Chapter 11
Farmed and Wild Fish

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11.1 Aquaculture and Proteomics

Aquaculture, or the farming of aquatic animals in enclosures or confinements, is an ancient practice used by man to ensure a constant supply of fresh seafood. The origin of aquaculture is credited to Chinese populations, and flourished well before 1,000 BC. The earliest document describing aquaculture, *The Classic of Fish Culture* by Shan Li, dates back to 475 BC, and reports the structure of ponds, the method of propagation of the common carp, and the growth of fry. Other sources indicate the bas-relief found in the tomb of Akihetep in Egypt, dating back to 2,500 BC, as one of the most ancient pieces of evidence of this practice. Here, a man is depicted while gathering tilapia, catfish, and other fish from a square-edged enclosure; however, whether this may or may be not be a pond is still debated. Along the centuries, men have kept fish in ponds and enclosures both for religious purposes or as reservoirs of fresh seafood, but farming techniques did not evolve substantially until the latter half of the nineteenth century and the beginning of the twentieth, when marine fish farming started to gain interest. Then, in the late 1960s, with the advent of modern technologies and materials such as plastic and fiberglass, aquaculture underwent a massive expansion, giving rise to the current widespread and extensive market. New and more efficient techniques were developed, enabling large-scale farming of freshwater and saltwater fish, from inland tanks to off-shore floating cages, and many new species were brought into culture (C.E. Nash 2011). The extensive diffusion of aquaculture enterprises was also spurred by the oil crisis, that made fish capture less economical, by the declining fish population due to uncontrolled fishing, and by the considerable increase in the global population demand for aquatic food products. In this respect, aquaculture can significantly contribute to preservation of marine biodiversity, and

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promote a more rational exploitation of marine resources. Currently, aquaculture contributes to about half of the fish consumed worldwide, reaching a production of over 50 million tons in 2006 (FAO, the state of world fisheries and aquaculture 2008) (www.FAO.org), with a growth higher than any other animal food-producing sector. Recently, driven also by the oncoming market saturation for some of the most popular species, fish farmers are increasingly interested in quality improvement and certification, and in attracting consumer interest in higher-level products. The evolution of aquaculture techniques and the considerable increase in our knowledge of fish physiology, health, and nutrition can contribute to the production of fish with higher quality, safer to eat, and nutritionally more similar to wild fish, and contribute to lowering the production costs for the farmer without compromising quality.

Sensorial, textural, and nutritional properties of farmed fish depend on many factors, and the influence of farming conditions on the quality of the final product is the subject of an active and lively field of research. During farming, fish are exposed to a plethora of stimuli and environmental constraints that differ from those of their free-ranging counterparts: diet, feeding regimens, physical activity, crowding, and water quality (salinity, temperature, currents, number and variety of microbial pathogens) can be quite different from those experienced in the wild. Moreover, their extent and impact can differ significantly depending on the fish species and the farming techniques, which range from inshore recirculated tanks, to offshore cages, to rearing in coastal lakes and lagoons in semi-wild conditions. Under a nutritional perspective, investigations of quantity and composition of fatty acids have played a major role in studies assessing the impact of aquaculture techniques on fish species of economical interest. In fact, these are raised on artificial diets and have frequent access to unlimited food sources with concomitant reduced physical activity, leading to an increased amount of fat in farmed as compared to wild fish, with negative consequences on sensorial qualities and shelf-life. However, the nutritional quality of farmed fish products is generally considered to be equally elevated due to the presence of polyunsaturated fatty acids, including omega 3, which are beneficial to the heart and the circulatory system of humans. As far as proteins are concerned, farmed fish are traditionally considered as having the same quantity and quality of protein as wild fish in terms of gross composition. Nevertheless, it is now well known that farming can modify the protein expression profiles of several tissues (Addis et al. 2010a; Gornati et al. 2004a, b; Martin et al. 2001, 2003; Forné et al. 2010; Hall et al. 2003; Martinez et al. 1995; Martinez and Pettersen 1992). Being able to track and detect variations in protein expression against similar genetic backgrounds, proteomics holds considerable promise in unveiling the alterations and differences occurring between wild and farmed fish, and in understanding the underlying physiological and pathological mechanisms. The knowledge gathered by proteomics has the potential to identify and help understand these factors, and it is hoped, to provide the means to control them, enabling production of higher quality fish (Forné et al. 2010; Melamed et al. 2002; Piñeiro et al. 2003).

This chapter provides an overview of the proteomic studies that have directly assessed the impact of farming on fish tissues, focusing on muscle, liver, blood serum, kidney, and testis proteomes. Table 11.1 provides an overview of the methods applied for assessing changes in protein expression, and Table 11.2 summarizes the most relevant findings that emerged from these studies.

Table 11.1 Overview of proteomic methods used for assessing expression changes induced by farming on fish tissues

Species	Factor	Lysis conditions	Buffer conditions	Separation	Identification	References
Muscle						
S. aurata	Farming	Scalpel fragmentation and homogenization by bead beating	8 M urea, 2% CHAPS and 0.5% ampholines	1DE: SDS-PAGE 2DE: 24 cm, IEF on pH 3–10 NL strips, SDS-PAGE on 10–18% gradient gels; Coomassie and MS-compatible silver	MALDI-MS and LC-MS/MS (ESI-Q-TOF)	Addis et al. 2010a
S. aurata	Farming	Mechanical homogenization of tissue	50 mM KCl, followed by extraction in 300 mM KCl, 0.1 mM DTT, 150 mM phosphate buffer, 5 mM mgATP, dialysis and resuspension in 500 mM KCl	1DE: SDS-PAGE 2DE: IEF on pH 3–10 rods, SDS-PAGE on 15% gels; Coomassie	Peptide mapping and Western immunoblotting	Carpené et al. 1998
G. morhua	Farming	Tissue scraping and homogenization in ice	ReadyPrep Sequential Extraction Kit (Bio-Rad)	2DE: IEF on 11 cm, pH 3–10 strips, SDS-PAGE on 12.5% gels; Silver	None	Martinez et al. 2007
D. labrax	Farming	Fragmentation and mincing with a Stomacher	Water	1DE: SDS-PAGE Microfluidic separation on an Agilent 2100 Bioanalyzer	LC-MS/MS (LCQ ion trap)	Monti et al. 2005
G. morhua	Farming	Sonication	30 mM HEPES, 5 mM K_2HPO_4, 0.25 mM sucrose, 0.5 mM EDTA, 0.5% ASB14 and protease inhibitor cocktail	2DE: IEF on 13 cm, pH 5–8 strips, SDS-PAGE on 12% gels; Coomassie	LC-MS/MS (ESI-Q-TOF)	Olsson et al. 2007
Liver						
O. mykiss	Starvation	Dounce homogenization	9 M urea, 2% CHAPS, 25 mM Tris HCl pH 7.5, 3 mM EDTA, 50 mM KCl, 50 mM DTT, 2% ampholines and protease inhibitors	2E: IEF on 7 cm 4–7 strips; SDS-PAGE on 10–15% gels; Coomassie	MALDI-MS	Martin et al. 2001

(continued)

Table 11.1 (continued)

Species	Factor	Lysis conditions	Buffer conditions	Separation	Identification	References
O. mykiss	Dietary substitution	Dounce homogenization	9 M urea, 2% CHAPS, 25 mM Tris HCl pH 7.5, 3 mM EDTA, 50 mM KCl, 50 mM DTT, 2% ampholines, protease inhibitors	2DE: IEF on 7 cm 4–7 strips; SDS-PAGE on 10–15% gels; Coomassie	MALDI-MS	Martin et al. 2003
S. aurata	Handling and crowding	Ultra-Turrax homogenization and sonication	7 M urea, 2 M thiourea, 4% CHAPS buffer, 0.3% DTT, 1% ampholines, protease inhibitors	2DE: IEF on 11 cm 3–10 strips; SDS-PAGE on 12% gels; Coomassie	LC-MS/MS (ESI-Ion trap)	Alves et al. 2010
S. aurata	Temperature	Pulverization in liquid nitrogen	7 M urea, 2 M thiourea, 2% CHAPS, 80 mM DTT, 50 mM Tris HCl, pH 7.4	2DE: IEF on 24 cm, pH 3–11 NL strips, SDS-PAGE on 12.5% gradient gels; Coomassie	MALDI-MS/MS and LC-MS/MS (CapLC-QTOF)	Ibarz et al. 2012
Serum						
P. fluviatilis	Domestication	Dilution in buffer	7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% ampholines	2DE: IEF on 24 cm, pH 4–7 strips; SDS-PAGE on 12.5% gels; RuBPs	LC-MS/MS (CapLC-QTOF2)	Douxfils et al. 2011
Testis						
S. senegalensis	Domestication	Mechanical distruption and sonication	Sonication in TNE buffer, addition of 1% NP-40, 1 mM PMS, and 2 mM DTT, protein precipitation and resuspension in 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris HCl, pH 8.5	2-D DIGE: IEF on 18 or 24 cm, pH 3–10 strips; SDS-PAGE on 12% gels	MALDI-MS	Forné et al. 2009
				CyDye labeling	IT-MS/MS (LCQ MS)	
Kidney						
S. aurata	Bacterial infections	Scalpel fragmentation and homogenization by bead beating	8 M urea, 4% CHAPS, 0.5% ampholines	2DE: IEF on 24 cm, pH 3–10 NL strips, SDS-PAGE on 10–18% gradient gels; Coomassie	MALDI-MS and LC-MS/MS (ESI-Q-TOF)	Addis et al. 2010b

Table 11.2 Summary of the most relevant findings emerged from proteome studies on farmed fish tissues

Species	Factor	Tissue	Main observations	References
G. morhua	Farming	Muscle	Differences in protein expression and protein degradation. Presence of additional spots between 100 and 50 kDa in wild, and between 45 and 35 in farmed cod Differential expression of energy metabolism enzymes with mitochondrial alterations in farmed individuals	Martinez et al. 2007; Olsson et al. 2007
D. labrax	Farming	Muscle	Overexpression of carbohydrate metabolism enzymes and underexpression of proteins involved in muscular development as a consequence of farming	Monti et al. 2005
S. aurata	Farming	Muscle	Only minor differences due to environmental factors in farmed vs wild individuals of comparable size. Significant overexpression of metabolic versus structural proteins in smaller versus larger farmed individuals	Addis et al. 2010a
S. aurata	Handling and crowding	Liver	Alterations in expression levels of proteins involved in aminoacid, carbohydrate and lipid metabolism, folding, signaling and cytoskeleton upon exposure to stress	Alves et al. 2010
O. mykiss	Dietary substitution	Liver	Increase in general catabolism and in protein turnover, downregulation of Apolipoprotein AI, downregulation of structural proteins, and alteration of nitrogen metabolism following dietary substitution with vegetable sources	Martin et al. 2003
P. fluviatilis	Domestication	Serum	Differences in abundance of the C3 complement component, transferrin and Apolipoprotein E in F4 fish versus F1 fish. Increase in production of immune response proteins in response to chronic stressors following domestication	Douxfils et al. 2011
S. aurata	Bacterial colonization	Kidney	Alterations in expression of mitochondrial enzymes and oxidative stress responses upon bacterial colonization	Addis et al. 2010b
S. senegalensis	Domestication	Testis	Alterations in protection against oxidative stress, protease inhibition mechanisms and iron and glucose metabolism in captive F1 fish versus wild fish	Forné et al. 2009

11.2 Muscle

Skeletal muscle is the edible part of fish, and is therefore the most interesting tissue from a commercial perspective. In addition, it is one of the most easily accessible for reproducible sampling and is available for analysis along the whole production chain, even in processed products. Therefore, the identification of traits enabling us to differentiate fish produced by means of aquaculture techniques in this tissue matrix would enable its monitoring "from the farm to the fork."

Muscle composition contributes strongly to quality, inasmuch as number and integrity of muscular fibers have a considerable influence on texture, elasticity, and water-holding capacity (Johnston 1999). The structural unit of muscle is the sarcomere, a supramolecular contractile assembly that accounts for about half of the total muscle proteins. This machinery is mainly composed of myosin, actin, tropomyosin, troponins, and myosin light chains, the giant proteins titin and nebulin, and several other proteins having structural or regulatory functions. Another relevant portion of the total muscle protein composition is represented by sarcoplasmic proteins, which account mainly for glycolysis and energy metabolism, providing the "fuel" for contraction. The characterization of muscle tissue proteomes and their comparative evaluation in farmed and wild fish can provide useful information on the influence exerted by farming practices, environmental variables, diet, and other factors, and assist the improvement of aquaculture productions (Reddish et al. 2008). In fact, confinement, size, diet (Kiessling et al. 1991), crowding (Gornati et al. 2004a, b), amount and intensity of physical activity (Johnston and Moon 1980; Totland et al. 1987), and water quality and temperature (Johnston et al. 1998) can all have significant effects on protein expression levels, also depending on the farming technique used, on the quality of feeds, and on dimensions and exposition to marine currents within rearing spaces. Several economically relevant farmed species have been subjected to proteomic studies of muscle tissues aimed at assessing the variations induced by farming, including Atlantic cod (*Gadus morhua*) (Martinez et al. 2007; Olsson et al. 2007), Mediterranean sea bass (*Dicentrarchus labrax*) (Monti et al. 2005), and Gilthead sea bream (*Sparus aurata*) (Addis et al. 2010a). Figure 11.1 illustrates a representative 2D-PAGE map of farmed sea bream muscle tissue.

11.2.1 Atlantic Cod: Molecular and Structural Alterations Induced by Farming

Atlantic cod (*G. morhua*) is not yet considered to be a fully domesticated species. In fact, numerous significant differences are observed between farmed and wild-captured Atlantic cod, including size and shape of the head, hepatosomatic index, skin pigmentation and flesh discoloration, also accompanied by mineral-induced melanin deposition in blood vessels located between muscle

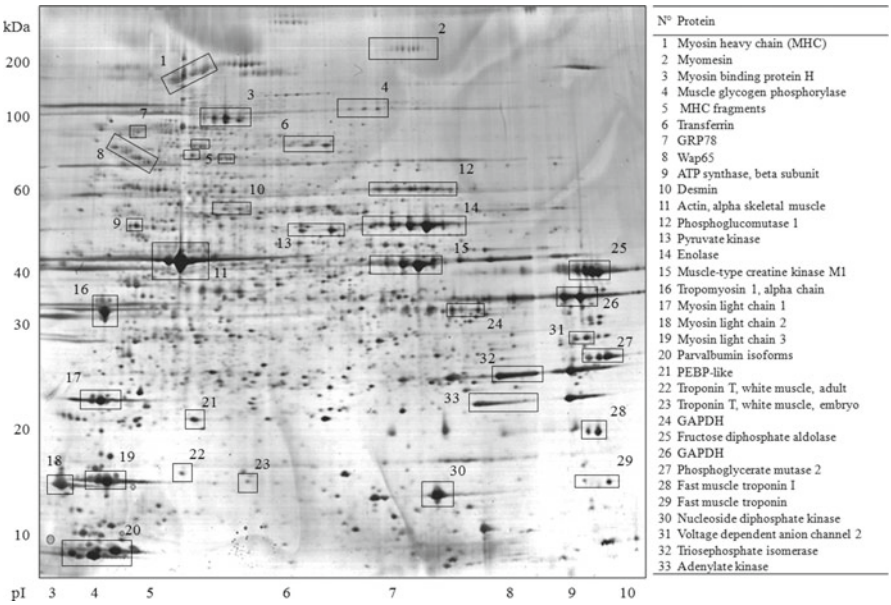

Fig. 11.1 2-D PAGE map of farmed *Sparus aurata* muscle. *Numbers* indicate the main protein groups subjected to mass spectrometry identification. Protein identities are reported on the *right*. The following conditions were applied: IEF on 24 cm, pH 3–10 IPG strips; SDS-PAGE on 8–16% polyacrylamide gradient gel; silver staining. Protein identification was performed by LC-MS/MS on a nanoLC-nanoESI-Q-TOF instrument

segments (Cooper and Midling 2007). Muscle structure and storage characteristics of farmed cod differ from those observed in wild individuals (Olsson et al. 2007). In addition, post-mortem degradation has been shown to be faster in farmed than in wild cod muscle (Ofstad et al. 1996). As the farmed and the wild cod are not yet genetically distinct, the most likely sources for these differences are environmental.

11.2.1.1 Atlantic Cod: Molecular Alterations

Martinez et al. (2007) examined the suitability of muscle proteins to differentiate farmed from wild cod by means of two-dimensional electrophoresis. Neither farming conditions nor size of fish were detailed in this work. Protein extracts were obtained from wild and farmed cod by homogenization and sequential extraction with the ReadyPrep Sequential Extraction Kit from Bio-Rad Laboratories, generating a Tris fraction and a CHAPS-urea fraction. Proteins from pools of five individuals were separated using 11-cm, pH 3–10 Immobiline strips for the first dimension, and 12.5% polyacrylamide gels for the second

dimension, followed by silver staining. According to differential gel analysis, the main difference among wild and farmed fish profiles was the presence of additional spots between 100 and 50 kDa in wild fish, and between 45 and 35 kDa in farmed fish. Protein identification was not carried out; however, the authors suggested that some of these spots resulted from a differential expression induced by farming conditions, and others resulted from differences in the proteolytic degradation of high molecular weight proteins.

In general, this work indicated that a different protein expression and/or a different post-mortem degradation can be seen in farmed cod compared to wild cod muscle. As far as differential expression is concerned, this is in line with the numerous characteristics of farmed cod that deviate from those considered to be normal in the wild fish, including higher condition factor, larger liver, smaller head, and backbone malformations. Faster post-mortem deterioration in farmed fish is also a plausible option; other authors (Olsson et al. 2003) had already reported a faster post-mortem deterioration in farmed fish compared to wild fish. In farmed halibut, post-mortem degradation proceeds faster, and the flesh has lower functional properties than its wild counterpart. In the case of cod, this differential degradation is supported by the findings of Ofstad et al. (1996), who reported that farmed cod flesh is less firm and has a higher water content, lower functional properties, and a different muscle fiber structure. The factors responsible for these differences were suggested to be related to the post-mortem change of parameters such as pH, which is altered by food intake before death, or to the different abundance or expression of endogenous proteases involved in post-mortem muscle tenderization. Characterization of these proteins and their dynamics was indicated as an opportunity for improving farming conditions in order to obtain higher-quality fish with optimal muscle composition.

11.2.1.2 Atlantic Cod: Structural Alterations

In order to investigate the physiological mechanisms originating the phenotypic differences observed between farmed and wild cod, Olsson et al. (2007) performed a study using proteome analysis and electron microscopy, revealing that several metabolic disorders are present in muscle tissues of farmed Atlantic cod. Twenty farmed (2,663+/− 462 g) and nineteen wild cod (4750 +/− 870 g) were examined in this study. Protein lysates were obtained by sonication, and protein extracts were separated on 13-cm, pH 5–8 gradient strips. The second dimension was performed on 12% polyacrylamide gels, and spots were visualized using a commercial Coomassie blue stain. Upon differential analysis, 11 differentially expressed protein spots were detected between farmed and wild cod muscle, and were subjected to identification by LC-MS/MS with a Q-TOF instrument. Unfortunately, due to the lack of sequence information at the time of the analysis, only five of these produced viable data, and were identified as adenylate kinase, triosephosphate isomerase, aldolase, creatine kinase, and

isocitrate dehydrogenase. All these enzymes are involved in energy metabolism, and mitochondria are the main cellular organelle where these processes take place. In keeping with these observations, ultrastructural analysis of farmed cod muscle revealed a number of intrafibrillar mitochondria showing clear cristae malformations, with ring structures formed by the inner membrane. Furthermore, significant intermyofibrillar fat deposition was observed in muscle from farmed cod, whereas no interfibrillar muscle fat was present in wild fish. Disturbances in mitochondrial–oxidative capacity in skeletal muscle can play a role in insufficient oxidation of fatty acids, and therefore in their abnormal deposition in this tissue (He et al. 2001; Vogel 2001; Petersen et al. 2003). The enzymatic alterations identified in the proteome analysis revealed the existence of alterations in energy metabolism, and supported these observations.

11.2.2 European Sea Bass: An Integrated Microfluidic Approach for Assessing Muscle Protein Profiles in Farmed Individuals

The European sea bass (*D. labrax*) is currently one of the main products of European aquaculture, and is considered to be a fish of high economical value. Monti et al. (2005) evaluated the impact of farming on its muscle proteins by using a proteomic approach based on SDS-PAGE separation followed by LC/MS-MS for protein identification, combined with relative quantification of proteins by denaturing capillary electrophoresis in farmed and wild sea bass. The authors implemented this integrated approach based on microfluidic electrophoresis and proteomic procedures in order to enable rapid detection of protein expression changes induced by aquaculture on *D. labrax* muscle.

Information on size of fish, feeding regimens, or aquaculture conditions were not reported in this work. Protein extracts were obtained by fragmentation of muscle tissues, resuspension in MilliQ water, and mincing in a Stomacher 400 circulator. Water-soluble proteins were then subjected to either SDS-PAGE or microfluidic separation and detection on an Agilent 2100 Bioanalyzer instrument. Protein 50 and Protein 200 Plus chips were used to enable separation over a wide mass range. Such analysis revealed statistically significant and reproducible differences in the protein profiles of farmed and wild muscle tissues. SDS-PAGE analysis confirmed these differences, enabling the detection of at least nine proteins with a statistically significant variation between wild and farmed *D. labrax*. Upon LC-MS/MS analysis using an LCQ ion trap instrument, identification of the differentially expressed proteins was accomplished. Among these, many enzymes involved in carbohydrate metabolism were found overexpressed in farmed sea bass, such as glyceraldehyde-3-phosphate dehydrogenase (+92%) and aldolase (+75%). In addition, the impact on creatine kinase (−45%), nucleoside diphosphate kinase B (−70%), and parvalbumin (−22%) suggested an influence of aquaculture conditions on the muscular development of farmed fish.

11.2.3 Gilthead Sea Bream: An In-depth Comparative Muscle Protein Profiling of Differently Sized Wild and Farmed Individuals

The gilthead sea bream (*S. aurata*) is the leading product of Mediterranean aquaculture (Gómez-Requeni et al. 2004), accounting for 133,026 t in 2008 (FAO – Fisheries and Aquaculture Information and Statistics Service). Although farmed for centuries, this saltwater fish has seen a considerable increase in production during the past 20 years. To obtain information on muscle protein expression profiles in this species along the production cycle, and to compare them with those seen in wild individuals, a 2DE-MS study was performed in fish from four different offshore floating cage plants in Sardinia, Italy. Interesting insights on proteome variability and on protein expression profiles associated with fish size and environmental conditions were provided. Moreover, a detailed reference 2-D map of farmed *S. aurata* muscle proteins was generated (Addis et al. 2010a).

The study was carried out on a large number of wild and farmed Gilthead sea bream, taking into account several parameters such as season, water temperature and salinity, and size of fish. Protein extracts were generated from 540 samples collected for 2 years, by fragmentation of frozen tissue, resuspension in 8-M urea–2% CHAPS buffer, and homogenization by repeated cycles of mechanical bead beating. All extracts were subjected to SDS-PAGE, and total profiles were compared by computerized densitometric analysis. Interestingly, as opposed to farmed Atlantic cod and sea bass, there were no obvious differences in muscle protein profiles that could be directly related to the farmed or wild status. Protein abundance differences were mostly either individual or related to factors other than the wild or farmed condition. Although it might appear surprising, this was not so unexpected. In a dedicated study, Carpenè et al. (1998) did not find any significant differences in the protein composition of muscle from farmed and wild sea bream, although differences in the fatty acid profiles were seen. As also observed by Martinez et al. (2007), this may be related to several factors such as the farming conditions (offshore sea cages with low rearing densities vs. crowded inland tanks), as well as the selection that occurred during the years; in fact, sea bream have been reared in captivity for a longer time, and this species might be either more adapted or best suited to be kept in captive conditions for biological or lifestyle reasons, this as opposed to Atlantic cod, for which several evident morphological alterations are still seen in captivity fish.

However, although not directly linked to the farmed or wild status, several differences were indeed present among several sample groups. To assess the identity of these proteins and to shed light on the drive for their differential expression, farmed and wild fish samples were subjected to an in-depth 2DE study followed by MALDI-MS and nanoLC-ESI Q TOF-MS. As a further classification, fish were grouped into size classes for comparison. This further analysis revealed two important sources of protein expression variability: fish size and geographic location of the farming plant. Shorter farmed (ca. 16 cm) and longer wild (ca. 26 cm) sea bream displayed significant conserved differences in their protein profiles: in smaller fish

there was a higher expression of proteins involved in metabolism (sarcoplasmic proteins), whereas in larger fish there was a higher expression of proteins involved in structure and contraction (sarcomeric proteins). Interestingly, individual variations were also seen in proteins involved in seafood allergy, such as parvalbumins. Many of these observations were in line with those seen by Monti et al. (2005) in farmed sea bass. However, when differential comparison was carried out on wild and farmed fish having comparable sizes, these differences were no longer apparent. Instead, other proteins were seen to undergo statistically significant variations among groups, but in this case these were mainly associated with water temperature, such as warm temperature acclimation-related 65 kDa protein (Wap 65), to presence of infection or stressful conditions, such as transferrin, or to phosphorylation states, such as myosin binding protein H isoforms. Therefore, it must be kept in mind that in studies aimed at assessing differential expression profiles among farmed and wild fish muscle tissues, size and farming conditions are extremely significant factors to be considered in order to obtain meaningful results.

11.3 Liver

Liver functions are essential for life, as this organ performs a remarkable number of essential metabolic functions. Over 10,000 biochemical reactions are estimated to occur in this organ at any given time point, including carbohydrate, fat, and protein metabolism, storage of vitamins and minerals, and regulatory functions that control blood sugar and hormone levels. The liver is the primary organ for the synthesis of many different proteins, such as transferrin, fibrinogen, apolipoproteins, and globulins, of phospholipids and cholesterol, and of bile acid production and excretion (Gazzana and Borlak 2009). This organ is exposed to a wide range of xenobiotics and toxins, and is considerably influenced by factors such as diet, environment, and stress. All these things considered, as opposed to muscle, where less targeted studies have been performed in most cases, the consequences of aquaculture on the liver proteome have been studied by evaluating the effect of specific influencing factors. The species investigated for comparative wild/farmed liver protein expression include the rainbow trout (*Oncorhyncus mykiss*), where changes in protein profiles were studied under different feeding regimens (Martin et al. 2001, 2003), and the gilthead sea bream (*S. aurata*), where manipulation stress was evaluated (Alves et al. 2010). Figure 11.2 illustrates a representative 2-D-PAGE map of farmed sea bream liver tissue.

11.3.1 Rainbow Trout: Influence of Dietary Substitution with Vegetable Sources

Martin and coworkers (2003) applied proteomics to investigate the proteins undergoing differential expression in the liver of rainbow trout subjected to different feeding regimens, to gain insights to the biochemical processes occurring as a result of an altered

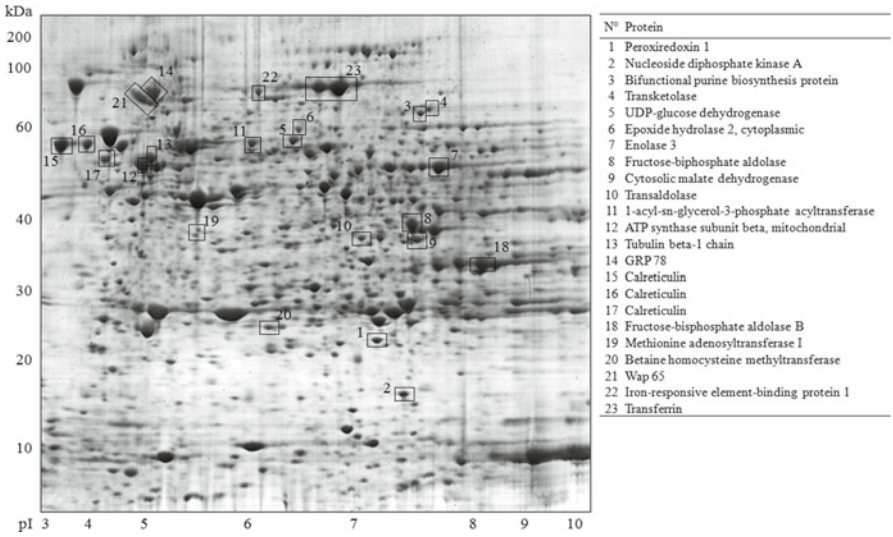

Fig. 11.2 2-D PAGE map of farmed *Sparus aurata* liver. *Numbers* indicate the main protein groups subjected to mass spectrometry identification. Protein identities are reported on the *right*. The following conditions were applied: IEF on 24 cm, pH 3–10 IPG strips; SDS-PAGE on 8–16% polyacrylamide gradient gel; colloidal Coomassie staining. Protein identification was performed by LC-MS/MS on a nanoLC-nanoESI-Q-TOF instrument

metabolism due to dietary substitution with vegetable sources. The two diets were formulated with ingredients commonly used in aquaculture feeds. Both contained fish meal and plant ingredients, but one (S) was higher in soybean meal, believed to induce adverse effects on different metabolic pathways, whereas the other (C) was higher in fish meal. The feeding trial was carried out for 12 weeks. Among other parameters, such as nitrogen metabolism, protein utilization, and selected enzyme activities, a 2DE analysis was performed on liver. For protein extraction, frozen tissue was homogenized in a 9-M urea–2% CHAPS buffer using a Teflon Dounce homogenizer. Soluble proteins were focused in a 7-cm pH 4–7 strip, followed by SDS-PAGE onto 10–15% gradient gels. Proteins were stained with colloidal Coomassie blue G250.

Following differential analysis of liver proteins from three fish fed diet S and three fish fed diet C, 33 protein spots were shown to be significantly altered in abundance between the diets, and 17 were successfully identified by MALDI TOF-MS. A variety of proteins including heat shock proteins (HSPs), enzymes, fatty acid binding protein, structural proteins, and proteins involved in the immune response were found to be differentially regulated. Structural proteins, such as keratin and tubulin, were downregulated in diet S, as well as HSPs. However, these displayed a differential expression of two HSP 108 isoforms. Many enzymes involved in anabolism were also downregulated, indicating an emphasis on catabolism relative to anabolism following dietary substitution with soybean. On the other hand, an increase in aldolase B and protein phosphatase A2 was detected in diet S compared to diet C, likely indicating an

increase in metabolism and general turnover of proteins. Finally, an isoform of Apolipoprotein AI-1 (Apo A I) was significantly downregulated in fish fed diet S. Apo AI is involved in the removal of free cholesterol to the liver for excretion.

In conclusion, by using proteomics, this study showed for the first time that rainbow trout fed with diets containing vegetable sources undergo alterations in nitrogen metabolism and display alterations in liver protein expression profiles. This study opens the way to using this technique for designing feeds with well-balanced cost/quality ratios, and to gain insights on compatibility of feed composition with fish metabolism.

11.3.2 Gilthead Sea Bream: Influence of Handling and Crowding as Chronic Stressors

Apart from the dietary regimen, farming conditions pose many other constraints that can have a significant impact on protein expression profiles when compared to life conditions experienced in the wild. Among the most important factors, crowding and repeated handling are considered to act as significant chronic stressors. Alves and coworkers (2010) applied comparative proteomics of liver to characterize the metabolic molecular indicators of chronic stress in gilthead sea bream. Liver proteome profiles were compared in fish stressed by repetitive handling (HND), in fish crowded at high stocking densities (HSD), and in control fish (CTRL). The experiment was performed using a pooling strategy in order to minimize the effect of individual variations; to level this out, four technical replicates were analyzed for each condition (Westermeier et al. 2008). Protein extracts were obtained in a buffer containing 7-M urea–2-M thiourea–4% CHAPS buffer, and homogenization was accomplished by Ultra-Turrax treatment followed by sonication. 2DE was carried out on 11-cm pH 3–10 immobiline strips, followed by SDS-PAGE on 12% polyacrylamide gels. Staining was performed with Coomassie G250. Protein identification was carried out by LC-ESI-Ion trap MS/MS.

As a result, a total of 164 differential spots was detected, including 79 upregulated and 85 downregulated by stress; 37 were unique to handled fish (HND) and 40 were unique to crowded fish (HSD). Of these, 12 were provided a protein identification, revealing the involvement of several cellular processes related to adaptation to stressful conditions: amino acid, carbohydrate and lipid metabolism, folding, signaling, and cytoskeleton.

Concerning proteins involved in energy metabolism, the expression levels of triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, alpha enolase, and pyruvate dehydrogenase were all affected by chronic stress. The authors explained this observation with the mobilization of energetic reserves involving the activation of liver glycogenolysis and gluconeogenesis to guarantee glucose supply to brain and muscles (Moyle and Cech 1996). Expression levels of fatty acid binding protein were also increased in stressed fish; this was explained as a mechanism for supporting higher rates of lipid consumption by liver cells in order to sustain the increased energetic requirements. Another observation was the decrease in calmodulin abundance upon chronic stress. The authors suggested that this might be an adaptation to chronically

increased intracellular calcium levels, induced by repetitive epinephrine stimulation, aimed at preventing the triggering of apoptotic mechanisms.

Interestingly, a halving of heat-shock cognate protein 70 (hsc70) was detected in both groups subjected to chronic stress compared to the control group. HSPs are well known to be involved in the cellular response to stress (Ali et al. 2003; Basu et al. 2001; Boone and Vijayan 2002). Although belonging to the HSP class, hsc70 is constitutively expressed in cells without stress, and the observation of its downregulation is in line with previous studies (Gornati et al. 2004b; Poltronieri et al. 2007). Another downregulated protein seen in both groups was glutamine synthase. This observation was also in line with previous findings, both in fish (Hopkins et al. 1995) and in mice (Vats et al. 1999). Finally, the increase in hemoglobin expression seen in stressed fish is also in line with previous data in various fish species (Acerete et al. 2004; Biron and Benfey 1994; Montero et al. 1999; Roche and Bogé 1996), as this protein is a known indicator of response to stress due to the increase in efficiency of oxygen transportation (Bone et al. 1996; Randall et al. 1997).

In summary, liver proteomics coupled with conventional stress monitors, such as plasma cortisol levels, enabled the identification of several proteins associated with stress in fish, that may hold promise for the development of more practical tests, such as ELISAs, useful for evaluating and monitoring the existence of chronic stress factors in aquaculture plants.

11.4 Blood Serum

Blood serum (or plasma) is a biological fluid of primary importance, typically considered to be a "river" of proteins and peptides that flows through cells and tissues of the whole organism (Zhou et al. 2005). As such, it conveys and collects proteins and peptides of the organs it bathes, reflecting variations occurring in physiological and pathological conditions in distant organs and body districts. In humans and mammals, blood serum has become the "gold standard" biological matrix for discovery and diagnosis, also for its ease of sampling and the ability to acquire numerous samples during a study. In fish, the difficulties in obtaining blood samples from live animals and the stress associated with sample collection have often outweighed the practical aspects of working with serum when compared to other tissues. However, this biological fluid is frequently collected and investigated when fish are to be sacrificed, and many proteomic studies on immune response have been performed using this biological matrix. Concerning proteomic studies aimed at investigating differences between farmed and wild fish, studies are less numerous, and are also mostly focused on the immune system. However, a good example of an application of blood serum proteomics to this topic is the study by Douxfils and coworkers (2011) on the influence of the domestication process on the immune status of Eurasian perch (*Perca fluviatilis*). Figure 11.3 illustrates a representative 2-D-PAGE map of farmed sea bream serum.

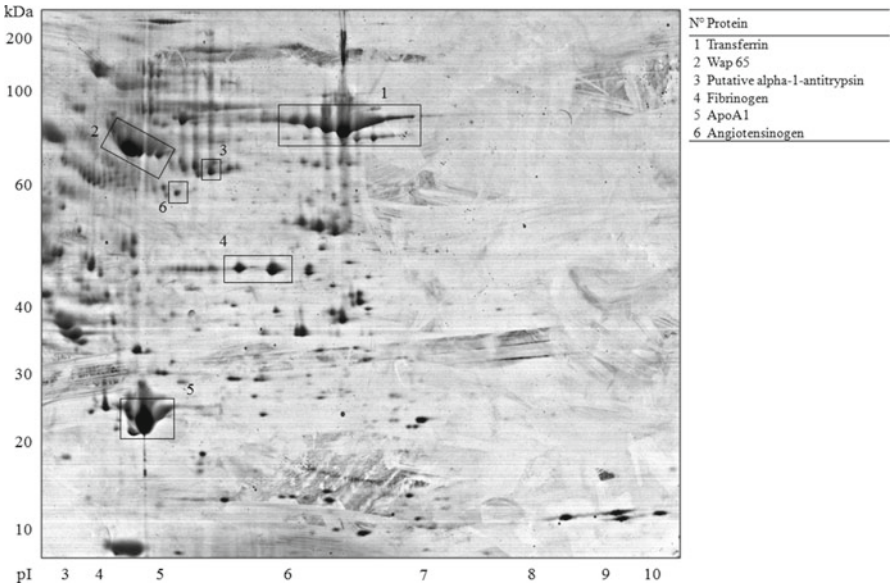

Fig. 11.3 2-D PAGE map of farmed *Sparus aurata* serum. *Numbers* indicate the main protein groups subjected to mass spectrometry identification. Protein identities are reported on the *right*. The following conditions were applied: IEF on 24 cm, pH 3–10 IPG strips; SDS-PAGE on 8–16% polyacrylamide gradient gel; colloidal Coomassie staining. Protein identification was performed by LC-MS/MS on a nanoLC-nanoESI-Q-TOF instrument

11.4.1 Eurasian Perch (P. fluviatilis): Modulation of the Immune Status as a Consequence of the Genetic Selection Incurred During the Domestication Process

The transition from the native to the captive habitat (domestication) imposes an adaptation to situations that are different from those experienced in the wild, and animals have to adapt to the new conditions (Price 1999). Therefore, a genetic selection occurs in captivity even without any intentional pressure by humans. In fish, a reduced genetic diversity has been reported in captive stocks when compared to wild populations, attributable to founder effects, inbreeding, artificial selection, and natural selection mechanisms due to survival of the fittest (Alarcon et al. 2004; Blanchet et al. 2008; Jackson et al. 2003; Koljonen et al. 2002; Pampoulie et al. 2006; Porta et al. 2006; Withler et al. 2007). In fact, natural selection in captivity acts through exclusion of the individuals unable to survive the many stressors posed by the captive conditions (Mignon-Grasteau et al. 2005). These physiological, morphological, and behavioral changes induced by domestication lead animals to be better adapted to captivity and to the presence of humans (Driscoll et al. 2009). In this context, Douxfils and coworkers (2011) combined the measurement of conventional stress indicators

with microsatellite analysis and proteomic investigation of serum profiles in a wild population of Eurasian perch during their domestication process, with the aim of evaluating its influence on the stress physiology and associated immunomodulation. Specifically, the effect of chronic confinement stress on immune capacity was evaluated on two distinct generations (F1 and F4) of 1-year-old Eurasian perch juveniles reared at different stocking densities in recirculated tanks. The authors conducted the study without operating any particular morphological, physiological, or behavioral trait selection on the population under study. A combination of parameters was evaluated, including usual stress indicators, parameters related to innate and adaptive immunity, microsatellite analysis, and differential proteomic analysis of blood serum.

Proteomic experiments were performed by using pooled sera, on four replicate pools of serum per condition (unconfined F1 fish, confined F1 fish, unconfined F4 fish, confined F4 fish). Pooled sera were diluted six times and reduced in 7-M urea–2-M thiourea–4% CHAPS buffer, and proteins were subjected to isoelectric focusing after cup-loading onto rehydrated pH 4–7, 24-cm IPG strips. The second dimension was carried out in 12.5% polyacrilamide gels, and RuBPs staining (ruthenium II tris bathophenanthroline disulfonate) was performed (Rabilloud et al. 2000). Differentially expressed spots were subjected to protein identification by LC-ESI-MS/MS on a CapLC Q-TOF2.

As a result, statistically significant differences were observed in the abundance of C3 complement component, transferrin, and apolipoprotein E (ApoE) in the sera of confined F4 fish when compared to confined F1 fish. Specifically, confinement caused a decrease in transferrin abundance at day 7 in F1 fish, but not in F4 fish. Transferrin is known for its role as a positive acute phase protein, and in fish it has been demonstrated to be an activator of the macrophage antimicrobial response (Stafford and Belosevic 2003; Stafford et al. 2004). Concurrently, F4 fish showed an elevated abundance of apoE when compared to F1 fish. The authors suggested that this might provide fish with the ability to face the increased energetic demand associated with stress, as reported in the common carp *Cyprinus carpio* (Concha et al. 2003). Concerning complement C3, several differentially expressed spots produced this protein identification, although none of them matched the expected entire molecular weight, suggesting the presence of numerous fragments. Among these, a general decrease in expression was observed in F4 fish subjected to confinement stress when compared to F1 fish in the same conditions. In general, therefore, the expression level of several immune response proteins in response to chronic stressors was higher in F4 than in F1 fish, highlighting the positive effects of domestication on the immune status and therefore on the ability to fight disease.

In conclusion, by applying serum proteomics, the study by Douxfils and coworkers (2011) enabled the demonstration that, using this animal model and this farming technique, even the maintenance of fish for few generations in captivity exerts a selective pressure on the immune capacity of fish and on their ability to fight chronic stressors, although at the price of a genetic drift and the reduction of genetic diversity, allelic richness, and heterozygosity.

11.5 Kidney

In fish, the kidney is a composite organ with three different functions: excretory, hematopoietic, and endocrine. As such, it becomes an interesting organ for monitoring health and wellness of farmed fish. However, only sparse proteomic studies have been performed to assess the impact of aquaculture on the fish kidney. Figure 11.4 illustrates a representative 2-D-PAGE map of farmed sea bream kidney tissue.

11.5.1 Alterations of the Protein Expression Profile upon Bacterial Colonization of the Kidney in Gilthead Sea Bream

In a study aimed to monitor the health status of gilthead sea bream farmed in offshore floating cages (Addis et al. 2010b), the most frequent bacteriological finding was internal organ colonization by *Moraxella* spp, a Gram-negative opportunistic bacterium. The kidney was the most affected organ in this survey, and a proteomic study was carried out in order to monitor alterations in its protein expression profile and to identify possible biomarkers linked to bacterial colonization. Protein extracts

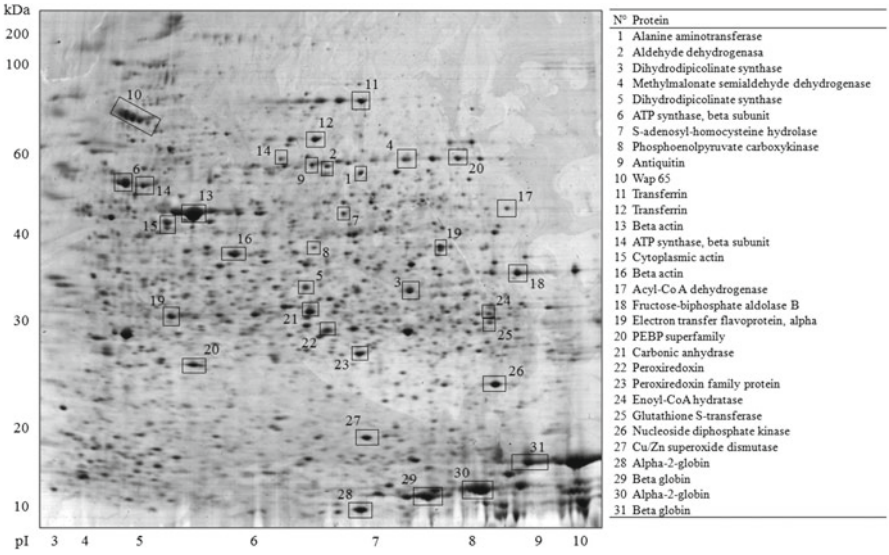

Fig. 11.4 2-D PAGE map of farmed *Sparus aurata* kidney. *Numbers* indicate the main protein groups subjected to mass spectrometry identification. Protein identities are reported on the *right*. The following conditions were applied: IEF on 24 cm, pH 3–10 IPG strips; SDS-PAGE on 8–16% polyacrylamide gradient gel; colloidal Coomassie staining. Protein identification was performed by LC-MS/MS on a nanoLC-nanoESI-Q-TOF instrument (Adapted from Addis et al. 2010b)

were generated from 7 positive and 28 negative kidney samples by fragmentation with a scalpel and homogenization by bead beating in 8-M urea-2% CHAPS buffer. Isoelectric focusing was carried out onto 24-cm, pH 3–10 NL IPG strips, and SDS-PAGE was performed on 8–16% polyacrylamide gradient gels, which were stained by colloidal Coomassie. Computerized image analysis identified several differential protein spots, which were identified with a combination of MALDI-MS and NanoHPLC-nanoESI-Q-TOF-MS. In particular, ten spots were found to be consistently overexpressed in proteomic maps of Moraxella-positive kidneys. Interestingly, seven of these spots corresponded to six mitochondrial enzymes, including alanine aminotransferase, aldehyde dehydrogenase, dihydrodipicolinate synthase, methylmalonate semialdehyde dehydrogenase, ATP synthase beta subunit, and Acyl-CoA-dehydrogenase. Among other proteins, peroxiredoxins, S-adenosyl-homocysteine hydrolase, wap 65, transferrin, glutathione S-transferase, carbonic anhydrase, and Cu/Zn superoxide dismutase were also identified. Mitochondrial proteins are key to many metabolic activities, and alteration of their expression is often associated with disease (Palmfeldt et al. 2009), whereas many of the other upregulated proteins are related to oxidative stress responses, infection, inflammation, or programmed cell death processes. In summary, even infection by opportunistic bacteria can lead to mitochondrial enzyme alterations in kidney tissues, producing a stressful condition potentially influencing farmed fish metabolism, and opening the way to infection by other bacterial pathogens. Although the impact on zootechnical performance remains to be demonstrated, the identification of a panel of proteins with altered expression levels might be useful as a tool for detecting and containing infections by Gram-negative pathogens before these spread to the whole farming plant.

11.6 Reproductive Organs

Another topic concerning adaptation to captivity of farmed fish, which has been addressed using proteomics, is fertility and reproduction. However, only few studies evaluated these aspects in a comparative manner between farmed fish and their wild counterparts. In this respect, Fornè et al. (2009) applied the 2-D-DIGE technology to investigate on the molecular basis for the loss of fertility consequent to captivity in Senegalese sole (*Solea senegalensis*).

11.6.1 Senegalese Sole (S. senegalensis): An Investigation of Fertility in Wild Caught Versus F1 Captive Fish by Means of Testis Proteomics

The flatfish Senegalese sole has an elevated commercial value, making its optimal reproduction in captivity of considerable interest. However, the process is not yet completely controlled; although spontaneous reproduction in fish adapted to

captivity has been observed for several years (Anguis and Cañavate 2005), males from the F1 generation have a poor reproductive performance due to a reduced sperm production, sometimes resulting in the complete absence of egg fertilization (Agulleiro et al. 2006; Porta et al. 2006; Cabrita et al. 2006). Forné and coworkers applied comparative protein profiling by 2-D-DIGE to investigate the changes occurring in the testis proteome of wild-caught and F1 Senegalese sole males reared in captivity, with the aim of identifying proteins potentially involved in the low sperm production and reduced fertilization capacity of F1 males (Miura et al. 1999).

Testis samples from F1 males were collected after a treatment aimed to induce teleost spermatogenesis by means of implants containing synthetic analogues of gonadotropin-releasing hormones (F1GnRHa), a combination of GnRHa and 11-ketoandrostenedione (F1GnRHa + OA), or saline solution as a control (F1C). Testis samples were also obtained from wild-caught males at the spermiation stage (F0Mat). All testis samples were mechanically disrupted in saline buffer and then subjected to sonication. Extracted proteins were precipitated and resuspended in a pH 8.5, 7-M urea–2-M thiourea–4% CHAPS buffer. After cyanine labeling and sample mixing, samples were focused into linear pH 3–10 IPG strips, followed by SDS-PAGE into 12% polyacrylamide gels. Images were generated by scanning with a confocal laser scanner and analyzed with dedicated software. Differentially expressed spots were subjected to protein identification by analysis with a MALDI-TOF mass spectrometer, selecting the peptides to be analyzed by a following round of nanoESI IT MS/MS with an LCQ MS.

As a result, a total of 58 spots showed statistically significant differences in the analyzed groups, and 45 of them were successfully identified as the products of 31 genes. In this set of proteins, many were present in several differential spots, indicating the presence of potential differentially espressed isoforms or carrying various post-translational modifications. These included alpha-2-macroglobulin-1, hemopexin, keratin, adenosine triphosphate synthase b subunit, peroxiredoxin 6, and keratin 18, having different patterns of regulation depending on the developmental stage. It is worth noticing that such observations are facilitated and their validity is reinforced by the 2-D-DIGE technology that, combined with software analysis, provides more robust information on the presence and relative abundance of isoforms differing by isoelectric point. In total, the differential proteome analysis of testis tissue from captive F1 and wild-caught Senegalese sole males showed a significant difference in expression of 24 proteins involved in cytoskeletal organization, catabolic processes, and redox or antioxidant activities. Application of the differential proteomics approach enabled the authors of this work to discover that F1 males experience alterations in protection against oxidative stress, protease inhibition mechanisms, and iron and glucose metabolism when compared to wild fish, and these might be responsible for their impaired sperm production and reduced fertilization rates. Moreover, several new proteins not previously described in the testis of teleost fish were reported. The results of this work open the way to other investigations aimed at understanding the physiology of Senegalese sole, as well as identifying the mechanisms that impair production of viable sperm in these and other farmed teleost fish.

11.7 Concluding Remarks

The studies carried out thus far have demonstrated the potential of proteomics to identify pathways and functions influenced by farming in many fish tissues. The impact of factors such as feeding regimens, crowding, handling, temperature, growth rates, farming techniques, and many others has been characterized in most fish tissues, widening our knowledge of fish biology and providing useful tools for increasing efficiency and quality of aquaculture productions.

Nevertheless, the informative power of many differential proteomic investigations carried out on fish still suffers from the limited availability of annotated sequence data. It is hoped that in the future this problem will become less important, especially with the progress made on model organisms as well as on genome sequencing and annotation technologies. Furthermore, the integration with gene expression techniques, lipidomics, and metabolomics will enable us to fully exploit the potential of proteomics in supporting the future production of healthier and higher quality fish.

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