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Omics Methodologies:
New Tools in Aquaculture Studies

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1. Introduction

According to the FAO, a growing percentage of world aquatic production is derived from aquaculture, whose importance is increasing dramatically due to commercial overfishing and a growing demand for seafood (FAO, 2010). In 1980, aquaculture production represented 9% of fishery resources; by 2010, it had increased to 43%. It is thought that such a production will need to double in the next 25 years. The FAO is promoting aquaculture because it is an important source of income and employment and also because of its great contribution to food security and the development of many countries. Currently, there are three main challenges for developing productive, feasible and sustainable aquaculture: 1) diversification of the proteins used for the feeds, 2) resolution of problems derived from stressful conditions, diseases and/or deterioration of environmental conditions, and 3) introduction of new species to make this industry less vulnerable to market demand (COM, 2002). The Senegalese sole (*Solea senegalensis*) is a flatfish species with a high potential for use in marine aquaculture diversification. The cultivation of sole has been successful under several husbandry conditions, but the frequent occurrence of opportunistic diseases and its high sensitivity to different stressors, such as manipulation, pollutants, etc., make sole unable to be produced industrially (Cañavate, 2005; Dinis et al., 1999). Consequently, the identification of biomarkers responsive to pathological situations and pollutants will help to prevent health problems and to improve their farming.

Biomarkers provide evidence of alterations by physiological or environmental conditions (López-Barea, 1995a). The so-called "classic" biomarkers are suggested *a priori* by virtue of their biological roles but are rather biased because they concentrate on a small number of proteins, excluding others that are also altered in the same conditions but whose relationship with the physiological or environmental changes is unknown (López-Barea & Gómez-Ariza, 2006). In 1989, a group at the University of Cordoba (UCO) began to develop a battery of biomarkers sensitive to physiological or environmental changes in several bioindicator species, including bivalves, crustaceans, fish, mammals and mammalian cell lines. A variety of biochemical parameters were included, such as phase I (ethoxyresorufin-*O*-deethylase, EROD) or phase II biotransforming enzymes (GSH transferase, GST), antioxidative defences (superoxide dismutases, SOD; catalase, CAT; glutathione

peroxidases, GSHPx, glucose-6P and 6P-gluconate dehydrogenases, glutathione reductase, GSSGrase), neurotransmission-linked esterase activities, such as acetylcholine (AcChE) and carboxyl esterases (CbE), oxidative damages to biomolecules, including DNA (8-oxo-dG), proteins (protein-SSG mixed disulphides), lipids (malondialdehyde, MDA), and the glutathione content and redox status (total glutathione, GSSG/GSH). The UCO group also developed new biochemical indicators that are altered by physiological or environmental changes, such as the levels of individual GST and SOD isoenzymes, the activation of promutagens to genotoxins by exposure to extracts of reference or exposed animals –a global measure of biotransforming capacity– and the metallothionein (MT) levels using a new and extremely sensitive HPLC-based fluorescent assay.

The utility of these "classic" biochemical biomarkers was later validated by the UCO group in studies carried out preferentially in natural sites in Spain, Slovakia and Tunisia, and contrasted with experimental exposures to model contaminants carried out under controlled conditions. These studies were reported in the following publications, limited in this review to those made in fish, and listed here by their date of publication: Rodriguez-Ariza et al. (1992, 1993, 1994a, 1994b), Martínez-Lara et al. (1992, 1996, 1997), Pedrajas et al. (1993, 1995, 1998), López-Barea (1995b), Lenartova et al. (1997), López-Barea & Pueyo (1998), Cousinou et al. (1999, 2000), Alhama et al. (2006, 2010), Romero-Ruiz et al. (2003, 2008), and Jebali et al. (2008). These "classic" biochemical biomarkers also responded to physiological changes, including oxidative alterations promoted by different feeding schemes, as described in Pascual et al. (1995a, 1995b, 1997, 2003) and Cánovas-Conesa et al. (2007).

While genes typically exert their functions at the protein level, genetic responses to stress are often regulated at the transcriptional level. Therefore, the determination of transcriptional profiles has become an essential approach in understanding the coordinated gene response to various physiological and pathological variables. The construction of cDNA libraries by *suppression subtractive hybridization* (SSH) (Prieto-Álamo et al., 2009; Williams et al., 2003) is a fundamental methodology used in differential expression studies with non-model species because it enables the identification of genes with no previous knowledge of their sequences. SSH is a PCR-based technique for generating cDNAs enriched in differentially expressed genes, useful for large-scale gene identification in non-model organisms (Diatchenko et al., 1996). Unlike SSH, which only provides qualitative results, *DNA microarrays* give semiquantitative (fold-variation) data, and more importantly, permit, in a single experiment, the analysis of the levels of thousands of transcripts, making them a valuable high-throughput methodology in Functional Genomics. Moreover, heterologous hybridization allows the use of microarrays made from transcripts of one species to probe gene expression in other related species. *Real-time qRT-PCR* has become a reference method to detect and quantify transcripts and to validate the results obtained with other techniques such as subtractive libraries or microarrays.

The UCO team gained wide experience in quantifying changes occurring at the mRNA level by RT-PCR. Of relevance is the devise of new approaches for the quantification of the exact number of transcript molecules and their application to a wide variety of organisms and conditions. This team developed, validated and optimised relative quantifications using complex multiplexed RT-PCR (Gallardo-Madueño et al., 1998; Manchado et al., 2000; Michan et al., 1999; Monje-Casas et al., 2001; Prieto-Álamo et al., 2000; Pueyo et al., 2002) and absolute quantification by real-time RT-PCR (Jiménez et al.,

2005; Jurado et al., 2003, 2007; Montes-Nieto et al., 2007; Prieto-Álamo et al., 2003, 2009). They also developed a quantitatively rigorous approach based on a combination of multiplexed and real-time RT-PCR to increase the number of transcripts to be quantified simultaneously without compromising the sensitivity, reliability and repetitiveness of the absolute measurements (Jurado et al., 2003; Michan et al., 2005; Monje-Casas et al., 2004; Ruiz-Laguna et al., 2005, 2006). These studies have demonstrated the potential benefits of absolute transcript quantifications in studies of tissue-specific expression profiles (Jurado et al., 2003, 2007; Prieto-Álamo et al., 2003, 2009; Ruiz-Laguna et al., 2005), of changes associated with growth stages or with the age or sex of an individual and have been particularly useful in studies with free-living animals (Jiménez et al., 2005; Michan et al., 2005; Monje-Casas et al., 2004; Prieto-Álamo et al., 2003; Ruiz-Laguna et al., 2005, 2006). We have demonstrated that the main drawback of relative quantifications is the variability of most popular internal standards. By comparing the differences in the transcript molecules with the conventional fold variations, we have also shown that relative quantifications grossly overestimate changes affecting poorly transcribed genes in comparison with highly abundant mRNAs.

Proteomics addresses the post-genomic challenge of examining the entire complement of proteins (proteome) expressed by a genome in a cell, tissue or organ at a given time under defined conditions (James, 1997). Protein expression is modulated at different levels from transcription to the maturation of the polypeptides produced by the translation of mature mRNAs. Proteins were initially separated by *two-dimensional electrophoresis* (2-DE; Wilkins et al., 1996), and their expression was analysed by 2D software (Melanie, etc.). Proteins were identified by mass spectrometry analysis of their peptide mass fingerprint (MALDI-TOF-PMF) or *de novo* sequencing of some peptides (nESI-MS/MS), comparing the results with public databases (Simpson, 2003). 2-DE, which is labour-intensive and has low reproducibility, requires a large amount of sample, and its narrow dynamic range is problematic with proteins of extreme Mr/pI. Shotgun proteomic methods allow the analysis of complex protein mixtures after full digestion by *multidimensional separation* coupling tandem liquid chromatography (LC/LC) and MS/MS (Washburn et al., 2001). The application of proteomic technology faces the problem of the lack of genomic information on most non-model sentinel organisms. This makes it difficult to identify differentially expressed proteins by high-throughput methods such as MALDI-TOF-PMF (López-Barea & Gómez-Ariza, 2006).

2. Conventional aquaculture studies with *Solea senegalensis*

2.1 Early studies

Studies of sole aquaculture began in Faro (S Portugal) and Cádiz (SW Spain) to produce good quality larvae and juveniles (Dinis et al., 1999). Broodstock spawning studies established optimal feeding regimes by combining squid (*Loligo vulgaris*) and polychaetes (*Hediste diversicolor*) at the final maturation stages. Spawning was studied in terms of temperature (stopped <16 ºC), duration (4-6 months), egg fertilisation rate (20-100%) and viable egg rate (72%). Larvae hatch at 2.4 mm and accept *Artemia nauplii* as the first prey two days after hatching (DAH). Metamorphosis spans from 11 to 19 DAH, at which point the fish are fed live *Artemia metanauplii*. They reach 16 mm at 40 DAH and 35 cm/450 g after 1 year, with 8% survival. Pasteurellosis can cause pigmentation abnormalities and

malformations associated with eye migration and can progress to death (Dinis et al., 1999).

The potential of sole for aquaculture was reviewed some time later (Cañavate, 2005). Although important progress in reproduction techniques was reached, much basic knowledge remained lacking. Ongrowth was successfully carried out, but progress was limited by opportunistic diseases due to suboptimal rearing conditions resulting in an inability of the sole to achieve an adequate physiological status for resistance. Growth, survival and pigmentation were studied during sole growth in tanks with three bottom types (Rodiles et al., 2005). The final length and weight was similar in the sand, white and dark conditions, but different pigmentation patterns appeared on the sand (clear, dark) and white bottoms (clear, brown, dark). The homogeneous dark pattern, preferred by markets, is only obtained in tanks with a dark bottom. A lower survival rate was found on sand bottoms due to pathologies derived from the difficulties in maintaining the sand bed.

2.2 Organ development and reproductive studies

Digestive tract development was studied in larvae until 30 DAH, which involved the assessment of histology, digestive enzymes, lipids, proteins and carbohydrates in the buccopharyngeal cavity, oesophagus, early stomach, anterior and posterior intestine, pancreas and liver (Ribeiro et al., 1999a). The digestive tract elongates in metamorphosis, increasing absorption. Phosphatases, lipase and aminopeptidase have been detected starting at 2 DAH and the levels increase during development. Proteins abound in the intestinal epithelium and exocrine pancreas, and neutral lipids are found at the yolk sac intestinal epithelium and liver. After 31 DAH larvae ingest, digest and absorb nutrients because they now have a complete digestive tract. A time course of pancreatic and intestinal enzymes was studied in larvae until 31 DAH (Ribeiro et al., 1999b). Digestive enzymes increase until 10 DAH then decrease until 18 DAH, a pattern typical of developing animals. Alkaline phosphatase abounds from 21-27 DAH, during the development of brush border membranes, with a parallel decrease in the cytosolic enzyme, Leu–Ala peptidase.

Thyroid development was studied in sole larvae by histo- and immunohistochemistry to synchronise larval development and improve fish production (Ortiz-Delgado et al., 2006). The first follicle is visible by the first feeding; increases during metamorphosis and has adult characteristics by 30 DAH. Thyroid hormones decrease to undetectable levels at yolk-sac reabsorption. T3 and T4 are detected by 6 DAH and increase during metamorphosis.

Seasonal profiles of sex steroids –17β-estradiol (17β-E), testosterone (T), 11-ketotestosterone (11-KT), and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P)– were studied in *S. senegalensis* in an attempt to achieve steroid-induced maturation (García-López et al., 2006a). Females have six maturation stages, as follows: early, intermediate and final ovarian development, then partially, mid and spawned out. By summer´s end, a new gonadal cycle starts, as demonstrated by increased reproductive parameters. By mid-autumn some females reach advanced maturation stages, which coincide with a peak of running males. By the start of spring, ovarian development reaches its peak, and plasma steroid levels are maximal at the start of the spawning period, which occurs from March to June. In parallel with oocyte and sperm release, the proportion of spawned out fish and non-running males increases, and steroid levels decline. The high levels of 17,20β-P during spawning make it a candidate for a maturation-inducing steroid.

Testicular development was also studied (García-López et al., 2006b). The spermatogenetic cycle consists of the following five stages: early (I), mid (II), and late (III) spermatogenesis, maturation (IV), and recovery (V). In the summer, stage I and V testes are found with low values of sex steroids and I_G (gonadosomatic index). Recrudescence begins in autumn, with an initial increase of I_G 11-KT and T and the appearance of stage II and III testes. In the winter, 11-KT and T peak and soon decrease, and I_G slightly declines. In the spring, 11-KT and T decline further, while I_G slightly increases and running males peak with stage IV testes. Sperm production and quality was assessed in wild-captured and F1 broodstock fish (Cabrita et al., 2006). Males produce motile sperm from February to November, with specific peaks of high spermiation and fluent males. Sperm volume and cell density is lower in F1 males than in wild-captured broodstock.

Ovarian development was also studied (García-López et al., 2007). In the autumn/winter, oocytes progress to vitellogenic stages in parallel with high levels of K (condition factor), I_G, and plasma 17β-E and T. In the late winter/early spring, development is maximal, with females at intermediate and final maturation and K, I_G, 17β-E and T peaking. Steroid levels are lower in cultured sole than in naturally spawning females, leading to atresia and lack of oocyte maturation, thus reducing ovary size with declining K, I_G, and 17β-El and T levels and many perinucleolar oocytes. The amount of circulating 17,20β-P, the putative maturation-inducing steroid, remains near constant through the period, suggesting that oocytes are unresponsive to its stimulation.

Skeletal development and malformations are a bottleneck in sole aquaculture. Maturation and abnormalities of the vertebral column and caudal skeleton have been studied in sole (Gavaia et al., 2002). Different defects are found in the caudal complex and the vertebral column, and 44% of fish show at least one defect. While the causes are unknown, their high incidence may reflect rearing and/or feeding problems. The tissue distribution and evolution of bone Gla (Bgp) and matrix Gla proteins (Mgp) and Ca^{2+} deposition were studied in zebrafish during larval development and in adult tissues as well as sole metamorphosis (Gavaia et al., 2006). In zebrafish, Bpg and Mpg accumulate mainly in the matrix of skeletal structures already calcified or under calcification. In sole metamorphosis, Bpg and Mpg increase in parallel to the calcification of the axial skeleton. In both species, Mpg also accumulates in non-mineralised vessel walls.

2.3 Nutrition studies

Studies on the requirements, catabolism and assimilation of amino acids (AAs) were carried out in early larval, metamorphic and post-larval sole. Initial studies on indispensable (IAA) and dispensable (DAA) amino acids (Rønnestad et al., 2001), showed that sole assimilated most (85%) of the dietary IAAs and catabolised most of the DAAs. Such results were confirmed after studying the bioavailability of several AAs in larvae (Conceição et al., 2003). The demand and availability of AAs and proteins in relation to digestive capacity were reviewed, and AAs sources were described, highlighting the regulatory role of cholecystokinin and peristaltic activity (Rønnestad et al., 2003). A balanced AA profile improved amino acid assimilation in post-larval sole (Aragão et al., 2004a). Changes in AA requirements and dietary imbalances were studied in *Sparus aurata* and *S. senegalensis* (Aragão et al., 2004b); the AA profiles of both changed during ontogeny, especially in sole due to its marked metamorphosis. AA imbalances were found during development. In both

species, Phe/Tyr addition was studied to assess the effects on metamorphosis after their conversion into thyroid hormones (Pinto et al., 2010). While Phe did not affect sole metamorphosis, dietary Tyr increased the production of thyroid hormones, which was beneficial for sole metamorphosis.

The nutritional physiology of sole development was studied to optimise diets and understand limiting factors in weaning (Conceiçao et al., 2007). Larvae have a high capacity to digest live prey, even at the early stages. Use of inert microdiets in co-feeding with *Artemia* resulted in the development of intestinal activity and enhanced survival, although it was also accompanied by low growth and high size dispersal. Fatty acid absorption increases with their degree of unsaturation, and larvae spare DHE from catabolism. Rotifers and *Artemia* are deficient in one or more AAs, such as His, Lys, Arg, Thr, or those containing sulphur and aromatic rings, depending on the larval stage; balancing the dietary AA profile with dipeptides increases retention and decreases catabolism in *Artemia*-fed larvae.

The effects of non-protein energy levels on growth and oxidative status were studied in sole fed diets with 4 energy levels (Rueda-Jasso et al., 2004). Cellular energy allocation showed differences in liver, but not in muscle. TBARS were higher in fish fed a diet with high lipid content, in parallel to high CAT and SOD activity. Yet, the protein source or energy levels had no major impact on sole growth, nutrient utilisation or fatty acid composition (Valente et al., 2011). Quantitative lipid imbalances and a low protein/neutral lipid ratio increased the accumulation of lipid droplets in the enterocytes and lowered fatty acid absorption in larvae (Morais et al., 2005). The effects of a neutral lipid level and source were studied in marine fish larvae (Morais et al., 2007). A growth-depressing effect of high neutral lipids, as assessed by lower digestive enzyme activity, absorption and/or food intake was reported. In larvae, lipid transport from enterocytes to the body is more critical than lipolytic activities. Phospholipid digestion is more efficient than that of neutral lipids, whose excess leads to the accumulation of large lipid droplets in the enterocytes, reducing fatty acid absorption and growth.

The feed transit, protein and energy digestibility of practical feed were assessed in sole (Dias et al., 2010). Protein digestibility is high for fishmeal and corn gluten, intermediate for soybean meal, and moderate for wheat meal. Energy digestibility varies from 88 to 93% for soybean meal, corn gluten and anchovy fishmeal and is 73% in wheat meal. Thus, flatfish, despite its high dietary protein requirement, digest vegetable ingredients quite well, suggesting that the development of practical feeds with high levels of plant-protein sources would be beneficial.

2.4 Conventional biomarker studies in *S. senegalensis* aquaculture

Nearly 15 years after the studies of the UCO group, the use of biomarkers to follow fish physiology and pollution effects has become popular and is now applied by most groups. Antioxidant enzymes, stress proteins, lipid peroxides and histology were studied in sole larvae (Fernández-Díaz et al., 2006) fed on the following 3 diets: live *Artemia nauplii*, microcapsules, and vitamin A-supplemented microcapsules. Live-fed larvae grow larger and undergo faster metamorphosis than microcapsule-fed larvae, although all groups have near 80% survival. Vitamin A improves the growth and development compared to an inert diet. *Artemia*-fed larvae have organs with normal development, but histological alterations

are seen in larvae fed an inert diet. Catalase (CAT), superoxide dismutase (SOD), total GSH-peroxidase (t-GPX), lipid peroxides (MDA) and stress proteins (HSP70, not HSP60) are diet- and age-dependent. Inert diet-fed larvae have similar biomarker responses, but different (p<0.05) from *Artemia*-fed larvae. Higher antioxidant defences are attributed to the start of metamorphosis and the use of inert food.

A similar approach was used by Cañavate et al. (2007) to assess the effect of light on the development of sole larvae with or without adding β-carotene-rich *Dunaliella salina*. SOD, CAT, t-GPX and MDA were used as biomarkers. Growth and survival after metamorphosis were unaffected by light or *D. salina*. Light affects CAT and t-GPX throughout development but does not affect MDA, and SOD is only affected in metamorphosis. *D. salina* does not affect SOD, CAT or t-GPX and no interaction with light intensity was found. MDA lowers significantly only when *D. salina* is added, and its effect was found only in metamorphosing larvae, whose MDA levels are much higher than in earlier stages. These results confirm the antiperoxidative effect of β-carotene from live algae in the larval rearing process.

2.5 The Pleurogene project

After the initial studies on the growth and reproduction of the Senegalese sole, "Genoma España" and "Genome Canada" promoted the "Pleurogene" project, in order to develop new technology to assess gene and protein expression during the reproduction and breeding of two flatfish, Senegalese sole and Atlantic halibut. The project [http://www.gen-es.org] aimed to improve basic knowledge of reproduction, larval development and survival, and had de following objectives: 1) Establishment of an EST database and shotgun proteome analysis; 2) Construction of a microarray for high-throughput analysis of gene expression; 3) Construction of genetic linkage map; 4) Development of methods for gene expression profiling through laser capture microdissection RNA; 5) Identification of changes in gene expression during gamete development and maturation; 6) Genomic analysis of sex determination and differentiation; 7) Determination of the pattern of gene expression during larval metamorphosis, the ontogeny of the gastrointestinal tract and the effects of dietary treatments; and 8) Development of E-mold, an integrative bioinformatics platform for genomic, proteomic and morphological information from flatfish.

The Pleurogene project developed genomic and proteomic tools to help achieve these goals (Douglas et al., 2007; Cerdà et al., 2008), and also had the following major research results: 1) Development of genomics tools for the Senegalese sole, including 10 different cDNA libraries from adult and larvae tissues and 1 normalised multi-tissue library, 10,300 new sole EST sequences and nearly 500 peptides, and a sole oligonucleotide microarray with probes to detect 4550 different RNAs; 2) Development of a hormone treatment to increase sperm motility in sole; 3) Generation of a sole genetic linkage map; 4) Development of a progeny test or paternity kit; 5) Development of indirect approaches for sex control; 6) Production of recombinant gonadotropin hormone; 7) Generation of gene and protein expression maps associated to larval development, metamorphosis, and nutrition; 8) Generation of gene and protein expression maps of testes producing high quality sperm; 9) Generation of gene expression maps for sexual differentiation and maturation; and 10) Development of the "Solea-mold" that allows for new data to be included, *in silico* experiments to be performed, and comparative studies to be undertaken.

3. "Classic" biomarkers versus "*omics*" methodologies

3.1 Assessing pollution effects in fish

After the initial studies of the UCO group, the use of "classic" biomarkers became increasingly popular to assess the effects of pollutants on fish. One example was the "Prestige" oil spill in November 2002 off the Galician coast (NW Spain). Due to the heavy nature of the crude oil and its low solubility in sea water, its dispersion was low and it remained *in situ* in oil patches adhered to rocks and sediments. The biological effects of these oil patches were tested in *S. aurata* (Morales-Caselles et al., 2006), using MT levels, EROD activity and histology as biomarkers, without significant results. Biomarkers including CAT, GSHPx, GSSGrase and DT-diaphorase, AcChE, CbE, GST and MDA were also analysed in *S. senegalensis*, with modest alterations (Solé et al., 2008).

Antioxidative and phase II and III biotransforming enzymes were used to follow the effects of linear alkylbenzene sulphonates in sole via an *in vivo* continuous-flow assay (Alvarez-Muñoz et al., 2007) that was also used in *S. aurata* and *S. senegalensis* to assess the toxicity of sediments from littoral areas in northern and southern Spain (Jiménez-Tenorio et al., 2007), using MTs, EROD and histopathology. Jimenez-Tenorio et al. (2008) assessed the sediment toxicity caused in *S. senegalensis* by acute or chronic spills using histopathology, EROD and GST as biomarkers. Oxidative stress biomarkers and PAH contents were studied in Huelva soles near a petrochemical plant (Oliva et al., 2010). Significant correlations were found among the levels of GST, GPx and CAT, hepatic levels of PAH metabolites and PAH contents in sediments.

The effect of waterborne copper (Cu) was studied in *S. senegalensis* in static conditions (Fonseca et al., 2009) by assessing the following characteristics: biomarkers such as MTs or MDA, mass indices, and biochemical condition indices such as the RNA/DNA ratio and the lipid and protein content. Cu triggers a biomarker response and lowers growth and condition, without changes in morphometric indices. Decreased condition shows that lipid reserves enable fish to respond to toxicity and to maintain growth and protein synthesis, although with lower rates than control fish.

The effects of Mexel®432 and NaClO antifoulings were studied in *S. senegalensis* by assessing osmolality, Na^+/K^+-ATPase, stress, histology, oxidative damage, antioxidant defences and detoxification (López-Galindo et al., 2010a,b). NaClO increases plasma cortisol, glucose and lactate after an acute stress, with a later recovery. Gill GST and AChE are sensitive to NaClO. Hepatic markers initially respond to NaClO but longer exposures are toxic. Mexel®432 initially increases cortisol, which later returns to basal values, but glucose, lactate and triglycerides decresase. Gills have a lowered Na^+,K^+-ATPase activity, causing an imbalance in osmoregulation. Moderate changes are found in KAT, GSSGrase and GPX but not in MDA, GST or CbE. A multi-biomarker approach was used in *Dicentrarchus labrax*, *S. senegalensis* and *Pomatoschistus microps*, from the Aveiro and Tejo (Portugal) estuaries (Fonseca et al., 2011), which were affected by anthropic activities, without highly significant results.

Detection of DNA damage by biomarkers for genotoxicity was used in *S. senegalensis* (Costa et al., 2008a) exposed to sediments from three Sado Estuary (W Portugal) sites. The two blood parameters used were erythrocyte nuclear abnormalities (ENA) and DNA strand-breaks (DNA-SB). The levels of metals, PAHs, PCBs and DDTs were determined in the

sediments. Scarcely polluted sediments are weaker inducers of genotoxic damage, whereas those under urban, industrial or agricultural influences significantly increase ENA and DNA-SB. A strong correlation exists between PAH and PCB content and genotoxicity, while metals have a weaker correlation. In a parallel study, *S. senegalensis* were exposed to Sado Estuary sediments (Costa et al., 2008b). Livers had more histological lesions than gills, and sediments contaminated by organics caused more damage to both organs than those contaminated by metals. Two "classic" biomarkers, MT and CYP1A, were also assessed. Lethality and biomarker responses do not linearly depend on the cumulative levels of contaminants but rather of their bioavailability and synergistic effects (Costa et al., 2009). In a parallel study, exposure to contaminated sediments induced DNA fragmentation and clastogenesis (Costa et al., 2011). Still, the most contaminated sediment revealed an antagonistic effect between metals and organics, enhanced by higher bioavailability. The laboratory assay caused a more pronounced increase in ENA, whereas a significant increase in DNA-SB exists in field-tested fish exposed to reference sediment.

3.2 The advent of "omics" methodologies

In contrast to the "classic" biomarker strategy, "*omics*" approaches, plus *in vitro* and *in silico* methods, are becoming a powerful multidisciplinary strategy. Their use is still at an early stage because most popular bioindicators are poorly represented in gene/protein sequence databases (Ruiz-Laguna et al., 2006; González-Fernández et al., 2008). *Omics* includes *genomics* to study DNA variations, *transcriptomics* for genome-wide characterisation of gene expression by measuring mRNAs, *proteomics* to assess the cell and tissue-wide expression of proteins, and *metabolomics* for global assessment of metabolite concentrations. These technologies provide detailed molecular information that helps to identify response pathways and to define mechanisms and modes of action without requiring previous knowledge. In 2003, the UCO group began a search for new biomarkers through the use of "omics" methods, which allowed the study of the Aznalcóllar spill and the status of Doñana National Park and its surroundings (SW Spain). These studies were collected in the following publications, listed here by publication date: Rodriguez-Ortega et al. (2002, 2003), Ruiz-Laguna et al. (2005, 2006), Bonilla-Valverde et al. (2004), López-Barea & Gómez-Ariza (2006), Romero-Ruiz et al. (2006), Montes-Nieto et al. (2007, 2010), Vioque-Fernández et al. (2007a, 2007b, 2009a, 2009b), González-Fernández et al. (2008), Abril et al. (2011), and Pueyo et al. (2011).

All organisms are adapted to certain extracellular salinity ranges. Osmoregulatory mechanisms are central to adaptation. Osmotic stress was studied in tilapia *Oreochromis mossambicus*, spiny dogfish shark *Squalus acanthias*, and an intertidal sponge *Tetilla mutabilis*, by genomics and proteomics methods (Kültz et al., 2007). SSH, RACE-PCR and proteomics allowed the identification of genes and proteins involved in adaptation to salinity or other environmental stresses. Algorithms based on sequence homology searches (MSBLASTP2) are powerful tools for protein identification. Gene ontology and pathway analysis can subsequently use identified genes and proteins for modelling molecular mechanisms of environmental adaptation. The dependence on information about biochemical pathways and gene ontology databases for model species is a severe barrier for work with non-model species. To minimise this dependence, focusing on a single biological process is key when applying "omics" methods to non-model organisms.

Environmental metabolomics allows for the characterisation of the metabolism of organisms from the natural environment and of those reared under laboratory conditions. Viant (2007) used this approach to characterise the responses of organisms to natural and anthropogenic stressors, discussing the challenges of measuring metabolites and highlighting the dynamic nature of the metabolome, whose variability is a challenge in environmental studies. The normal metabolic operating range (NMOR) is defined as the region in metabolic space in which 95% of individuals reside, and stress is a deviation from NMOR. The importance of genotypic and phenotypic anchoring (e.g., knowing species, gender, and age) is emphasised to facilitate the interpretation of multivariate metabolomics data.

An NRC-UK sponsored international consortium from government agencies, academia and industry in Canada, Japan, the UK, and the USA was carried out on fish toxicogenomics (Van Aggelen et al., 2010). The following three topics were addressed: progress in ecotoxicogenomics, perspectives on roadblocks for practical implementation of toxicogenomics into risk assessment, and dealing with variability in data sets. Although examples of successful application of "omic" technologies were identified, it is critical to perform studies that relate molecular changes to ecologically adverse outcomes. Although there are hurdles to pass on the road to regulatory acceptance, "omics" are already useful for elucidating modes of action of toxicants and can contribute to the risk assessment process as part of a weight-of-evidence approach.

A qRT-PCR approach was used to assess how *Lactobacillus rhamnosus* IMC 501 added to *Amphiprion ocellaris* larvae, alters development, and also to study the responses after probiotic exposure (Avella et al., 2010). Larvae and juveniles had 2-fold higher weight after probiotics were supplied. Metamorphosis occurs 3 days early, and factors involved in growth and development (I-l GF I/II, myostatin, PPAR α/β, vitamin D receptor α, and retinoic acid receptor γ) have higher gene expression. Probiotics lessen the severity of the general stress response as demonstrated by lower levels of glucocorticoid receptor and 70-kDa HSP expression. Improved development of the skeletal head was also found, with 10–20% less deformities in probiotic-treated juveniles.

"Omics" have also been used in chemical screening and perturbation studies in zebrafish (Sukardi et al., 2010). Pharmacological efficacy and selectivity have been evaluated by chemical-induced phenotypic effects, although this has limitations in the identification of action mechanisms. "Omics" also facilitates the translatability of zebrafish studies across species by comparing conserved chemically induced responses. Thus, De Wit et al. (2010) characterised the estrogenic and metabolic effects of 17α-ethinylestradiol (EE2) in *D. rerio*, following the concern regarding the effects of endocrine-disrupting compounds. Oligo microarrays, with 3479 zebrafish-specific oligos, were used to generate differential gene expression levels, and proteomic responses were evaluated by DIGE and MALDI-TOF/TOF. Assessment of the differentially expressed transcripts and proteins showed that both individual platforms could profile clear estrogenic interference and multiple metabolism-related effects and stress responses. Cross-comparison of transcriptomics and proteomics datasets have limited concordance, but a revision of the results shows that transcriptional effects project at the protein level as downstream effects of the affected signalling pathways.

Public databases of coexpressed gene sets are valuable resources for many studies, including gene targeting for functional identification and investigations of regulatory mechanisms or protein–protein interactions. While coexpressed gene databases are highly popular in plant

biology, those with animal data are limited due to the lower reliability of coexpression data. The COXPRESdb (http://coxpresdb.jp) represents the coexpression relationship in humans and mouse (Obayashi & Kinoshita, 2011). Updates focusing on enhancing the reliability of gene coexpression data in animals have been reported. A new coexpression measure, Mutual Rank, has been implemented, and five other animal species, such as the rat, chicken, zebrafish, fly and nematode, were included to assess the conservation of coexpression. In addition, different layers of "omics" data have been added into the integrated network of genes. Functioning as a gene network representation, COXPRESdb can help researchers to clarify the functional and regulatory networks of genes in a broad array of animal species.

4. Transcriptomic studies in *Solea senegalensis*

Cultivation of the Senegalese sole is hampered by its sensitivity to different stresses and infectious diseases that can cause high mortality. Consequently, there is a need to identify sole genes responsive to stress, infections and pollutants in order to improve productivity, management and fish welfare. Transcriptomic responses of sole stimulated with lipopolysaccharide (LPS), a mimetic of bacterial infections, and copper sulphate, a zoosanitary compound, were studied by different experimental and methodological approaches, such as SSH libraries, DNA microarrays and real-time qRT-PCR (Prieto-Álamo et al., 2009; Osuna-Jiménez et al., 2009).

4.1 SSH libraries

The construction of subtractive libraries in *S. senegalensis* allowed the identification of differentially expressed genes in response to LPS in the head-kidney, a hematopoietic and lymphoid organ involved in immune response and to $CuSO_4$ in the liver, a central metabolic organ in xenobiotic detoxification and in the defence system (Prieto-Álamo et al., 2009). In both cases, forward (F) and reverse (R) libraries were designed to obtain clones of genes that were up- or down-regulated in response to LPS of $CuSO_4$ relative to the PBS control. To offset inter-individual variations and temporal differences in the responses, the libraries were constructed with total RNA from pooled head-kidney or liver (≥ 10 fish/condition) of soles treated with LPS or $CuSO_4$ for 6 and 24 h. Four hundred sixty clones were sequenced and the products of the ESTs were identified by comparison with the open access databases. A total of 222 unique sequences were detected, and 185 were identified as related to major physiological functions (Table 1).

A high percentage of identified ESTs were related to immune response (Figure 1). Their presence in a sole head-kidney library stimulated with LPS agreed with the immune role of this organ and the immunostimulating effects of LPS (Swain et al., 2008). The number of genes classified as being immune-related was even larger in the liver than in the head-kidney. Most of these immune-related ESTs coded for acute phase proteins (e.g., lysozyme, coagulation factors, proteinase inhibitors, complement components, and Fe transport/homeostasis proteins) according to several genomics studies indicating that, in teleost, the liver is an important source of immune transcripts (Ewart et al., 2005), mediating a powerful acute phase response (Bayne & Gerwick, 2001). In addition, these results supported the capacity of Cu (like other metals) to alter immunological competence, in agreement with a report showing Cu up-regulation of the cytokine TGF-β in striped bass (Geist et al., 2007).

	LPS (head-kidney)	CuSO$_4$ (liver)
Number of clones sequenced	231	229
Number of clones analysed	222	226
Average sequence length (bp)[a]	411 ± 168	414 ± 155
Number of unique ESTs	133	89
Up-regulated ESTs	62	48
Identified ESTs	49	44
Non-identified ESTs	13	4
Down-regulated ESTs	71	41
Identified ESTs	58	34
Non-identified ESTs	13	7

Table 1. Characteristics of the sole SSH libraries and sequences. [a]Mean ± SEM
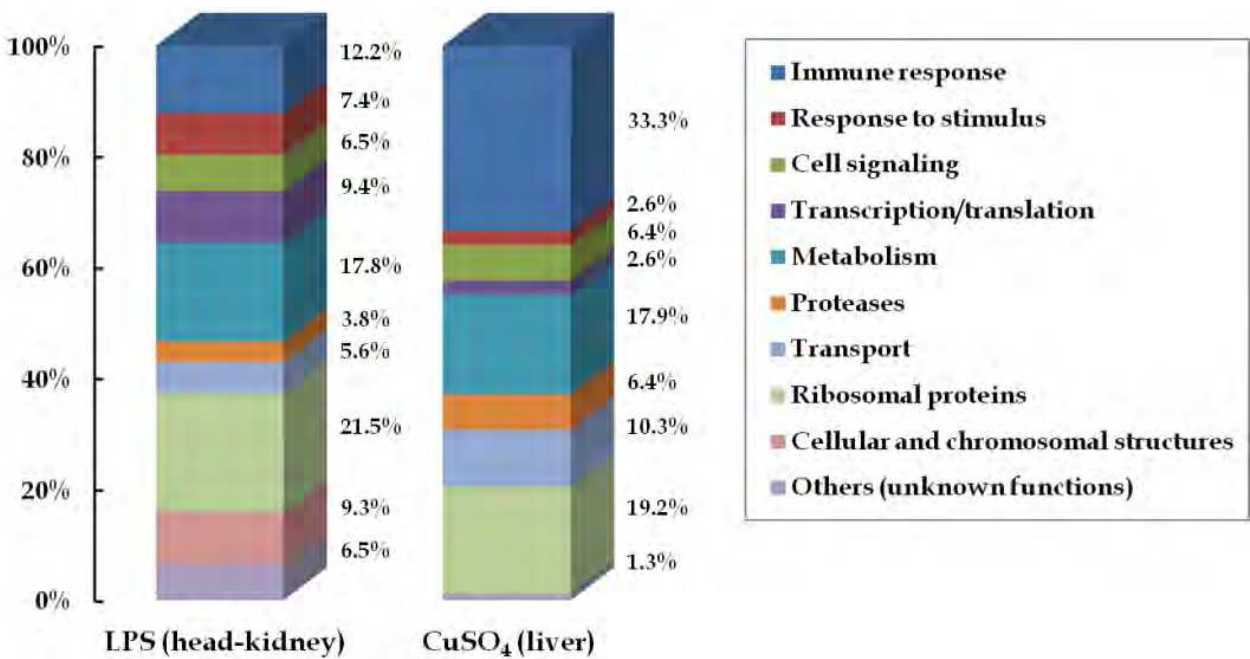
Fig. 1. Functional classification of unique ESTs obtained in SSH libraries from LPS or CuSO$_4$-treated soles. "Others" indicates genes that had significant identities to databases entries of unknown function.

Genes coding for products involved in osmoregulation and nitrogen excretion (e.g., liver angiotensinogen, sodium potassium ATPase beta subunit, kininogen 1, angiotensin I converting enzyme 1, and alanine-glyoxylate aminotransferase 2-like) were also identified in libraries from the livers of CuSO$_4$-treated soles, in agreement with the previously reported effects of copper on osmoregulation, acid-base balance and nitrogen excretion (e.g., Blanchard & Grosell, 2006; Evans et al., 2005).

4.2 DNA microarrays

The microarray developed for the European flounder *Platichthys flessus* (GENIPOL platform, Williams et al., 2006) had been used to assess hepatic gene expression of many species of flatfish, confirming that heterologous microarray analyses between closely related species is

a suitable approach (Cohen et al., 2007). This platform turned out a very useful tool for analysing the transcriptional expression of *S. senegalensis*.

First, the hepatic response of soles exposed to $CuSO_4$ or LPS was studied using pooled samples. Statistical analyses showed that 405 genes were differentially expressed after Cu treatment at 6 h, 468 with Cu at 24 h, 271 with LPS at 6 h and 664 with LPS at 24 h (Table 2).

	LPS		$CuSO_4$	
	6 h	24 h	6 h	24 h
Up-regulated genes	172	251	101	341
Down-regulated genes	233	217	170	323
Total	405	468	271	664

Table 2. Number of differentially expressed genes in *S. senegalensis* liver in response to LPS or $CuSO_4$ treatments that were identified by heterologous DNA microarrays.

The functional analysis of the results (Blast2GO software) permitted the identification of response-specific genes to $CuSO_4$ (cell junction and cell signalling), to LPS (glutathione transferase and immune response) or to both treatments (immune response, digestive enzymes, unfolded protein binding, intracellular transport and secretion, and proteasome) (Table 3). This way, the functional category cell junction was statistically significantly over-represented amongst the genes induced by copper at 6 h. This term grouped genes related to cellular adhesion, such those coding for claudins (CLDN26) and genes related to cell signalling, such as GIT2 that encodes G-protein-coupled receptor kinase interactor 2, which is in agreement with the ability of copper to alter the tight junction permeability in human intestinal mucosa (Ferruzza et al., 2002). Glutathione-S-transferases were more prevalent amongst the transcripts down-regulated by LPS at 24 h. The capacity of LPS and bacterial infection to down-regulate biotransformation activities such as GSTs has been described in a number of fish species (Reynaud et al., 2008). As shown in Table 3, genes related to the immune response were specifically induced by LPS. These included the antimicrobial peptide hepcidin (HAMP), TNFα-induced protein 9 (TNFAIP9), cytokines (IL8, IL25) and chemotaxins (LECT2).

Other immune-related genes were induced by both LPS and $CuSO_4$ treatments. This is the case for classic piscine acute phase proteins like haptoglobin (HP) or C7 (Bayne & Gerwick, 2001). C7 is a component of the complement system, whose up-regulation by copper is in line with complement proteins being engaged in novel biological functions distinct their well-established role in innate immunity (Mastellos et al., 2005). Although soles were fasted prior to and during the experiments, digestive enzymes such as trypsin (PRSS2), chymotripsin (CTRB), elastase (ELA4) and carboxypetidase A (CPA1) and B (CPA2) were down-regulated in response to both treatments. This might be due to a general stress caused by the treatment (Auslander et al., 2008). Furthermore, LPS and copper treatments resulted in the up-regulation of genes encoding unfolded protein-binding, which are induced in fish in response to different kinds of stress conditions (e.g., bacterial infection or exposure to heavy metals) (Basu et al., 2002), intracellular transport and secretion, in order to accommodate the rapid onset of cytokine secretion and for membrane traffic associated with the phenotypic changes of immune activation (Pagan et al., 2003), and proteasomal proteins in agreement with previous results in mammalian cells (Qureshi et al., 2003; Fernandes et al., 2006).

	Response to LPS	Response to CuSO$_4$	Selected genes
Cell junctions	---	Up-regulation	CLDN26, GIT2
Glutathione-S-transferases	Down-regulation	---	GST-A, GST1, GST3
Immune response	Up-regulation	---	HAMP, TNFAIP9, IL8, IL25, LECT2
	Up-regulation	Up-regulation	C7, HP
Digestive enzymes	Down-regulation	Down-regulation	PRSS2, CTRB, ELA4, CPA1, CPA2
Unfolded protein binding	Up-regulation	Up-regulation	GP96, HSP70
Intracellular transport/secretion	Up-regulation	Up-regulation	ARF5, TMED7, SEC22
Proteasome	Up-regulation	Up-regulation	PMSD3, MSUG1

Table 3. Selected genes that were significantly differentially expressed in the liver of *S. senegalensis* during LPS or CuSO$_4$ treatments.

Because the GENIPOL cDNA microarray was constructed from ESTs derived from flounder liver and had been used in studies of hepatic expression (Cohen et al., 2007; Williams et al., 2006, 2007, 2008), we investigated whether this platform would be valid for analysing the transcriptional response in the head-kidney. To this end, the GENIPOL microarray was used to compare the basal transcriptional expression in the *Solea senegalensis* head-kidney and liver. We determined that 1004 genes were statistically differentially expressed in both organs: 418 transcripts were more abundant in the liver than in the head-kidney and 586 transcripts were more abundant in the head-kidney than in the liver. Thus, although the microarray was constructed with hepatic ESTs, the number of genes identified as over-expressed is similar in both organs, though slightly higher in the head-kidney than in the liver. The analysis of transcriptional patterns showed that the most represented biological processes amongst the genes up-regulated in the liver in comparison with the head-kidney were those involved in innate immune response, digestion, lipid transport, and monooxygenase activity (Table 4). The category "innate immune response" grouped genes coding for acute phase proteins because, as has been previously discussed, the liver is the main source of these plasmatic proteins. Amongst the genes related to lipid transport that were particularly remarkable were those coding for several apolipoproteins. The term "monooxygenase activity" encompassed genes belonging to the cytochrome P450 family, in agreement with what is known about the detoxifying capacity and the biotransformation activity of the liver in teleosts (Thorgaard et al., 2002).

Functional terms over-represented in the list of transcripts that were more abundant in the head-kidney than in the liver related to cellular division and protein turnover (protein degradation and the proteasome and ribosomal proteins), among others (Table 5). These results agree with the role of the head-kidney as a major haematopoietic organ in teleosts and with its function as a secondary lymphoid organ in the clearance of soluble and particulate antigens from circulation (Whyte, 2007). It is worth noting that when the lists of genes that are up-regulated in both organs were compared, two sequences corresponding to

Innate immune response

> alpha-1-antitrypsin, alpha-2-macroglobulin, anticoagulant protein C precursor, coagulation factor VIIc, chemotaxin, complement component C3, complement component C8, complement component C9, complement regulatory plasma protein, fibrinogen alpha, fibrinogen beta chain precursor, fibrinogen gamma chain precursor, haptoglobin, hepcidin precursor, interleukin 8 precursor, kininogen 1, plasma protease C1 inhibitor precursor, prothrombin precursor, putative complement factor, transferrin

Digestive enzymes

> chymotrypsinogen 1, chymotrypsinogen 2, trypsinogen 2 precursor

Lipid transport

> apolipoprotein A-I, apolipoprotein A-IV, apolipoprotein C-I precursor, apolipoprotein E, apolipoprotein H, 14kDa apolipoprotein, fatty acid-binding protein

Monooxygenase activity

> cytochrome P450 2F2, cytochrome P450 2X, cytochrome P450 3A, cytochrome P450 3A45, cytochrome P450 8B1, cytochrome P450 monooxygenase

Table 4. Selected genes up-regulated in the liver in comparison with the head-kidney in *S. senegalensis*.

Cellular division/cytoskeleton

> alpha-tubulin, actin-related protein 3 homolog, actin related protein 2/3 complex subunit 4, beta-actin, cofilin 2, coronin 1A, lamin B1, microtubule-based motor protein, mitotic spindle assembly checkpoint protein, myosin regulatory light chain 2, nuclear movement protein PNUDC, thymosin beta-4

Protein degradation/proteasome

> polyubiquitin, proteasome alpha 1 subunit isoform 2, proteasome (prosome, macropain) subunit alpha type 7, proteasome beta-subunit C5, proteasome subunit beta type 3, proteasome (prosome, macropain) subunit beta type 5, proteasome 26S ATPase subunit 5, proteasome subunit N3, ubiquitin carboxyl-terminal hydrolase isozyme L, ubiquitin specific protease 9

Ribosomal proteins

> 40S ribosomal protein S3a, 40S ribosomal protein S4, 60S ribosomal protein L3, 60S ribosomal protein L4, 60S ribosomal protein L13

Table 5. Selected genes up-regulated in the head-kidney in comparison with the liver in *S. senegalensis*.

the GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene, but with different expression patterns, were detected. The first of these sequences was more abundant in the liver, while the second was prevalent in the head-kidney. *Solea senegalensis* possesses two different GADPH paralogous genes that exhibit different tissue expression patterns, with GAPDH1 being more abundant in the liver and the GAPDH2 isoform more prevalent in the head-kidney (Manchado et al., 2007). A detailed analysis of the sequences detected in the microarray study revealed that these sequences match the two described isoforms. Altogether, these results demonstrated that the GENIPOL microarray platform was valid for analysing the transcriptional response of the sole head-kidney, discriminating between genes coding for transcripts with specific patterns of hepatic or renal expression.

Consequently, the GENIPOL platform was used to assess the response to LPS in the head-kidney. After 24 h of LPS treatment, a total of 224 genes was statistically differentially expressed in the head-kidney (117 up-regulated and 107 down-regulated). The functional analysis of the results revealed that the biological processes altered by LPS treatment in the head-kidney were very similar to those detected in the liver. Most notably amongst the up-regulated genes were the functional groups immune response, unfolded protein-binding, intracellular transport/secretion and proteasome, and digestive enzymes in the list of down-regulated genes. In contrast, the glutathione transferases category, whose transcript levels were down-regulated by LPS in the liver, was not affected in the head-kidney.

4.3 Real-time qRT-PCR

Although the determination of absolute transcript abundance (the exact number of molecules of a transcript in all samples of the study) is the only adequate procedure to accurately assess the expression of a gene (Prieto-Álamo et al., 2003), these kinds of studies are infrequent due to the experimental difficulties that impair this type of analysis. A set of more than 20 sole transcripts was selected for the quantification of their levels by this rigorous approach (Table 6) (Prieto-Álamo et al., 2009; Osuna-Jiménez et al., 2009), based on the physiological relevance of their products and on the magnitude and specificity of their responses.

Immune Response

   complement component C3, complement component C7, transferrin, haptoglobin, ferritin M, natural killer enhancing factor, tumor necrosis factor alpha-induced protein 9, hepcidin, nonspecific cytotoxic cell receptor protein-1, angiotensinogen, sequestosome 1, tumor necrosis factor receptor associated factor

Response to stress

   CCAAT/enhancer binding protein, cold inducible RNA binding protein, DNA-damage-inducible transcript 4-like, NHP2 non-histone chromosome protein

Energetic Metabolism

   glyceraldehyde-3P-DHase, transketolase, NADH-DHase 1 alpha subcomplex 4

Protein Synthesis, Folding and Degradation

   asparaginil-tRNA synthetase, heat shock protein GP96, proteasome 26S non-ATPase subunit 3, cathepsin Z

Transport

   α-globin

Table 6. Biological functions of proteins encoded by the sole transcripts selected to be validated by real-time qRT-PCR (Prieto-Álamo et al., 2009; Osuna-Jiménez et al., 2009).

The absolute quantification of the selected transcripts was not limited to the samples used for global analysis, but rather extended to samples coming from different individuals, organs, treatments and exposure times. Consequently, this gene-to-gene analysis provided valuable additional information on transcriptional expression patterns of the selected genes. Individual quantification is also mandatory to prevent biased interpretations of specimens with abnormal expression levels. The absolute real-time qRT-PCR on individual samples

demonstrated that inter-individual variations of most examined transcripts in treated animals was in the range of that in control fish, indicating similar susceptibility to LPS or $CuSO_4$ challenge among individuals. The qRT-PCR quantifications confirm, in general, the results obtained with the subtractive libraries and the DNA microarrays. As expected, substantial differences in abundance were found depending on the transcript and tissue examined. In general, each transcript displayed a characteristic expression profile, distinguishing between constitutive or up-/down-regulated, early or late responsive, stressor-specific or not, etc., as a function of the organ analysed.

5. Proteomics studies of GBD

Fish reared in earth ponds are eventually affected by Gas Bubble Disease (GBD) outbreaks if ponds are not correctly handled, particularly under high temperature and radiation conditions. GBD is a non-infectious pathology occurring when the partial pressures of atmospheric gases in solution exceed their respective partial pressures in the atmosphere. GBD was initially observed in farmed species, although outbreaks in wild fish, both fresh-water and marine animals, have also been reported. GBD can have serious adverse economic repercussions in fish cultures by reducing productivity and the commercial value of the fish as well as the farm profitability. Thus, the development of biomarkers responsive to hyperoxia stress would be a valuable tool to apply in systems where oxygen supersaturation might be possible, and would also contribute to basic knowledge of oxidative stress. Oxygen supplementation is a common practice in intensive fish farming, in order to allow high density cultivation while reducing the amount of water demanded in aquaculture facilities. It is also required during fish transportation. In intensive aquaculture, the use of oxygen is regulated by sophisticated mechanisms to keep its concentration close to desired values. However, in open ponds, the likelihood of oxygen supersaturation conditions is higher, because primary producers are in a high nutrient environment, occasionally combined with high temperature and radiation. Photosynthetic oxygen overproduction is a factor of concern for pond aquaculture, particularly when species such as sole, which exhibit nocturnal and benthic habits, are considered. These features complicate water management compared to pond operation in pelagic fish farming.

Environmental and physicochemical conditions inducing hyperoxia, such as radiation, temperature and dissolved O_2, were monitored in two independent land-based ponds of an aquaculture research centre (IFAPA Centro El Toruño, Puerto de Santa María, Cádiz, Spain) in which *S. senegalensis* were reared, after a GBD outbreak was detected in some of these animals (Salas-Leyton et al., 2009). Fig. 2 (upper) shows the appearance of the earth pond used as control (100 m², water renewed 4-fold/day) and of that in which GBD developed (900 m², water unrenewed) in which algal blooms can be observed at its border. As shown in Fig. 2 (centre), the dissolved oxygen profile detected in the hyperoxic earth pond was typical of that in environments dominated by macroalgal biomass and high photosynthetic activity, where extreme oxygen levels are reached during a great part of the daily cycle (including night hours), always above saturation without a desaturation phase. As shown in Fig. 2 (lower), the following typical GBD symptoms were detected in fish from this pond: exophthalmia caused by retrobulbar bubbles (A), subcutaneous emphysemas, obstruction of gill lamellas (B), big bubbles located at caudal (C) and dorsal
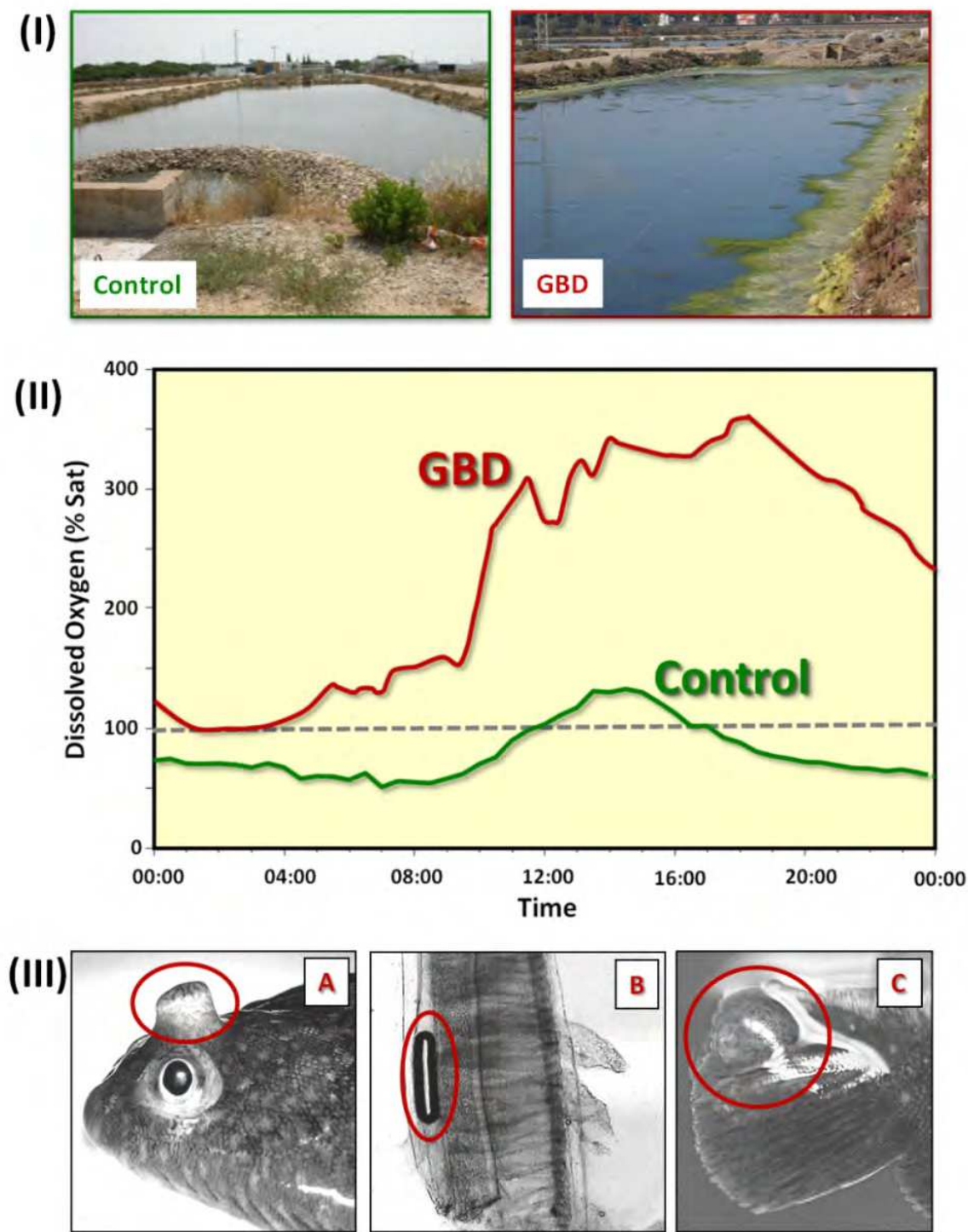
Fig. 2. Earth ponds, dissolved oxygen monitoring and visible symptoms in GBD-affected soles. (I) The experiments were carried out in two independent land-based ponds, one a 900 m² rectangular pond where oxygen supersaturation developed spontaneously, named the GBD pond (right) and the other a 100 m² control pond (left) in which water was renewed four times per day. (II) Dissolved oxygen was simultaneously monitored throughout the daily cycle in both the control (green line) and GBD ponds (red line). (III) Visible symptoms observed in GBD-affected soles included the following: exophthalmia caused by retrobulbar bubbles (A), a bubble obstructing a gill lamella (B), and a large bubble located at caudal fin (C).

fins, haemorrhages, anomalous swimming accompanied by loss of orientation, near-lethargy status and individual isolation were the main effects of O_2 supersaturation (Salas-Leyton et al., 2009).

Under the described aquaculture conditions, a parallel proteomic study was carried out in search of protein alteration patterns that might be used as potential new and unbiased biomarkers of hyperoxic stress (Fig. 3) (Salas-Leyton et al., 2009). The following three health statuses were studied in sole individuals: (1) healthy control fish, (2) GBD-affected but asymptomatic fish, and (3) GBD-affected fish with visible symptoms. Protein expression profiles were studied by 2-DE in cytosolic fractions of gills and livers. A total of 1,525 and 1,632 spots were detected in the four gill and liver gels, respectively, that were run in each of the three situations studied. Fig. 3 (upper) shows the master gels of cytosolic gill (left) and liver (right) proteins. A total of 205 protein spots were differentially expressed in the gills and 498 in the liver in each health status. Fig. 3 (middle) shows the number of spots which are present only, absent, increased or diminished in each of the studied conditions, and the total number of changes found. A significantly higher number of differentially expressed spots were found in GBD-affected soles, mainly in fish with visible symptoms. Of these, 25 spots in the gills and 23 in the liver were selected for identification using tandem mass spectrometry (nESI-IT MS/MS), *de novo* sequencing and a bioinformatics search. Fig. 3 (lower) shows the percentage of the relative intensity of each spot in each health status. Sequence tags were obtained from 9 (gills) and 5 (liver) of the selected spots, resulting in a total of 14 identified spots in the GBD status, which are indicated in Fig. 3 (lower).

Due to the central role of gills in oxygen exchange, the proteins identified in the gills of GBD-affected fish were, unsurprisingly, related to oxidative alteration of the cytoskeleton structure or function (β-tubulin and β-actin), motility (light myosin chain and α-tropomyosin), regulatory pathways (calmodulin, Raf kinase inhibitor protein [RKIP]) and carbohydrate metabolism (glyceraldehyde 3-P dehydrogenase). The hyperoxia-linked effects of these proteins and higher-level responses, related to inflammatory response, apoptosis or cell death, or derived from alterations in the regulatory proteins, have been discussed in depth (Salas-Leyton et al., 2009). In gills, only RKIP was most intense in GBD-affected animals without symptoms, while the other seven identified proteins were most intense in GBD-affected fish with symptoms. Proteins identified in the liver were related to protein oxidative damages (β-globin and fatty acid-binding protein [FABP]), protection from oxidative stress (dicarbonyl/L-xylulose reductase and glycine N-methyltransferase) and inflammatory response (complement component C3), in agreement with the predominant metabolic role of this organ. In the liver, only FABP was most intense in healthy fish, while the other four identified proteins were most intense in GBD-affected fish with visible symptoms. Approximately 50% of the identified proteins corresponded to "unusual" proteins not often found in proteomics screens, while the rest were preferential targets of oxidative stress. Most of these latter proteins were found as truncated forms of oxidised proteins that might trigger cellular defences against hyperoxygenation preceding GBD outbreaks. Some of the identified proteins might be considered to be good hyperoxia stress biomarkers and could be used in the early-warning detection of GBD outbreaks. Massive proteomic approaches have been applied for the first time to the study of this non-infectious pathological condition, gas bubble disease, in fish.
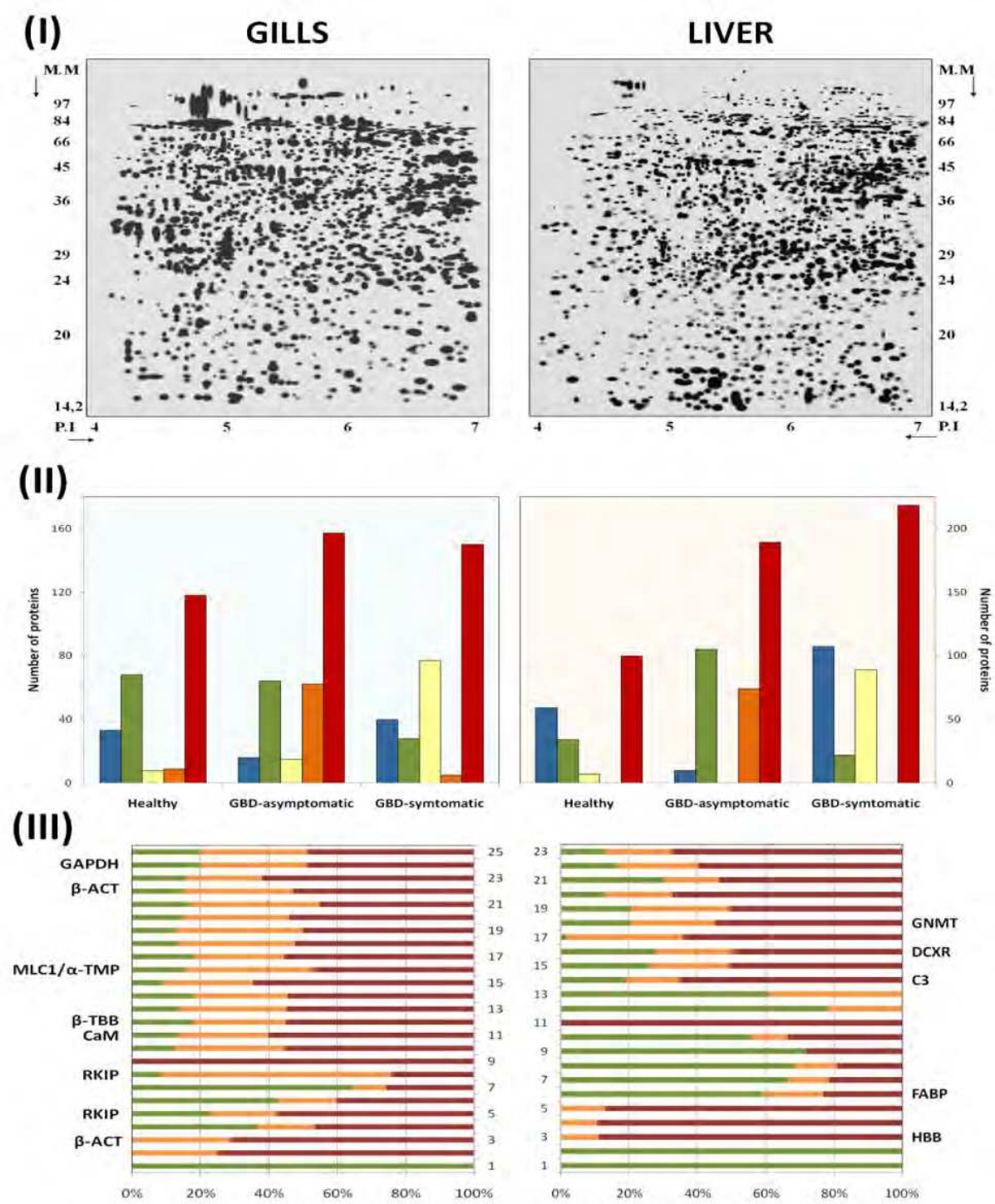
Fig. 3. Proteomic study of GBD-affected juvenile soles after 2-DE of cytosolic gill (left) and liver (right) proteins. (I) Master gels. (II) Number of proteins showing distinct protein expression patterns in healthy, GBD-asymptomatic and GBD-symptomatic fish. Bars represent the number of protein spots present only (■), absent (■), increased (■), diminished (■) and the total number of changes (■) found in each of the conditions. (III) Spots selected for possible identification by mass spectrometry analysis. Bars represent the percentage of the relative intensity of each spot in every status: healthy (■), GBD-asymptomatic (■) and GBD-symptomatic (■) animals. Successfully identified proteins are highlighted.

6. Conclusions

Given the economic importance of *S. senegalensis*, an aquaculture species that remains largely unexplored at the genomic level, the application of postgenomic methodologies provides results that may be highly relevant at a genetic, immunological and toxicological level, contributing to the improvement of management and welfare of this organism in aquaculture. The utility of high-throughput proteomic methods for unveiling the molecular basis of a cumbersome disease in aquaculture has been also demonstrated.

7. Acknowledgments

8. References

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Aquaculture

Edited by Dr. Zainal Muchlisin

This book provides an understanding on a large variety of aquaculture related topics. The book is organized in four sections. The first section discusses fish nutrition second section is considers the application of genetic in aquaculture; section three takes a look at current techniques for controlling lipid oxidation and melanosis in Aquaculture products. The last section is focused on culture techniques and management, ,which is the larger part of the book. The book chapters are written by leading experts in their respective areas. Therefore, I am quite confident that this book will be equally useful for students and professionals in aquaculture and biotechnology.

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