression of peroxiredoxin and heat shock proteins of the HSPA8 family in muscle. Peroxiredoxins are antioxidant proteins, while heat shock proteins prevent protein aggregation, having a role in protein

folding. Regarding HSPA8 isoforms, they are usually constitutive and not stress-inducible, which makes the interpretation of the results difficult, especially if we take into account the non-proteomic observations. As mentioned before, we should be careful in directly interpreting these results as increased signs of oxidative stress, since we have no other indicators that glycerol-fed fish were indeed more stressed. It is important to note that both peroxiredoxin and HSPs generally have a protective and anti-apoptotic effect, so these results could also imply that these fish have a higher capacity to cope with oxidative stress-induced protein misfolding and aggregation. Still in reference to the pathways of cellular response to stress, we observed lower abundance of transferrin spots in glycerol-fed fish compared to control. Transferrin is mainly associated to iron transport to peripheral tissues. Also, due to the fact that transferrin helps to maintain low concentrations of free iron, it most likely also has a role in reducing the generation of ROS via Fenton reactions. Experiments in mammals and fish have shown transferrin expression in liver and release into the bloodstream is increased as part of the primary stress response[371-373]. It is also relevant to note we have previously seen increased transferrin abundance in gilthead seabream muscle in response to harvesting and slaughter stress[358]. Also, a recently published study on the effects of prolonged hypoxia on the sarcoplasmic proteome of rainbow trout muscle also demonstrates an immediate increase in transferrin isoforms after the onset of hypoxia[374]. These observations would again suggest that control fish were generally more stressed than those fed the glycerol-containing diet.

Finally, we also observed an increased expression of a phosphohistidine phosphatase (PHP) in the muscle of glycogen-fed fish, which is a protein involved in signaling, acting in opposition to nucleoside diphosphate kinase in phosphohistidine signaling. Although the topic of phosphohistidine signaling is still a young research field, there are already some studies in vertebrates which can help elucidate its relevance in this context (for a review see [375]). Some possible targets are known for PHP, but two are of particular importance, since they have been shown to be specifically affected by PHP activity[376-377]. One of these is ATP-citrate lyase, a protein involved in the regulation of the cytosolic acetyl-CoA pool and therefore responsible for regulating lipid biosynthesis. A logic possibility is that increased dephosphorylation rate of ATP-citrate lyase (and, therefore, inactivation) might be a compensatory mechanism to prevent overly active lipogenesis due to increased availability of glycerol. A reduction of the metabolic flow of Krebs cycle carbon (probably originated by starvation-induced β-oxidation of fatty acids) towards lipogenesis should increase efficiency of energy-generating catabolic processes and gluconeogenesis. This suggests the muscle

glycerol-fed fish apparently displayed a more appropriate response towards the experimental challenges (starvation, slaughter stress, hypoxia and post-mortem energy depletion) by effecting a change which confers higher metabolic efficiency. The other highly likely target of PHP is the G_β subunit of heterotrimeric G proteins, a component of the glucagon transduction pathway, among others. Phosphatase activity on this subunit apparently decreases the basal (i.e. ligand-independent) levels of signal transduction[376]. This suggests the decreased levels of PHP in fish fed the control diet might induce higher glucagon-independent stimulation of glycogenolysis during fed states, contributing towards lower glycogen deposition.

4.4.5. Effects on enzymatic activities and lipid oxidation in muscle

Besides the proteomic results, directed biochemical assays suggest increased levels of active glycogen phosphorylase and slightly lower levels of lipid oxidation (as assessed by the amount of TBARS present in muscle) in glycerol-fed fish. This is consistent with information on glycogen metabolism in rainbow trout, where glycogen phosphorylase activity in the muscle was seen to increase in response to epinephrine and glucocorticoids, but only when glycogen stores were above 5 μmol/g[378]. Looking at all of these signals together, and taking into account that glycerol-fed fish showed otherwise clear signals of improved muscle energy status, we are led to interpret these observations as a sign of higher response magnitude and coping ability of glycerol-fed fish towards harvesting/slaughtering stress, since all mentioned proteins have a cytoprotective effect and glycerol-fed fish displayed signals of generally lower levels of oxidative stress. Analysing these results within an allostatic framework[89], we are therefore inclined to consider the observed proteomic changes as mostly adaptive, rather than maladaptive, specifically given the relatively low number of affected proteins.

Another interesting observation regards glycogen phosphorylase activity and TBARS abundance at 6 h post-mortem: although control *vs.* glycogen-fed differences for these parameters are negligible for most samples, a substantial difference in these parameters was seen for this time-point. Given that samples obtained at 6 h post-mortem were collected from the same fish sampled at 0 h post-mortem, we assume higher TBARS levels measured can be attributed to this repeated manipulation of the same fish for the first two samplings. What is relevant to note is that only fish from the control group displayed an induction of lipid oxidation due to post-mortem manipulation, which again suggests that glycerol-fed fish appear to have improved stress coping abilities. In the same way, glycogen phosphorylase activity in control fish seems to have been suppressed for this time-point, which could be explained by inactivation due to increased oxidative stress. This seems particularly plausible,

since we observed a relevant inverse correlation between glycogen phosphorylase activity and TBARS abundance, at this time-point ($\rho \approx 0.45$).

4.4.6. Impact of possible contaminants present in crude glycerol

A major concern regarding the use of biodiesel-derived crude glycerol in animal feeds is the fact that it often contains significant amounts of salts, fatty acids and some methanol (or, less commonly, ethanol) residues, due to the transesterification process. While the impact of salts and fatty acids can be mitigated through adequate diet formulation, the presence of methanol in crude glycerol is of higher concern, as it is known to induce oxidative cellular stress in most animals. On the other hand, methanol is not as toxic towards fish as it is towards primates[379], possibly due to a lower metabolic rate (and therefore lower endogenous production of formaldehyde and formate), since fish should be able to excrete methanol and its metabolites more efficiently than land animals. Additionally, due to the high temperature of the feed extrusion and drying process, it is very likely that any methanol present would evaporate. Experiments with inclusion of biodiesel derived crude glycerol in pig feeds confirm that the usual levels of methanol present do not induce any symptoms associated with methanol-induced acute metabolic acidosis and oxidative stress (e.g. optic nerve lesions)[340, 342]. Also, we have no reason to believe chronic exposure to low amounts of methanol is dangerous by itself, as its production by normal metabolic processes is unavoidable and its presence in the environment is ubiquitous; for this reason, it is also very unlikely that it bioaccumulates in any way. Given that no signs of oxidative stress or mitochondrial damage due to acute methanol poisoning were observed in glycerol-fed fish, it seems safe to assume inclusion of crude glycerol with less than 0.03 % methanol in gilthead seabream feeds has no observable deleterious effect. Nevertheless, and given that crude glycerol composition can vary between different sources, batches and according to the type of fats used as raw material for biodiesel production, it might be relevant to enforce strict quality control criteria on feed-grade crude glycerol, to ensure it does not contain undesirable amounts of methanol.

4.5. Final considerations

Concluding, glycerol seems like a promising choice for (at least partial) replacement of starch and other carbohydrate sources for gilthead seabream diets, both in terms of

economic and sustainability criteria as in terms of fish nutrition, health, meat quality and nutritional value. Furthermore, the original hypothesis of using glycerol as a dietary supplement to improve the energy status of gilthead seabream muscle seems quite plausible, having seen in this experiment clear signals of increased glycogen and ATP abundance in muscle.

In the future, it would be interesting to explore how glycerol improves the gilthead seabream's stress coping capabilities, through experiments using explicit challenges or during situations when the energetic requirements of fish are particularly demanding (e.g. grow-out phase, sexual maturation, winter). Also, it would be relevant to assess the optimal inclusion levels for a given diet, depending on its purpose (e.g. grow-out vs. finishing diets).

Supporting information available

This information is available in the CD-ROM version of this dissertation and online, at "http://dx.doi.org/10.1021/jf3023244".

Chapter 5.

Characterization of the impact of crowding and repeated handling stress on gilthead seabream liver proteome.

This chapter has been previously published as:

Ricardo N. Alves, Odete D. Cordeiro, Tomé S. Silva, Nadège Richard, Mahaut de Vareilles, Giovanna Marino, Patrizia Di Marco, Pedro M. Rodrigues, Luís E.C. Conceição. **Metabolic molecular indicators of chronic stress in gilthead seabream (*Sparus aurata*) using comparative proteomics.** Aquaculture **2010**, 299 (1-4), 57-66. (doi:10.1016/j.aquaculture.2009.11.014)

Abstract

The aim of this study was to identify possible molecular stress indicators in gilthead sea bream *Sparus aurata*. Two potential stressful conditions were tested: repeated handling and crowding at high stocking density. Gilthead seabream kept under optimized rearing conditions were used as control fish. Cortisol was measured as primary stress indicator and the liver proteome of stressed fish was compared to that of control fish using comparative proteomics. Plasma cortisol levels in sea bream repeatedly handled and crowded at high stocking density were significantly higher than in undisturbed control fish. A total of 560 *spots* were detected and the statistical analysis revealed a differential expression in about 50% of all detected proteins. Spots with greater than 2-fold or lower than 0.5-fold changes were identified by liquid chromatography- tandem mass spectrometry (LC- MS/MS). Proteins like fatty acid binding protein (lipid transport and antioxidant role), heat shock cognate protein (chaperoning), calmodulin (Ca^{2+} signaling), mitochondrial porine – voltage-dependent anion channel (lipid oxidation), glutamine synthetase (ammonia metabolism), cofilin and beta-tubulin (cytoskeleton), haemoglobin and several other proteins involved in carbohydrate metabolism (triose-phosphate isomerase, pyruvate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, alfa-enolase) were differentially expressed in fish under chronic stress. Some of these proteins may be used in the future as chronic stress and/or fish welfare biomarkers, after validation studies using RT-PCR and ELISA assays.

5.1. Introduction

Farmed animals welfare is increasingly becoming a relevant and significant societal topic[380]. Both consumer awareness and ethical reasons make this issue a top priority on animal farming conditions. On the other hand, animal stress and welfare are also an important issue for producers, since growth and reproductive performances, health status and susceptibility to diseases are strictly related to stress conditions[381]. Welfare studies on terrestrial farmed animals are nowadays common in scientific research (e.g., [382-384]). However, only recently, welfare of farmed fish has emerged[381, 385-386]. Defining the welfare status of fish is difficult and the identification of reliable welfare indicators represents the main challenge. A wide range of physical, physiological and behavioral measures are used to assess fish welfare[387], and an accurate evaluation can only be made through the integration and interpretation of several indicators[68, 74].

An objective approach for welfare evaluation is the study of the stress response[388]. The stress response in fish occurs at three levels. The primary response involves the rapid activation of the brain-sympathetic-chromaffin cell axis (BSC-axis) and the hypothalamic-pituitary-interrenal axis (HPI-axis) with the release of catecholamines and cortisol into the bloodstream. The secondary response is defined by the hormonal effects on blood and tissues, including changes in metabolism, hydromineral balance, cardio-respiratory and immune functions. Tertiary response includes physiological effects related to whole-animal performance such as growth, disease resistance, behaviour and survival[76-78].

Avoidance of adverse consequences of exposure to chronic stressors is a central welfare goal in aquaculture[68, 381]. It is well reported that some aquaculture practices such as repetitive handling, confinement and crowding of fish represent potential chronic stressors affecting fish physiology and the welfare status[389-392]. Therefore, improvement of aquaculture practices in order to minimize chronic stress conditions during farming and preserve fish welfare is one of the main challenges in aquaculture research. In line with this, recently the European Food Safety Authority identified some hazards and risk factors potentially affecting welfare of several farmed species, included gilthead seabream[393].

Usually stress response in fish is evaluated by measuring levels of hormonal, metabolic, haematological and hydromineral parameters in the blood; growth and feeding parameters, and organosomatic indexes are generally used as tertiary stress indicators[76]. Beside the reliability of clinical biochemical parameters, including plasma cortisol, glucose,

lactate, electrolytes as primary and secondary stress indicators, their use in some circumstances, poses some difficulties in the interpretation of results because stress response is subjected to modulation by several factors, both intrinsic and extrinsic to the fish[76, 388, 394-395]. For example, traditional parameters such as plasma cortisol, may be not sufficient to assess physiological and welfare conditions when chronic stress occurred, due to HPI-axis acclimation[391, 396-398].

Nevertheless, recent developments in cutting edge technologies such as proteomics, genomics and metabonomics could give rise to a better insight of the mechanisms involved in stress-related processes in fish, thus facilitating the identification of stress and/or welfare indicators. Indeed these technologies are probably the best approaches as they 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 a tissue at once, in terms of their presence and relative abundance, without "a priori" knowledge. Gene expression analysis has recently begun to emerge as an alternative approach to assess stress and welfare conditions[399-404]. Proteomic techniques, such as bidimensional gel electrophoresis, are also promising and potentially new approaches as alternatives to conventional methods[280, 405-407].

The main objective of the present study was the identification of potential metabolic stress and welfare molecular biomarkers in gilthead seabream. Liver proteome was compared in fish stressed by repeated handling and by crowding at high stocking density and in control fish using comparative proteomics. Gilthead seabream was used as a model due to its economic importance and to the available knowledge on its stress physiology[391-392, 396, 408-414]. The liver was chosen as the target organ for comparative proteomic analysis, due to its major role in several key metabolic processes.

5.2. Materials and methods

5.2.1. Animals, experimental conditions and sampling procedure

The experiment was carried out at the Ramalhete Research Station (CCMAR, University of Algarve, Faro, Portugal). The trial lasted from September 18 until October 16 of 2007, after a 4 month adaptation period in experimental tanks. Fish were supplied by a commercial fish farm.

In this study, two potential chronic stressful conditions were tested: repeated handling (HND) and crowding produced by high stocking density (HSD). A control group (CTRL) was also reared in optimized rearing conditions at low stocking density and without any disturbance. The experimental treatments were tested in duplicate. To induce handling stress, fish were netted, and air exposed for 1 min, twice a week. It has been reported that such a short air exposure is an acute stressor, from which seabream recovers easily[392].

Forty-four specimens of 376.37 ± 43.37 g (mean ± standard deviation) were distributed among six polyethylene tanks of 1000 L. Tanks were supplied with in flow-through Ria Formosa Lagoon seawater. Temperature (22.45 ± 1.55 ºC), salinity (36.92 ± 0 .90), and oxygen saturation level (> 80 % saturation) were daily monitored.

Six fish (initial density - 2.52 kg/m^3) were used for each CTRL and HND tanks and, eleven fish (initial density – 46.15 kg/m^3) for each HSD tank. A cage system was mounted inside the HSD tanks in order to reduce the rearing volume at approximately 100 L with a water flow system allowing a good water quality.

At initial sampling, fish were anesthetized with 250 ppm of 2-phenoxyethanol (Sigma) for weight and length measurement.

During the trial, fish were fed twice a day (10:00 am and 02:30 pm) *ad libitum* with a diet based on 94.63% of dry matter (DM), 50.73 % DM crude protein, 20.26% crude fat and crude energy 24.36 Kj/g DM. HND fish were fed once a day when subjected to handling treatment. .

At the end of the experiment, fish were anaesthetized and euthanized with an overdose of 2-phenoxyethanol (1500 ppm) for blood sampling and biometric measurement. The blood was collected from the caudal vein with a heparinized syringe, placed on ice, incubated at 4ºC and centrifuged at 3000 rpm for 10 min (as described by [396]). Plasma samples were collected, quickly frozen in liquid nitrogen and then stored at -80ºC.

Plasma cortisol concentration was measured using a commercial solid-phase competitive chemiluminescent enzyme immunoassay Immulite on 10 µl of plasma sample (Siemens Medical Solution Diagnostic, Los Angeles USA). Chemiluminescence was measured by the Immulite One analyzer. The immunoassay was previously compared to ^{125}Iodine radioimmunoassay Coat-A-Count Cortisol (D.P.C. Los Angeles, CA) on 22 plasma samples, obtaining a significant linear correlation (R^2=0.98). Cortisol analyses were performed at Fish Physiology and Health Laboratory of Institute for Environmental Protection and Research (ISPRA, Italy).

For proteome analysis, the liver was quickly removed, washed with MilliQ water, frozen immediately in liquid nitrogen, and kept at -80ºC. To avoid contaminations, extra care was taken during liver handling, to prevent protein degradation by liver proteases.

5.2.2. Liver proteome analysis by 2DE

Four technical replicates were done for each experimental condition, using a pooling strategy to reduce the influence of inter-individual variation (and, thus, of biological outliers) enabling to focus on consistent global changes in protein expression[280].

Protein extraction and purification

Prior to protein extraction, liver samples were always handled in liquid nitrogen (to prevent protease activity). For protein extraction, 100 mg of tissue (pooled from all livers for each condition) was dissolved in 1 mL of extraction buffer containing 7 M urea (Sigma-Aldrich), 2 M thiourea (Sigma-Aldrich), 4% w/v CHAPS (Sigma-Aldrich), 0.3% w/v DTT (Sigma-Aldrich), 1% v/v ampholytes IPG Buffer pH 3-10 (GE Healthcare) and 1% v/v protease inhibitor cocktail (Sigma-Aldrich) and then incubated on ice for 30 min. After incubation period, the lysate was homogenized with an Ultra-Turrax IKA T8 (IKA-WERG), sonicated four times for 4 s, and centrifuged at 19000 *g* for 5 min at 4ºC. Considering the high fat content of the samples, two extra centrifugation steps were performed, at the same speed and temperature, during 10 and 5 min, respectively.

The protein extracts were purified with ReadyPrep 2-D Cleanup Kit (Bio-Rad), through quantitatively precipitation of proteins. The obtained pellets were ressuspended in a rehydration buffer (RB) composed by 8 M urea, 2% w/v CHAPS, ampholytes – Biolyte pH 3-10, 0.2% w/v DTT 50 mM and bromophenol blue (trace), (Bio-Rad). All protein concentration was measured with Quick Start™ Bradford Protein Assay with BSA as a standard (Bio-Rad).

Two-dimensional gel electrophoresis

200 µg of protein were diluted in RB to a total volume of 200 µL and applied to Immobiline™ Drystrip pH 3-10 11 cm (GE Healthcare). The strips were rehydrated overnight (passive rehydration).

The first dimension was carried out in an Ettan IPGphor (GE Healthcare) at 20ºC and below 50 µA per strip with a constant voltage of 250 V for 3 h. Voltage was then increased to 1000 V for 1 h, and 3500 V for 3:30 h. Finally, a constant 3500 V was applied for 30 min. As

a result, the proteins were focused for 8:13 h to a total of 12000 Vh. After first dimension, the strips were equilibrated using a standard Bio-Rad buffer (EB) composed of 6 M urea, 20% v/v glycerol, 2% w/v SDS and 0.375 M Tris-HCl (pH 8.8) (Bio-Rad). The equilibration was executed in two 30 min steps (reduction and alkylation). For the first step, 14.81 µl of a reduction solution (2.7 mg/uL DTT) was added, while for the alkylation step, 57 mg of iodoacetamide (GE Healthcare) was introduced in EB.

After equilibration, the strips were transferred into SDS-PAGE gels and fixed against the gels with agarose. For second dimension, 11 cm Bis-Tris Criterion™ XT Precast Gels (Bio-Rad) and running buffer containing MOPS (50 mM MOPS (Fluka), 50 mM Tris, 0.1 w/v SDS, 1 mM EDTA) were used. The electrophoresis was run at 200 V in a Criterion™ Dodeca™ Cell (Bio-Rad) using a PowerPac™ Universal power supply (Bio-Rad). Gels were stained with Coomassie Brilliant Blue G-250 - EZBlue™ Gel Staining solution (Sigma).

Gel image analysis

The gels were scanned with a GS-800 calibrated densitometer (Bio-Rad) at a 42.3 µm/pixel resolution. Gel images were cropped and background subtracted for differential expression analysis. Isoelectric points were estimated based on the linearity of the IPG strips and, molecular masses determined by co-electrophoresis with standard protein markers.

To study differential expression pattern between the three conditions (CTRL, HND, HSD), the PDQuest™ 2-D Analysis Software Version 8.0 (Bio-Rad) was used. Spot detection, matching and quantification are the main tasks of this software. After matching, all gel spots were normalized by local regression model (LOESS) method as implemented in PDQuest. The results were then statistically analyzed**.**

Statistical analysis

The statistical analysis was performed using the R software environment[299]. To evaluate the possibility of using parametric tests, the Shapiro-Wilk W-test and Levene's test were used. Since normality and homoscedasticity assumptions were not valid for the present dataset, a one-tailed Mann-Whitney U-test ($p < 0.05$) was used to assess differentially expressed proteins and to verify significant differences in mean plasma cortisol concentrations between different experimental groups.

5.2.3. Protein identification by LC-MS/MS

Spots with a greater than 2-fold or lower than 0.5-fold statistically significant expression change between conditions were considered significant and manually excised from the gels. Identification by LC-MS/MS was carried at the Aberdeen Proteomics facilities (University of Aberdeen, UK).

The spots were reduced, alkylated (with DTT and iodoaceamide, respectively) and subjected to tryptic digestion using an autolysis-resistant modified trypsin. The resultant peptides were extracted with acetonitrile and formic acid, and separated by liquid chromatography (UltiMate 3000 LC System, Dionex Ltd). This LC system was coupled to an electrospray ionization (low-flow nebulizer – Bruker Daltonics Ltd) – ion trap (HCTultra PTM Discovery System – spherical ion trap) mass spectrometer.

The MS/MS CID data were acquired in data-dependent AutoMS(2), averaged from three spectra with a scan range of 100 to 2200 m/z. Up to 3 precursor ions were selected from the MS scan (300 to 1500 m/z) in each AutoMS(2) cycle. Precursors were actively excluded within a 1.0 min window with singly-charged ions also excluded. Peptide peaks were detected and deconvoluted automatically using DataAnalysis software (Bruker Daltonics, Ltd.).
The mass lists generated were used in a «MS/MS Ion Search», through the MASCOT search engine (Matrix Science, www.matrixscience.com) to identify tryptic peptides by peptide fragment fingerprinting (PFF). The search was done on the NCBInr database limited to *Actinopterygii* (ray-finned fish), assuming the following parameters: tryptic digestion (one missed cleavage allowed), formation of double or triple charged peptides, carbamidoethylation of cysteine residues, possible oxidation of methionine residues, a 0.5 Da MS/MS mass tolerance and a 1.5 Da MS mass tolerance.

5.3. Results

5.3.1. Growth and cortisol response

No fish mortality occurred throughout the experiment. There were no significant differences in mean total length and mean total weight among the experimental groups, at the beginning and at the end of the experiment (ANOVA, $p > 0.05$), (Table 5.1).

High stocking density and handling treatments elicited an increase in plasma cortisol levels compared to baseline level measured in control group (2.23 ng/mL) (Figure 5.1). Mean

plasma cortisol concentration in HND (16.55 ng/mL) and HSD fish (16.30 ng/mL) was similar and approximately 7-fold higher than CTRL fish.

Table 5.1 – Total length and wet weight of *Sparus aurata* in a control group (CTRL), repeated handling (HND) and crowding produced by high stocking density (HSD) at the beginning and at the end of the experiment.

	Total length (cm)		Wet Weight (g)	
	Initial	Final	Initial	Final
CTRL	28.21 ± 1.20[a]	29,93 ± 1.16	377.75 ± 46.73	425.92 ± 51.94
HND	28.17 ± 1.40	28.25 ± 1.13	370,58 ± 50.38	400.67 ± 51.55
HSD	28.23 ± 0.85	28.93 ± 1.03	377,92 ± 47.31	412.33 ± 41.91
ANOVA (p value)	0.99	0.1	0.45	0.91

a) mean ± standard deviation. n = 6 (CTRL and HND), n = 11 (HSD).

Despite the similar cortisol values in HND and HSD groups a significant difference were detected only between HND and CTRL fish (one-tailed Mann-Whitney U-test, p = 0.0036), probably due to high data variability. No differences were found between the two HND and HSD stressful conditions (one-tailed MW U-test, p = 0.4225).

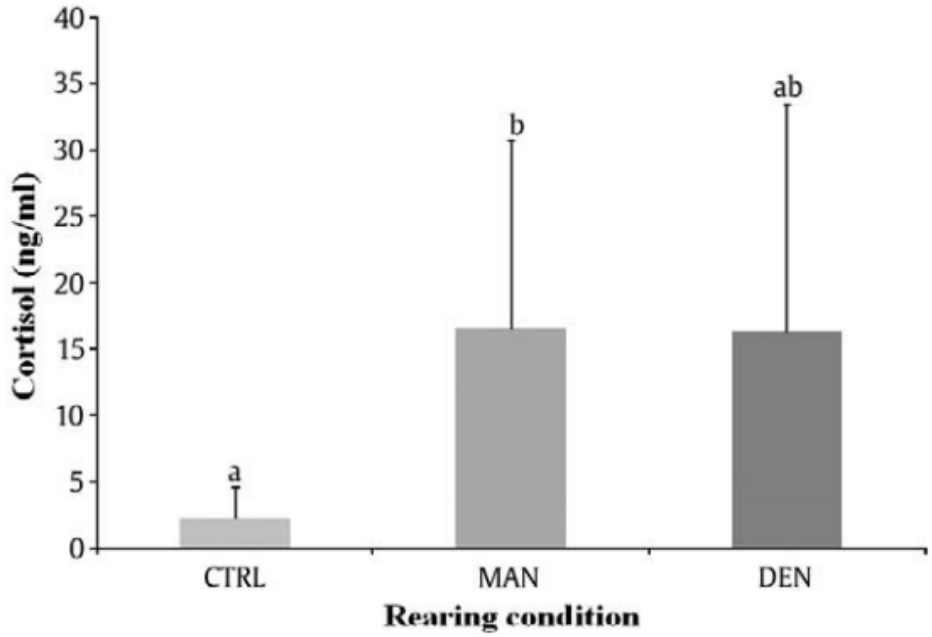

Figure 5.1 – Mean plasma cortisol concentration in control (CTRL), repeatedly handled (HND), and crowded (HSD) gilthead seabream. Different letters indicate significant differences among treatments (p-value < 0.05).

5.3.2. Comparative 2-DE

In order to identify potential biomolecular welfare indicators for cultured *S. aurata*, the liver proteome of the three experimental groups (CTRL, HND, HSD) was compared using 2-DE. One representative gel for each experimental condition is shown in Figure 5.2. A total of 560 spots were acquired in a reproducible way across all gels and after statistical analysis (one-tailed MW U-test, $p < 0.05$) significant differences in a high number of spots were detected between the experimental groups. About half of the detected proteins (299 spots) were expressed significantly different between the CTRL group and stressed fish (HND and HSD). 235 spots showed differences among CTRL and HND and 223 spots differed between CTRL and HSD. 70 spots were affected by both treatments.

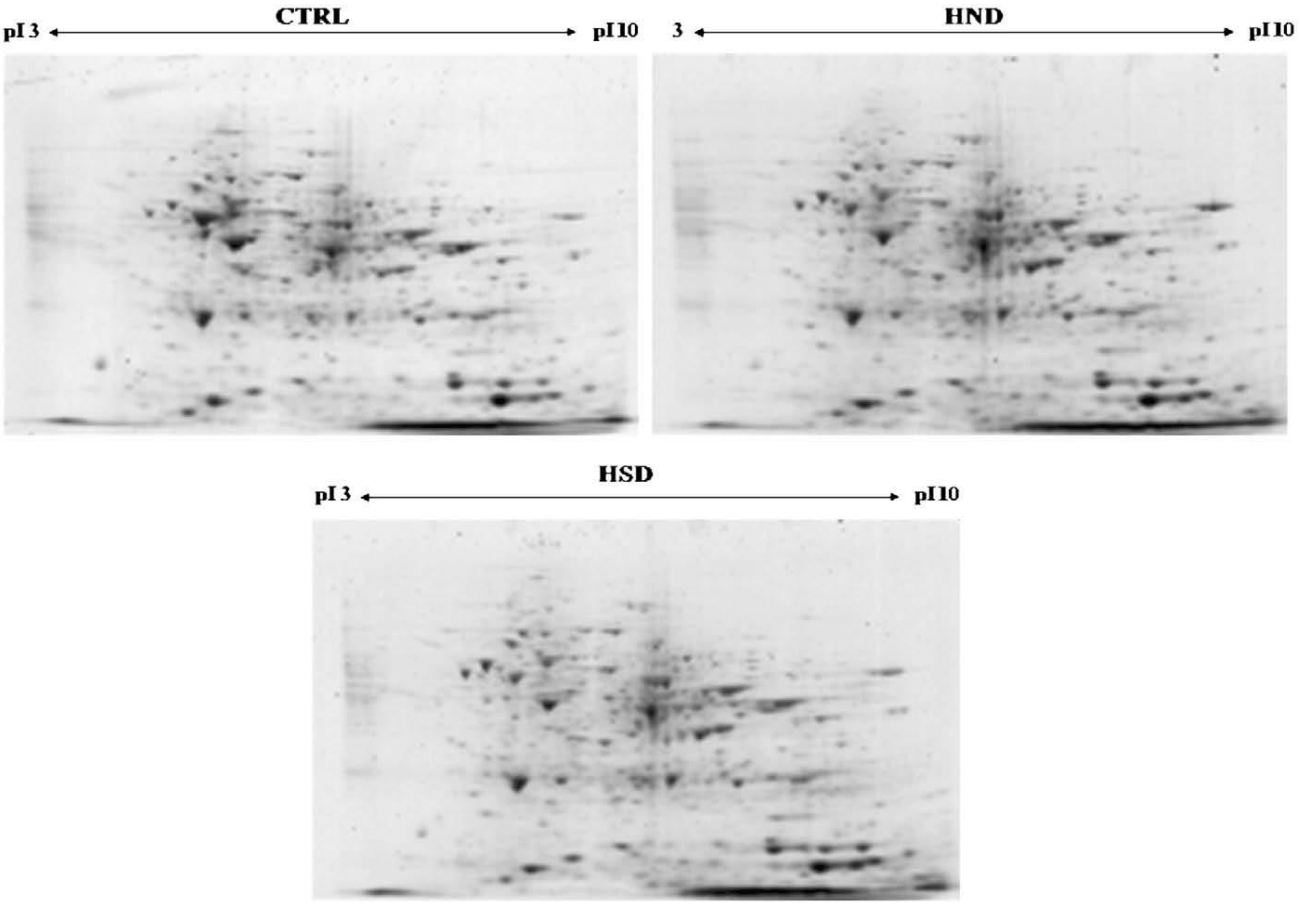

Figure 5.2 – Colloidal CBB-stained 2-DE gels (strips pH 3–10 linear — 12% Bis–Tris) from S. aurata liver (one gel by condition).

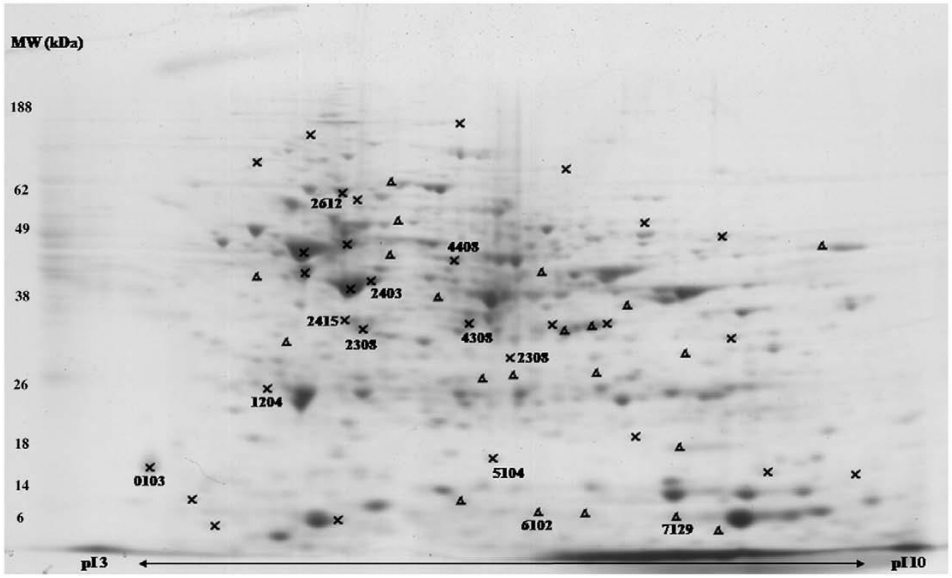

Figure 5.3 – Reference gel representing some of the relevant spots. Spots marked with a cross (×) represent down-regulated proteins in stressed fish (Mann–Whitney test, p-value < 0.05). Spots marked with a triangle (Δ) represent up-regulated proteins in stressed fish (Mann–Whitney test, p-value < 0.05). Numbered spots show protein spots identified by mass spectrometry.

Only spots with a greater than 2-fold or lower than 0.5-fold were considered as relevant in this study. A total of 164 relevant spots were detected, with 79 being up-regulated and 85 being down-regulated as a response to stress. Of the 164 spots, 37 only changed in handling fish (HND) and other 40 only changed for fish grown under high stocking conditions (HSD). Figure 5.3 provides an overview of the most relevant changes in protein expression. The numbered spots in figure 5.3 represent the ones excised from the gels.

5.3.3. Peptide fragment fingerprinting using LC/MS-MS

Excised spots were digested with trypsin and characterized by LC/MS-MS. The resultant MS-MS peptide spectra were analyzed in the non redundant data base NCBInr (taxonomic level – Actinopterygii) by PFF. The obtained spectra were used in a PFF search against the NCBInr database.

Table 5.2 shows the results of protein spot identification. 12 of these proteins were positively identified: 2 proteins were up-regulated (fatty acid binding protein; haemoglobin) and 10 down-regulated (calmodulin; triose-phosphate isomerase; beta-tubulin; glutamine synthetase; pyruvate dehydrogenase; heat shock cognate protein 70; glyceraldehyde 3-phosphate dehydrogenase; alfa-enolase; voltage-dependent anion channel I; cofilin).

Figures 5.4 and 5.5 summarize the main changes in expression of the 12 proteins described in Table 5.2 (one-tailed MW U-test, $p < 0.05$). Identified proteins were found to be involved in several metabolic pathways, such as glycolysis, gluconeogenesis, ammonia metabolism, cytoskeleton proteins, signalizing proteins and lipid transport.

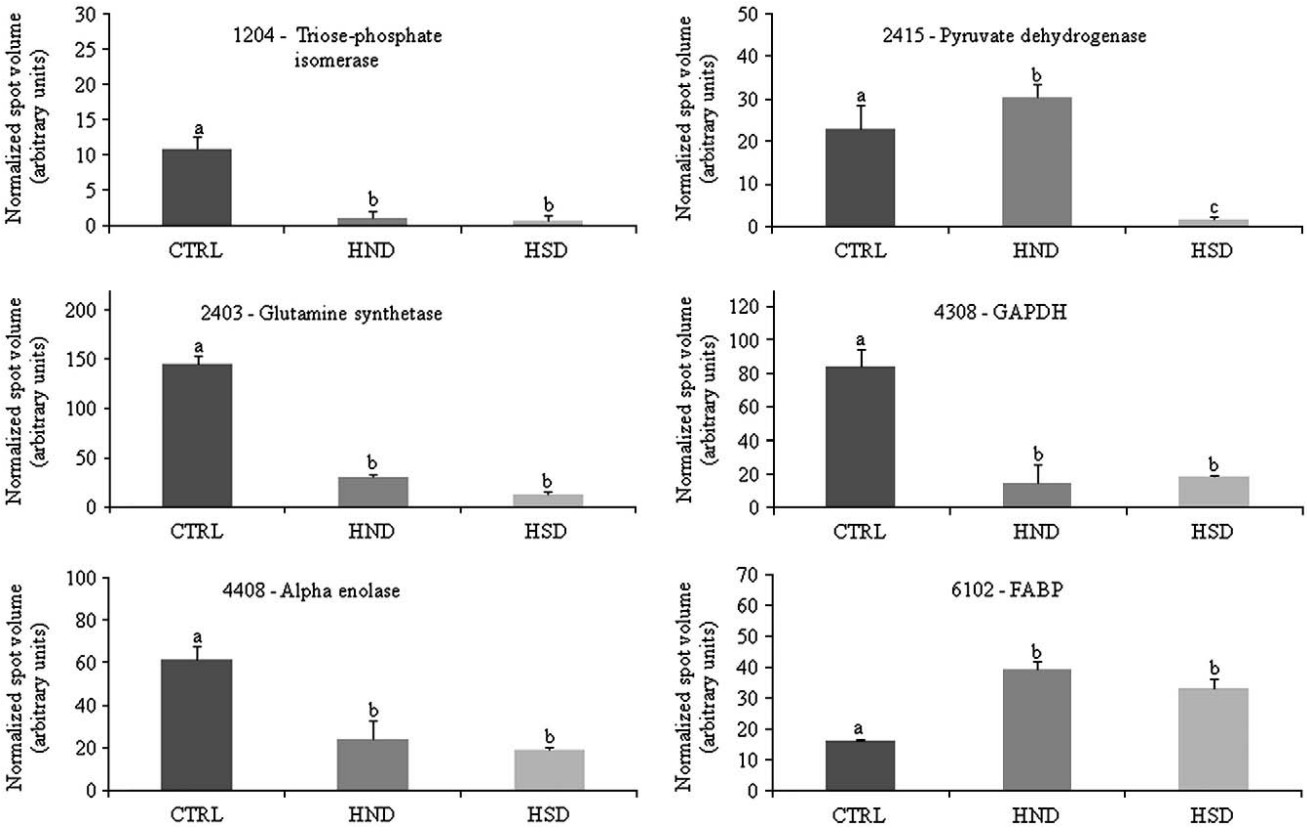

Figure 5.4 – Differential expression patterns of proteins involved in carbohydrate metabolism, lipid transport and ammonia metabolism identified by LC-MS/MS. Each bar represents the abundance median for every spot in each condition (normalized amount of protein) ± median absolute deviation (mad). GAPDH - glyceraldehyde 3-phosphate dehydrogenase; FABP – fatty acid binding protein. Different letters indicate significant differences among conditions ($p < 0.05$). In each histogram is represented, for the three culture conditions (four gels by condition), the normalized spot volume median and related median absolute deviation (mad). The statistical test results are also represented above each bar of the histograms.

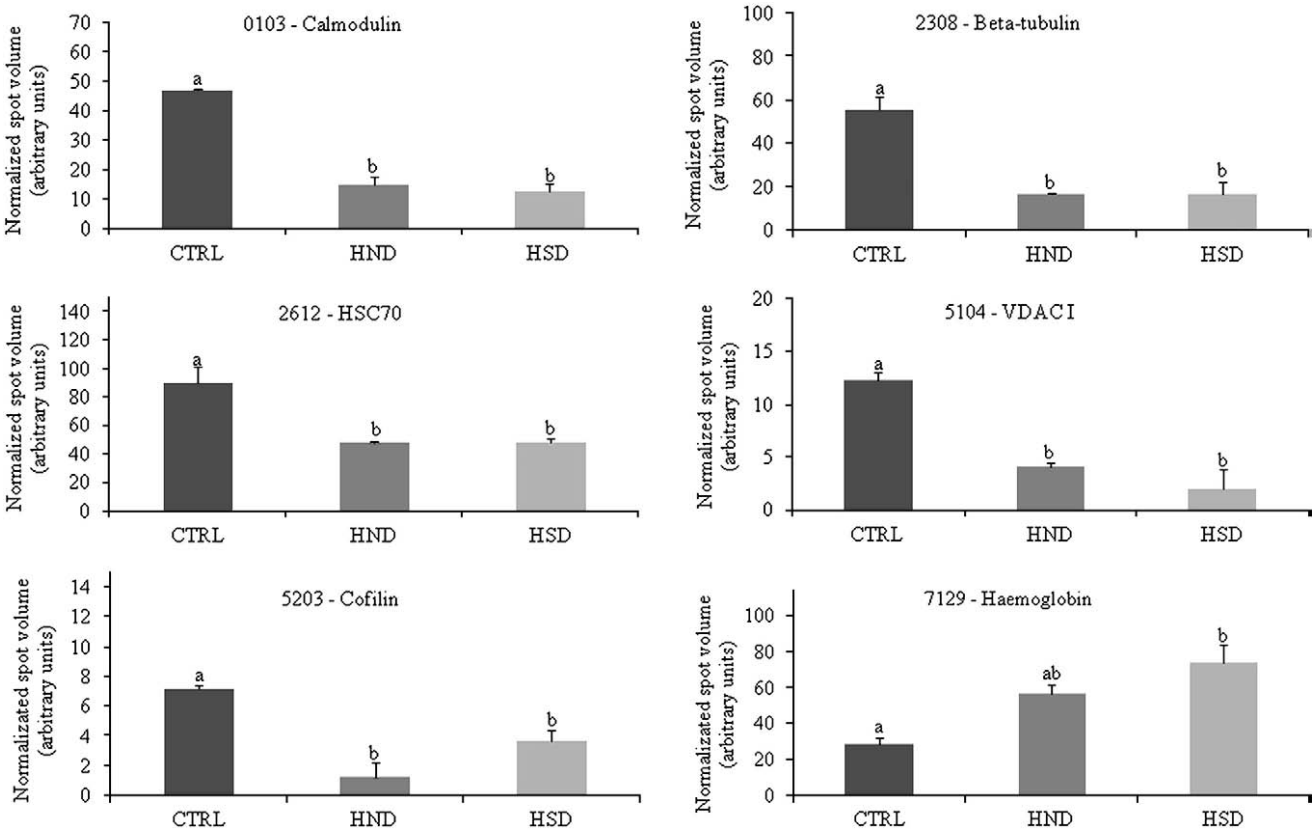

Figure 5.5 – Differential expression patterns of calmodulin, cytoskeleton proteins, heat shock cognate protein 70, hemoglobin and voltage-dependent anion channel identified by LC-MS/MS. Each bar represents the abundance median for every spot in each condition (normalized amount of protein) ± median absolute deviation (mad). HSC70 – heat shock cognate protein 70; VDAC I - voltage-dependent anion channel I. Different letters indicate significant differences among conditions ($p < 0.05$). In each histogram is represented, for the three culture conditions (four gels by condition), the normalized spot volume median and related median absolute deviation (mad). The statistical test results are also represented above each bar of the histograms.

Table 5.2 – Main identified proteins by LC MS/MS. The identification was made by peptide fragment fingerprinting (PFF) in the option MS/MS Ion Search from the bioinformatics application Mascot. The PFF was made in the non redundant NCBInr data base for the Actinopterygii taxonomic level (p-value < 1E-05).

Spot no.	Protein (species)	GI number[a]	Mw_{th}/pI_{th} [b] (Mw_{exp}/pI_{exp} [c])	Sequence coverage (%)[d]	# peptides matched (E-value < 0.05)[e]	Best peptide match: Sequence, Charge state, E-value[f]	Combined Mowse Score[g]
0103	Calmodulin (*Oncorhynchus sp.*)	gi|71664	16.70/4.09 (18.00/4.10)	10	2	VFDKDGNGYISAAELR, 2+, 9.2E-06	89
1204	Triose-phosphate isomerase (*Acipenser brevirostrum*)	gi|15149246	27.10/6.30 (25.00/4.60)	9	1	VVLAYEPWAIGTGK, 2+, 5.30E-04	59
2308	Beta-tubulin (*Danio rerio*)	gi|82658236	50.10/4.81 (33.00/5.72)	39	31	GHYTEGAELVDSVLDVVR, 2+, 2.7E-12	922
2403	Glutamine synthetase (*Bostrychus sinensis*)	gi|20799646	42.0/5.75 (42.50/5.80)	25	6	RLTGHHETSNNINEFSAGVANR, 3+, 7.1E-07	255
2415	Pyruvate dehydrogenase (*Danio rerio*)	gi|47085923	39.60/5.78 (35.00/5.60)	5	1	VFLLGEEVAQYDDGAYKVSR, 3+, 2.50E-02	46
2612	Heat shock cognate protein 70 (*Oryzias latipes*)	gi|157278337	76.58/5.80 (73.00/5.48)	43	28	SINPDEAVAYGAAVQAAILSGDK, 2+, 3.8E-10	1156
4308	Glyceraldehyde 3-phosphate dehydrogenase (*Pagrus major*)	gi|15146358	36.38/6.36 (36.00/6,67)	50	18	VPVADVSVVDLTCR, 2+, 8.3E-07	529
4408	Alfa-enolase (*Acipenser baerii*)	gi|98979415	47.47/5.95 (49.00/6.45)	47	28	SGETEDTFIADLVVGLCTGQIK, 2+, 4.1E-10	1274
5104	Voltage-dependent anion channel I (*Tetraodon nigroviridis*)	gi|47221743	30.65/6.53 (31.00/6.50)	41	11	SENGLEFTSTGSANTETSK, 2+, 1.9E-12	500
5203	Cofilin (*Tetraodon nigroviridis*)	gi|47225287	18.86/6.82 (18.40/5.50)	15	3	YALYDATYETK, 2+, 1.50E-03	108
6102	Fatty acid binding protein (*Tetraodon nigroviridis*)	gi|47222259	13.40/6.32 (10.00/6.80)	29	1	MISSENFDDYMK, 2+, 6.9e-05	68
7129	Haemoglobin (*Pagrus major*)	gi|37778990	15.81/8.55 (12.00/8.33)	7	1	SADIGAEALGR, 2+, 2.4E-06	84

a)GI number - NCBI acess number.

b) Mw_{th}/pI_{th} - Theoretical molecular weight and isoelectric point. Mw_{th} is given in KDa.

c) Mw_{exp}/pI_{exp} - Experimental molecular weight and isoelectric point. Mw_{exp} is given in KDa.

d) Sequence coverage (%) - percentage of the entire protein covered by matched peptides.

e) # matched peptides (E-value < 0.05) - number of peptides matched to entry with significative E-value (when E-value < 0.05).

f) Best peptide match with the lower E-value: sequence, charge state and E-value.

g) Combined Mowse Score – a non-probabilistic protein score, derived from the ions score.

5.4. Discussion

5.4.1. Physiological response to chronic stressors

Fish welfare may be compromised under chronic stress farming conditions[68]. Regular handling and high stocking densities are regarded as chronic stressors leading to a series of physiological, biochemical and behavioral changes in gilthead seabream[392, 396, 411], thus affecting welfare.

Results of the present study show that gilthead seabream repeatedly handled and crowded at high stocking density experienced chronic stress as indicated by the increase in plasma cortisol levels compared to level measured in undisturbed control fish.

Plasma cortisol concentrations in control fish are consistent with baseline values reported in literature for this species[410, 414-416]. Also the quantitative plasma cortisol increase in HSD sea bream is concordant with mean values reported for chronic stressed sea bream reared for 15 weeks at about 40 kg/m^3 and also in fish confined for at 26 kg/m^3 for 14 days[391, 396].

The cortisol stress response observed in gilthead seabream subjected to repeated netting and 1 min air exposure biweekly for 4 weeks, is also in accordance with cumulative effect on plasma cortisol levels, of some repetitive factors, including netting and 3 min air exposure, performed twice daily for 10 days, on turbot *Scopthalmus maximus*[390].

Analysis of plasma cortisol in this study, suggests the reliability of this parameters as indicator of neuroendocrine stress response to repetitive handling and high stocking density in gilthead seabream, as already reported in several previous stress studies [391-392, 396, 408, 410, 412, 414-415]. However, a high individual variability was found in the cortisol level of both stressed groups, probably related to the individual difference in the stress response[76], and to the possible adaptation, at least of some individuals, to chronic stress conditions with consequent plasma cortisol reduction[391]. Therefore, plasma cortisol is still to be confirmed as reliable welfare indicator in *Sparus aurata* exposed to chronic stress.

On the other hand, not even significant differences in fish growth among experimental conditions were detected, suggesting that the applied chronic stress conditions did not affect fish growth performance and health during the 1 month experimental period. However, the duration of the rearing experiment may not be long enough to detect differences in fish growth or long-term effects in the health status.

This is to our knowledge the first study to use comparative proteomics techniques, and 2-DE in particular, as an alternative to the traditional methods to assess chronic stress / welfare status in fish. From the 164 detected spots with relevant down- and up-regulated expression patterns in stressed fish, 12 have been identified by peptide fragment fingerprinting using the Mascot search engine (http://www.matrixscience.com) in "MS/MS Ion Search" mode. All identified proteins had high homologies with proteins species in the database. These proteins are involved in several cellular processes related to stress adaptation, including amino acid, carbohydrate and lipid metabolism, folding processes, signaling and cytoskeleton.

5.4.2 Expression of proteins involved in energy metabolism

In the present study the proteins pyruvate dehydrogenase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, enolase and fatty acid binding protein were modulated by chronic stress conditions. Not surprisingly, all these enzymes play important roles in energy metabolism since one of the first aspects of fish adaptation to stress consists in the mobilization of energetic reserves, involving the activation of liver glycogenolysis and gluconeogenesis, in order to guarantee a continuous supply of glucose to essentials organs such as brain and muscles[417]. When this happens, glycolysis is generally inhibited in the liver[418].

In the current study, the 13-fold abundance reduction of pyruvate dehydrogenase (spot 2415) in stressed fish can be explained as a long-term adaptation to reduce the conversion of pyruvate to acetyl-CoA, favouring the gluconeogenic route and, thus, glucose production. The biological importance of this adaptation should be related to the expected high levels of pyruvate due to conversion in the liver from lactate.

Several studies have confirmed increased glucose export capacity in fish liver reared under high density conditions, resulting in higher blood glucose levels [392, 396, 410-411] noticed a 20% increase in plasma glucose, 60% decrease in liver glycogen and 20% increase in gluconeogenic potential in the liver of high stocking densities *S. aurata*. These authors suggest that the increasing of capacity of the liver to export glucose was related to 100% increase displayed by glucose-6-phosphatase (G6Pase) activity.

Since carbohydrates reserves in the liver are probably not enough to maintain plasma glucose levels for prolonged periods of stress, the mobilization of amino acids and lipids becomes energetically relevant[419]. In addition, several studies demonstrate that adverse

growth conditions such as high stocking densities induce changes in metabolism of lipids[396, 420].

The 2-fold increase in the expression of fatty acid binding protein in the liver of stressed fish observed in this study can be explained as a mechanism to sustain high rates of lipid usage in liver cells, in order to respond to the increased energetic requirements due to stress. The fatty acid binding proteins (FABPs) represent a family of proteins responsible for the transport and intracellular maintenance of lipid-derived fatty acids, and are involved in the uptake of dietary fatty acids by intestinal cells, the accumulation of fatty acids in the cytosol, the targeting of fatty acids to specific metabolic pathways, and genetic regulation and fatty acid signaling[421-425].

High lipid utilization by liver cells, in response to stress, as suggested in the present study, is supported by previous works. Montero et al. verified that high culture densities cause a decrease in the hepatosomatic index in the gilthead seabream, associated with an increase in lipid mobilization[396]. These authors also verified a decrease in enzymes related with lipid synthesis (i.e. glucose 6-phosphate dehydrogenase). Vijayan et al. have shown that rearing *Salvelinus fontinalis* under high stocking densities cause an increase in hydroxyl-CoA dehydrogenase, a lipid oxidation-related enzyme[420]. In addition, a study in *Mus musculus* has shown that the liver FABP has determinant role in the fatty acid oxidation[426].

During lipid oxidation, triacylglycerides are converted into glycerol and fatty acids[427]. In the present study, a down-regulation of 13-fold in the expression of pyruvate dehydrogenase in stressed fish reveals a metabolic shift from glycolysis to gluconeogenesis and the simultaneous reduction in expression of triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase and of enolase shows a general reduction of these pathways in favor of the ketogenic pathway. This pathway is based on mitochondrial β-oxidation of fatty acids for production of ketone bodies to be exported from liver for reconversion to acetyl-CoA and use as metabolic fuel in the peripheral tissues[427].

Furthermore, the reduction of expression triose phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in stressed fish is especially interesting given the fact that glycerol (converted to dihydroxyacetone phosphate and enter gluconeogenesis to produce glucose) enters the glycolytic/gluconeogenic pathway at this step[419]. These results suggest a long-term adaptation so as to redirect the surplus of glycerol to glucose production, as opposed to pyruvate production.

The observed reduction in the levels of voltage-dependent anion channel (VDCA) in the liver cells of stressed fish also supports the idea of a long-term adaptation process to

situations of exposure to chronic stress to increase metabolic efficiency. This protein is a mitochondrial porin that regulates the outer membrane's permeability, especially to polyanionic molecules (such as phosphoenolpyruvate and adenosine triphosphate)[428]. The β-oxidation of palmitate and palmitoyl-CoA is dependent on the activity of VDCA[428]. The down-regulation in this protein in stressed fish may help to prevent the occurrence of mitochondrial oxidative stress due to the production of ROS (reactive oxygen species) during β-oxidation, by limiting its rate. An increase in FABP (which has been shown to reduce the amount of hydrogen peroxide-induced ROS in rat hepatocytes) (Wang et al., 2005) also supports the idea that an adaptative long-term metabolic response to chronic stress may involve mechanisms to mitigate the production and impact of these reactive species[429]. These findings together with the presented results, suggest that FABP and VDAC are potential biomarker candidates for fish welfare.

5.4.3. Expression of calmodulin

A 3.8-fold decrease in calmodulin abundance in the liver of chronically stressed fish was observed. Calmodulin (CaM) is a highly conserved[430] calcium-binding protein that acts as a mediator of calcium-induced intracellular signaling. It interacts with other proteins involved in the regulation of important cellular processes[431]. In stress situations, epinephrine triggers a series of reactions which increase intracellular levels of calcium, inducing an activation of the Ca^{2+}/CaM-dependent kinases.

Glycogen phosphorylase is one of the key enzymes in this glycogenolysis pathway, catalyzing the degradation of glycogen to glucose-1-phosphate monomers, which can be converted to glucose-6-phosphate and used as fuel[419]. The reduction of glycogen reserves commonly observed in the stressed fish liver is associated to the activation of glycogenolysis by the action of catecholamines[392, 411]. One of the regulation mechanisms of this enzyme is through phosphorylation by a Ca^{2+}/calmodulin-dependent protein kinase[432]. This enzyme will, in turn, phosphorylate glycogen phosphorylase, favoring glycogenolysis[432]. Thus, the 3.8-fold decrease in calmodulin abundance in the liver of chronically stressed fish observed in the current study may result from a long-term adaptation to the increased intracellular levels of calcium, reducing the sensibility of hepatocytes to repetitive epinephrine stimulation, in order to prevent the triggering of apoptotic signals.

The down-regulation of calmodulin and cytoskeleton proteins in stressed fish may also be related to lipid metabolism. Carnitine pamitoyltransferase I (CPT-I) regulates the influx of fatty acids to the mitochondrial matrix. Besides the classical allosteric regulation

mechanism[427, 433], recent studies show that CPT-I can also be regulated by Ca^{2+}/calmodulin-dependent protein kinase II (Ca^{2+}/CaM-PK II), through cytoskeleton components[434-436]. These authors suggested, that the activation of Ca^{2+}/CaM-PK II induces a disruption of the intermediate filaments and subsequent increase in CPT-I activity and in the rate of β-oxidation. A reduction in the expression levels of tubulin and cofilin in stressed fish seem to indicate a lower degree of cytoskeletal reorganization activity in liver cells. This may simply reflect a slow adaptation in hepatocytes due to chronic exposure to stress situations, away from ATP-intensive cellular processes that involve cytoskeletal reorganization and protein synthesis, in order to maximize metabolic efficiency. A clear relationship between the changes in cytoskeletal components observed during the current study and the biological processes involved cannot be established.

5.4.4. Expression of heat shock cognate protein 70

Spot 2612 was identified as heat shock cognate protein 70 (hsc70). This protein was shown to be down-regulated to half its abundance in both stressed groups. The heat shock proteins (HSPs) constitute a family of extremely conserved proteins, being part of the cellular response of fish to stress[437-442].

Hsc70 belongs to the HSPs70 class, and is expressed constitutively in cells without stress, in contrast to hsp70 which is induced in response to stressing agents[443]. The HSPs70 have a very important role in cells, as they act as chaperones and have a crucial role in protein folding, regulation, secretion and degradation[444-445].

The observed hsc70 down-regulation in chronic-stressed fish is in line with observations of previous studies. Gornati et al.[399] verified higher gene expression of hsc70 in the brain and liver of control fish (*Dicentrarchus labrax*), compared to the fish subjected to high rearing densities (80 e 100 kg/m^3). In addition, stress caused by transport, changes the hsc70 liver expression in *D. labrax* adult individuals. Immunohistochemical techniques also reveal that this protein only accumulates in the control fish liver[446]. Others studies in fish subjected to heat shock and exposure to cortisol verified degradation and a subsequent decrease in synthesis and accumulation of HSPs70[440, 447]. Both results suggest that hsc70 is a very good candidate to be used as a biomarker for fish welfare.

5.4.5. Expression of glutamine synthetase

Glutamine synthetase (GSase, spot 2403) was another identified protein with down-regulation levels in stressed fish, having a ratio HND/CTRL and HSD/CTRL of 0.21 and 0.09

respectively. This enzyme catalyzes the ATP dependent reaction of glutamine formation from ammonia and glutamate. It is responsible for the intra-hepatic and inter-tissue ammonia elimination/detoxification occurring in the liver[448-449]. Other functions of GSase include nucleotide biosynthesis and amino acid turnover[450].

The down-regulation of the GSase expression in stressed fish suggests that an increase in glutamine synthetase accumulation/expression may have occurred in the beginning of the present study, followed by a feedback mechanism to reduce this accumulation/expression after some time. These results can be supported by previously works on GSase activity, and its importance in the nitrogen metabolism control during the initial response to stress. In fish, exposure to confinement stress for 24 hours, results in an increasing of the hepatic GSase activity only after two exposure hours to stress[451]. In mice, chronic stress by hypoxia reveals an increasing of glutamine synthetase activity in the first days, and a subsequent drop to basal levels[452]. Enzyme activity and mRNA expression studies, as well as the plasma cortisol and ammonia estimate throughout the experiment would be necessary to sustain this hypothesis.

5.4.6. Expression of hemoglobin

An increase in the hemoglobin expression in stressed fish (spot 7129, HSD/CTRL – 2.55) is in line with previous studies with a wide variety of fish species[396, 453-455]. Hemoglobin is already a common indicator for the assessment of a stress response. An explanation is based on the necessity to increase the efficiency of oxygen transportation, due to the increased energetic requirements in stress conditions[418, 456].

5.5. Final considerations

This work consisted in a new approach on gilthead seabream welfare, more specifically in terms of chronic stress. Mostly proteins founded in this study proved that related with metabolic stress, most specifically with metabolic and energetic adjustments,

With this preliminary study, it can been concluded that proteins FABP, hsc70, GSase, haemoglobin, calmodulin, triose-phosphate isomerase, GAPDH, alfa-enolase, beta-tubulin, cofilin, pyruvate dehydrogenase and VDCA are potential biomarkers to be used in the evaluation of fish stress/welfare. However, validation studies on gene expression (RT-PCR), metabonome expression, ELISA assay, and enzymatic activities, as well as in a broader range

of chronic stress situations, are required before these biomarkers can be used as welfare indicators. Such studies will also allow a better understanding of the role of these proteins in chronic stress situations and fish welfare.

The most promising molecular markers identified were the hsc70, FABP, calmodulin, pyruvate dehydrogenase and VDCA, as their role in stress response is better understood. In addition, it would be interesting to explore the connections between FABP, calmodulin and the cytoskeletal proteins.

To have a general overview on stress/welfare of gilthead seabream and for improve the rearing conditions of this species, several studies must be realized. For example, behavioral analyses, physical appearance, water quality and proteomics studies on others tissues, like head kidney, plasma, brain, gills are important to obtain more relevant data on stress/welfare biomarkers. In the future, commercial kits like genomic tools and practical tests (e.g. ELISA assays) may be developed to study the welfare/stress of fish in aquaculture with aid of such molecular markers.

Chapter 6.

Characterization of the impact of repeated handling stress on Senegalese sole liver proteome.

This chapter has been previously published as:

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Abstract

The Senegalese sole, a high-value flatfish, is a good candidate for aquaculture production. Nevertheless, there are still issues regarding this species' sensitivity to stress in captivity. We aimed to identify potential molecular markers for Senegalese sole that indicate a physiological response to chronic stress.

Two groups of fish were reared in duplicate for 28 days, one of them weekly exposed to handling stress (including hypoxia) for three minutes, and the other left undisturbed. Two-dimensional electrophoresis enabled the detection of 287 spots significantly affected by repeated handling stress (Wilcoxon-Mann-Whitney U-test, $p < 0.05$), 33 of which could be reliably identified by peptide mass spectrometry. Chronic exposure to stress seems to have affected protein synthesis, folding and turnover (40S ribosomal protein S12, cathepsin B, disulfide-isomerase A3 precursor, cell division cycle 48 and five distinct heat shock proteins), amino acid metabolism, urea cycle and methylation/folate pathways (methionine adenosyltransferase I α, phenylalanine hydroxylase, mitochondrial agmatinase, serine hydroxymethyltransferase, 3-hydroxyanthranilate 3,4-dioxygenase, betaine homocysteine methyltransferase), cytoskeletal (40S ribosomal protein SA, α-actin, β-actin, α-tubulin and cytokeratin K18), aldehyde detoxification (aldehyde dehydrogenase 4A1 family and aldehyde dehydrogenase 7A1 family, carbohydrate metabolism and energy homeostasis (Fatty acid-binding protein, enolase 3, enolase 1, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, aconitase 1, mitochondrial ATP synthase α-subunit, electron-transfer-flavoprotein α polypeptide), iron and selenium homeostasis (transferrin and selenium binding protein 1), steroid hormone metabolism (3-oxo-5-β-steroid 4-dehydrogenase) and purine salvage (hypoxanthine phosphoribosyltransferase). Further characterization is required to fully assess the potential of these markers for the monitoring of fish stress response to chronic stressors of aquaculture environment.

6.1. Introduction

Aquaculture is an increasingly important human activity, already accounting for the production of about 50% of all fish consumed worldwide[457]. This trend is expected to continue, with aquaculture soon becoming the main supplier of fish for human consumption[458]. As production rapidly increases to meet demands, the welfare of farmed fish is a growing concern, not only due to public perception, marketing and product acceptance, but also often in terms of production efficiency and quality[68, 459-460].

Although fish welfare cannot be objectively measured, it is usually evaluated using measures of physiological response to prolonged, repeated or unavoidable stress (chronic stress)[68]. One of the main challenges in aquaculture research is the development of optimized rearing techniques to reduce the deleterious effects of long-term exposure to stressful agents[76]. Physiological responses to stress are grouped as primary, which include endocrine changes such as in measurable levels of circulating catecholamines and corticosteroids, and secondary, including changes in features related to metabolism, hydromineral balance, and cardiovascular, respiratory and immune functions. Tertiary or whole-animal changes in performance, such as in growth, reproductive function, disease resistance and behavior, can result from the primary and secondary responses and possibly affect survival[76]. Chronic stress measurements largely concern these tertiary responses which usually arise from repeated exposure to situations that are inherent to aquaculture production, such as fish handling, transport, food withdrawal (during/prior to sampling, vaccination and transport), slaughter techniques and culturing under high stock densities[68, 76, 381, 391, 396].

Many physiological and biochemical studies performed with the purpose of understanding the metabolic response mechanisms to chronic stress have been undertaken. The classical parameters used as indicators of a stress response include the levels of cortisol, glucose, lactate and total protein in the plasma, haematocrit and haemoglobin values and some enzymatic essays[392, 410, 453, 461-463]. In terms of chronic stress these are not the most reliable parameters to consider, since fish have variable susceptibility to acute stress[394] and will therefore respond differently to a repeated exposure. Accordingly, cortisol levels can often return to basal levels due to an adaptation process[453, 464]. On the other hand, the use of molecular indicators such as the products of stress-related genes has recently begun to emerge as an alternative approach to diagnose exposure to stress conditions and to measure welfare. Proteomic techniques, such as two-dimensional gel electrophoresis and mass spectrometry

(MS), are promising approaches to obtain a better understanding of the underlying molecular mechanisms related to short and long-term adaptations to stress and for screening new potential indicators of fish welfare[406-407, 465-466].

For this study, juvenile Senegalese sole (*Solea senegalensis*) was selected for use as a model species due to its resistance to stress in terms of survival, when in its natural habitat[467]. Additionally, sole is a very attractive candidate for marine aquaculture in Southern Europe and has considerable potential as a future species for farming[468]. This species is a common high-value flatfish in Southern European countries well adapted to warm climates and increasingly farmed in Portugal and Spain[469]. Several aspects of its rearing have been developed and optimized (larviculture and weaning techniques), resulting in significant advances over recent years[470-471]. On the other hand, this species has not yet become a commercial success, as its cultivation is still hampered by its high sensitivity to stress and to several infectious diseases that can cause large mortalities, when in captivity[472].

Consequently, there is a specific need to identify candidate biomarkers responsive to the usual handling techniques employed during cultivation of soles that may affect them negatively. We selected the liver for proteomic analysis as it is a central organ in regulation of primary metabolism and is likely to respond to chronic stressors, due to its responsiveness towards glucocorticoids like cortisol.

The aim of this study was therefore to use repeated handling (including mild hypoxia) on Senegalese sole as a model of chronic stress exposure and proteomic techniques (2DE and MS) to increase the wealth of information regarding this species' responses to chronic stressors. Together, this should allow finding robust indicators of chronic stress/fish welfare which can be used to improve rearing techniques and, therefore, growth performance and economical viability of Senegalese sole as a commercial aquaculture species.

6.2. Materials and methods

6.2.1. Fish trials and sampling

The experiment took place at the Ramalhete Research Station (CCMar, Faro, Portugal), using a partially-recirculated seawater system (temperature: 20 ± 1 ºC; salinity: 36 $g.L^{-1}$; dissolved oxygen above 90 % saturation level), comprised by flat-bottom sand-coloured

fiberglass tanks (0.21 m^2 area, 20 L volume, 54 L h^{-1}constant water flow rate). Senegalese sole (*Solea senegalensis*) juveniles (24.2 ± 0.4 g wet weight) were used as the model species. Before the experiment, fish were anaesthetized with 2-phenoxyethanol (1000 ppm; Sigma-Aldrich, Germany) and individually measured, weighed, and tagged with water ink on the ventral side.

Two treatments were carried out in quadruplicates and compared during this experiment: "control" (CTRL) and "handling" (STR). Eleven sole juveniles were introduced in each of the eight tanks, having been subjected to a 6-7 days acclimatization period prior to the beginning of the trial. Initial density inside the tanks was 1.25 kg m^{-2}.

In STR treatments, fish were removed from the tanks and exposed to air for three minutes (handling-type stressor). This exposure was applied once a week, inside a covered container. Individuals from the CTRL groups were reared without any disturbance, except for daily tank cleaning procedures.

Feeding of all treatments was done using automatic feeders, supplying eight meals a day. Measurements of temperature, salinity, and dissolved oxygen were performed daily, besides measurements of ammonia and nitrite levels (twice a week). Ammonia and nitrite levels in water were measured twice a week. Fish were kept at a photoperiod of 12 h light: 12 h dark and the light intensity at water surface was 200 lux.

Four weeks (28 days) after beginning the experiment, fish were fasted for 24 h prior to final sampling. On the sampling day, five individuals of each tank were anaesthetized with 2-phenoxyethanol (1000 ppm) for blood sampling, biometric measurements and liver sampling. The blood was collected from the caudal vein with a heparinized syringe, placed on ice, incubated at 4 ºC and centrifuged at 1500 × *g* for 2 min. Plasma samples were collected for a companion study [473], quickly frozen in liquid nitrogen and then stored at -80 ºC. Liver was dissected on a glass placed over an ice bed, weighed, frozen in liquid nitrogen, and kept at -80 ºC for further analysis.

6.2.2. Protein extraction and purification

Liver tissue was pooled from individuals of the same treatment (n = 20 per treatment): each liver was split in four pieces, with each piece distributed over four eppendorf (for a total of 100 mg of tissue per pool). Pooling was performed to reduce the impact of inter-individual variations on the estimate of mean protein abundance levels, having done 4 technical

replicates (representative of each treatment's "average liver profile", rather than a specific individual) per pool.

Each 100 mg of tissue was resuspended in 1 mL of extraction buffer containing 7 M urea (Sigma-Aldrich), 2 M thiourea (Sigma-Aldrich), 2 % w/v CHAPS (Sigma-Aldrich), 50 mM DTT (Sigma-Aldrich), 1 % v/v ampholytes IPG Buffer pH 3-10 (GE Healthcare) and 1 % v/v protease inhibitor cocktail (Sigma-Aldrich) and then incubated on ice for 30 min. The hepatic tissue was homogenized with an Ultra-Turrax IKA T8 (IKA-WERG), sonicated five times for 5 s, and centrifuged two times at 19000 × *g* for 10 min at 4 ºC. Non-protein contaminants were removed from the protein extracts using the ReadyPrep 2-D Cleanup Kit (Bio-Rad). Precipitated proteins were resuspended in a standard 2DE rehydration buffer (8 M urea, 2 % w/v CHAPS, ampholytes – Biolyte pH 3-10, 0.2 % w/v DTT 50 mM and traces of bromophenol blue, Bio-Rad). All protein quantifications were performed using Quick StartTM Bradford Protein Assay (Bio-Rad).

6.2.3. Two-dimensional gel electrophoresis

250 µg of total protein from each sample was diluted in Rehydratation Buffer (Bio-Rad) to a final volume of 200 µL and loaded overnight on to 11 cm Immobiline™ Drystrip pH 4-7 (GE Healthcare), by passive rehydration.

In the first dimension, proteins were isoelectric focused using an Ettan IPGphor (GE Healthcare), at 20 ºC, applying increasing voltage until 250 V for 2 h, raising this value to 3500 V over a time period of 4 h, and then keeping the applied voltage at a constant 3500 V for 8 h (for a total of about 35760 Vh). After the first dimension, strips were equilibrated using standard Bio-Rad reducing/alkylating buffers (30 min each step). For the reduction and alkylation step, 40 mg of DTT (GE Healthcare) and 57 mg of iodoacetamide (GE Healthcare) were added to reducing and alkylating buffers, respectively.

The strips were transferred on to 13 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 (Fluka), 50 mM Tris, 0.1 % (w/v) SDS, 1 mM EDTA). The electrophoresis proceeded at 180 V in a Criterion™ DodecaTM Cell (Bio-Rad) with a PowerPac™ Universal power supply (Bio-Rad). At the end of the electrophoretic run, gels were stained using EZBlue™ colloidal CBB solution (Sigma).

The obtained gels were scanned with a GS-800 calibrated densitometer (Bio-Rad) at a 42.3 µm/pixel resolution. PDQuest™ 2-D Analysis Software v8.0 (Bio-Rad) was used to

study protein differential expression between the different conditions by performing semi-automated spot detection, matching and protein volume estimation. The values obtained for the spot volumes were normalized by expressing protein abundances as a ratio to the total volume of valid spots in ppm (part per million).

6.2.4. Statistical analysis

Statistical analysis of the results obtained with PDQuest™ was performed using the R software environment[299]. The Shapiro-Wilk W-test and Levene's test were used to evaluate the possibility of using the usual parametric methods (i.e. Welch's t-test, ANOVA) for the assessment of statistically significant variations. Since the normality and homoscedasticity assumptions were not valid for our dataset, a non-parametric test (one-tailed Wilcoxon-Mann-Whitney U-test, $p < 0.05$) was chosen to detect significant variations in protein expression between the studied groups. In addition, exploratory multivariate analysis by Principal Component Analysis, as well as clustering of the gels according to correlation-based metrics, was performed as a complement to the Wilcoxon-Mann-Whitney univariate test.

6.2.5. Protein identification by MS

LC/MS-MS

Spots deemed relevant by statistical analysis were manually excised from preparative gels and protein identification by LC-MS/MS carried out at the Aberdeen Proteomics facilities (University of Aberdeen, UK).

The spots were reduced, alkylated (with DTT and iodoaceamide, respectively) and subjected to tryptic digestion using an autolysis-resistant modified trypsin. The resultant peptides were extracted with acetonitrile and formic acid, and separated by liquid chromatography (UltiMate 3000 LC System, Dionex Ltd). This LC system was coupled to an electrospray ionization (low-flow nebulizer – Bruker Daltonics Ltd) – ion trap (HCTultra PTM Discovery System – spherical ion trap) mass spectrometer.

The MS/MS CID data was acquired in data-dependent AutoMS(2), averaged from three spectra with a scan range of 100 to 2200 m/z. Up to 3 precursor ions were selected from the MS scan (300 to 1500 m/z) in each AutoMS(2) cycle. Precursors were actively excluded within a 1.0 min window, and singly-charged ions were also excluded. Peptide peaks were

detected and deconvoluted automatically using DataAnalysis software (Bruker Daltonics, Ltd.).

MALDI-TOF-TOF

Some of the excised proteins spots were washed first in 50 % acetonitrile and then in water. The spots were then dried and in-gel digested with trypsin (porcine sequencing grade modified trypsin, Promega, Southampton, UK), redissolved in 0.1 % TFA and finally desalted and concentrated on microcolumns with POROS R2 resin (Applied Biosystems, Foster City, CA). The microcolumns were packed in a GELoader tip (Eppendorf AG, Hamburg, Germany) as previously described [474]. 0.8 mL matrix solution (5 mg mL^{-1} α-hydroxycinnamic acid in 70 % v/v CH3CN (acetonitrile) and 0.1 % TFA) was used to elute the peptides from the microcolumns directly onto the MALDI target.

An Applied Biosystems 4800 Proteomics Analyser with TOF/TOF™ optics (Applied Biosystems, Darmstadt, Germany) in positive reflector mode was used for the acquisition of data. Identification of peptides was exclusively based upon MS/MS spectra. Collected MS spectra in the manual set up were therefore used to select peaks for MS/MS analysis. Depending on the individual sample, the number of laser shots and laser intensity was varied to obtain optimal MS/MS. When analyzing the spectra, peaks originating from human keratin and trypsin autolysis products were identified and eliminated using the software PeakErazor (http://www.gpmaw.com) [475]. All other peaks with intensities that made it possible to produce an acceptable MS/MS spectrum were used for MS/MS.

Database searching

The mass lists generated were used for protein identification in a «MS/MS Ion Search», through the MASCOT search engine (Matrix Science, http://www.matrixscience.com/), for the identification of the tryptic peptides by peptide fragment fingerprinting (PFF). The search was done on the NCBInr database (June 2010) limited to *Actinopterygii* (ray-finned fish), assuming the following parameters: tryptic digestion (one missed cleavage allowed), formation of double or triple charged peptides (in the case of LC/ESI-IonTrap-MS) or single charged peptides (in the case of MALDI-TOF-TOF), carbamidoethylation of cysteine residues, possible oxidation of methionine residues, a 0.5 Da MS/MS mass tolerance and a 1.5 Da MS mass tolerance (for LC/ESI-IonTrap-MS) or a 0.5 Da MS/MS mass tolerance and a 70 ppm MS mass tolerance (for MALDI-TOF-TOF).

Spots were considered identified when at least one tryptic peptide was unambiguously identified, taking a E-value threshold of 0.05 as indicative of low probability of peptide being erroneously identified.

6.3. Results

6.3.1. Growth and classical physiological measurements of stress response

At the end of the experiment, there were no significant differences in mean total length and weight among treatments (data not shown). No fish mortality occurred throughout the experiment.

Plasma cortisol levels increased significantly (Mann-Whitney U-test, p=0.001865) by about 7.5 fold (smoothed bootstrap estimate, n=1000, noise level=[σ]/10) in handled fish compared to the control group. The same trend was observed for lactate (Mann-Whitney U-test, p=0.02962, 1.5 fold increase) and glucose (Mann-Whitney U-test, p=0.3554, 1.1 fold increase), although high dispersions were observed for both metabolites in terms of individual response to handling stress[473].

6.3.2. Comparative 2-DE of liver proteome

Computer-assisted analysis of the gels resulting from the two-dimensional electrophoresis of *S. senegalensis* liver enabled the detection and quantification of 744 protein spots across all gels. Before looking at values of normalized quantity obtained for each spot individually, a preliminary multivariate approach was undertaken in order to assess the quality of the dataset.

As can be observed from the biplot for the first three PCA (Principal component analysis) components (see Supporting information – Figure A), the gels are separated according to treatment, with about 50 % of all variation explained along PC1. Agglomerative clustering (by the complete linkage method) and a low-dimensional embedding of the gels by metric multidimensional scaling (MDS) using a distance metric based on the non-parametric Kendall tau correlation coefficient confirms the information provided by the PCA projections,

showing that in general gels from the same treatment are more similar, according to the chosen criterion (Figure 6.1).

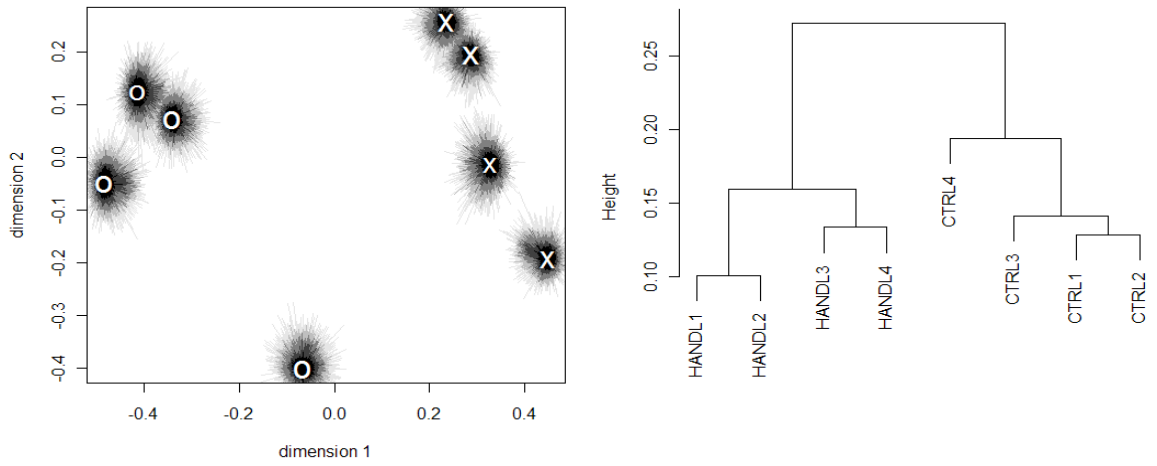

Figure 6.1 – Multidimensional scaling (MDS) plot showing the inter-gel distance calculated as $(1-\tau)/2$, where τ is the Kendall correlation coefficient between the two gels; stability assessment was performed by repeating MDS calculations, randomly truncating 1/3 of the dataset's variables and overlaying the deviation using the procrustes algorithm (O, control; X, handling), on the left. The same inter-gel distances calculated were used to construct a dendrogram (CTRL, control; HND, handling), on the right.

Univariate analysis of the normalized protein quantity using a one-tailed Wilcoxon-Mann-Whitney U-test ($p < 0.05$) revealed 287 spots displaying a statistically significant "handling effect", 142 of which are down-regulated with handling while 145 are up-regulated. Application of PCA to this reduced set of variables (thereby eliminating the influence of spots with inconsistent patterns of variation) shows a reduction in spurious variations, as PC1 then accounts for about 70 % of the total variance and a removal of the variables which are orthogonal to the vector that goes from the control to the handling centroid can be seen (see Supporting information – Figure B). The results obtained for agglomerative clustering of gels and multidimensional scaling confirm a higher degree of separation between the stressed and unstressed clusters (Figure 6.2).

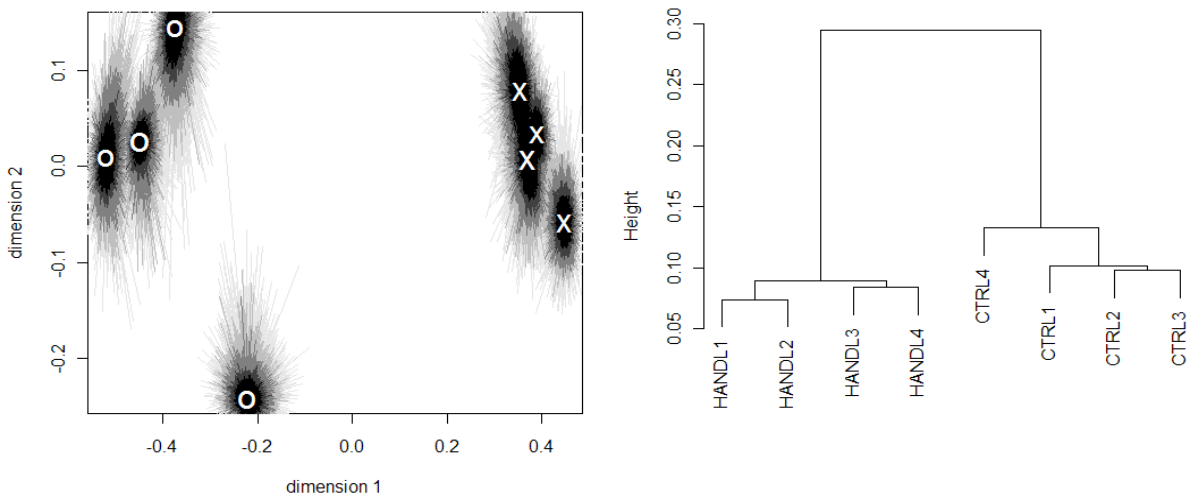

Figure 6.2 – Multidimensional scaling (MDS) plot showing the inter-gel distance calculated as (1-τ)/2, where τ is the Kendall correlation coefficient between the two gels, when reducing the analysis to the 287 spots deemed significant by univariate analysis; stability assessment was performed by repeating MDS calculations, randomly truncating 1/3 of the dataset's variables and overlaying the deviation using the procrustes algorithm (O, control; X, handling), on the left. The same inter-gel distances calculated were used to construct a dendrogram (CTRL, control; HND, handling), on the right.

Of all relevant spots which displayed a significant effect in response to handling, 33 could be excised, digested with trypsin and sequenced by MS/MS (Figure 6.3).

Table 6.1 shows the results of protein identification as well as the ratio between their abundance in stressed and control fish. The expression patterns of these proteins across experimental groups are also presented in (See Supporting information – Figure C-F). 17 proteins (α-actin; β-actin; 40S ribosomal protein SA; 40S ribosomal protein S12; 3-hydroxyanthranilate 3,4-dioxygenase; fatty acid-binding protein; phenylalanine hydroxylase; heat shock protein 70 HSPA5 family; heat shock protein 70 HSPA8 family; heat shock protein 90 HSP90B family; mitochondrial ATP synthase α-subunit; hypoxanthine phosphoribosyltransferase; serine hydroxymethyltransferase; betaine homocysteine methyltransferase; phosphoglycerate kinase; 3-oxo-5-β-steroid 4-dehydrogenase; cathepsin B) were up-regulated and 16 (α-tubulin; cytokeratin K18; cell division cycle 48; disulfide-isomerase A3 precursor; electron-transfer-flavoprotein α polypeptide; heat shock protein 70 HSPA9 family; mitochondrial agmatinase; aconitase 1; aldehyde dehydrogenase 4A1 family; aldehyde dehydrogenase 7A1 family; glyceraldehyde-3-phosphate dehydrogenase; enolase 3;

enolase 1; transferrin; selenium binding protein 1; methionine adenosyltransferase I α) down-regulated in stressed fish compared to the control fish.

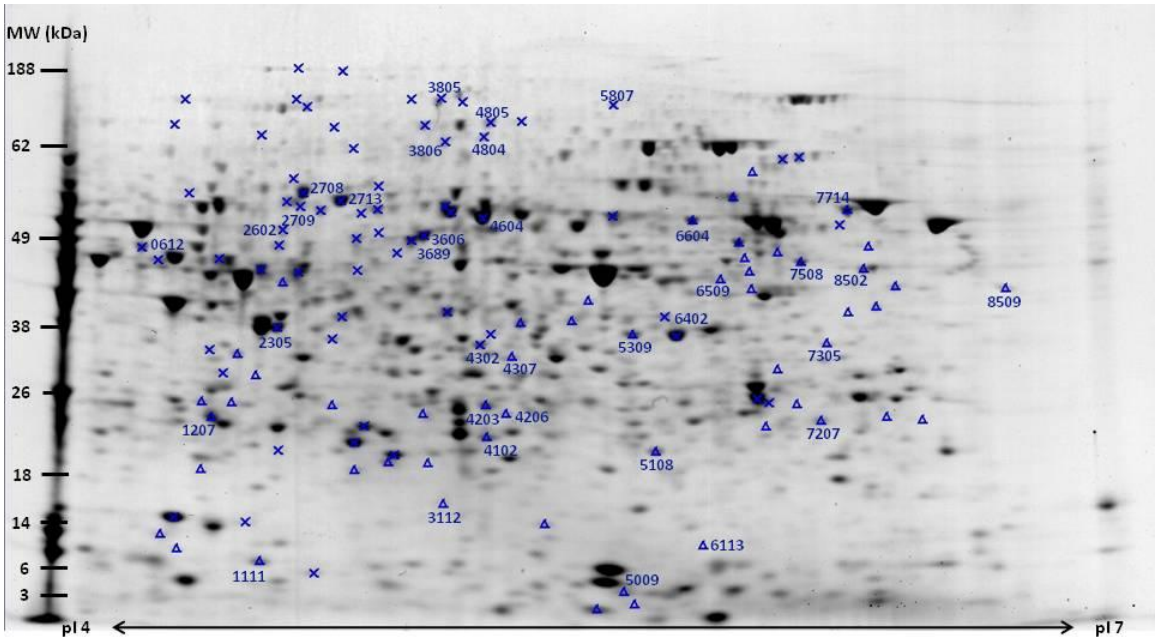

Figure 6.3 – Colloidal CBB-stained 2-DE gel (11 cm strips, linear pH 4-7 gradient – 12% Bis-Tris polyacrylamide gels) from *S. senegalensis* liver. Reference gel representing some of the relevant spots: Spots marked with a cross (×) represent down-regulated proteins in stressed sole (Mann-Whitney test, $p<0,05$). Spots marked with a triangle (Δ) represent up-regulated proteins in stressed sole (Mann-Whitney test, $p<0,05$). numbered spots show protein spot identified by mass spectrometry.

6.4. Discussion

During the course of this study, we identified several cellular functions that were affected as a result of chronic exposure of *Solea senegalensis* to handling stress (Table 6.1). We sought to interpret the observed changes in the hepatic proteome of chronically stressed sole, taking into account that these changes could be considered either long-term allostatic adaptations to repeated handling stress or indications of impaired liver health status. Figure 6.4 shows a diagram detailing a simplified model of how observed hepatic proteome changes can direct or indirectly result from the exposure to chronic stress.

Table 6.1 – Main identified proteins by LC MS/MS. The identification was made by peptide fragment fingerprinting (PFF) in the option MS/MS Ion Search from the bioinformatics application Mascot. The PFF was made in the non redundant NCBInr data base for the Actinopterygii taxonomic level (E-value < 0.05).

Spot no.	Protein (species)	GI (number)[a]	MW_{th}/pI_{th} [b](MW_{exp}/pI_{exp}[c])	Sequence coverage (%) [d]	# matched peptides (E-value<0.05) [e]	Best peptide match: Sequence, Charge State, E-value [f]	Combined Mowse Score [g]	Ratio expression STR/CTRL	Metabolic pathways
1111	40S ribosomal protein S12 (*Oreochromis niloticus*)	gi|3122783	14.7/6.30 (6.5/4.60)	20	1	K.LGEWVGLCK.I, 2+, 0.0093	49	2.19	
5807	cell division cycle 48 (*Larimichthys crocea*)	gi|213054513	90.0/5.16 (92.0/5.8)	38	18	R.QAAPCVLFFDELDSIAK.A, 2+, 7.5e-06	603	0.34	
7714	heat shock protein 90 HSPA90B (*Puralichthys olivaceus*)	gi|110226526	92.1/4.70 (53.0/6.5)	21	25	R.EEEAIQLDGLNAAQIK.D, 2+,3.4e-11	931	1.98	
3806	heat shock protein 70 HSPA9 (*Sparus aurata*)	gi|119692141	69.1/5.57 (62.1/5.25)	28	9	K.IIAVYDLGGGTFDISVLEIQK.G, 2+, 4.9e-11	497	0.43	Protein synthesis, folding and turnover
4804	heat shock protein 70 HSPA9 (*Sparus aurata*)	gi|119692141	69.1/5.57 (62.4/5.35)	14	5	K.VQQTVQDLFGR.A, 2+, 3.3e-06	211	0.73	
7508*	heat shock protein 70 HSPA8 (Salmo salar)	gi|7960186	71.8/ 5.28 (47.5/6.25)	6	2	K.SINPDEAVAYGAAVQAAILAGD K.S, 1+, 1.4e-08	134	3.00	
8502*	heat shock protein 70 HSPA5 (*Danio rerio*)	gi|39645428	72.1/5.04(47.0/6.40)	8	3	R.IEIESFFEGEDFSETLTR.A, 1+, 2.4e-12	335	2.56	
7207*	cathepsin B (*Lutjanus argentimaculatus*)	gi|226821413	37.16/5.69 (24.5/6.31)	10	2	K.NGPVEGAFTVYEDFVLYK.S, 1+,3.4e-13	259	2.43	
5009	heart-type fatty acid-binding protein (*Fundulus heteroclitus*)	gi|150072477	14.8/5.74 (6.00/5.75)	16	2	K.LGEEFDETTADDR.K, 2+, 4.5e-07	114	4.35	Carbohydrate metabolism and energy homeostasis
3606	enolase 1 alpha-like (*Oreochromis mossambicus*)	gi|93115123	37.8/5.61 (50.0/5.20)	43	21	K.VNQIGSVTESLQACK.M, 2+, 5.2e-09	894	0.70	
2602*	enolase 3 protein (*Danio rerio*)	gi|47551317	47.84/6.25 (51.0/4.75)	4	1	R.AAVPSGASTGVHEALELR.D, 1+, 9.5e-06	73	0.03	
5108*	phosphoglycerate kinase (*Oryzias latipes*)	gi|46849381	41.65/6.04 (20.0/5.84)	4	1	K.QIVWNGPVGVFEWENFAK.G, 1+, 9.5e-13	141	2.57	
6402 *	glyceraldehyde phosphate dehydrogenase (*Oncorhynchus mykiss*)	gi|13936898	36.42 /7.23 (39.0/5.85)	4	1	K.LVTWYDNEFGYSNR.V,1+, 0.014	40	0.40	
4102	mitochondrial ATP synthase alpha-subunit (*Cyprinus carpio*)	gi|47207317	60.0/9.23 (20.0/5.35)	27	23	K.GMSLNLEPDNVGVVVFGNDKL + Oxidation (M), 2+, 3e-10	1178	1.95	
4302	electron-transfer-flavoprotein, alpha polypeptide (*Danio rerio*)	gi|38707985	35.3/6.90 (36.5/5.30)	38	13	K.DPEAPIFQVADYGLVADLFK.V,2 +, 2.4e-14	517	0.79	
3805 *	aconitase 1 (*Danio rerio*)	gi|77993336	99.45/6.45 (93.0/5.25)	4	2	K.FVEFFGPGVAQLSIADR.A, 1+, 1.7e-06	110	0.18	
612	methionine adenosyltransferase I, alpha (*Danio rerio*)	gi|47223302	43.0/6.60 (48.5/4.3)	17	5	R.FVIGGPQGDAGVTGR.K, 2+, 7e-09	256	0.17	Amino acid metabolism, urea cycle and methylation/folate pathways
2305	agmatinase, mitochondrial precursor (*Salmo salar*)	gi|209148871	40.6/6.60 (38.0/4.75)	16	3	R.CVEEGLLDCK.R , 2+, 1.6e-05	97	0.54	
4307*	phenylalanine hydroxylase (*Takifugu rubripes*)	gi|47218328	52.5/5.45 (35.0/5.35)	18	8	K.SEVISCIFSLK.E, 2+, 1.9e-05	219	2.17	
4203*	serine hydroxymethyltransferase (*Salmo salar*)	gi|47220449	55.44/ 9.00 (25.5/5.32)	3	1	R.YYGGAEIVDQIELLCQK.R, 1+, 0.012	40	1.42	
4206*	betaine homocysteine methyltransferase (*Sparus aurata*)	FF291611	27.1/4.99 (25.0/5.35)	8	2	R.LNADEVVIGDGGFVFALEKR.G, 1+, 1.4e-11	248	3.25	
5309	3-hydroxyanthranilate 3,4-dioxygenase (*Osmerus mordax*)	gi|47221724	33.3/5.79 (37.5/5.83)	23	4	K.DYHIEEGEELFYQLK.G, 2+, 2.4e-06	153	1.24	

Table 6.1 (continued) – Main identified proteins by LC MS/MS. The identification was made by peptide fragment fingerprinting (PFF) in the option MS/MS Ion Search from the bioinformatics application Mascot. The PFF was made in the non redundant NCBInr data base for the Actinopterygii taxonomic level.

6501	beta-actin (*Pungitius pungitius*)	gi|66731680	40.5/5.29 (47.0/5.85)	54	13	K.SYELPDGQVITIGNER.F, 2+, 1.1e-08	472	2.56		
7305*	40S ribosomal protein SA (*Osmerus mordax*)	gi|225708482	34.44/4.92 (37.0/6.3)	19	3	R.ADHQPLTEASYVNIPTIALCNTD SPLR.Y, 1+,1.6e-12	152	2.80		
3603*	K18, simple type I keratin (cytokeratin Type I) (*Oncorhynchus mykiss*)	gi|47210018	36.36/7.03 (49.0/5.19)	5	1	R.MNLESEIEALKEELIHLK.K + Oxidation (M), 4.5e-08	113	0.70	Cytoskeletal system	
6113*	alpha-cardiac actin (*Oncorhynchus mykiss*)	gi|20799652	16.64 /5.25 (9.52/5.94)	7	1	K.IWHHTFYNELR.V, 1+, 0.0088	43	10.12		
6604*	tubulin, alpha 8 like 3 (*Danio rerio*)	gi|54261795	50.72/4.93 (53.1/5.9)	14	4	R.FDGALNVDLTEFQTNLVPYPRI, 1+, 3.2e-11	362	1.47		
2708*	aldehyde dehydrogenase 4A1 precursor (*Danio rerio*)	gi|41055855	61.95/6.85 (55.5/4.84)	2	1	R.ASGTNDKPGGPHYVLR.W, 1+, 6.5e-05	65	0.04	Aldehyde detoxification	
2709*	aldehyde dehydrogenase family 7 member A1 homolog (*Salmo salar*)	gi|213514574	59.45/7.10 (54.0/4.86	9	3	K.GSDCGIVNVNIPTSGAEIGGAFG GEK.H, 1+, 2.1e-10	232	0.54		
3112*	3-oxo-5-beta-steroid 4-dehydrogenase (*Salmo salar*)	gi|226442824	37.72/6.38 (15.3/5.20)	11	2	R.HFDGALVYFNEHEVGQAIR.D, 1+, 3e-08	144	2.28	Purine salvage	
4604*	selenium binding protein 1 (*Danio rerio*)	gi|41055742	51.52/5.83 (53.1/5.34)	5	2	R.FLYFSNWLHGDIR.Q, 1+, 5.9e-05	115	0.03	Iron and selenium homeostasis	
4805*	transferrin (*Solea senegalensis*)	gi|237784636	18.64/5.37 (70.0/5.36)	16	2	R.AFFGASCVPGAPR.D, 1+, 2.6e-05	126	0.37		
1207	hypoxanthine phosphoribosyltransferase (*Solea senegalensis*)	gi|169643691	25.3/5.98 (25.5/4.5)	37	4	K.FVVGYALDYNEYFR.D,2+, 4e-07	225	1.88	Steroid hormone metabolism	

a)GI number - NCBI acess number.

b) Mw_{th}/pI_{th} - Theoretical molecular weight and isoelectric point. Mw_{th} is given in KDa.

c) Mw_{exp}/pI_{exp} - Experimental molecular weight and isoelectric point. Mw_{exp} is given in KDa.

d) Sequence coverage (%) - percentage of the entire protein covered by matched peptides.

e) # matched peptides (E-value < 0.05) - number of peptides matched to entry with significative E-value (when E-value < 0.05).

f) Best peptide match with the lower E-value: sequence, charge state and E-value.

g) Combined Mowse Score – a non-probabilistic protein score, derived from the ions score.

* spots sequenced by MALDI TOF-TOF; all others were sequenced by LC ESI IonTrap

6.4.1. The primary stress response – hypothalamic-pituitary-interrenal (HPI) axis and cortisol

A key observation during this experiment was the measurement of higher levels of cortisol in the plasma of fish from the STR group, confirming that this group did experience a higher degree of stress compared to the CTRL group. At the proteome level, an increase in the abundance of 3-oxo-5-β-steroid 4-dehydrogenase (aldo-keto reductase 1D1, AKR1D1) was also observed in the liver of chronically stressed fish. AKR1D1 has an important role both in bile acid synthesis and in the metabolism of C21 and C19 steroid hormones (aldosterone, testosterone, progestrone, cortisol, cortisone), catalyzing the first step in cortisol and cortisone catabolism (their conversion to 11β, 17α, 21-trihydroxy-5β-pregnane-3,20-dione and 17α,21-dihydroxy-5β-pregnane-3,11,20-trione, respectively)[476]. It is therefore plausible that this effect is a direct response to the higher levels of cortisol observed in chronically stressed fish.

6.4.2. Hypoxia-normoxia injuries and the cellular response to redox stress

Hypoxia-normoxia cycles and high rates of fatty acid β-oxidation induce the formation of ROS (reactive oxygen species) and lipid peroxides.

A decrease in protein disulfide-isomerase (PDI) abundance was observed in the livers of chronically stressed fish. It has both a disulfide-isomerase activity which helps the correct formation of disulfide bridges between cysteine residues and a chaperone activity, preventing proteins from misfolding in the endoplasmic reticulum[477]. This reduction might be interpreted as a sign of impaired liver function in response to chronically stressed fish.

We also observed a decrease in cell division cycle 48 (CDC48/p97/VCP) abundance stressed fish livers. This protein is a cytosolic chaperone-like AAA ATPase which has several known biological functions. Although most of its cellular functions are related to its segregase activity (which enables disassembly of oligomeric protein complexes and extraction of protein embedded in lipid membranes), its specific function at any given time is defined by its interaction with a multitude of adaptor proteins (so far, more than fourteen distinct adaptors are known to interact with and modulate CDC48 activity)[478]. Its main role appears to be related to the endoplasmic reticulum-associated degradation (ERAD) pathway, which is activated as part of the UPR, when the accumulation of misfolded proteins occurs in the ER. Specifically, the CDC48-UFd1-Np14 complex has been implicated in the translocation of ubiquitinated proteins from the ER to the cytosol for proteasome-mediated degradation[479].

Due to CDC48's importance in ERAD and its association with Hsp70 during the UPR, the observed decrease in CDC48 abundance in stressed fish may indicate an impaired capacity in chronically stressed fish for an appropriate response to ER stress[480]. A recent experiment involving exposure of Senegalese sole to model toxicants also shows a decrease in CDC48 protein abundance in liver cells is response to such treatment[481].

The heat shock protein (HSP) family is composed of proteins which are involved in protein folding, being usually upregulated in response to stressful stimuli. On the other hand, some HSP isoforms are constitutively expressed (these are usually named "heat shock cognate proteins" or HSC), suggesting an essential role in ER health maintenance even in non-stressed states[482].

During our experiment, we observed statistically significant variations in the abundance of 5 HSP isoforms in response to chronic handling stress: three of the proteins spots (identified as Hsp70 of the HSPA8 and HSPA5 families and a Hsp90 of the HSP90B family) had higher intensity and two of the protein spots (both Hsp70 of the HSPA9 family) had lower intensity in the STR group compared to control.

Hsp90 is a family of roughly 90 KDa multifunctional proteins with a chaperone function, assisting in the prevention of protein misfolding and aggregation[483-484].

In our case, the identified protein was of the HSP90B family (also known as Grp94), an ER isoform of Hsp90, suggesting the increased accumulation observed should not be attributed to Hsp90's relation to the GR, but to its function as a protein chaperone in the ER. In general, several studies have established the induction of this Hsp90 isoform in response to numerous types of stress (including ischemia-reperfusion types of injury)[485-487], as well as a direct interaction between Hsp90 and proteins of the Hsp70 family[488].

Hsp70 are also a multifunctional family of chaperone proteins with molecular weight around 70 KDa, which contribute to the prevention of protein misfolding[489-490]. Hsp70 also interacts with CHIP (an E3 ubiquitin ligase), delivering to it misfolded proteins for ubiquitination and proteolysis[491].

Unfortunately, there is still much confusion of nomenclature regarding HSPs (both in literature as in annotated bioinformatic databases), making it difficult to correctly interpret observations described by others regarding changes in the expression/accumulation of proteins of the HSP family in response to different types of stress. Fortunately, the information obtained by mass spectrometry was enough to reliably identify each HSP as belonging to one specific family (see Supporting information). For consistency, we use the *Homo sapiens/Danio rerio* gene names, which have been well characterized[492].

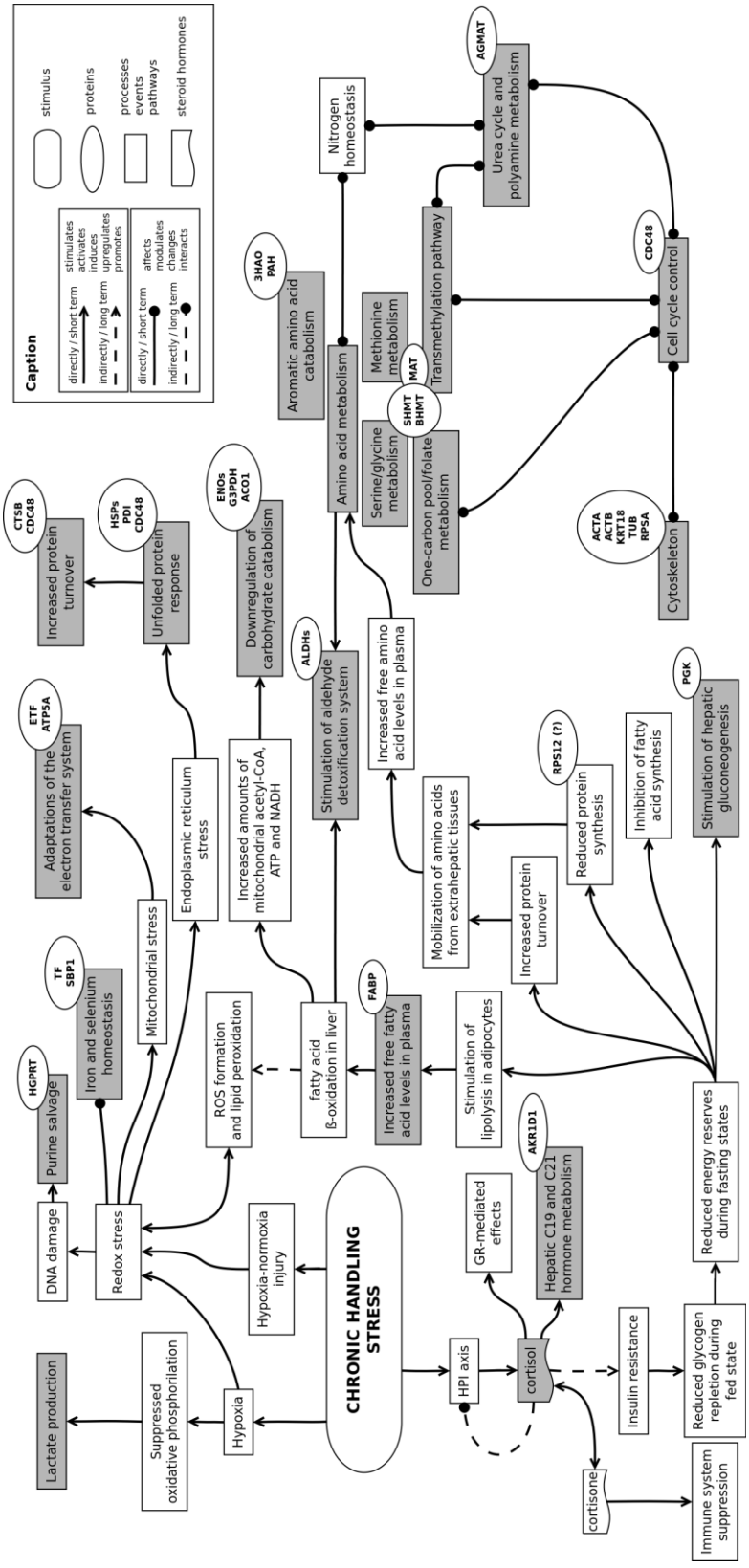

Figure 6.4 – Diagram detailing a simplified model of how observed hepatic proteome changes can result direct or indirectly from the exposure to chronic stress. Observed events and affected pathways are coloured in grey.

One of the upregulated HSP (of the HSPA5 family) is an ER chaperone known to be upregulated by both ER stress[493-494] and starvation[495]. Proteins of the HSPA5 family (often named Grp78 or glucose regulated protein 78), along with PDI, are known to be required for folding of immunoglobulin chains[495]. The observed increase should therefore be related to its function as an ER protein chaperone and as regulator of the UPR[496], constituting a protective adaptation.

The other upregulated protein of the Hsp70 family belongs to the HSPA8 family. Proteins of this family are generally known as being constitutive chaperones, meaning they are usually undisturbed or even downregulated by exposure to stress[437-438, 444, 497]. On the other hand, experiments with turtles, rats and gerbils showed that heat shock cognate expression can be induced by heat shock stress and hypoxia[498-499], suggesting that the often observed reduction in HSC expression after exposure to heat shock may occur due to reduced translation rate (competition with transcripts involved in the protein response for translation) combined with increased rates of protein turnover in stressful situations. This elicits further investigation, to explore the possibility that this specific protein is stress-inducible in Senegalese sole. Regardless, the increase in its abundance observed in stressed fish should constitute part of the unfolded protein response.

Finally, the two downregulated HSP protein spots correspond to proteins of the HSPA9 family. This is a family of heat-uninducible constitutively expressed mitochondrial HSPs (being, therefore, a heat shock cognate) involved in protein transport into the mitochondrial matrix[500], which possibly explains why their abundance was lowered in stressed fish. Additionally, it may also be a sign of increased mitochondrial stress, since this is a mitochondrial-specific protein.

During the course of our work, we also observed an increased in cathepsin B abundance in STR compared to CTRL. Cathepsin B is a lysossomal cysteine protease involved in hepatocyte cell death caused by ischemia-reperfusion injury[501-502]. In our specific case, the increase in cathepsin B can be interpreted as a general higher level of apoptosis and/or hepatic tissue remodelling in stressed fish, a sign of increased liver injury due to oxidative stress[501-506].

Selenium and iron are biologically important metals that in their free form can act as pro-oxidants[507-508]. Their redox properties are the basis of their biological functions. In general, selenoproteins seem to be related to mechanisms of protection against redox stress, suggesting that the reduction in SBP1 abundance in stressed fish may reflect lower hepatic levels of anti-oxidative enzymes[509-510].

Transferrin is mainly synthesized in the liver. Its main function is to transport iron absorbed in the intestine to other tissues[511-513]. Additionally, due to its ability to chelate free iron, it has an important role as extracellular antioxidant[514]. The observed decrease in hepatic transferrin abundance can be either an adaptation to reduce enteric iron absorption (to prevent further aggravating redox stress via Fenton reactions) or just a general sign of reduced hepatic capacity for iron processing.

6.4.3. Interaction between chronic stress and fasting on the regulation of energy homeostasis

Given that the fish were fasted for 24 hours pre-slaughter, some of the observed changes in protein abundance should be viewed as a possible interaction between the hormonal actions of cortisol and glucagon[515]. During fasting, low plasma glucose levels induce glucagon synthesis. This peptidic hormone stimulates glycogenolysis, enabling mobilization of energy stored as glycogen. As glycogen reserves become depleted (usually a few hours after feeding), lipolysis is induced in adipose tissues, increasing plasma levels of glycerol and non-esterified fatty acids (NEFA). The observed increase in Fatty Acid Binding Protein (FABP) abundance in stressed fish could reflect a higher degree of lipid mobilization, since this protein is required for their uptake, transport and metabolism, preventing oxidative damage from high levels of free NEFA[497]. This is consistent with the known effects of cortisol in the stimulation of lipolysis in adipose tissue and the inhibition of peripheral glucose uptake during fed states[516], which would lead to reduced glycogen stores and impaired resistance to pre-slaughter fasting without resorting to alternative energy sources.

In the present study, the only protein of the glycolysis/gluconeogenesis pathway which displayed increased abundance in stressed fish was phosphoglycerate kinase (PGK). Several studies have already shown, in hepatocytes and other cellular types, an increase in PGK expression in response to hypoxia-type stressors[517-519]. On the other hand, every other protein identified in this pathway during this study (enolase 1, enolase 3 and glyceraldehyde-3-phosphate dehydrogenase) displayed lower abundance in the liver of stressed fish, suggesting a metabolic shift from glycolysis/gluconeogenesis to β-oxidation and ketogenesis. Additionally, a sharp reduction in the abundance of aconitase 1, which catalyses the citrate-isocitrate equilibrium in the Krebs cycle, was also apparent in stressed fish, further supporting a flux reduction through these energetic pathways in favor of β-oxidation and ketogenesis.

During this study, we saw a decrease in the abundance of electron transfer flavoprotein (ETF) in the liver of chronically stressed sole.

During fed states, most of the electrons entering the mitochondrial electron transport system (ETS) Q-junction do so through complex I (oxidation of mitochondrial NADH) or complex II (oxidation of succinate to fumarate); as metabolism shifts away from carbohydrate catabolism towards lipid catabolism, ETF dehydrogenase and phosphoglycerol dehydrogenase start to gain more relevance in feeding the Q-junction with electrons. Therefore, the observed reduction in ETF abundance indicates a lower capacity for lipid catabolism in chronically stressed fish, since the availability of oxidized ETF is a limiting factor in the rate of fatty acid β-oxidation[520]. This is probably an adaptative response to higher levels of oxidative stress, in an attempt to reduce the rate of electron transfer into the ETS and maintain a lower membrane potential, helping to reduce ROS formation and lipid peroxidation[521].

We also observed a higher accumulation of an ATP synthase (complex V) subunit. In mitochondria, this proton pump generates ATP from ADP and organic phosphate using the proton-motive force, lowering the membrane potential. Therefore, it seems this increase in ATP synthase may constitute another adaptation towards the maintenance of a lower charge gradient across the mitochodrial membrane, in an effort to prevent ROS formation due to hypoxia-normoxia cycles and to high rates of fatty acid β-oxidation[521]. Another potentially positive effect of enrichment in ATP synthase in the mitochondrial membrane is an increase in ATP production by competing with other membrane transporters for the transport of charge across the membrane. This is consistent with previous observations in the response of gilthead seabream to chronic stress, where a sharp decrease in the abundance of the mitochrondrial voltage-dependent anion channel was observed[497].

Elevated plasma levels of aromatic amino acids (specifically, phenylalanine and tyrosine, but also tryptophan) are usually indicative of compromised hepatic function[522-524]. The observed increase in phenylalanine hydroxylase and 3-hydroxyanthranilate-3,4-dioxygenase expression in fish subjected to chronic handling stress possibly constitute a long-term adaptation to increased steady-state levels of phenylalanine and tryptophan.

Both amino acid catabolism and fatty acid β-oxidation generate aldehydes that have to be processed by enzymes of the ALDH family. Reduction in the abundance of some ALDH enzymes in the liver of fish subjected to chronic handling stress may suggest that their capacity to process aldehydes was partially compromised and, consequently, the effects of oxidative stress could be aggravated[525-527].

6.4.4. Urea cycle, polyamine synthesis, methionine metabolism and folate carbon pool

A reduction in the abundance of agmatinase and methionine adenosyltransferase (MAT) was observed in response to chronic handling stress. Both these proteins have important functions in the interface between the urea cycle, methionine metabolism and polyamine synthesis. Since agmatinase abundance is reduced in stressed fish, it is expected that putrescine production via this route is reduced and that both higher steady-state concentrations of agmatine can be reached and/or the response to transient increases in agmatine production is delayed. Agmatine is known to reduce ornithine decarboxylase (ODC) activity through the induction of antizyme (polypeptidic ODC inhibitor)[528], which suggests a generally lower production of putrescine and other polyamines (spermidine and spermine) should occur in the liver of chronically stressed sole.

A marked reduction in the abundance of MAT (s-adenosyl-methionine-producing enzyme) in the liver of stressed fish should also contribute to the lower synthesis rates of polyamines from putrescine, since this is a process dependent on the presence of decarboxylated s-adenosyl-methionine[529]. Besides an effect on polyamine synthesis, such a sharp change in MAT abundance should have multiple effects in the liver tissue, as S-adenosyl-methionine (SAM) is the most important co-factor in methylation reactions, having an essential role in hepatic metabolism[530].

Another relevant fact related to the methionine-homocysteine methylation cycle was an observed increase in betaine-homocysteine S-methyltransferase (BHMT), an enzyme directly involved in the resynthesis of methionine from homocysteine (alternatively to the folate-dependent methionine synthase route).

It is known that dihydrocortisone increases BHMT expression, which is particularly interesting due to the fact that this is an immediate metabolite of cortisol catabolism that is generated by AKR1D1[531-532], one of the enzymes whose expression was increased in the liver of chronically stressed fish during our study. It is, therefore, unsurprising that elevated levels of BHMT were observed in chronically stressed fish. An interesting side-effect of increased BHMT activity would be an increased availability of dimethylglicine and sarcosine as carbon sources for the mitochondrial folate metabolism.

Regarding folate metabolism, we observed an increase in the abundance of mitochondrial serine hydroxymethyltransferase (SHMT), which catalyzes the reversible conversion of serine and THF (tetrahydrofolate) to glycine and 5,10-methylene-THF. Due to

the fact that the metabolism of methionine, polyamines, choline, betaine, dimethylglycine, sarcosine, glycine and serine are so tightly coupled to the folate one-carbon pool and the urea cycle, it becomes challenging to exactly predict the effect of an increase in mitochondrial SHMT. There is extensive evidence that folate metabolism is quite adaptable to the metabolic requirements of cells[533] and in silico modelling of the hepatic folate metabolism seems to suggest that this tight coupling might contribute to a high robustness towards sharp variations of the concentrations of these metabolites in response to the dietary intake of proteins[534].

The observed upregulation of mitochondrial SHMT in the liver of stressed fish is not surprising given that (at least in humans) the promotor region of its gene also contains glucocorticoid-responsive elements (GRE) and thyroid hormone-responsive elements (TRE), as well as consensus sequence motifs for AP-2, PuF-1, PEA3, Sp-1, UBP1, octB2, Adh1, FSE2, HC3, and IE1[535]. It therefore seems likely that increases in BHMT and mitochondrial SHMT co-occur.

Concluding, several changes were noted at the level of polyamine, folate and methionine metabolism that suggest a generally lower level of cellular proliferation, lower level of glutathione synthesis, higher degree of utilization of choline/betaine/dimethylglycine/sarcosine as carbon sources for mitochondrial folate metabolism and possibly a higher production of serine in mitochondria to fuel cytoplasmic folate metabolism and gluconeogenesis. Taken together, these should be signs of reduced resistance to hepatic stress. A diagram showing the relationships between these pathways and the role of the mentioned proteins in these pathways can be seen in the Supporting information (Figure G).

6.4.5. Cytoskeleton and cell cycle control

The cellular stress response is usually accompanied by reorganization of the cytoskeleton[536-538]. During our experiment, changes in the abundance of several cytoskeletal proteins were noted (α and β-actin, α-tubulin, cytokeratin K18). Although these changes can be difficult to interpret due to the different range of functions displayed by cytoskeletal proteins, it is plausible that these may result from cell cycle-related processes.

Cytokeratin K18 (KRT18) is a component of the intermediate filaments. Protein interaction studies show KRT18 interacts with Mrj, a Hsp40 homolog. Additionally, KRT18 is known to interact with TRADD (Receptor-Associated Death Domain), implying a modulatory function related to apoptosis[539]. Recently, a proteomic experiment on rainbow trout *(Oncorhynchus mykiss)* fibroblasts also showed a decrease in KRT18 abundance in

response to sodium azide-induced anoxia followed by reoxygenation[466]. Although these changes can be difficult to interpret due to the different range of functions displayed by cytoskeletal proteins, it is plausible that changes in the cytoskeleton may result from cell cycle-related processes. In fact, one of the above mentioned proteins (p97/VCP/CDC48) is known to be involved in cytoskeletal reorganization during mitosis[540].

Ribosomal protein SA (also known as 37kDa laminin receptor precursor and p40 protein) is a bifunctional protein, being both an essential component of ribosomes and a receptor for laminin and other ligands[541-542]. This protein was shown to be upregulated by testicular heat shock[543], UV radiation exposure in melanocytes[544] and heat stress in Jurkat cells[545]. It has also been associated with the regulation of cytoskeletal dynamics[546]. Furthermore, it has been shown (in gastric cancer cells) that the promoter of the gene that codes for this protein contains hypoxia-responsive elements[547], which implies that it is activated in the presence of HIF-1 (Hypoxia-Inducible Factor). This is consistent with the type of stressor used during the experiment (hypoxia/handling).

Ribosomal protein S12 is also one of the ribosome components, being responsible for selection efficiency of tRNA molecules, during translation[548]. This protein has been shown to be upregulated by osmotic and cold stress in cyanobacteria[549] and by UV exposure in keratinocytes[550].

6.5. Final considerations

During this experiment, we were able to elicit a chronic stress response in Senegalese sole using a handling/hypoxia-type stressor. This was confirmed by generally higher plasma levels of cortisol, lactate and glucose in STR fish and by consistent variations in protein abundance in about 1/3 of all detected proteins, even though the chosen stressor was applied sparingly (weekly) and for a short duration (4 weeks total duration). The proteins which changed in response to stress were involved in several distinct molecular pathways, most of which seem to be tightly related with energy homeostasis, cell cycle control and the cellular response to redox stress.

Several proteins identified in this study seem to have a good potential to be validated as stress biomarkers for Senegalese sole. Nevertheless, due to the wide range of observed responses to hypoxia and handling-type stressors, it is important to contextualize the results,

since it is clear that the choice of biological models (in vivo vs. in vitro, chosen species), type and duration of applied stress have a significant impact on the type and magnitude of observed responses. For this reason, we compiled a list of comparative proteomic/transcriptomic studies similar to ours (Supporting information, Table A) in an attempt to realistically characterize the position of each identified protein as a candidate for biomarker validation. This type of meta-analysis is often difficult due to nomenclature and data presentation inconsistencies between studies and the fact that many of the observed changes are usually specific to a certain isoform or post-translationally modified version of a protein.

Some of the identified proteins (like HSPA90B, HSPA5, AKR1D1, PGK, ALDH7A1, PAH and 3HAO) immediately seem to be the most relevant targets for further validation, as their relation to cellular stress in general has already been somewhat explored and comparative studies are generally in agreement on the effect of stress in the expression of these proteins.

In addition to these, proteins such as HSPA9, HSPA8, PDI, BHMT, SBP1 and FABP seem to be strongly affected by stress-like events, although their relations to chronic stress and possible roles as biomarkers are still not clear. Despite their abundance seeming subjected to the action of several unknown processes or stimuli, which impairs the use of these proteins per se as sure signs of stress, they could still be useful as co-biomarkers, specifically if they are found to be predictive of specific types of stress. FABP is especially interesting, since we have also observed in a prior study on gilthead seabream an increase in the hepatic abundance of this protein in response to repeated exposure to an identical handling-type stressor[497] and it seems to be upregulated specifically in handling/confinement situations (Figure E). Besides these, we were able to identify some proteins which have not yet been directly associated to induced stress in any reported comparative studies, like ATP5A1, CDC48, cathepsin B, ACO1, SHMT, ALDH4A1 and RP SA, that could also be useful as possible co-biomarkers if their relation to chronic stress is confirmed.

Finally, the other identified proteins generally cannot be seen as good candidates for validation as safe biomarkers of chronic stress due to the fact that they are either known to be highly responsive to a wide range of cellular stimuli, making them unreliable for accurately assessing the presence of chronic stress (which is the case for enolases, GAPDH, actins, tubulins, transferrin), or the result of comparative stress studies for these proteins has so far been inconclusive and ambiguous (RP S12, ETF, MAT1, AGMAT1, cytokeratin K18 and HGPRT).

In conclusion, it seems gel-based comparative proteomics is a useful tool for screening potential stress biomarkers, as long as the obtained results are further validated using expression analysis (RT-PCR, cDNA microarrays), immunological assays (immunohistology, immunoblotting) and metabolite concentration data. Extrapolation of the present results from Senegalese sole to other fish/animal species will also require a broader validation in several biological models.

Supporting information available

This information is available in the CD-ROM version of this dissertation and online, at "http://dx.doi.org/10.1007/s10126-012-9437-4".

Chapter 7.

Integrated proteomic and metabolic profiling of gilthead seabream liver to track interactions between dietary factors and seasonal temperature variations.

Abstract

Gilthead seabream is sometimes affected by a metabolic syndrome, known as the "winter disease", which has a significant economic impact. It is caused, among other factors, by the thermal variations that occur during colder months and there are signs that improved nutritional status can mitigate the effects of this thermal stress. For this reason, a trial was undertaken where the effect of a fortified diet on the impact of thermal stress was assessed through metabolomic and proteomic analysis of gilthead seabream hepatic tissue.

For this trial, four groups of 25 adult gilthead seabream were reared for 8 months, being fed either with a control diet (CTRL, commercial formulation) or with a diet dubbed "Winter Feed" (WF, fortified formulation). Fish were sampled at two time-points (at the end of winter and at the beginning of summer), with liver tissue being taken for FTIR spectroscopy and DIGE analysis.

Results have shown that seasonal temperature variations constitute a metabolic challenge for gilthead seabream, with hepatic carbohydrate stores being consumed over the course of the inter-sampling period. Regarding the WF diet, results point towards a positive effect in terms of performance and improved nutritional status. This diet seems to have a mitigating effect regarding the seasonal challenge, not only in terms of carbohydrate depletion, but also in terms of the observed accumulation of lipids in the later sampling. This suggests this diet is indeed a good candidate for a "golden standard" diet against which to compare alternate (possibly more cost-effective) formulations.

7.1. Introduction

Gilthead seabream (*Sparus aurata*), a species of high commercial value reared in the Mediterranean coast, is sometimes affected by the "winter disease" (or "winter syndrome"). This syndrome often induces chronic mortality during the low temperature months, followed by acute mortality when the temperature progressively rises, with significant economical impact. It is characterized by both behavioural (e.g. erratic swimming, voluntary fasting, hyposensitivity to stimuli) and physiological changes (e.g. impaired growth, pale and fatty liver, tissue necrosis, infections) that can ultimately lead to systemic dysfunction and death[551-558].

In etiological terms, although the occurrence of low environmental temperatures (i.e. thermal stress) seems to be the main common underlying factor, other factors also seem relevant to the onset of this "winter disease", namely the occurrence of metabolic distress, nutritional imbalances and/or deficits, immunosuppression and the presence of opportunistic pathogens[558]. It is important to underline the multifactorial nature of this syndrome, as it seems to display higher incidence on fish which are particularly small and susceptible to stress. Nevertheless, most of these factors can be seen as a consequence of gilthead seabream's lack of adaptation (or misadaptation) to cold temperatures while in captivity. While seabream is often subjected to environmental temperatures as low as 11 ºC, in the wild, it adapts by swimming to deeper (and warmer) waters. In captivity, this behavioural adaptation is not possible, and, generally, gilthead seabream refuse to eat when water temperatures reach below 13 ºC, increasingly displaying slower metabolic rates as temperatures decrease[559-562]. It is therefore thought that it is the synergistic effect of thermal stress, nutritional deficit and metabolic depression that induce the secondary effects (e.g. disruption of ionic balance, altered hepatic composition and metabolism, changes in blood metabolites, immunosuppression) that ultimately contribute towards a higher susceptibility to other sources of stress, opportunistic infections, metabolic failure and, eventually, death.

Although the exposure of reared gilthead seabream to low temperatures might be unavoidable during cold months, there are indications that the administration of specifically-formulated diets prior, during and after low temperature periods might mitigate the ultimate impact of thermal challenge on fish nutritional status and health, helping to prevent the occurrence of full-blown "winter disease"[563-564]. Within this context, a special fortified diet

(dubbed “Winter Feed” or WF) was designed to serve as reference for a high-quality feed, appropriate to maintain improved nutritional and metabolic status in gilthead seabream during the cold months. Although hardly economically feasible, this diet was formulated to constitute a “golden standard” (against which other possible diets can be compared), containing a higher proportion of marine-derived ingredients and supplemented with phagostimulants, marine phospholipids, antioxidant vitamins, taurine and soy lecithin, compared to a quite challenging diet with low levels of fishmeal and a concomitant partial replacement of fish oil by rapeseed oil (dubbed “CTRL” diet).

The purpose of this study was therefore to validate this WF diet as a “golden standard” for the mitigation of seasonal challenges. This was done taking into account not only zootechnical performance measures, but also proteome and metabolome profiling technologies. Furthermore, it was also important for us to assess the usefulness of these screening/profiling technologies in the assessment of fish nutritional and metabolic state, providing additional tools for directed, knowledge-based dietary formulations.

For this purpose, we used difference gel electrophoresis (DIGE) for proteomic analysis and solid-state transmissive Fourier transform infrared (FT-IR) spectroscopy for metabolomic profiling. DIGE is based on multiplex two-dimensional gel electrophoresis (2DE), enabling separation and quantification of hundreds to thousands of proteins in a complex mixture[565]. FT-IR spectroscopy is a method of metabolic profiling based on the differential absorption of IR radiation by chemical functional groups that, although does not possess a particularly high resolving power, is relatively high-throughput and very inexpensive[178]. These two techniques provide complementary and untargeted information that is essential to understand how gilthead seabream is affected by seasonal thermal stress and how dietary factors interact with that effect.

7.2. Material and Methods

7.2.1. Experimental diets

A control feed (CTRL) was formulated with low-fishmeal levels (15%), a significant amount of plant-protein sources and a blend of fish and rapeseed oils (Table 7.1). This diet was supplemented with an inorganic phosphorus source (dicalcium phosphate) and a

crystalline essential amino acid (L-lysine) to guarantee that known nutritional requirements of the species were covered. The CTRL diet contained 48.3% crude protein, 19.6% crude fat, 22.5 kJ/g gross energy. Comparatively, the experimental winter feed (WF) had a much higher proportion of marine-derived protein sources (45.8%), consequently lower level of plant-proteins and the totality of the oil fraction associated to fish oil and krill phospholipids. This WF diet was further supplemented with betaine as a phagostimulant, soy lecithin to facilitate fat emulsification during digestion, vitamin C and vitamin E as antioxidants and non essential amino acid taurine given its role as antioxidant and involvement on bile acid conjugation. The WF diet contained 50.6% crude protein, 19.7% crude fat, 22.4 kJ/g gross energy.

Main ingredients were ground (below 250 micron) in a micropulverizer hammer mill (Hosokawa Micron, SH1, The Netherlands). Powder ingredients and oil sources were then mixed accordingly to the target formulation in a paddle mixer (Mainca RM90, Spain). Diets were manufactured by temperature controlled-extrusion (pellet size: 5.0 mm) by means of a low shear extruder (Italplast P55, Italy). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 2 hours at 60°C. Throughout the duration of the trial, experimental feeds were stored at room temperature, but in a cool and aerated emplacement. Samples of each diet were taken for analysis of proximate composition (Table 7.1).

7.2.2. Fish rearing and sampling

The experiment was conducted at the Experimental Research Station of CCMAR (Faro, Portugal) and took place between November and June of the following year. Four homogenous groups of 25 seabream each, with a mean initial body weight of 87 ± 5 g were stocked in 1000 L circular plastic tanks supplied with flow-through seawater. Temperature (14.8 ± 2.1 °C, with minimum and maximum values of 9.5 and 19.5 °C, respectively) and other physicochemical parameters varied within the natural ranges (salinity: 33 ± 2 ‰, dissolved oxygen above 5 $mg.L^{-1}$). Each dietary treatment was tested in duplicate tanks over 213 days. Fish were fed to apparent satiety, by hand, twice a day (10.00 and 16.00h) and feed intake was recorded.

Over its course, two samplings were performed (Sampling 1/March, during the lowest temperature regime, and Sampling 2/June, during the temperature rise period), where fish were weighted, measured and liver samples (10 samples/tank) taken and immediately frozen in liquid nitrogen for proteomic and metabolomic analysis. At sampling, macroscopic

observation of liver allowed identification of abnormal organs characteristics (discoloration, firmness, exudation). Additionally, at each sampling five fish from each tank were sampled for analysis of whole-body composition. Prior to samplings, fish were starved for 48 h (common practice in the industry).

Table 7.1 – Ingredients and proximate composition of the experimental diets.

Ingredients, %	**CTRL**	**WF**
Fishmeal 70 LT	10.0	30.0
Fishmeal 65	5.0	10.8
Krill protein hydrolysate	0.0	5.0
Soy protein concentrate	8.0	0.0
Pea protein concentrate	4.0	0.0
Wheat gluten	8.4	0.0
Corn gluten	16.0	5.5
Soybean meal 48	16.5	7.0
Rapeseed meal	4.0	0.0
Dehulled pea grits	2.0	3.0
Wheat meal	5.0	12.6
Fish oil	10.0	8.0
Rapeseed oil	5.7	0.0
Krill phospholipids	0.0	12.5
Vitamin and mineral premix	0.2	0.2
Vitamin C	0.0	0.3
Vitamin E	0.1	0.5
Choline chloride	0.1	0.1
Guar gum	0.5	0.5
Soy lecithin	0.0	1.0
Dicalcium phosphate	4.0	1.0
L-Lysine	0.5	0.0
L-Taurine	0.0	1.0
Betaine	0.0	1.0

Proximate composition	**CTRL**	**WF**
Dry matter (DM), %	97.48	94.27
Crude protein, % DM	48.33	50.57
Crude fat, % DM	19.62	19.70
Ash, % DM	8.23	10.93
Gross Energy, MJ/kg	22.53	22.44
Phosphorus, % DM	1.52	1.68

7.2.3. Analytical methods for proximate composition of feeds and whole fish

Proximate composition analysis of the diets and whole fish was made by the following procedures: dry matter by drying at 105ºC for 24 h; ash by combustion at 550ºC for 12 h; crude protein (N×6.25) by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection (LECO FP428); fat after petroleum ether extraction by the Soxhlet method, total phosphorus according to the ISO/DIS 6491 method using the vanado-molybdate reagent and gross energy in an adiabatic bomb calorimeter (IKA).

7.2.4. Proteomic analysis by multiplex 2DE (DIGE)

Tissue protein extraction and CyDye labelling

Gilthead seabream liver tissue (100 mg) from five fish per tank (i.e. 10 fish per dietary treatment), sampled at the end of the winter period (i.e. Sampling 1), were individually homogenised with an Ultra-Turrax IKA T8 (IKA-WERG) in 10 volumes of ice-cold DIGE lysis/labelling buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 30 mM Tris, pH 8.5) containing 1 mM EDTA and 1% (v/v) protease inhibitor cocktail), followed by sonication. Homogenates were centrifuged twice at 13000 ×g for 10 min at 4 ºC to pellet insoluble material. The resulting supernatants were then depleted of non-protein contaminants using a ReadyPrep^{TM} 2-D Cleanup Kit (Bio-Rad) and resuspended in DIGE lysis/labelling buffer. All protein quantifications were performed using Quick Start^{TM} Bradford Protein Assay (Bio-Rad).

For DIGE minimal labelling, after pH adjustment of protein extracts to 8.5 by addition of 0.3 M NaOH, 50 µg of proteins were labelled with 400 pmol of fluorescent amine reactive cyanine dyes freshly dissolved in anhydrous dimethylformamide following manufacturer's instructions (5 nmol labelling kit, GE Healthcare). Labelling was performed on ice for 30 min in the dark and quenched with 1 mM lysine for 10 min. Five samples per dietary treatment were labelled with Cy3 and five with Cy5 to reduce the impact of label differences, while an internal control consisting of equal quantities of protein from all samples was labelled with Cy2.

Two-dimensional gel electrophoresis

Labelled liver proteins were first separated according to their isoelectric point onto 24 cm long Immobiline^{TM} Drystrip with a pH 4-7 linear (GE Healthcare). For each strip, 50 µg of liver protein of one sample from each dietary treatment plus 50 µg of internal standard were diluted in rehydration buffer (ReadyPrep 2-D starter kit, Bio-Rad) to a final volume of 450 µL and were loaded overnight by passive rehydration. Isoelectric focusing was performed using an Ettan^{TM} IPGphor^{TM} 3 isoelectric focusing unit (GE Healthcare), at 20°C, for a total of 52000 V.hrs, at maximum 50 µA per strip. After separation of proteins in the first dimension, proteins focused in Immobiline strips were reduced for 30 min in 6 mL of equilibration buffer (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol) with 2% (w/v) DTT, and then alkylated for 30 min in 6 mL of equilibration buffer with 2.5% (w/v) iodoacetamide. The equilibrated strips were then placed onto 12.5% acrylamide gel cast between low fluorescent glass cassettes (EttanDALTsix gel caster system, GE Healthcare). The strips were sealed with 0.5% (w/v) agarose in 250 mM Tris, 192 mM glycine, 0.1% (w/v) SDS and a trace of Bromophenol Blue, and proteins were separated according to their size in a second dimension by SDS-PAGE. The second dimension was run in the EttanDALT system under a constant current of 10 mA per gel for 1h, followed by 40 mA per gel until the bromophemol blue dye front reached the bottom of the gels. The electrophoresis running buffer, containing 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, was used at 1X in the lower chamber and 2X in the upper chamber.

Gel image acquisition and analysis

After electrophoresis, gels were immediately scanned with a Typhoon Trio^{TM} Variable Mode Imager (GE Healthcare) using three laser emission filters (520BP40 for Cy2, 580BP30 for Cy3 and 670BP30 for Cy5) at 500 PMT and at a resolution of 100 µm. Image analysis was performed using the Progenesis SameSpots™ software (Nonlinear Dynamics) and exported for analysis.

7.2.5. Metabolomic analysis by solid-phase transmissive FTIR spectroscopy

Tissue preparation

Liver tissue samples from 10 fish per tank (i.e. 20 fish per dietary treatment), which were collected at the two distinct sampling times (Sampling 1 and Sampling 2), were

lyophilised, ground in liquid nitrogen and lyophilised again, reducing them to a fine dry powder.

Using a quartz/agate pestle and mortar, each liver sample was then mixed with KBr (following a volume ratio of 4 parts KBr per 1 part of dry liver powder) until homogeneous. Small amounts of these mixtures were then pressed into pellets for further analysis by transmissive IR spectroscopy.

IR spectrum acquisition and post-processing

Four spectra per pellet were acquired (at distinct points of the pellet) using a "TENSOR" FTIR equipment (Bruker) coupled to the OPUS control/analysis software (Bruker). Each of these spectra was obtained by averaging 25 spectra covering the 400 to 4000 cm^{-1} range at a 4 cm^{-1} resolution. Spectra were then post-processed by the application of a baseline subtraction algorithm (rubberband correction, 64 points) and exported from OPUS for statistical analysis.

After a preliminary analysis step, five of the samples were deemed clear outliers, so pellet preparation and spectral acquisition was repeated for these samples, to ensure atypical behaviour is not due to technical reasons (but, rather, due to actual biological differences). Furthermore, an "average" pellet was prepared for each sampling/tank combination (to represent a virtual "average fish" for each tank), by sample pooling, to improve estimation of the multivariate centroid for each group.

Total number of spectra used in the analysis was therefore 383 (each of them obtained, as stated above, by averaging 25 IR spectra).

7.2.6. Univariate and multivariate data analysis

All data analyses were performed with the R statistical computing software[299]. Proteomic data was just imported and used as-is (since SameSpots already applies a valid normalization procedure). The result dataset's dimensions were 20 channels × 1637 spots per channel. For the metabolomic data, spectra were truncated to the 500 to 1800 cm^{-1} area-of-interest (due to high variability in the 1800 to 4000 cm^{-1} area), converted from transmittance to absorbance and normalized, by application of a simple SNV (standard normal variate) transform (i.e. mean centering each spectrum, then dividing each spectrum by its standard deviation and finally adding a constant across all spectra to ensure strictly positive values). The resulting dataset's dimensions were 383 spectra × 676 frequency bins per spectrum.

Metric multidimensional scaling (metric MDS) and principal component analysis (PCA) were performed using the `cmdscale` and `prcomp` functions, respectively. Heatmaps were plotted using the `heatmap` function. Sparse partial least squares (sPLS), sparse partial least squares discriminant analysis (sPLS-DA) and regularized canonical component analysis (rCCA) were performed using functions `spls`, `splsda` and `rcc`, respectively (from package *mixOmics*[566]). Networks of association were plotted using function `network` (from package *mixOmics*[566]). Overlay of distinct embeddings was performed using function `procrustes` (from package *vegan*[302]).

7.3. Results and Discussion

7.3.1. Trial results

The results of the fish trial appear to substantiate the positive effects of the WF diet on fish, with fish fed with this diet generally displaying higher growth rates, higher hepatosomatic indices and lower feed conversion ratios, compared to CTRL. No mortalities were reported for any of the tanks, which is a sign that the fish somewhat handled the seasonal challenge, displaying none of the behavioural symptoms of "winter disease". Nevertheless, some of the fish (particularly in the CTRL tanks) displayed at least one of the phenotypic traits of "winter disease": pale and friable liver.

Figure 7.1 shows how some performance parameters vary in function of fish weight. The first observation is that, as stated above, fish grew between the two sampling points, with fish fed with WF generally displaying higher growth both initially and during the period of time between the two samplings. We also observe that, although liver weight generally increases over time, there's a strong negative effect of season on hepatosomatic indices (HSI), suggesting that muscle growth outpaced liver growth during the two sampling periods. Furthermore, fish fed with WF generally display higher HSI values, particularly for the first sampling. Regarding visceral weight, we can see that it is generally proportional to body weight across all seasons, which implies a fairly constant viscerosomatic index (VSI). Nevertheless, fish fed with WF display slightly increased VSI over the ones fed with CTRL, though not significantly.

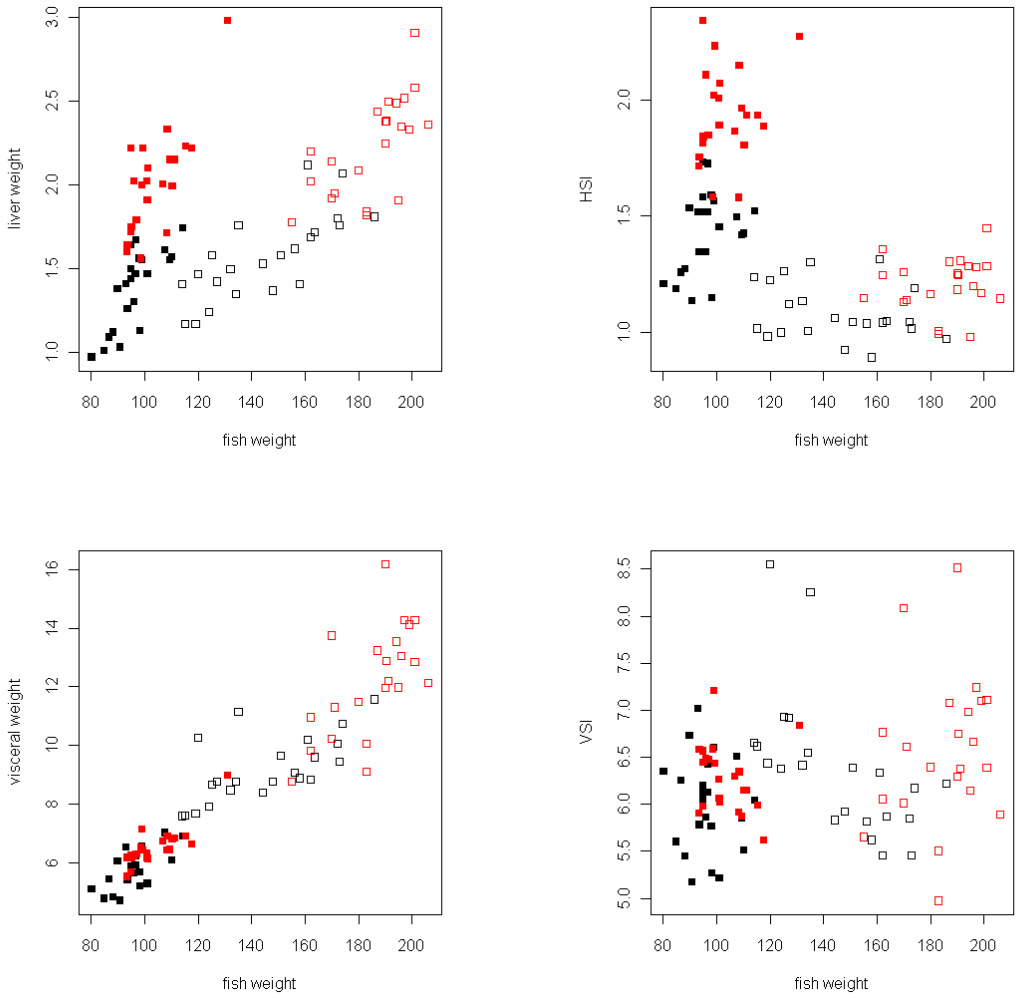

Figure 7.1 – Scatter plots showing the relation between fish weight and (from left to right) liver weight, HSI, visceral weight and VSI. Symbols correspond to season/sampling time (with closed boxes for 1st sampling and open boxes for 2nd sampling), while colours correspond to diet factor (black for CTRL and red for WF).

Principal component analysis (PCA) of these 5 performance parameters confirms that most of the observed variance is due to inter-season and inter-diet differences, as the first two components clearly separate between the 4 possible season/diet combinations (Figure 7.2). Given that these 5 parameters have only 3 degrees of freedom (fish weight, liver weight and visceral weight), the first three PCA components necessarily account for 100% of observed variance.

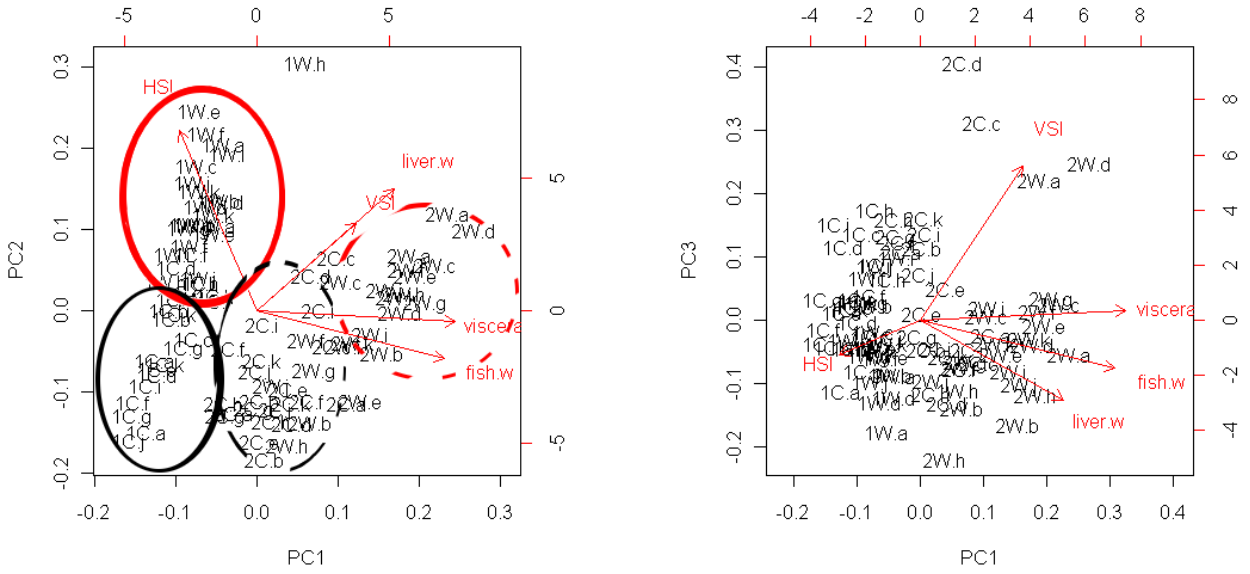

Figure 7.2 – Biplots showing the results of PCA analysis with the performance parameters (fish weight, liver weight, visceral weight, HSI and VSI) for PC1 and PC2 (left), as well as for PC1 and PC3 (right). Samples are identified according to their group ("1C" for CTRL/1st sampling, "1W" for WF/1st sampling, "2C" for CTRL/2nd sampling and "2W" for WF/2nd sampling). On the left graph, a visual aid showing the separation between groups can also be seen (black for CTRL, red for WF; full stroke for 1st sampling, broken stroke for 2nd sampling).

7.3.2. Proteomic response

DIGE analysis worked as expected, producing high-resolution, highly reproducible protein gel profiles for all samples. Representative images for the two different diet groups can be observed in Figure 7.3.

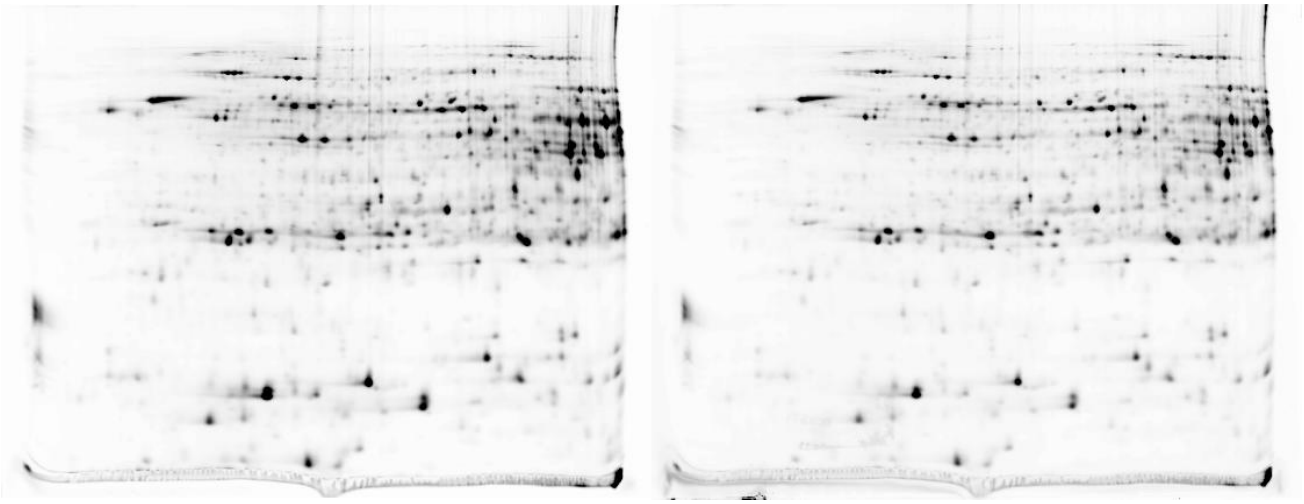

Figure 7.3 – Two grayscale images of CyDye channels showing representative hepatic proteome profiles for the Control (left) and Winter Feed (right) groups (50 µg protein/channel).

Analysis of the effect of the dietary treatment on the hepatic proteome (at Sampling 1) revealed distinct patterns for the two experimental diets (CTRL *vs.* WF): in fact, a quick univariate ANOVA test suggests that 500 spots (out of a total of 1637 detected spots) display differential expression in regards to the dietary factor. Out of these 500 spots, 300 are overexpressed in the WF group, while 200 are overexpressed in the CTRL group. Given the high number of univariate tests performed, we chose to use Benjamini-Hochberg's procedure to control the false discovery rate (at $\alpha = 0.05$), which implied taking only the 26 most significant spots (13 of which overexpressed, and 13 underexpressed, in the WF group) as such.

Looking at Figure 7.4, we can see that samples from the two CTRL-fed tanks are generally more similar between themselves and samples from the two WF-fed tanks are also generally more similar between themselves, showing that the tank effect is generally smaller than the diet effect, without any prior variable selection and regardless of the type of dissimilarity measure chosen. Obviously, this effect is even more pronounced if we restrict the dataset to the 500 most significant spots (Figure 7.5) and even more if we restrict the dataset to the 26 most significant spots (Figure 7.6).

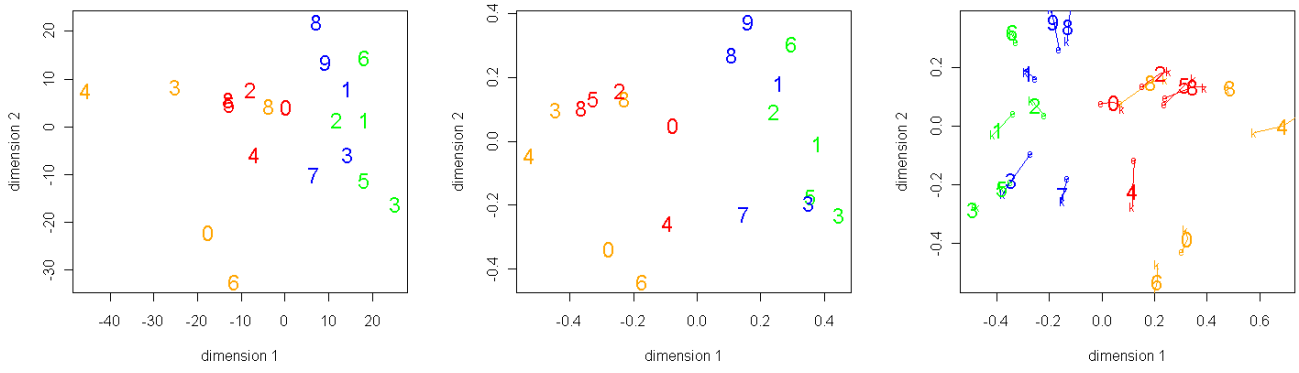

Figure 7.4 – Two-dimensional embeddings of the samples obtained by metric multidimensional scaling of the whole DIGE dataset, using (left) Euclidian distance as dissimilarity measure (i.e. similar to PCA projection), (center) a dissimilarity measure based on Kendall's correlation or (right) a mix of the two. Numbers correspond to different fish within a tank, while the colours correspond to the tanks themselves (orange and red for CTRL-fed tanks; blue and green for WF-fed tanks). In the last image, the first two embeddings are also overlayed, for comparison purposes (Euclidian distance, with "e", and Kendall's correlation, with "k").

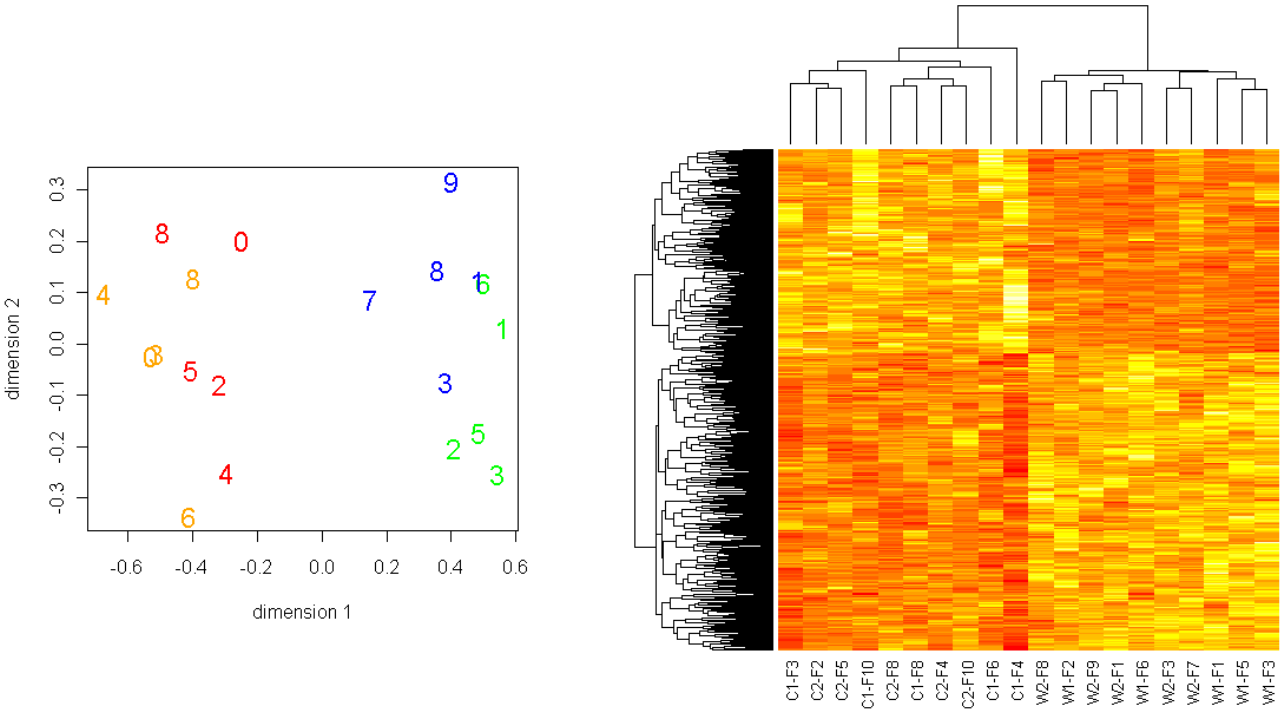

Figure 7.5 – On the left, two-dimensional embedding of the samples obtained by metric multidimensional scaling of the 500 most significant variables of the DIGE dataset, using a dissimilarity measure based on Kendall's correlation. Numbers correspond to different fish within a tank, while the colours correspond to the tanks themselves (orange and red for CTRL-fed tanks; blue and green for WF-fed tanks). On the right, a heatmap detailing the expression pattern of each sample, for the 500 most significant spots (red/orange means "below average expression", while yellow/white means "above average expression"). Furthermore, dendograms displaying hierarchical agglomerative clustering of samples and variables based on their Euclidian distance can also be seen, attached to the columns and rows (respectively).

In alternative to the univariate variable selection strategy, we also used a supervised multivariate method of variable selection. We therefore applied sPLS-DA (Sparse Partial Least Squares Discriminant Analysis) with 2 components to the dataset, using the "diet" factor as Y-vector and restricting the number of nonzero loadings for each component to 26. As can be seen in Figure 7.7, only the first component contains information related to inter-diet differences, so we extracted the variables with nonzero loadings from the first component, to compare with the results of the univariate variable selection strategy.

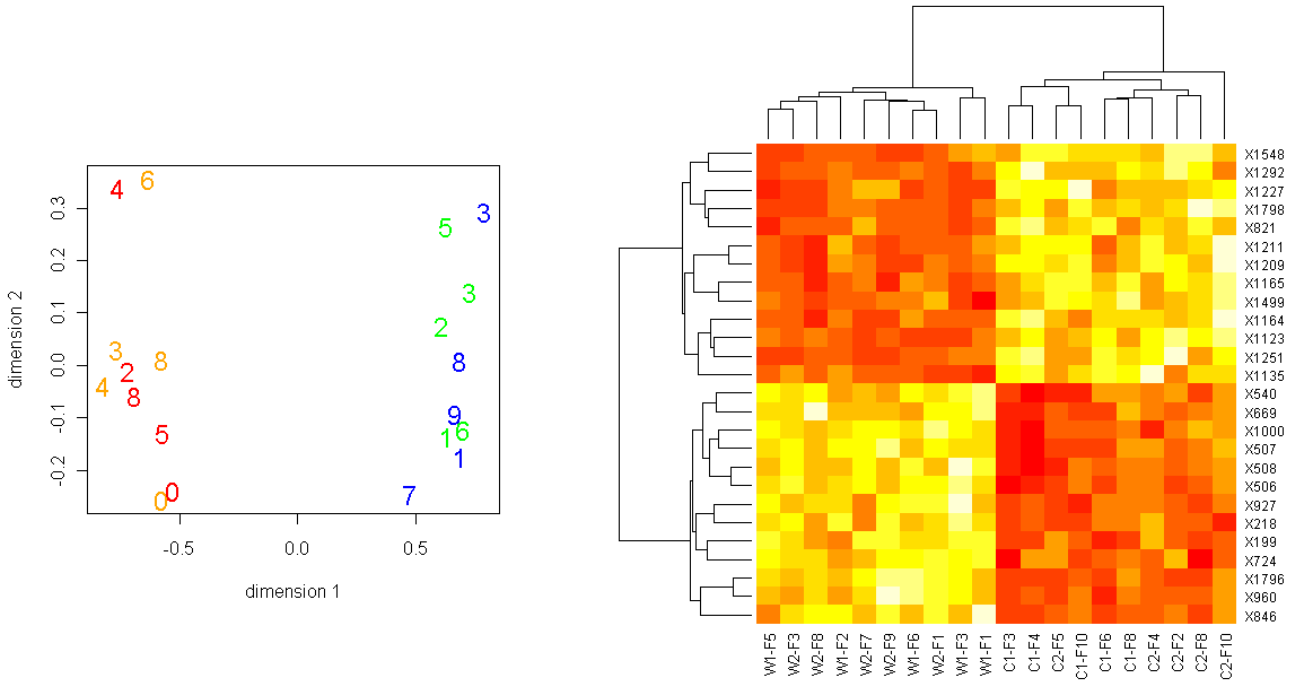

Figure 7.6 – On the left, two-dimensional embedding of the samples obtained by metric multidimensional scaling of the 26 most significant variables of the DIGE dataset, using a dissimilarity measure based on Kendall's correlation. Numbers correspond to different fish within a tank, while the colours correspond to the tanks themselves (orange and red for CTRL-fed tanks; blue and green for WF-fed tanks). On the right, a heatmap detailing the expression pattern of each sample, for the 26 most significant spots (red/orange means "below average expression", while yellow/white means "above average expression"). Furthermore, dendograms displaying hierarchical agglomerative clustering of samples and variables based on their Euclidian distance can also be seen, attached to the columns and rows (respectively).

Afterwards, we repeated the procedure, but using the "tank" factor as Y-vector and interestingly, as can be seen in Figure 7.8, the first two components do cleanly separate the four tanks into clusters, with the first component, again, containing information related to inter-diet differences, but now the second component containing inter-tank differences orthogonal (i.e. not related) to inter-diet differences. As before, we extracted the variables with nonzero loadings from the first component (to compare with other methods of variable selection for the "diet" effect), but this time we also extracted the variables with nonzero loadings from the second component (which should help account for inter-tank differences).

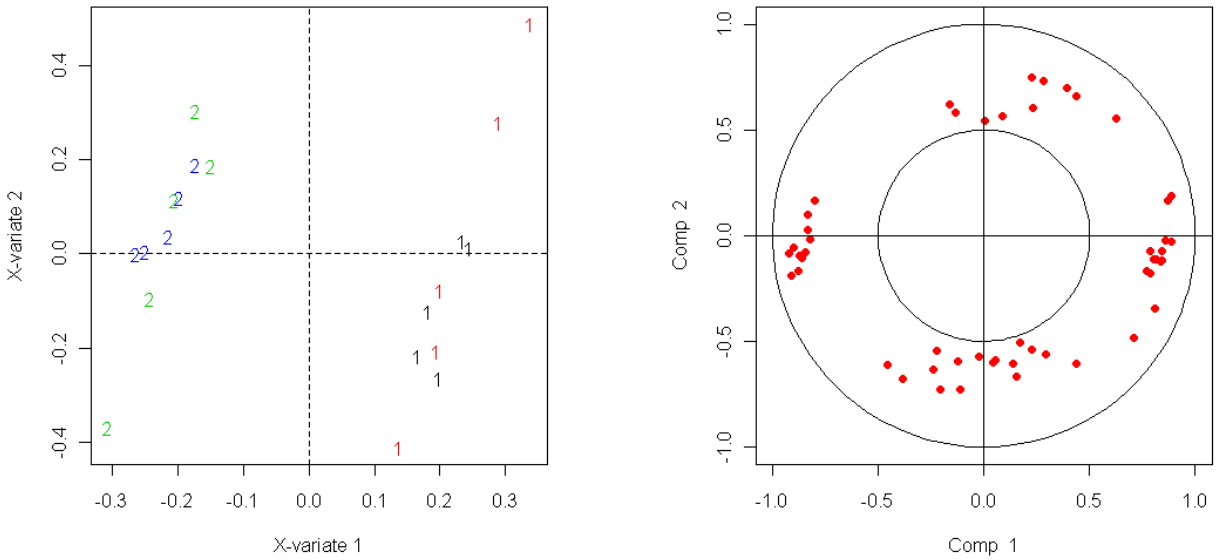

Figure 7.7 – Results of sPLS-DA analysis of the DIGE dataset, using the diet factor as Y-vector. On the left, projection of each sample on the two components ("1" means CTRL diet and "2" means WF diet, different colours for different tanks). On the right, loadings of selected variables for the two components.

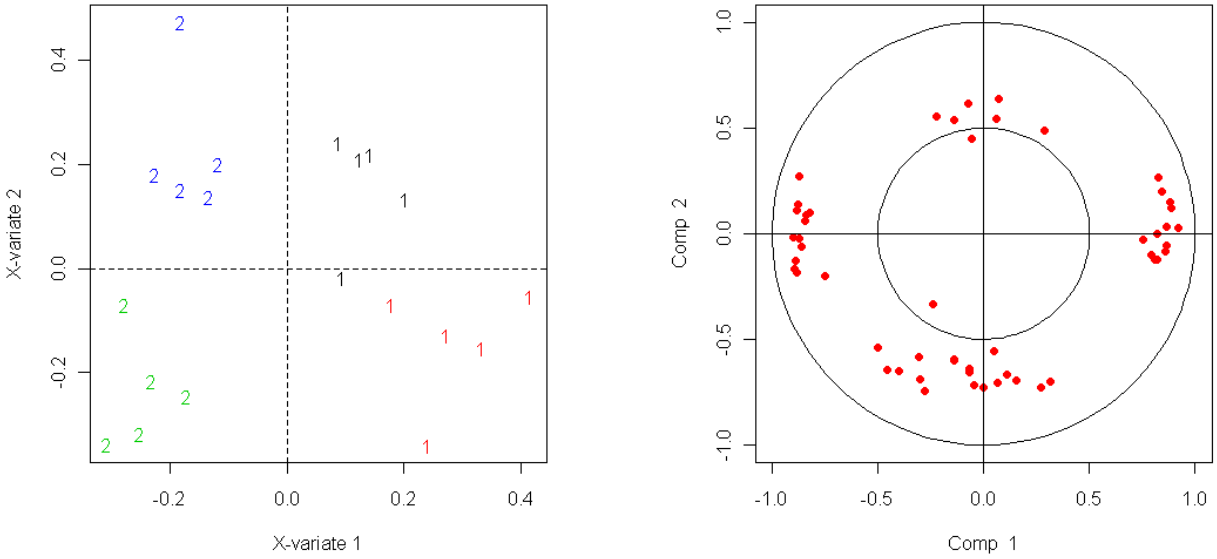

Figure 7.8 – Results of sPLS-DA analysis of the DIGE dataset, using the tank factor as Y-vector. On the left, projection of each sample on the two components ("1" means CTRL diet and "2" means WF diet, different colours for different tanks). On the right, loadings of selected variables for the two components.

Looking at the 26 selected spots from each of the three strategies (variables selected from ANOVA, variables selected from the PC1 of "diet effect"-focused sPLS-DA and variables selected from the PC1 of "tank effect"-focused sPLS-DA), we see that, first of all, all of them would pass the ANOVA test without multiple comparison correction at a $p < 0.05$ level (which is a good sanity check) and there is a good degree of overlap between the groups, as it can be seen in Figure 7.9.

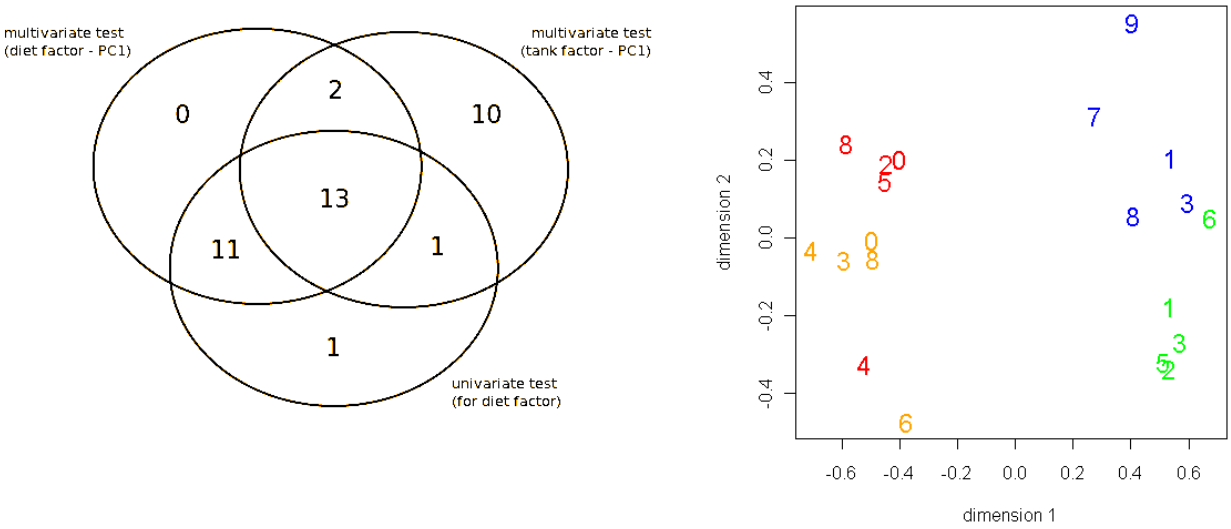

Figure 7.9 – On the left, Venn diagram showing the degree of overlap between the three variable selection strategies used to isolate the "diet effect" in the DIGE dataset. On the right, two-dimensional embedding of the samples obtained by metric multidimensional scaling of the 38 selected variables of the DIGE dataset for the "diet effect" (depicted in the Venn diagram) plus the 26 selected variables from the PC2 of the second multivariate strategy (for the "tank effect"), using a dissimilarity measure based on Kendall's correlation. Numbers correspond to different fish within a tank, while the colours correspond to the tanks themselves (orange and red for CTRL-fed tanks; blue and green for WF-fed tanks).

Finally, we also applied Sparse Partial Least Squares (sPLS) to select which protein spots (X-matrix) better help explain both the experimental factors and the performance parameters (Y-matrix). As can be seen in Figure 7.10, there is a group of proteins with strong positive correlation towards HSI, liver weight and the diet factor and a group of proteins with strong negative correlation towards the same variables. The same group of proteins also displays a similar (though weaker) correlation with fish weight, visceral weight and VSI. This is unsurprising, given what was seen above regarding the performance parameters for the first sampling: if we restrict analysis to this sampling time, the performance parameters are all generally positively correlated between themselves (and with the "diet" factor), except for the

VSI (which is fairly constant). Nevertheless, this colinearity is not perfect and some of the spots displaying strong correlation with HSI or liver weight do not display such a strong correlation with the "diet factor". This suggests yet another valid approach for selecting relevant protein spots: to take into account not only the experimental factors, but also auxiliary zootechnical measurements.

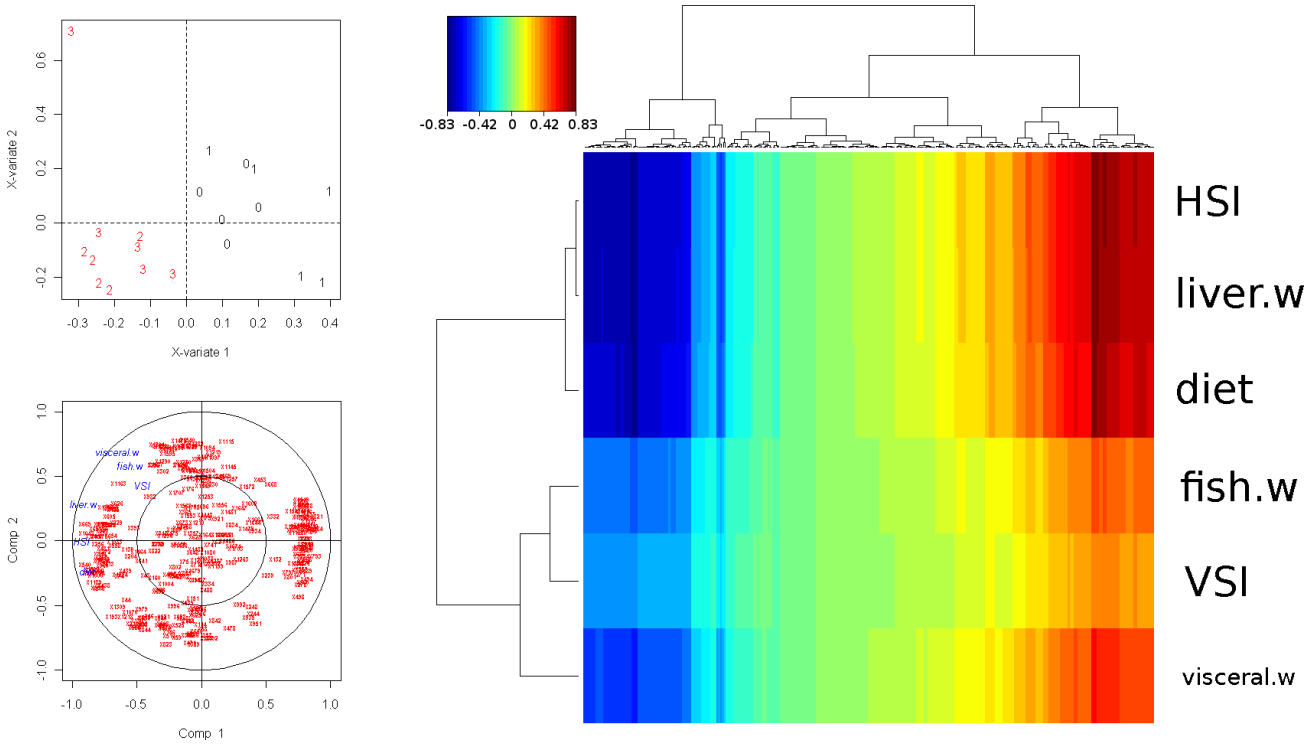

Figure 7.10 – Results of the sPLS analysis using the DIGE dataset as X-matrix and the performance parameters as Y-matrix. On the left, sample scores according to the first two components (above) and correlation circle containing both the selected variables from the DIGE dataset and all the variables of the Y-matrix (below). Samples are represented by symbols according to the respective tanks ("0" and "1" for the CTRL tanks, "2" and "3" for the WF tanks). On the right, heat map showing how selected DIGE variables and all performance variables co-variate.

Identification in the near future of protein spots deemed relevant will enable us to have a clearer and more defined view of the effects of the WF diet (and seasonal temperature variations) on the gilthead seabream's hepatic proteome, as well as possible biologically-relevant relations between protein expression and zootechnical performance parameters. This task is currently underway.

7.3.3. Metabolomic response

Preliminary analysis of the spectra showed relatively low intra-pellet variation, but a noticeable degree of inter-pellet variation (mostly due to a mix of pellet thickness variability and operator error). Nevertheless, although it might be advisable to run several technical replicates per biological sample to attenuate this effect, only one pellet was prepared per sample, which still provided quite interesting results.

Analysis of the FTIR dataset suggests, not only a diet effect, but an even stronger seasonal effect (i.e. comparing the metabolic fingerprints of the 1st sampling vs 2nd sampling). This can be seen in Figure 7.11, where spectra are plotted according to their (dis)similarity.

Looking at the spectra themselves and the results of univariate analysis (Figure 7.12), it becomes clear that the differences have to be attributed to several distinct spectral features. An overview of the different spectral features, along with their behaviour and associated functional groups can be seen in Table 7.2, where it is clear that observed changes can be mostly clustered into three types:

- Generally positive season effect and generally negative diet effect (type I): peaks 1, 2, 4, 6, 7, 8, 9, 10, 17 and 19;

- Generally negative season effect and generally positive diet effect (type II): peaks 12, 13, 14, 15, 16, 18, 20, 21 and 22;

- Generally negative season effect for control diet, generally positive season effect for winter feed diet, generally negative diet effect for 1st sampling and generally positive diet effect for 2nd sampling (type III): peaks 3 and 5.

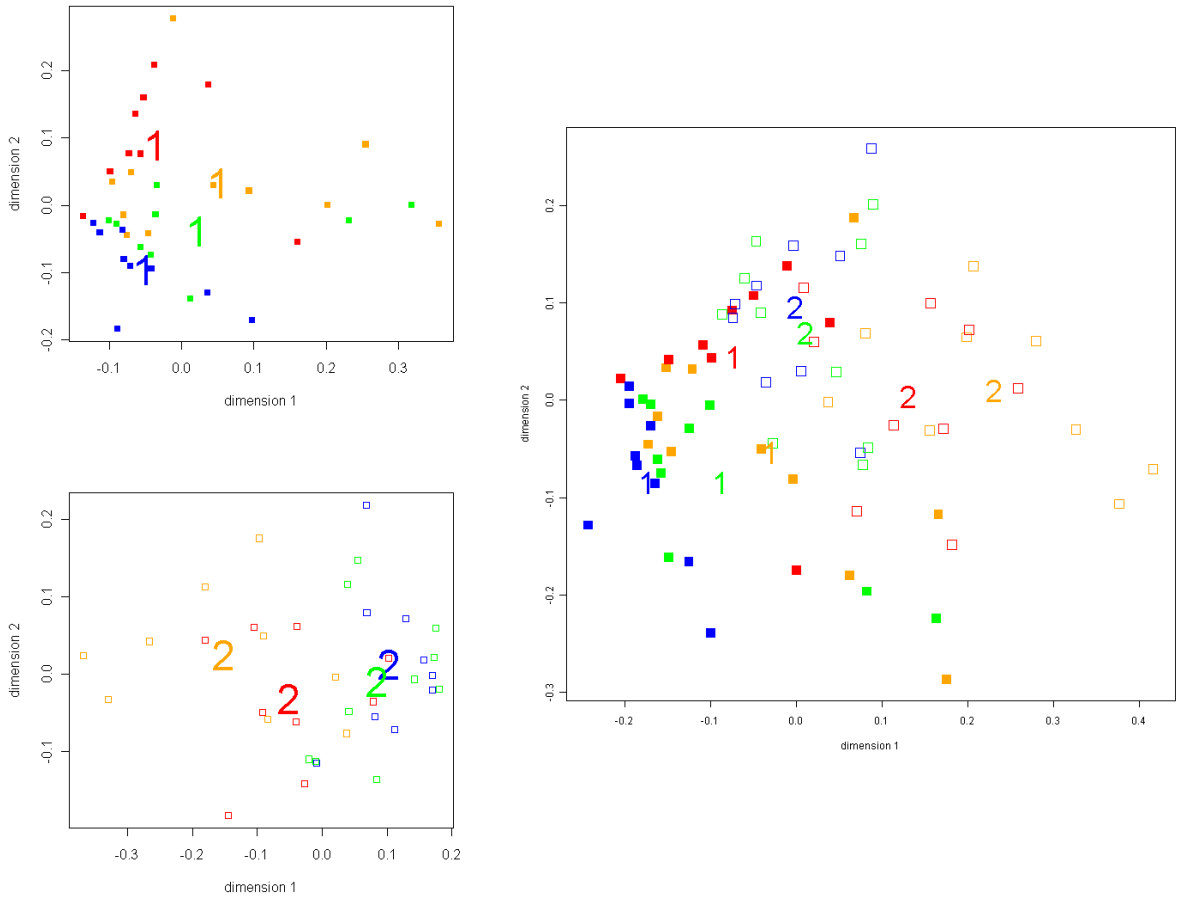

Figure 7.11 – Two-dimensional embeddings of the samples obtained by metric multidimensional scaling of the FTIR dataset, using a dissimilarity measure based on Kendall's correlation, for the two samplings separately (left) or together (right). The symbols correspond to the sampling times (closed box for 1st sampling and open box for 2nd sampling), while the colours correspond to the tanks themselves (orange and red for CTRL-fed tanks; blue and green for WF-fed tanks). The centroids for each group are marked with a number ("1" for 1st sampling and "2" for 2nd sampling) and the tank's respective colour.

The type III differences correspond to spectral features usually associated with proteins (amide I and amide II) and it is plausible that the effect of season and diet on the absorbance of these peaks might be negligible. To account for that, we have assessed the other features both with the normal normalization method as well as with a method that normalizes according to the area below the amide I and amide II peaks. If, on the other hand, we assume these effects are biologically significant, it would imply that, at the end of winter, WF-fed fish seem to have lower relative levels of protein in liver (compared to CTRL-fed fish), while, at the start of summer, WF-fed fish seem to have higher relative levels of protein in liver.

Table 7.2 – Spectral features detected in the 400-1800 cm^{-1} range, along with observed changes in response to season and diet, associated functional groups and main components. Other components which are thought to absorb in the same spectral range are also listed.

Peak ID	Wavenumber (cm^-1)	Season effect		Diet effect		Associated functional group vibration modes	Main components	Other components
		CTRL	WF	1st	2nd			
1	1740-1750	+	0	0	-	C=O stretching of esters and aldehydes	triglycerides, cholesterol esters	aldehydes, esters
2	1710	+	+	-	-	C=O stretching of ketones and carboxilic acids	fatty acids	ketones, carboxilic acids
3	1650	0	+	-	+	C=O stretching of amides (amide I peak); alkenyl C=C stretching	proteins	unsaturated fatty acids
4	1570-1610	+	+	-	0	conjugated C=C stretching	unsaturated fatty acids/lipids	aromatics
5	1540	0	+	-	+	N-H bending of amides (amide II peak)	proteins	aromatics
6	1460	+	0	0	-	methylene C-H bending	lipids	proteins, aromatics
7	1455	+	0	0	-	methyl C-H assymetric bending	lipids	proteins
8	1395-1415	+	+	-	-	COO^- symmetric stretching	fatty acids, amino acids	other carboxylates
9	1300-1310	+	0	0	-	methyne and olefinic C-H bending	unsaturated fatty acids/lipids	alcohols, aromatic amino acids, organic phosphates, carboxylates
10	1240	+	+	0	-	PO_2^- assymetric stretching	nucleic acids	phospholipids
11	1150-1155	0	-	+	-	CO-O-C assymetric stretching of glycogen and nucleic acids	carbohydrates, nucleic acids	aromatics, phospholipids, cholesterol esters
12	1100	-	-	+	+	C-O stretching of secondary alcohols	carbohydrates, glycerol	aromatics
13	1080	-	-	+	+	C-O stretching of glycogen; PO_2^- symmetric stretching	carbohydrates, nucleic acids	phospholipids, aromatics
14	1045	-	-	+	+	C-O stretching of oligo/polysaccharides	carbohydrates	aromatics
15	1025	-	-	+	+	inorganic phosphate; C-C skeletal vibrations	*unknown*	aromatics
16	930	0	-	+	0	$C\text{-}N^+\text{-}C$ stretch of nucleic acids	nucleic acids	aromatics, phosphatidylcholine, alcohols, carboxilic acids, amines
17	845-865	+	+	0	-	carbonate; C-C skeletal vibrations; peroxides	*unknown*	aromatics
18	760	-	0	0	0	methylene (CH2)n rocking; C-C skeletal vibrations	*unknown*	aromatics
19	700-720	+	+	-	-	methylene (CH2)n rocking; C-C skeletal vibrations; olefinic C-H; thiols	lipids	glutathione, alcohols, aromatics
20	650	-	0	0	+	*Unknown*	*unknown*	alcohols
21	610	-	0	0	+	Disulfides	*unknown*	glutathione, proteins, alcohols
22	575	-	0	0	+	*Unknown*	*unknown*	*Unknown*

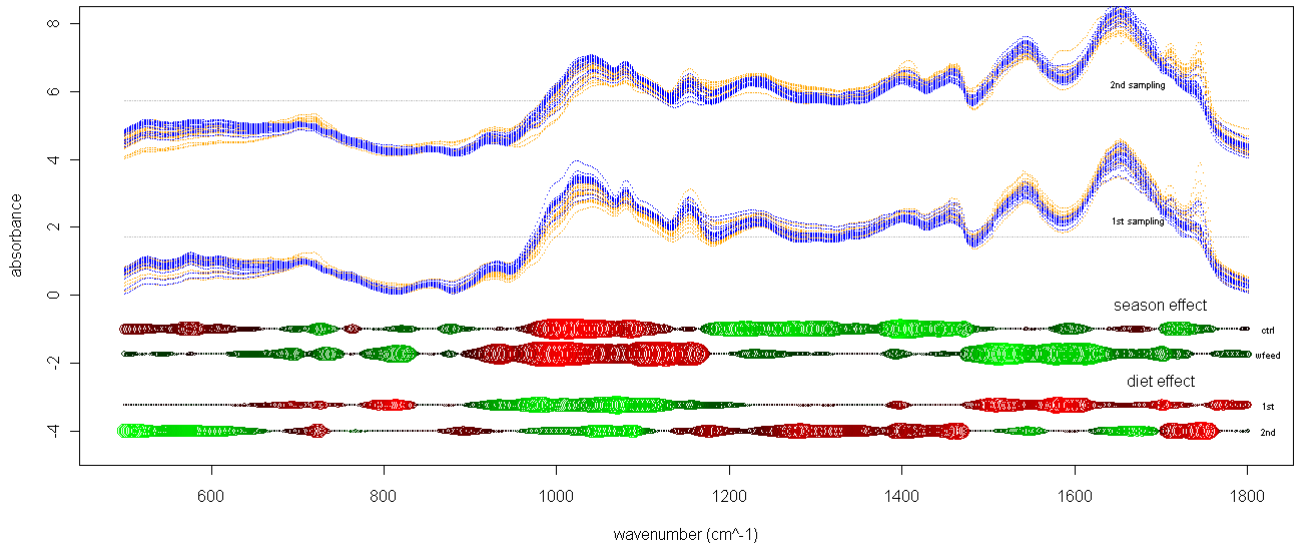

Figure 7.12 – Plots of the IR spectra in the 500-1800 cm^{-1} range, for the two samplings in separate (orange for CTRL-fed fish and blue for WF-fed fish). The line in grey corresponds to the mean value. Below, graphs depicting the season effect (separately for CTRL and WF fish) and the diet effect (separately for the two sampling times) for each spectral bin. Colour denotes effect's direction and strength, while thickness denotes statistical significance (as assessed by Wilcoxon-Mann-Whitney's U-test), being inversely proportional to the logarithm of the p-value.

Regarding type II peaks, we see that they mostly point towards carbohydrates (oligosaccharides, polysaccharides, glycogen), but also nucleic acids and phospholipids, which suggests that season had a strong negative impact on the hepatic glycogen reserves in gilthead seabream and, at the same time, suggests that the dietary treatment not only increased the amount of carbohydrate stores present at the end of winter, but this increase is still visible at the start of summer, despite observed depletion of carbohydrate stores between the two sampling points. This effect is still visible even if we assume no relevant changes in protein content occurred (i.e. if spectra are normalized according to the area under the amide I and amide II peaks).

Finally, type I peaks are mostly associated with lipids (both saturated and unsaturated fatty acids, triglycerides, cholesterol esters and phospholipids) as well as nucleic acids, implying that at the start of summer, levels of lipids in the liver are increased, particularly for the CTRL-fed groups. The diet effect seems to partially revert the season effect (as for the carbohydrates), generally decreasing the amount of present lipids (particularly in the 2nd sampling). This effect is still visible even after normalizing spectra according to amide I and

amide II area and would suggest that CTRL-fed fish appear to be mobilizing lipid stores (probably from adipose tissue) at the start of summer, to face increasing energy demands, while WF-fed fish do not display the same effect (suggesting an improved metabolic status). It should be noted that one of the specific symptoms of the "winter disease" is precisely the progressive hepatic accumulation of lipids resulting in a steatotic-like liver. This could be a sign that WF does indeed provide some protective or mitigating effect of the conditions that lead to the onset of "winter disease" in gilthead seabream.

Regarding nucleic acids, it is difficult to have a conclusive view, as some of the spectral features associated with nucleic acids behave according to the "carbohydrate cluster", while others behave according to the "lipid cluster". And, again, regarding proteins, it is difficult to say if the small observed variations are relevant or just a reflection of the changes in the hepatic carbohydrate (in the case of the WF group) and lipid content (in the case of the CTRL group).

These observations are generally supported by correlation plots that show how spectral features cluster in terms of behaviour (Figure 7.13).

As with the proteomic data, we also applied sPLS-DA to the FTIR dataset, in order to try to isolate the main features associated with the experimental factors (season, diet, tank). Looking at the results of this analysis (Figure 7.14), we see that they are mostly consistent with the observations mentioned above: most changes associated with the experimental factors can be mostly attributed to spectral features related to carbohydrates and lipids.

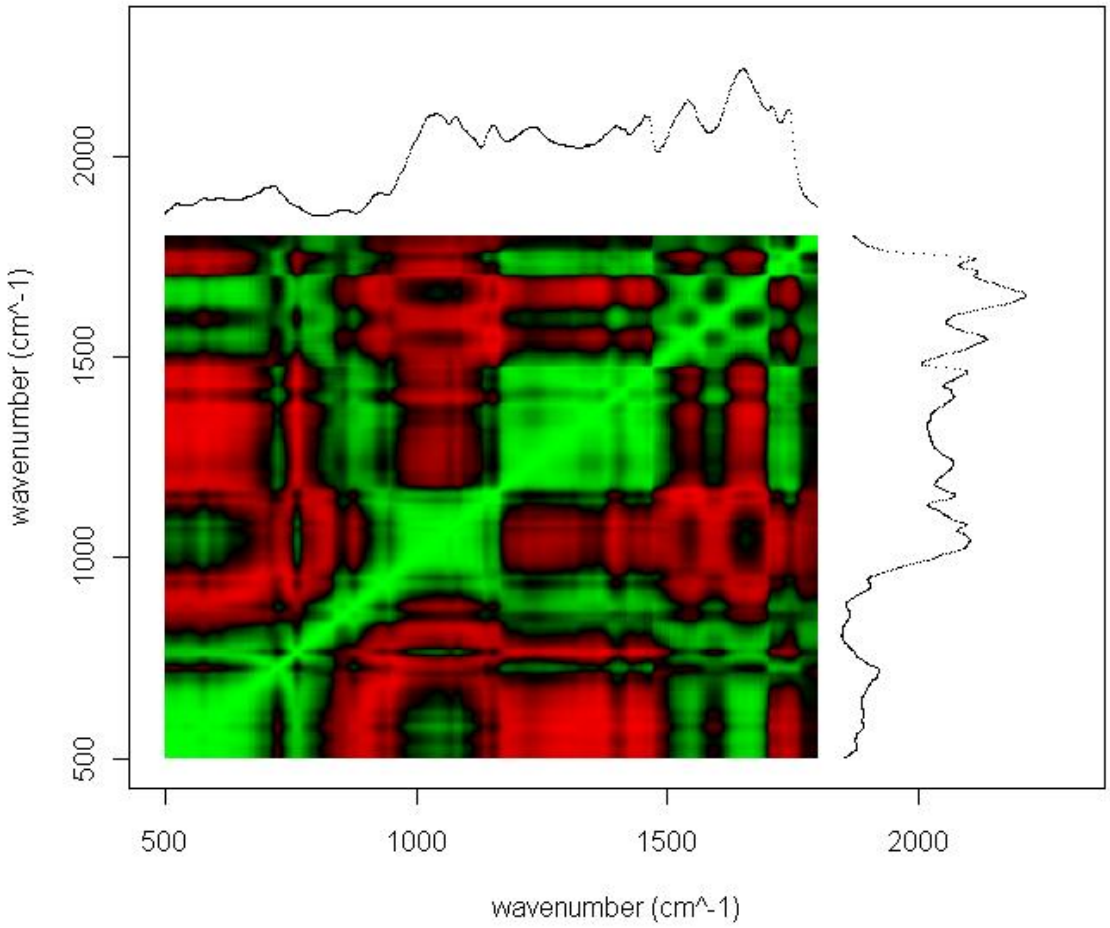

Figure 7.13 – Plot showing Pearson's correlation between spectral features (green for "positively correlated", black for "uncorrelated" and red for "negatively correlated").

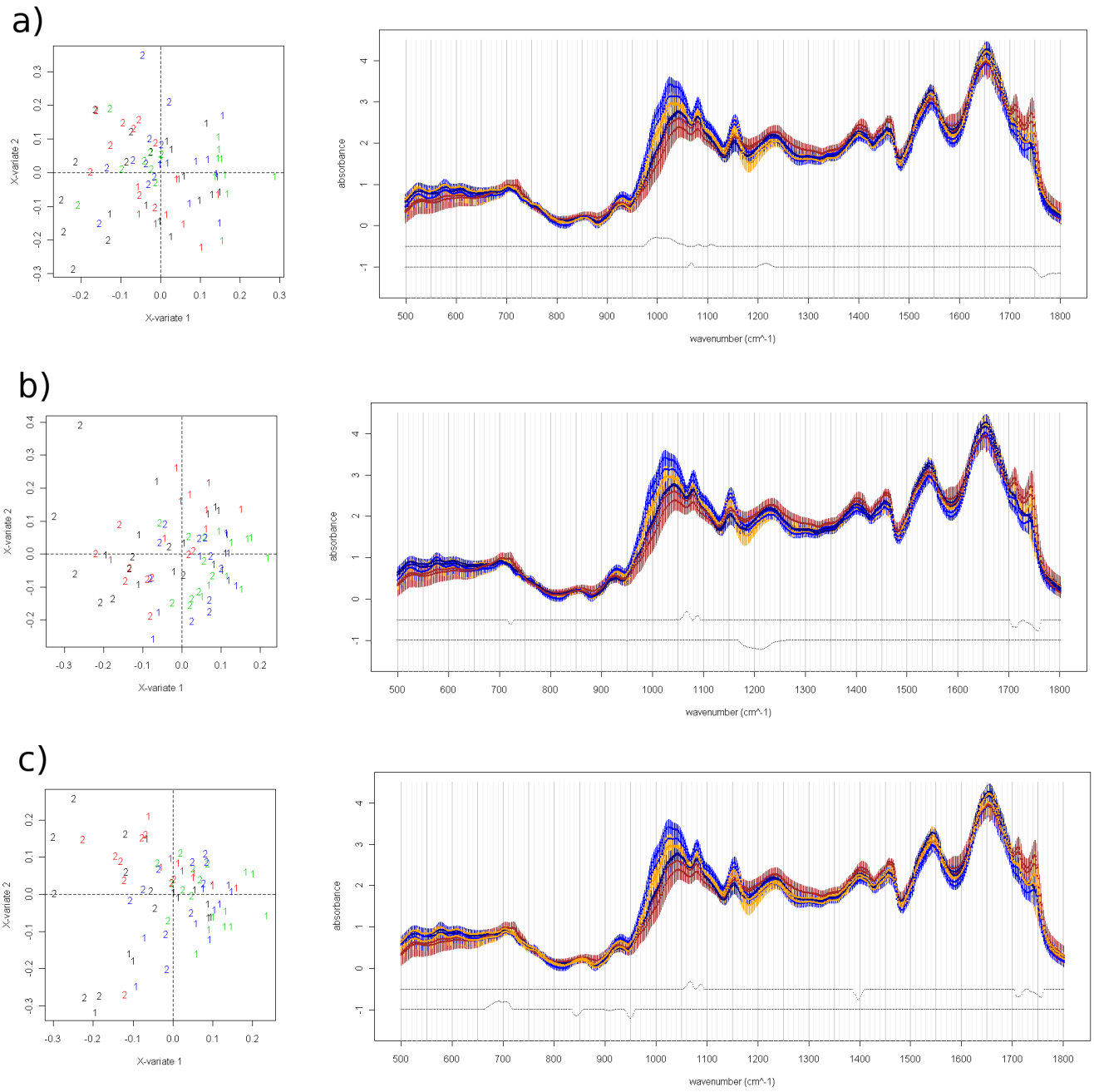

Figure 7.14 - Results of sPLS-DA analysis of the FTIR dataset, using the season (a), diet (b) and tank (c) factor as Y-vector. On the left, projection of each sample on the two components (numbers refer to the two different samplings times, while colors refer to the different tanks: black and red for CTRL, blue and green for WF). On the right, loadings of selected spectral features for the two components overlaid with the average spectra (and standard deviation) for each group ("orange" for 1st sampling CTRL, "blue" for 1st sampling WF, "red" for 2nd sampling CTRL and "dark blue" for 2nd sampling WF).

To confirm that the lipid fraction indeed increased for some of the experimental samples, we also looked at the 2840-3030 cm^{-1} spectral range (not shown), where lipid-related

IR absorptions related to CH stretching are expected to be present. Indeed, when the areas of the bands present in this range were estimated (and normalized against the area of the amide bands), it became clear that they also increase consistently with the other lipid-related bands.

In a nutshell, results from the FT-IR spectroscopy-based metabolic profiling suggest the major impact of the diet and season factors on gilthead seabream liver metabolome can be expressed mostly in terms of changes in relative carbohydrate and lipid abundance. This can be seen in Figure 7.15, which shows how the ratios between the areas of carbohydrate (950-1125 cm^{-1}), lipid (690-730, 1330-1475, 1700-1760, 2840-3300 cm^{-1}) and protein-related (1500-1700 cm^{-1}) IR absorptions almost cleanly separate the different experimental groups (particularly for the second sampling).

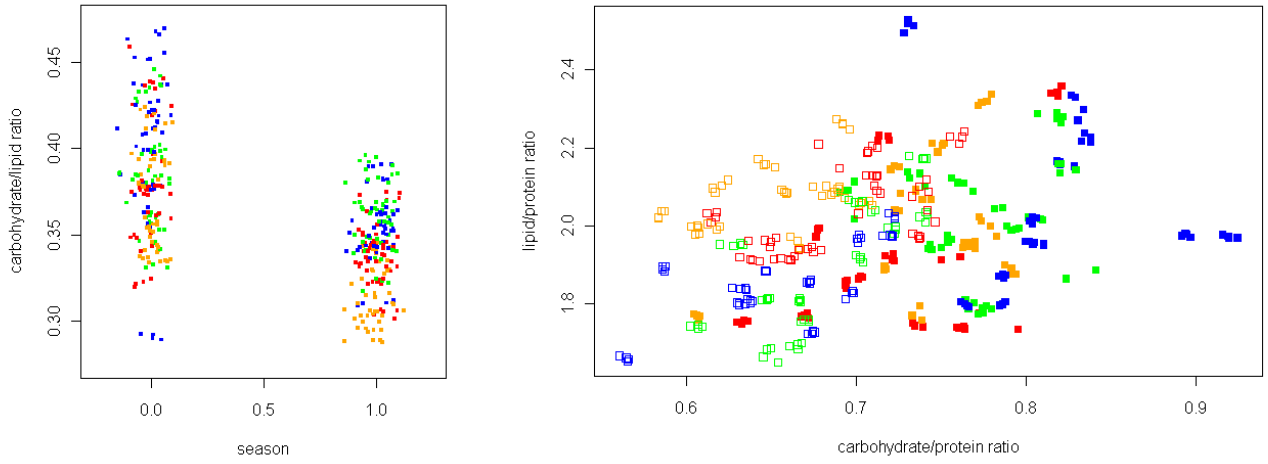

Figure 7.15 – On the left, plot showing the ratio between the area of carbohydrate-related IR absorptions and lipid-related IR absorptions, separately for the two seasons/sampling times, for every single technical replicate (coloured according to tank: red and orange for CTRL tanks, blue and green for WF tanks). On the right, scatter plot showing simultaneously the carbohydrate/protein ratio and the lipid/protein ratio (again, estimated based on excpected IR absorptions) for each single technical replicate. Samples are coloured similarly to the graph on the right, with symbols distinguish between sampling times (closed box for 1st sampling and open box for 2nd sampling).

Finally, we also applied sPLS to try to predict the experimental factors and performance parameters (Y-matrix) using the FTIR dataset. Looking at Figure 7.16, we see that the HSI displays a strong positive correlation with a specific cluster of variables (the "carbohydrate cluster") and a generally negative correlation with another specific cluster of variables (the "lipid cluster"). We also see the inverse situation for the season factor (as

previously discussed), as well as with fish weight and visceral weight, which is unsurprising as these two variables are positively correlated to the season factor. As already discussed, we can also see that the diet factor has the opposite effect of the season factor, although the magnitude of the season effect is larger than the magnitude of the diet effect. The other two variables (VSI and liver weight) display even weaker correlations with the FTIR dataset, but following the same trend as the HSI and the diet effect.

Looking in more detail into the correlation between HSI and hepatic carbohydrate content (Figure 7.17), it does seem clear that this correlation exists across all samples, while any eventual correlation with lipid content seems much weaker. This suggests that the variations in HSI observed could be mostly attributable to changes in hepatic carbohydrate content.

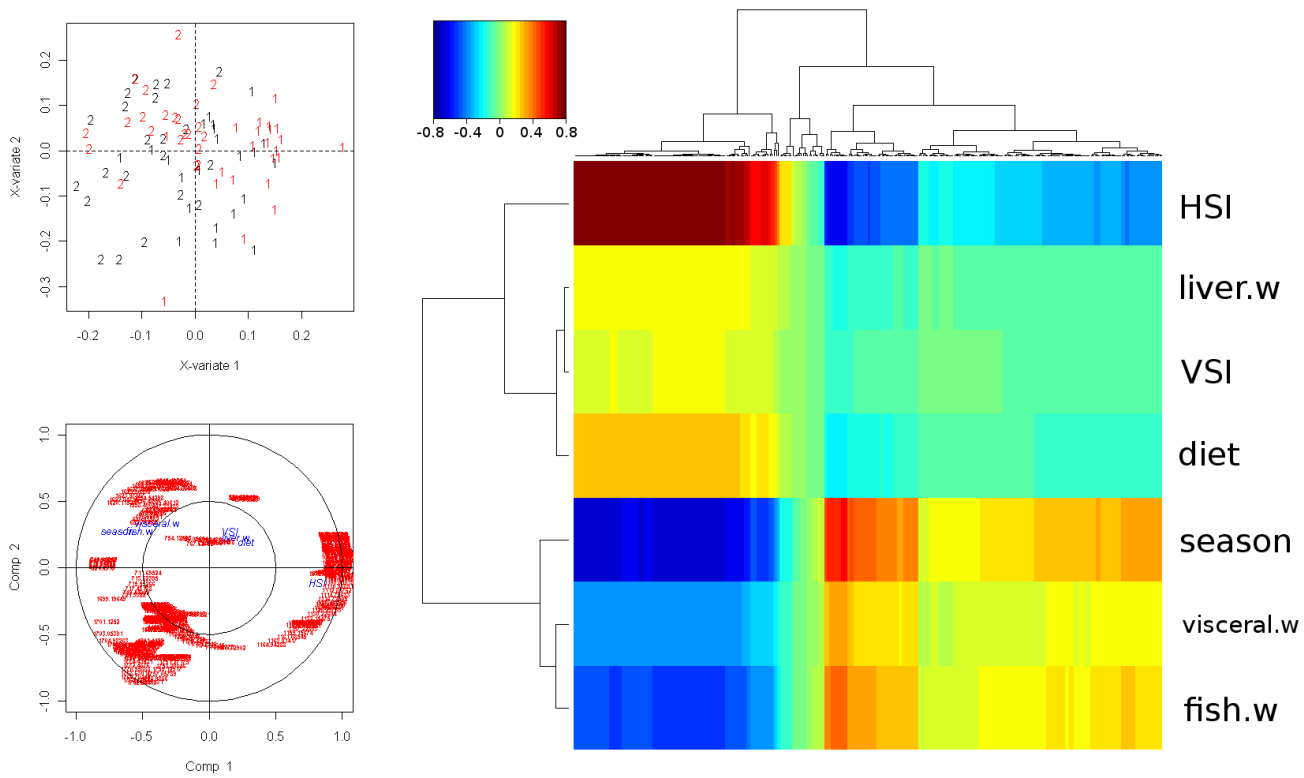

Figure 7.16 – Results of the sPLS analysis using the FTIR dataset as X-matrix and the performance parameters as Y-matrix. On the left, sample scores according to the first two components (above) and correlation circle containing both the selected variables from the FTIR dataset and all the variables of the Y-matrix (below). Samples are represented by symbols according to the sampling time ("1" for 1st sampling, "2" for 2nd sampling) and coloured according to diet (black for CTRL, red for WF). On the right, heat map showing how selected FTIR variables and all performance variables co-variate.

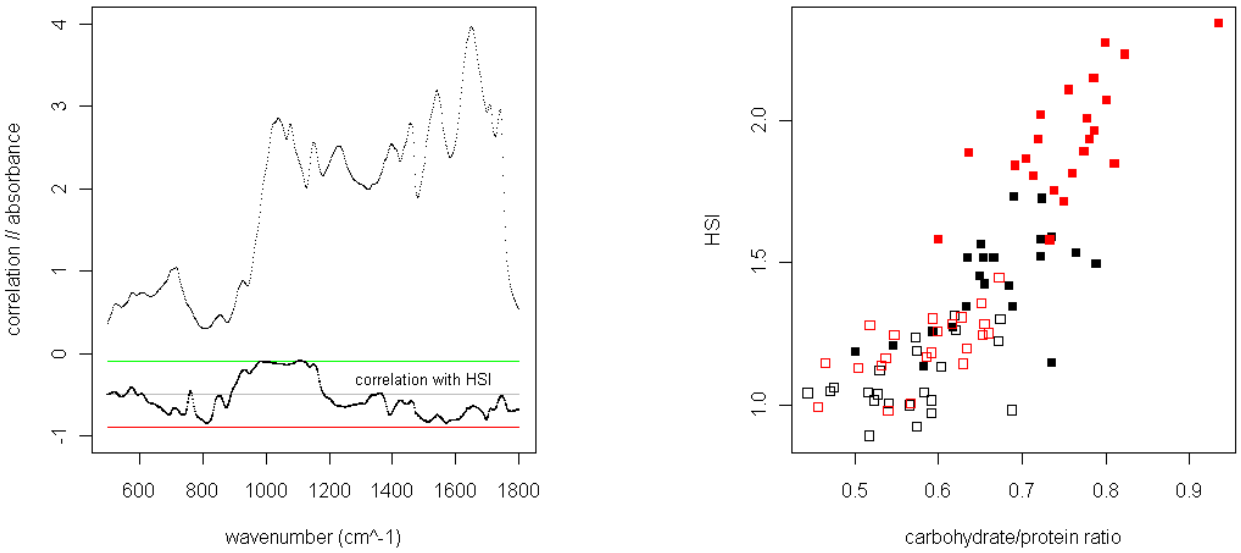

Figure 7.17 – On the left, plot showing a representative spectrum, as well as the correlation of the HSI with each of the frequencies, across all samples (green line denotes ρ = 0.8, red line denotes ρ = -0.8). On the right, scatter plot showing how the HSI relates to the amount of carbohydrates estimated by FTIR. Sampling time is identified with a symbol (closed box for 1st sampling, open box for 2nd sampling), while diet is identified with a colour (black for CTRL, red for WF).

7.3.4. Proteome-metabolome correlations

To look at correlations between the DIGE and FTIR datasets, we started by performing a reference metric multidimensional scale embedding of the samples using both datasets together and then re-doing the embeddings with the two datasets in separate to observe how the partial information distorts the inter-sample similarity relations (Figure 7.18). As it can be seen, there is a good consistency between the datasets, with disagreements generally occurring within the same diet and with the proteomic dataset displaying a better natural separation between the two diets (compared to the metabolomic dataset).

Afterwards, we applied sPLS in regression mode, with both datasets alternating between the role of X-matrix and Y-matrix. In Figures 7.19 and 7.20, plots of the scores and variable correlations for the first two components can be seen. It is interesting to note that, although the projections were obtained by an unsupervised method, the samples are very well separated according to the diet factor, which implies that the obtained latent variables that better explain the proteomic data from the metabolomic data (and vice-versa) also explain the differences between the diets.

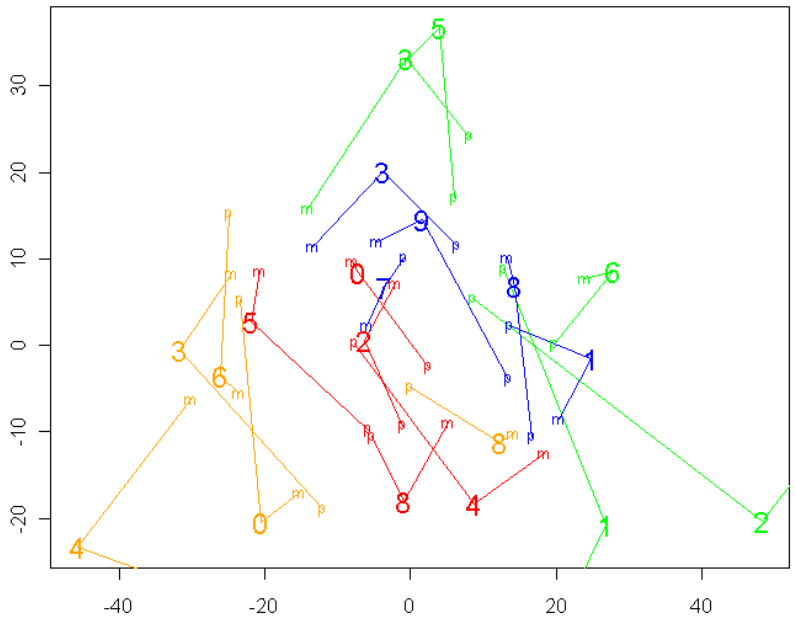

Figure 7.18 – Two-dimensional embeddings of the fish using both the DIGE and FTIR datasets to show sample dissimilarity, using Euclidian distance. The numbers represent the different fish (coloured according to tank: red and orange for CTRL, blue and green for WF) and their dis(similarity) relations according to their distance in both datasets. The letters "m" and "p" show the position the points would have if we just took into account either the metabolomic or the proteomic data, respectively.

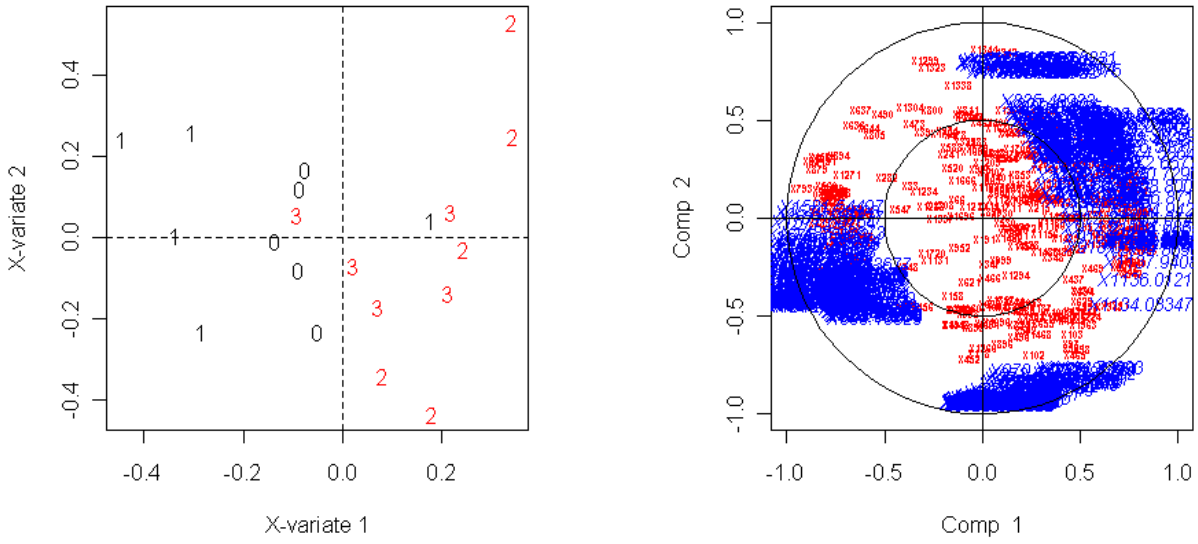

Figure 7.19 – Results of the sPLS analysis by using the DIGE dataset as X-matrix and the FTIR dataset as Y-matrix (i.e. try to predict the metabolome response from the proteome response). On the left, sample scores along the first two components. Each number represents a tank ("0" and "1" for CTRL tanks, "2" and "3" for WF tanks) and each colour represents a diet (black for CTRL and red for WF). On the right, correlation circle plot showing the correlation of each of the selected variables from the X and Y matrices with the first two components.

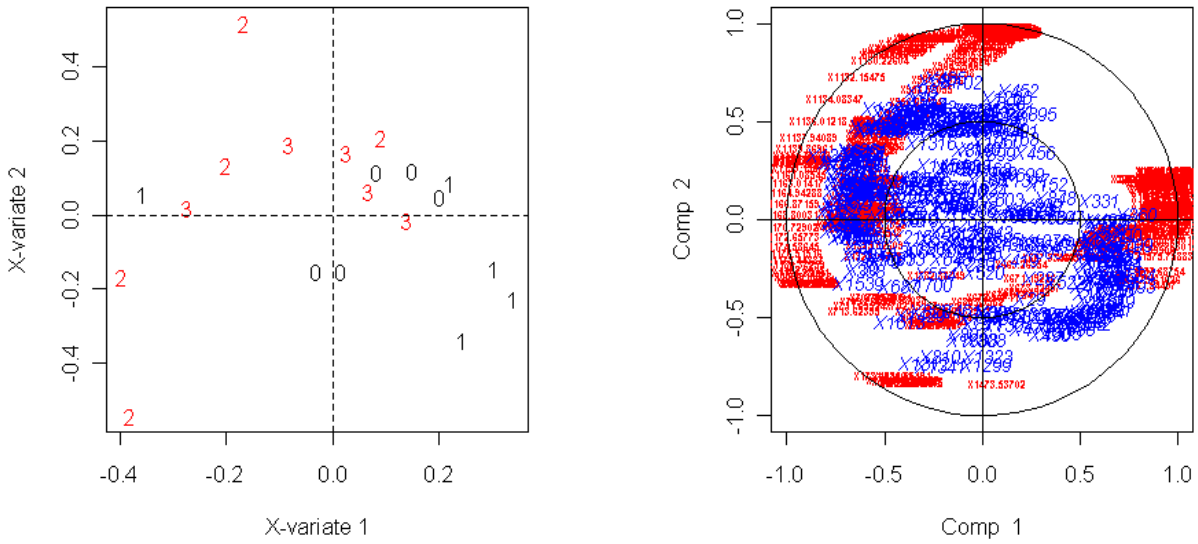

Figure 7.20 – Results of the sPLS analysis by using the FTIR dataset as X-matrix and the DIGE dataset as Y-matrix (i.e. try to predict the proteome response from the metabolome response). On the left, sample scores along the first two components. Each number represents a tank ("0" and "1" for CTRL tanks, "2" and "3" for WF tanks) and each colour represents a diet (black for CTRL and red for WF). On the right, correlation circle plot showing the correlation of each of the selected variables from the X and Y matrices with the first two components.

Additionally, we also applied regularized canonical correlation analysis (rCCA) as an alternate way of observing how the DIGE and FTIR datasets co-variate (Figure 7.21), obtaining similar results.

For each of these analyses, it is possible to construct a bipartite graph where each node represents either a DIGE or a FTIR variable and each edge represents the degree of association between two nodes. Once we identify most of the relevant protein spots, these will provide useful clues to understand how proteome can affect the metabolome (or vice-versa) and how these are co-affected by both seasonal challenges and dietary factors. In Figure 7.22, we can see small-resolution examples of the obtained networks, with vectorial versions being provided as Supporting information.

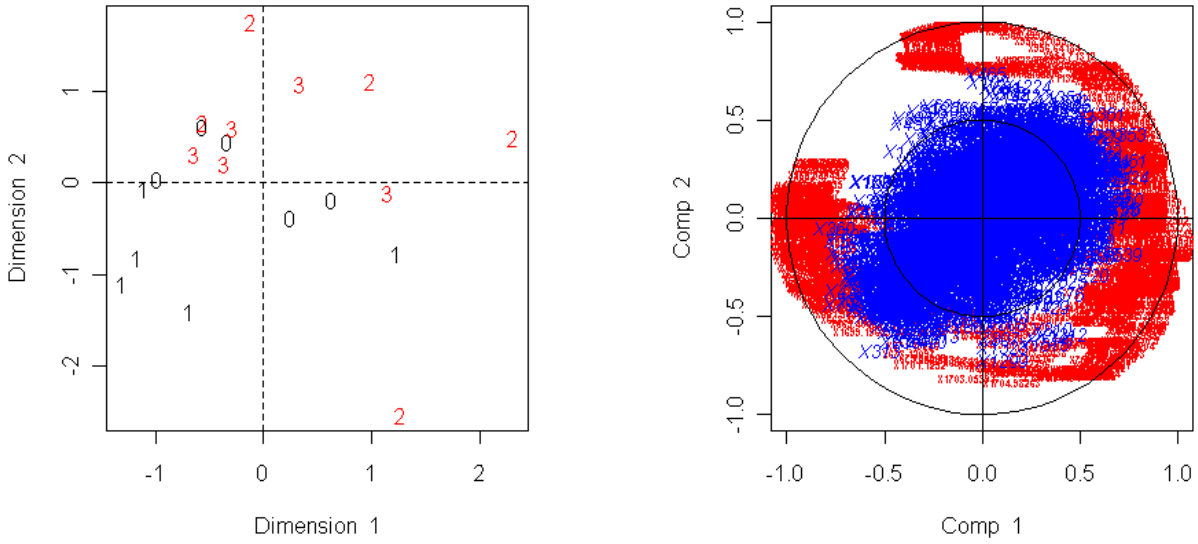

Figure 7.21 – Results of the rCCA analysis using the DIGE and FTIR datasets. On the left, sample scores along the first two components. Each number represents a tank ("0" and "1" for CTRL tanks, "2" and "3" for WF tanks) and each colour represents a diet (black for CTRL and red for WF). On the right, correlation circle plot showing the correlation of each of the selected variables from the X and Y matrices with the first two components.

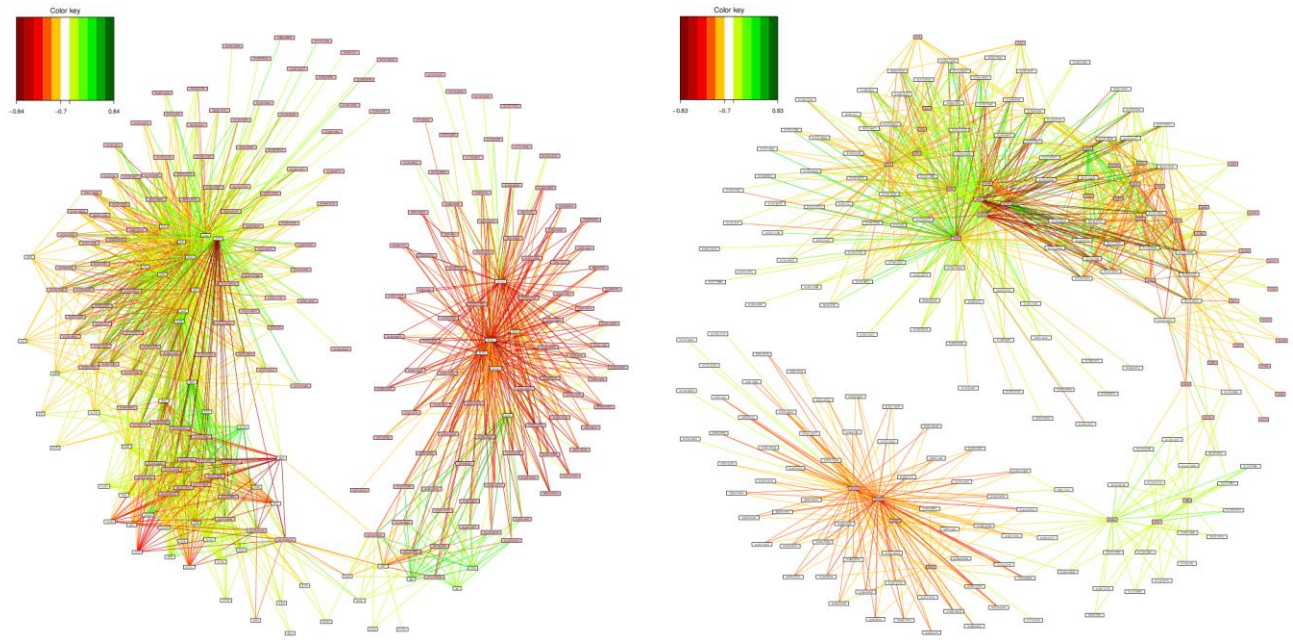

Figure 7.22 – Relevance association networks that show which variables correlate, either in the subspace defined by applying sPLS to predict the metabolomic dataset from the proteomic dataset (left) or in the subspace defined by applying sPLS to predict the proteomic dataset from the metabolomic dataset (right), and how. Relevance relations were restricted to those above 0.7 or below -0.7, to reduce visual clutter.

7.4. Final considerations

Over the course of this trial, we observed seasonal changes in performance parameters and in terms of metabolic fingerprint that suggest fish were challenged, displaying a progressive loss of hepatic carbohydrate stores. Nevertheless, under laboratory conditions, fish still displayed somatic growth over this period and none of the fish displayed all of the symptoms of "winter disease". Still, some of the fish, particularly in the CTRL group, displayed some possible early signs of the metabolic dysfunction associated to the "winter disease", namely hepatic accumulation of lipids.

Regarding the fortified WF diet, all obtained information points towards a generally beneficial effect in terms of performance and nutritional/metabolic status, with WF fish consistently displaying higher liver weight and HSI, at both sampling points, mostly due to a difference in the hepatic abundance of carbohydrates. The WF diet seems to have a mitigating effect regarding the seasonal challenge, not only in terms of carbohydrate depletion, but also in terms of the observed accumulation of lipids in the later sampling. This suggests this diet is indeed a good candidate for a "golden standard" diet against which to compare alternate (and possibly more cost-effective) formulations

Finally, we have confirmed that the use of metabolic and proteomic profiling technologies can be highly useful in such a context to obtain untargeted information on the nutritional and metabolic status of gilthead seabream, and we expect the information we will soon obtain in terms of protein identity to extend the preliminary results obtained so far. The next trial within this line of research will also take advantage of these technologies to assess the relative mitigating effect of several distinct possible formulations on this seasonal challenge, using the developed WF diet as "golden standard", to enable optimization of diets for this purpose taking into account both performance and cost.

Supporting information available

This information is available in the CD-ROM version of this dissertation.

Conclusions and Future Perspectives.

As already pointed out, the purpose of this work was to study the impact of short and long-term stressful situations on fish metabolism and post-mortem decomposition processes (and, therefore, their effect on fish welfare and quality), as well as the interacting effect of dietary factors, using proteomic technologies. For this effect, two different tissues were targeted: skeletal muscle, which constitutes the main edible part of fish; and the liver, which is the central organ in metabolism and regulation of metabolic processes.

Over the course of this work, several trials were focused on gilthead seabream muscle tissue, particularly revolving around immediately pre-mortem factors and their impact on decomposition processes and downstream quality attributes. Results have confirmed that it is possible to isolate the sarcoplasmic fraction of the muscle proteome in a reproducible way, without introducing levels of intra-batch noise and inter-batch bias that would compromise the measurement of proteomic responses (Chapter 2). Furthermore, the application of this methodology on the study of harvesting/pre-slaughter stress demonstrated a strong impact of this type of pre-mortem stress on the sarcoplasmic proteome, clearly hastening the transition from an early post-mortem profile to a late post-mortem profile (Chapter 3). Also, despite the fact that stress factors seem to induce broader and deeper changes than dietary factors, in terms of proteomic response, it also became clear that these (namely, glycerol supplementation) can positively affect gilthead seabream muscle, ensuring improved pre-mortem energy reserves and possibly having a mitigating effect on the impact of stress factors (Chapter 4).

Regarding the trials focused on the hepatic proteomic response to stress and dietary factors, results generally show a much higher sensitivity (compared to muscle tissue) towards external stimuli, with the stress factors, again, displaying a higher impact than dietary factors. Comparing gilthead seabream to Senegalese sole, it becomes clear that, despite the similarities between these two models regarding the hepatic proteome response to long-term stress, in terms of affected pathways, there is little overlap in terms of specifically affected proteins, pointing towards species-specific idiosyncrasies in their response to stress (Chapters 5 and 6). In fact, even the application of slightly different stressors on the same model (gilthead seabream), within the same experiment, has shown that the specific type and

duration of stressor applied also influences the proteomic response (Chapter 5). Finally, we have shown that data obtained with transmissive FTIR spectroscopy of gilthead seabream liver provides profiling information which is both consistent and complementary to proteomics-based profiling, enabling the study of seasonal challenges on fish metabolism, as well as the directed, evidence-based formulation of special fortified diets to mitigate the impact of these seasonal challenges (Chapter 7).

Concluding, this work has shown that both pre-mortem and chronic stress have a significant effect on muscle and hepatic proteomes of fish such as gilthead seabream and Senegalese sole. Interestingly, in some of the experiments, dietary factors were shown to partially mitigate some of the effects of induced stress (both at the level of muscle and hepatic tissue). Also, despite differences observed for stress and dietary factors on muscle proteome, few effects were seen in terms of impaired quality attributes (texture, aroma, appearance), which can be seen as positive, as it illustrates gilthead seabream's robustness, in terms of muscle phenotypical traits, but, on the other hand, also makes gilthead seabream a challenging model to study welfare-quality interactions.

Still, it is undeniable that these factors have an impact on fish welfare and metabolism, which is attested by the proteomic results described in this dissertation. Also, it is important to note that, although no differences were seen in terms of quality attributes measured within a relatively short period after slaughtering, the degradation-hastening effect of pre-slaughter (and slaughter) stress might still affect the rate of quality deterioration, possibly affecting quality attributes when assessed at later time-points or even inducing shorter product shelf-life. For this reason, it seems important that further studies regarding fish quality using proteomic technologies should explicitly incorporate "storage time" as experimental parameter. In the case of gilthead seabream muscle proteomics, it became clear that, although the soluble (i.e. sarcoplasmic) fraction can provide important information (even at the level of myofibril degeneration), interpretation of the results in a reliable way would be improved through a parallel analysis of the total proteome (i.e. using an extraction method that covers both the sarcoplasmic and myofibrillar fractions), which would help to distinguish between cases of differential expression *vs.* differential solubility.

In terms of possible future experimental setups within this context, it would make sense to refine the knowledge regarding the impact of stress factors on the specific endogenous proteolytic systems (e.g. caspases, calpains, cathepsins, proteasome), through factorial experiments that expand the model of Chapter 3 (i.e. pre-slaughter anesthesia vs pre-

slaughter stress) with slaughter stress factors and specific proteolytic inhibitors. Regarding the study of the impact of nutritional factors on muscle proteome (as seen in Chapter 4), an improvement of the methodology would require the introduction of an explicit "challenge" factor, allowing separability of the intrinsic nutritional effects from the nutritional-stress interaction effects.

Looking at the possibility of using purpose-focused dietary formulations within the context of stress mitigation (Chapter 7) or finishing strategies to improve the nutritional/quality profile of gilthead seabream (Chapter 4), this dissertation demonstrated its feasibility, also demonstrating the usefulness of both proteomic and metabolomic tools for the purpose of profiling. Future trials will benefit from information obtained, exploiting the use of both a negative (sub-standard diet) and positive (fortified diet) controls to understand the relative importance of the different supplementations. Nevertheless, one important lesson to take is that nutritional factors cannot fully prevent the effects of chronic stress on both welfare and metabolism, which underlines the utmost importance of harm reduction and management of avoidable sources of stress in aquaculture.

Regarding the possibility of obtaining specific and reliable biomarkers of welfare impairment or metabolic dysfunction, it seems like a complicated task, given the observed importance of e.g. the species (Chapters 5 and 6) and the type/duration of stressor (Chapter 5) on the specific hepatic response. In fact, this dissertation illustrates that even the "heat shock protein" (hsp) family of proteins, which are often considered classical indicators of cellular stress, seem to demonstrate tissue-specific differences in terms of expression of different hsp isoforms in response to stress and nutritional factors (Chapters 4, 5 and 6). It is clear that context is highly important. Even the known classification of hsp isoforms as "stress-inducible" *vs.* "constitutive" does not address all the observations expressed in this dissertation, which suggests the need for further study here (a concern already expressed by other people). On the other hand, results described in this work suggest several interesting candidates for further targeted studies to understand to which extent they can provide insight as measures of welfare or distress states, including DJ-1 protein, Raf kinase inhibitor protein, phosphohistidine phosphatase, among other regulatory proteins that have been shown to be affected by nutritional and stress factors. This underlines the usefulness of proteomics (and other screening or –omics technologies in general) within the context of aquaculture, particularly in the area of fish welfare, as a sensitive tool to detect signs of physiological/cellular stress or metabolic shifts, even when these might not be apparent through other targeted means.

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