Fish Metalloproteins as Biomarkers of Environmental Contamination

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Contents

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1 Introduction

In this review, we explore the fish metalloproteins that have been discovered by 'omic techniques, and their application as fish biomarkers of environmental contamination.

The fields collectively known as "'omics" have undergone tremendous development in the past decade. The best known among them is genomics, in which complete genome DNA sequences of living organisms are produced. Other "'omics" include structural genomics, proteomics, toxicoproteomics, and metallomics (Shi and Chance 2008). The information obtained by applying the fields of proteomics, toxicoproteomics, and metallomics can be utilized to establish biomarkers of exposure for organisms that are affected by environmental contaminants. Moreover, the likelihood of discerning viable biomarkers from proteomic and metallomic investigations is especially high, given that protein profiles appear to be specific to particular stressors (Martin et al. 2001; Shepard et al. 2000; Jellum et al. 1983; Blom et al. 1992; Powers 1989). Thus, environmental proteomics provides a more comprehensive assessment of the toxic and defensive mechanisms that are triggered by pollutants than do traditional biomarker studies (Gonzalez-Fernandez et al. 2008).

For several reasons, fish have attracted considerable interest in studies designed to assess the biological and biochemical responses that organisms have to environmental contaminants (Powers 1989). Fish are particularly useful for assessing water-borne and sediment-deposited toxins, and may provide advanced warning of the environmental contamination potential of new chemicals, or the status of environmental contamination by well-known toxicants. Fish are also particularly good models for studies in which biochemistry and comparative physiology are involved, because they live in diverse habitats and must adapt to environmental parameters and stress, both of which can be easily reproduced under laboratory conditions (Beyer 1996). The understanding of toxicant uptake, behavior, and responses in fish, therefore, has a high potential for ecological relevance. Because of these aspects, it is important that these organisms be studied in more detail; the development of 'omic techniques in recent years offers the possibility to perform such studies in new and useful ways.

2 Biomarkers and Proteomic Approaches to Their Discovery

Biomarkers have been extensively studied, and several comprehensive reviews and books on the subject are available, including ones that address biomarkers specific to fish (Adams 1987; NRC 1987; Schlenk 1999; Stegeman et al. 1992). Biomarkers are defined as measurements in body fluids, cells, or tissues that can indicate biochemical or cellular modifications resulting from the presence of toxicants or stress (NRC 1987). This original definition was later modified to take into account characteristics of organisms, populations, or communities, including behavior, in which measurable responses occur that reflect changes to the environment (Adams 1987; Depledge et al. 1992). In other words, the foundational concept of the biomarker

approach for assessing adverse effects or stress is based on the hypothesis that the effects of stress are typically manifested, first at lower levels of biological organization, before disturbances are realized at the population-, community-, or ecosystem-levels. This concept allows for development of early warning biomarker effect signals that may occur at later response levels (Bayne et al. 1985).

The emerging omics technologies, are well positioned to address such concerns by fomenting the discovery of mechanisms that underlay the toxic action of chemical pollutants, and by assisting in the identification of new biomarkers (Dowling and Sheehan 2006; López-Barea and Gómez-Ariza 2006; Quackenbush 2001). For example, biomarkers may consist of an integrated set of genes or proteins that are simultaneously expressed in certain situations. Biomarkers may be used to (1) characterize related functions of genes and gene products that have similar activity profiles or common mechanisms of regulation (Snape et al. 2004); (2) classify compounds that have similar modes of action by creating toxicological "fingerprints" for them (Oberemm et al. 2005; Miracle and Ankley 2005; Baker 2005); or (3) characterize different stress levels by integrating general and highly specific markers in one assay. Thus, the incredible amount of molecular-level information available from applying 'omic-technologies is rapidly fomenting the development of biomarkers, from singular biomarker measurements to highly complex multimarker genomic/proteomic panels (Miracle and Ankley 2005).

Proteomic analyses provide valuable information, when variations that occur within the proteome of organisms are compared as a consequence of biological perturbations or external stimuli. These stimuli often result in different protein expressions or the redistribution of specific proteins within cells (Martin et al. 2001, 2003; Vilhelmsson et al. 2004; Tyers and Mann 2003), which can be correlated with environmental contamination, and which may help identify proteins that are altered from pollutant exposure, or may help establish a protein-pollutant toxic mechanism relationship (López-Barea and Gómez-Ariza 2006). An added bonus in these studies is the fact that it is not absolutely necessary to establish the identity of a protein for it to become a successful biomarker of exposure. Indeed, the characteristics of a peptide and the specific conditions under which it occurs are the more pressing concerns (Hogstrand et al. 2002). Recent studies have produced protein expression signatures that were characterized in marine invertebrates, in response to changing salinities and temperatures, and in response to the presence of polychlorinated biphenyls and copper (Bradley et al. 1985; Shepard et al. 2000; Shepard and Bradley 2000; Kimmel and Bradley 2001).

3 Fish Proteomics in the Search for Biomarkers of Environmental Contamination

Functional genomic and proteomic technologies have allowed biological questions to be addressed in a broader approach by allowing simultaneous study of thousands of genes or proteins.

Applications of comparative proteome "'omic" techniques have proven useful for further examining the altered protein expression in fish development. These techniques have enhanced, for example, the understanding of the molecular mechanisms underlying normal and abnormal development (Kultz and Somero 1996; Kanaya et al. 2000), the effects of feeding habits on metabolism parameters and the liver proteome (Martin et al. 2003; Vilhelmsson et al. 2004), and the understanding of physiological mechanisms that explain the nature of phenotypic differences between farmed and wild fish (Olsson et al. 2007).

Comparing fish proteomes in different situations is appealing, because changes in proteome expression under complex field situations may disclose which gene products, metabolites, or proteins are most interesting to investigate (Albertsson et al. 2007). Such comparative proteomic studies are also useful as tools in the investigation of fish development and different ecological situations.

Within this context, Tay et al. (2006) used two-dimensional electrophoresis, followed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) to identify and to verify protein differences in the early development of zebrafish (*Danio rerio*). No dramatic protein changes were observed up to 18-h postfertilization, but significant changes occurred at subsequent developmental stages. The highest number of proteins was identified 6–10-h postfertilization. From 18 to 24-h postfertilization, the number of detectable spots above 50 kDa decreased dramatically, whereas there was a surge in lower molecular weight proteins at 24-h postfertilization. Interestingly, 49% of the proteins detected at 6-h postfertilization remained detectable when fish reached an age of 1-week. Most of the identified proteins in this study were cytosolic, cytoskeletal, and nuclear proteins, and these are involved in diverse functions such as metabolism, cytoskeleton, translation, and protein degradation. Identification of such proteins also produced additional insights on what constitutes normal fish development.

A similar study was conducted on two different age groups of Atlantic cod larvae (Sveinsdottir et al. 2008), but produced very different results from those observed in the zebrafish study. Interestingly, in this second study, despite the visible morphological and functional changes that occurred in larvae from 6 to 24-days posthatch, the pattern of the most abundant proteins in these Atlantic cod larvae was largely conserved. Such differences between the two species indicate significant differences in the normal development among different fish species.

The effects of starvation in certain species, such as rainbow trout, have also been studied by using comparative protein expression tools (Martin et al. 2001). Herein, protein extracts from whole rainbow trout liver were analyzed on high-resolution two-dimensional gels. The experimental group was starved for 14-days, during which time the control animals were fed a normal daily diet until satiated. Twenty-four proteins showed abundance differences between the two groups; 8 were increased in fed fish, while 16 increased in abundance as a result of food withdrawal. Five proteins that showed a difference in abundance levels in starved and fed fish were identified, including Cathepsin D, a known lysosomal protease. This study was the first to use protein profiling in a nonmodel organism (rainbow trout) to demonstrate, for the first time in teleosts, that proteomics have the potential to assist in studying cellular mechanisms involved in protein degradation.

In another context, comparing gene and proteome expression of fish, residing in contaminated versus noncontaminated environments, may also be useful. For example, Stentiford et al. (2005) reported significant differences in fish caught from polluted and nonpolluted sites. Their study was an important pilot study conducted in 2005 on flatfish *L. limanda* in which liver lesions and normal tissue from wild populations were investigated using proteomic techniques. Liver lesions had characteristic proteomic differences, and 56 proteomic features were upregulated in tumor tissue and 20 were downregulated. Twelve of these proteomic features exhibited the potential to act as biomarkers for neoplasic lesions. These authors concluded that the proteome of cancerous liver tissue is significantly different from that of nontumor liver tissue from the same fish. This was one of the first studies, in which comparative proteomic expression was utilized as a means to discriminate between tumorous and nontumorous livers in fish. Moreover, the preliminary data from this study was one of the first to suggest that proteomic approaches may be useful in an environmental contamination context for studying fish, and may have potential application to serve as a high-throughput screening approach for disease classification.

Another interesting environmental contamination study that utilized 'omic techniques disclosed protein differences in rainbow trout that were exposed to sublethal zinc doses for several days (Hogstrand et al. 2002). Zinc exposure induced the expression of the beta-chain of the trout complement C3-1 protein. This protein plays an important role in immune response and has an immunoregulatory function (Lambris et al. 1993). The authors concluded that the induction of this protein by zinc may constitute evidence of a stimulatory effect upon trout immune system.

Ling et al. 2009 performed another study in which the effects of metal (cadmium) contamination on the fish proteome were observed in gill tissue of *Paralichthys olivaceus,* by using two-dimensional electrophoresis. Compared to a control sample, significant changes were visualized in 18 protein spots that had been exposed for 24-h to seawater contaminated with 10.0 ppm of cadmium. Among these spots, two were upregulated, one was downregulated, seven showed low expression, and eight showed high expression. Ten of the 18 proteins identified on the 2D-PAGE gel included heat shock protein 70 and calcium-binding protein, and demonstrated a synchronous response to acute cadmium toxicity. These proteins may therefore be utilized as biomarker profiles for investigating cadmium contamination levels in seawater.

Other environmental contaminants, such as cyanotoxins, have similarly been addressed using 'omics techniques. Increased attention is being played to the cyanotoxins because they bioaccumulate in fish and other aquatic species and may induce subchronic and chronic toxicity. A certain class of cyanotoxins, the mycrocistins (MC), has been recently shown to produce significant effects on the proteome expression of several fish organs (Karim et al. 2011; Mezhoud et al. 2008). Mezhoud et al. (2008) force fed adult medaka fish either an MC solution (experimental) or water (controls). After 2-h of exposure, livers were extracted and prepared for 2D-SDS-PAGE analyses. After mass spectrometric analyses, 17 differentially regulated proteins were successfully identified. The identified proteins were assigned to several functional groups, such as the following: proteins involved in cell structures, in signal transduction, enzyme regulation, and oxidative stress. Examples of the identified proteins were methyltransferase, transmembrane, apolipoprotein

natural-killer-enhancing factor, and b-tubulin, among others. These altered proteins corroborated with data on studies that had mycrocistin toxic effects and modes of action, i.e., cytoskeleton disruption.

In a similar study, zebrafish were exposed to mycrocistin at two different concentrations (2 and 20 μg L^{-1}) and were then compared to a nonexposed control group. The liver proteome was also analyzed by 2D-SDS-PAGE. Compared to the 2D gels of the nonexposed zebrafish livers, the abundance of 22 protein spots from the MC-exposed zebrafish livers were significantly altered (≥2-fold or ≥0.5-fold). Among these altered proteins, three protein spots disappeared in both groups of MC-exposed zebrafish livers, seven protein spots were significantly upregulated, and ten protein spots were noticeably down-regulated in the livers of 2 μg L^{-1} MC-exposed zebrafish. In the 20 μg L^{-1} MC-exposed group, 4 protein spots were remarkably upregulated and 11 protein spots were markedly down-regulated. The identified proteins were distinguished to comprise 22 different proteins. Of these, nine were involved in metabolism and five in proteolysis. Two proteins were characterized as cell cytoskeleton proteins, corresponding to ones that were a-actinin-4- and profilin-2-like. The other six proteins were categorized as calcium/phospholipid binding, antioxidant defense, protein folding, and other functional proteins.

One of the most interesting proteomic studies was conducted directly in the field. Rainbow trout (*Oncorhynchus mykiss*) were caged upstream and downstream from a sewage treatment works (STW), which was releasing a complex mixture of contaminants. Two-dimensional gel electrophoresis was run on liver protein extracts from these two groups, and the results showed four significantly up- and down-regulated protein spots from the group caged downstream from the STW. The three downregulated spots contained betaine aldehyde dehydrogenase, lactate dehydrogenase, and an unidentified protein, respectively. The only upregulated spot consisted of both mitochondrial ATP synthase alpha-subunit and carbonyl reductase/20b-hydroxysteroid dehydrogenase (CR/20b-HSD). This was the first study in which these types of biological responses were produced in fish exposed to a complex contaminant mixture (i.e., STW effluents). However, the significance of the differentially expressed proteins from this study is still not clear and merits further investigation.

Such techniques have primarily benefited research on well-characterized species such as humans, mice, and yeast; until now, few proteomic studies have been performed in animals from natural ecosystems (Karim et al. 2011). Unfortunately, these well-characterized species may be inappropriate from an ecotoxicological and environmental perspective, since they may not be useful as sentinel species when investigating environmental contamination (Hogstrand et al. 2002). Nevertheless, several general biomarkers that are used in mammalian models are directly transferable to fish. Karim et al. (2011) recently summarized the merge of pathways involved in environmental stress in fish and mammalian models (Fig. 1), clearly showing that several pathways are shared by both groups, i.e., the physiological process of oxidative stress. Reviews and further studies of these pathways may assist researchers to discover new biomarkers in fish and further understand existing ones.

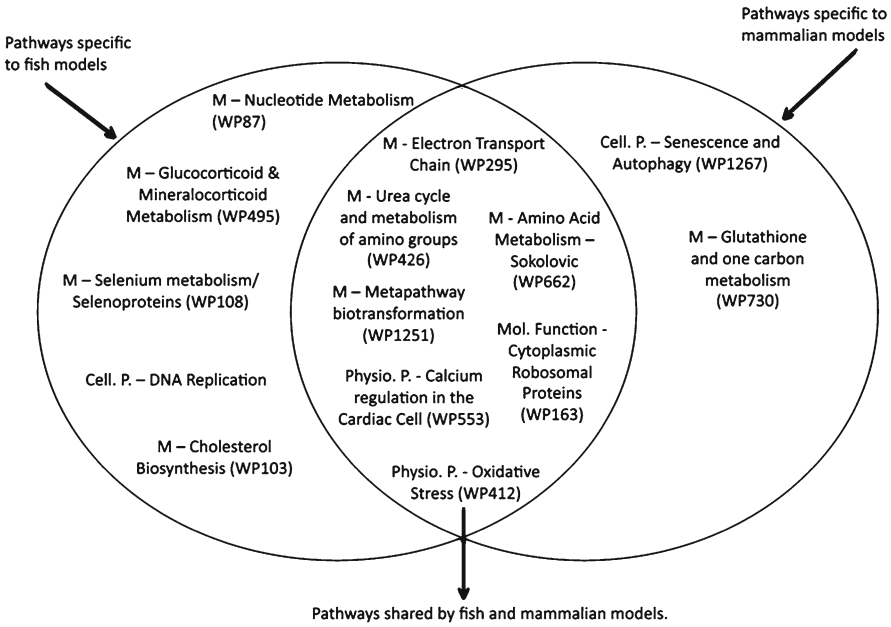

Fig. 1 Pathways involved in environmental stress in fish and mammalian models. *M* Metabolism, *Cell. P* cellular process, *Physio. P* physiological process (Adapted from Karim et al. 2011)

4 Metallomic Studies: Particularities

The recently developed field of metallomics, a term coined in the early 2000s, considers that biomolecules that bind metals and metalloids constitute a substantial portion of molecules involved in cell metabolism and behavior, and that identifying a metal cofactor in a protein can greatly assist in defining its function and placing it in the context of known cellular pathways (Haraguchi 2004). Metal-bound proteins, or metalloproteins, are now being used successfully as biomarkers of environmental exposure for some organisms (López-Barea and Gómez-Ariza 2006), although fewer studies and discoveries exist in metallomics, than for proteomics.

The late advent of the metallomic field and the paucity of metallomic findings results from several singular issues that occur when dealing with metalloprotein analyses. These issues include the absence of any protein amplification reaction similar to what exists for PCR (polymerase chain reaction) in genomics, the occurrence of post-translational changes in the biological entities, and finally, the low concentration of metallic trace elements present in biological tissues (usually <1 μg g^{-1}) and the complexity of the matrices (Gomez-Ariza et al. 2004). These deficiencies render metal-bound biomolecule analyses more difficult and challenging. Hence, metallomic analyses require sophisticated multidimensional analytical approaches. However, there has been a continuous development of new techniques

that combine atomic spectroscopy and standard biochemical or proteomic techniques that enhance future prospects for metallomic analyses; these include gel electrophoresis, multidimensional capillary or nanoflow chromatography, or capillary electrophoresis, and strategies for complementary applications of element and molecule-specific detection techniques (Prange and Profrock 2005). For example, mass spectrometry approaches such as inductively coupled plasma MS (ICP-MS), electrospray MS (ESI-MS), and matrix-assisted laser desorption ionization MS (MALDI-MS) are now routinely used alongside prior 2D-PAGE protein separation to rapidly and precisely identify the metalloid component of individual proteins. Incredibly large amounts of data can be collected using these techniques, and they allow for multiend point effect screening, and the potential for identifying unexpected adverse effects (Kling et al. 2008). Unfortunately, only a few fish metalloproteins have actually been discovered and applied as biomarkers, and their functional roles in fish physiology are not yet well understood. Therefore, the challenge, and a key future goal is to use the full potential of metallomic approaches and strategies to elucidate multiple new fish metalloproteins.

5 Metalloproteins as Biomarkers of Environmental Contamination in Fish

For 5 decades, the presence of environmental contaminants in biotic and abiotic samples has been measured using chemical analytical techniques. The analyses of metal concentrations and compartmentalization in fish have also been conducted for about that long (Marigomez et al. 2002). However, since the discovery that metal speciation has toxicity implications, and more recently, with the advent of metallomics, the validity of quantifying total metal levels in fish organs, and directly relating them to environmental contamination has been questioned (Moldovan et al. 2004). Total metal levels in fish, although providing clues to toxic exposure levels, can also be influenced by the amount of metals present in the form of metal-bound proteins. So, the idea that high metal levels in certain fish organs (i.e., liver) are indicative *only* of substantial on-site contamination is no longer regarded to be true. Further insights into normal regulatory functions of essential trace-elements and metal-bound proteins in fish have contributed to this attitude change. So, while on-site contamination is still a possibility when observing high metal levels in fish, other parameters, in addition to total metal concentrations, should be analyzed. With these new ideas in effect, metalloprotein studies in aquatic organisms have begun to gain attention, and metalloproteins are now recognized as being responsive to several environmental compounds. Although the use of fish metallomics has just started, several key fish metalloproteins have been discovered and have shown utility as biomarkers for environmental contamination (Table 1). Although some proteins, e.g., several selenoproteins, still do not possess any known function, they are known to be upregulated when exposed to environmental contamination (Karim et al. 2011), and such events merit further study. Metalloprotein levels

Table 1 Studies that have addressed metalloproteins as biomarkers in an ecological context

References	Metalloprotein type	Metalloprotein name	Fish organ or compartment	Fish species	Common name	Metalloprotein role
Kimura et al. (2001)	Matrix metalloproteinases (MMPs)	MMP-2; MMP-9	Oocytes	*Oryzias latipes*	Medaka fish	Follicular development; oocyte maturation; ovulatory or postovulatory processes
Hillegass et al. (2007)		MMP13	Whole embryo	*Danio rerio*	Zebrafish	Embryogenesis
Chadzinska et al. (2008)		MMP9	Immune organs; peritoneal and peripheral blood leucocytes	*Cyprinus carpio*	Common carp	Immune response
Gladyshev (2006)	Selenoprotein	Fep 15, SeIJ, SeIL, SeIP, SelU	Not described	Not described	Not described	Unknown
Geetha and Deshpande (1999)	Iron-binding protein	Ferritin	Liver	*Channa Punctatus*, *Labeo rohita, Scomberomorus cummerson*, *Scielutheronena Tetradactylum, Lates calcarifer*	Murrel, Rohu, Marine Spanish Mackerel, Indian Salmon, Brackish Water Perch	Important role in iron metabolism
Miguel et al. (1991)						
Kong et al. (2003)				*Dasyatis akajei*	Red stingray	

(continued)

Table 1 (continued)

References	Metalloprotein type	Metalloprotein name	Fish organ or compartment	Fish species	Common name	Metalloprotein role
Langston et al. (2002)	Metallothionein	–	Liver	*Anguilla anguilla*	Eel	Detoxification of heavy metals, internal homeostasis of Cu and Zn, participation in metabolic functions
Cinier et al. (1998)			Kidney, liver	*Cyprinus carpio*	Carp	
Pedrajas et al. (1993)	Superoxide dismutase	–	Liver, blood, muscle tissue	*Mugil* sp.	Mullet	Antioxidant
Li et al. (2008)	Mercury-containing protein, arsenoprotein, selenoprotein	–	Liver	*Hypophthalmichthys nobilis/ Ctenopharyngodon idella*	Bighead carp/grass carp	Detoxification processes

usually show interspecies variations and differential responses to different degrees of environmental contamination. Some of the metalloproteins that show promise as environmental biomarkers will be discussed further below.

5.1 Metallothioneins

Metallothioneins (MTs) are cysteine-rich heat-stable proteins that bind to metals entirely through metal–thiolate bonds (Kaegi and Schaeffer 1988). MTs are widely distributed, have been identified in all major classes of vertebrates, and play roles in organisms exposed to metal pollution (Roesijadi 1992; Mazzucotelli and Viarengo 1988). The mechanism of metal detoxification by MTs occurs via metal-initiated transcriptional activation of MT genes, resulting in increased MT synthesis, and subsequent binding of free metals to MT proteins. These proteins have three major physiological roles: (1) detoxification of heavy metals (Goering and Klaassen 1984); (2) internal homeostasis of Cu and Zn (Brouwer et al. 1986); and (3) participation in metabolic functions (Roesijadi et al. 1998). Several isoforms and different metals bound to these proteins exist in different fish organs (Vasak 2005).

MTs have been used successfully as biomarkers of the biological effects of heavy metals in aquatic organisms since the late 1980s (Viarengo et al. 1997). When exposed to different metals, some endogenous and exogenous factors modify MT levels in teleosts; i.e., reproductive steroids, stress hormones and season, temperature, salinity, and reproductive and dietary status (Olsson et al. 1995; Roesijadi 1992; Olsson et al. 1996; Hylland et al. 1998), and display marked variations in the inductive response among different species (Cinier et al. 1998). Therefore, stipulating what constitutes "normal" MT levels is difficult, because so many interspecies variations and differential responses to different environmental contamination levels have been observed. However, determining hepatic MT levels is an affirmed and appropriate biomarker for evaluating the biological significance of metal contamination; several studies indicate that the strength of relationships between metals and MT synthesis implies an induced response, primarily from metal exposure. Including a measurement of hepatic MT as part of a suite of sublethal effects measures, therefore, is likely to enhance environmental quality assessment (Langston et al. 2002).

MT has been often quantified, but rarely characterized, in fish tissues. The comparison between the characteristics of fish and mammalian MTs is only available in one study (Scudiero et al. 2005). What little is known indicates that fish MTs display several distinctive features in the primary structure, which includes the displacement of a cysteine residue located in the carboxy terminal half of the molecule, fewer lysine residues juxtaposed to cysteines, and the presence of three short segments of secondary structural elements. Fish MTs are also subject to heat effects, whereas mammalian forms are much less affected by heat. Moreover, fish metallothionein displays a better metal exchange capability than does mammal metallothioneins. Further 'omic studies are needed in the area of MT characterization; areas in which new work would be useful include deciphering structure, probing functional pathways, and further characterizing the nature of these metalloproteins.

5.2 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of calcium-dependent, zinc-containing endopeptidases (Bode and Maskos 2003). These proteins break down extracellular matrix components and facilitate both normal and pathological tissue remodeling, wound healing, embryo development, and tumor invasion (Matrisian 1992; Woessner 1994; Stetler-Stevenson 1996). The MMPs are important in normal physiological functions such as angiogenesis, wound healing, mammary gland and postpartum uterus involution, and cervical dilatation; however, excess MMP activity is correlated with diseases in mammalian models, such as tumor, arthritis, periodontal diseases, liver cirrhosis, atherosclerosis, and multiple sclerosis (Loftus et al. 2002; Nagase et al. 2006). Membrane-type matrix metalloproteinases (MT-MMPs) are apparently involved in cell–matrix interactions and also are implicated in a variety of immunomodulatory roles (Yang et al. 1996; Sato et al. 1997; Schonbeck et al. 1998). MMPs have also been identified in response to developmental and pathological changes in several organisms, primarily in the liver (Theret et al. 1998; Bueno et al. 2000). Despite the information that has been collected on the MMPs, their precise function remains unclear (Rath et al. 2001).

In fish, MMPs are not well studied, and they are only addressed in an ecological context in a few reports. MMPs were discovered in the oocytes of a small freshwater fish species, suggesting involvement during follicular development, oocyte maturation, or ovulatory processes (Kimura et al. 2001). These processes are directly linked to spawning and other events associated with ovulated oocytes or fertilized eggs, which are known to be affected by environmental contamination. However, the mechanism by which the MMPs affect these processes is still unknown. MMPs have also been implicated in normal and abnormal fish embryogenesis and development (Hillegass et al. 2007; Zhang et al. 2003). It has recently been demonstrated that glucocorticoid exposure alters craniofacial development and increases the expression and activity of MMPs in developing zebrafish (*Danio rerio*). Glucocorticoids are immunosuppressive and anti-inflammatory agents, used to treat autoimmune and inflammatory disorders and lymphoproliferative diseases, and are also potent teratogens (Almawi et al. 2002; Mandl et al. 2006). The mechanism by which they exert their teratogenic effects is unknown, but, in one study (Hillegass et al. 2007), zebrafish embryo glucocorticoid exposure increased the expression of two MMPs, i.e., MMP-2 (~1.5-fold) and MMP-9 (7.6–9.0-fold), at 72-h postfertilization. Further MMP activity was increased approximately threefold at 72-h, following glucocorticoid treatment, with craniofacial morphogenesis changes being observed. Cotreatment of zebrafish embryos with each glucocorticoid and the glucocorticoid receptor antagonist RU486 resulted in the attenuation of glucocorticoid-induced increases in MMP expression (52–84% decrease) and activity (41–94% decrease). Furthermore, the abnormal craniofacial phenotype observed following glucocorticoid exposure was less severe following RU486 cotreatment, demonstrating that in embryonic zebrafish, dexamethasone and hydrocortisone alter the expression and activity of MMP-2 and -9, and suggesting that these increases may be mediated through the glucocorticoid receptor.

MMPs also play a key role in fish immune responses. In one study (Chadzinska et al. 2008), sterile peritonitis was induced in common carp, when the expression of MMP-9 was evaluated in well-characterized fish immune organs, such as kidneys. The results showed that kidney MMP-9 expression was induced a mere 4-h after initial inflammation induction, and MMP-9 expression in kidney macrophages was significantly elevated until 48-h after induction. The inactivated MMP form of MMP-9, named pro-MMP-9, was also elevated up to 96-h of inflammation induction. The authors concluded that, in teleosts fish, MMP-9 should be considered as an active participant in the innate immune response, and it contributes to the resolution of the inflammation process. Although the mechanisms by which MMPs act are still unclear, they have been shown to act as useful biomarkers where inflammation processes occur.

5.3 Iron-Binding Metalloproteins: Transferrin and Ferritin

Iron-binding metalloproteins have also been discovered in fish. One main metalloprotein in this class is transferrin. It plays a crucial role in iron metabolism by binding and transporting Fe, thus making it unavailable for catalysis of superoxide radical formation. It is a single polypeptide chain of 70–80-kDa, comprised of two globular domains, resulting from an ancestral gene duplication and fusion, with each domain presenting an iron-binding site (Yang et al. 1984). Under normal conditions, most of the iron in blood plasma is bound to transferrin, and iron–transferrin complexes enter cells via a transferrin receptor-mediated endocytic pathway. Transferrin is abundant in nature and has been identified in a wide range of organisms, such as insects, crustaceans, fish, and mammals (Stafford and Belosevic 2003). Transferrin also has a close relationship with the immune system in several organisms and is recognized as a component of nonspecific humoral defense mechanisms against bacteria (Bayne and Gerwick 2001; Ellis 1999). This was first demonstrated in fish (Stafford and Belosevic 2003), wherein transferrin was expressed by activated goldfish (*Carassius auratus*) macrophages. It was also shown that transferrin significantly enhanced the killing response of goldfish macrophages exposed to different pathogens or pathogen products, such as lipopolysaccharides and several bacteria (*Mycobacterium chelonei*, *Trypanosoma danilewskyi*, *Aeromonas salmonicida*, and *Leishmania major*). This enhancement of the killing response indicated that transferrin is a primary activating molecule of macrophage antimicrobial response in fish, and is highly utilitarian as a biomarker for environmental contamination.

It has recently been discovered that transferrin is also a major cadmium-binding protein in blood plasma (De Smet et al. 2001). The authors indicate that fish transferrin shows binding affinities for cadmium in blood plasma that are comparable to human transferrin. The results of this study demonstrated that cadmium is primarily bound to two high molecular weight proteins in carp plasma, in which relatively small amounts are bound to a 60-kDa protein, and the major part is bound to transferrin. When humans and brown trout were compared to carp, differences between

cadmium transport in plasma were observed. These differences were explained by the absence or very low albumin concentrations present in carp plasma, since in humans and brown trout cadmium is mainly bound to albumin, not transferrin.

Ferritin, also an iron-binding metalloprotein, plays an important role in in vivo iron metabolism (Kong et al. 2003). It is a 450-kDa protein and is the main iron storage protein in both eukaryotes and prokaryotes, and keeps iron in a soluble and nontoxic form (Chasteen 1998; Harrison and Arosio 1996). Ferritin synthesis is known to be induced when iron is available, whereas under iron deprivation conditions, ferritin synthesis is repressed (Torti and Torti 2002). Moreover, upregulation of ferritin is observed under conditions of oxidative stress (Orino et al. 2001) and inflammation (Torti and Torti 2002; Torti et al. 1988), suggesting a link to immune response. Ferritin has been described in several species by using 'omic techniques, such as northern blotting, 2D electrophoresis, molecular cloning, and nucleotide sequencing (Yamashita et al. 1996; Andersen et al. 1995; Chen et al. 2004; Geetha and Deshpande 1999; Kong et al. 2003; Miguel et al. 1991). However, ferritin analyses in fish have gained less attention. Most researchers have focused on its isolation and characterization, rather than on its functional aspects. Few ecological-related studies are available on this metalloprotein. Geetha and Deshpande (1999) used native gel electrophoresis, SDS-PAGE electrophoresis, and immunoblotting to compare ferritin characteristics among different fish species. The results indicated that the iron content of ferritins from marine and brackish species was higher than those from fresh water species, the phosphate/iron ratio was higher than mammalian ferritins, and that ferritins in fish liver are monomeric.

In another study, conducted with sea bass, both ferritin and transferrin expression in fish brain and liver were analyzed by 'omic techniques (Neves et al. 2009), after the fish were either exposed to experimental bacterial infections or to iron modulation. The fish were divided into three groups: a group receiving iron overload, another iron deficiency, and a control group. In response to infection, transferrin expression was decreased in the liver and increased in the brain. Moreover, transferrin increased in the liver, in response to iron deficiency. Ferritin expression inversely reflected transferrin content of the liver. Ferritin also increased in infection and iron overload situations and decreased in response to iron deficiency. In contrast, ferritin expression in the brain was also increased in the presence of infection.

5.4 Selenoproteins

Few selenoproteins have as yet been discovered, and even fewer have been found in fish (Gladyshev 2006). Selenium-containing proteins can be divided into three groups: proteins into which the element is incorporated nonspecifically, specific selenium-binding proteins, and specific proteins that contain selenium in the form of genetically encoded selenocysteine. There are also proteins in which selenium has been detected but for which no information on its binding form is as yet available (Behne and Kyriakopoulos 2001). Mammal selenoproteins that have known function include

glutathione peroxidases, iodothyronine deiodinases, thioredoxin reductases, and selenophosphate synthetase 2. All of these are catalytically active in redox processes and are considered to be oxidoreductases that either repair or prevent damage to cellular components and regulate the redox state of proteins (Behne and Kyriakopoulos 2001; Surai 2006). Enzymatic functions for these selenium-binding proteins have been established, but information on their metabolic role and biological significance is incomplete. Mammal selenoproteins that have no known function include Selenoprotein P, Selenoprotein W, a 15-kDa selenoprotein, and an 18-kDa one.

Fish selenoproteins include the Selenoproteins U (SelU), P (SelP), J (SelJ), L (SelL), and Fep 15, the latter three of which are exclusively found in these organisms. None of these selenoproteins possess known functions, except for SelP (Gladyshev 2006). Unfortunately, little information is available on these metalloproteins.

Fep 15 is a 15-kDa selenoprotein, homologous to mammalian Sep15 and SelM, but detectable exclusively in fish, and it exhibits functions that are different from its mammalian counterparts (Novoselov et al. 2006). This selenoprotein was discovered in zebrafish, and seems to be an endoplasmatic-reticulum-resident protein that also occurs in the Golgi complex. The authors indicate that Fep 15 appears to be the first eukaryotic selenoprotein family with a highly restricted distribution. Since this protein was detected only in fish, it is suggested that Fep 15 has a specialized function unique to these organisms.

Two forms of SeIP are found in fish and have a high selenium content; both appear to play a role in selenium transport and utilization (see Fig. 1) because of this high Se content (Gladyshev 2006). Very little is known about how selenium is metabolized in fish. Gladyshev (2006) suggests that the abundance of natural selenoproteins contributes to selenium accumulation in fish, and that they may have benefited from a more uniform distribution of this trace element in the earth's water reserves. SelP is also the major plasma selenoprotein, which is synthesized in the liver and delivers selenium to other organs and tissues (Gladyshev 2006; Novoselov et al. 2006).

SelU has been demonstrated to be upregulated in the medaka fish (*Oryzias latipes*), when exposed to mycrocistin, which is believed to have environmental contamination implications. Mycrocistin is a toxin produced by freshwater cyanobacteria that exists in several of the world's contaminated areas, and fish are readily exposed to these compounds through both water and diet. Even when fish were exposed to as little as 1 μg mL^{-1} of mycrocystin for 30 and 60 min durations, they still displayed significant SelU upregulation.

5.5 Hg-. Se-, and As-containing Proteins

Hg–Se proteins in blood plasma have been discovered, and they are postulated to reduce the bioavailability of toxic Hg in several organisms (Yoneda and Suzuki 1997). Recently, mercury-, arsenic-, and selenium-containing proteins have been discovered in carp livers from a mercury-polluted area in China; these proteins seem to be related to detoxification processes in carp. In the Li et al. (2008) study, three

Hg-containing bands were detected in liver of the bighead carp and one such band was detected in grass carp. The proteins present in bighead carp showed significantly higher Hg content than did those in grass carp, which may reflect different feeding habits of these species; the former ingests mainly zooplankton, whereas the latter ingests aquatic plants. This behavior may have implications for the concept of bioaccumulation of toxic trace elements, such as Hg. Proteins that bind Hg may be present in higher amounts in higher trophic-level fish that exist in contaminated areas. The authors postulated that one of the proteins that contain high Hg levels occurs in bighead carp, and may act as an Hg-storage protein. Se and As were observed to coexist in one of the Hg-containing bands taken from bighead carp and from one band from grass carp. Se has an antagonistic effect against the toxicity of Hg and As, and its presence in Hg-containing bands may reflect organismal response to environmental Hg and As contamination. Moreover, the fact that Se and As were found to coexist in the Hg-containing electrophoretic bands suggested to the authors that these two elements may be involved in a detoxification process in fish liver.

5.6 Superoxide Dismutases

Superoxide dismutases (SODs) are antioxidant metalloenzymes, classified in three major families that bind to copper or zinc, iron or manganese, or nickel. Copper/zinc superoxide dismutase (Cu/Zn–SOD) catalyzes the dismutation of superoxide to hydrogen peroxide and molecular oxygen. SODs are important enzymes in neutralizing oxygen radical-mediated toxicity. Environmental pollution may enhance oxidative stress in exposed fish, and thus disturb this natural antioxidant enzyme system (Radi and Marcovics 1988).

The effect of several contaminants, either alone or in a mixture, has been studied for the effect they have on fish SODs. For example, in one lab exposure study, in which the endocrine disruptor tri-iodothyronine (T_3) in a teleost fish (*Anabas testudines*) was evaluated, proteomic analyses were conducted by native gel electrophoresis and Western blotting (Sreejith and Oommen 2008). The fish were injected with the hormone intraperitonially daily for 5-days, after which their liver and brains were excised for proteomic analyses. Liver and brain showed a significant decrease in SOD expression after T_3 treatment. This decrease is believed to result from the oxidative stress in the fish caused by the hypermetabolic state and prolonged exposure to oxygen-free radicals.

Ken et al. (2003) studied the role of SOD in protecting organisms against a widely used herbicide, paraquat (PQ). Paraquat produces oxidative stress by generating superoxide anions and is believed to be involved in the initiation of membrane damage through lipid peroxidation. Several studies with mammalian models have demonstrated that the over-expression of SOD in cells may be associated with PQ resistance (IPCS 1984; Komada et al. 1996), but few exist in which the relationship between PQ and fish SOD have been studied. Ken et al. (2003) performed a study on 8-day-old zebrafish, in which their larvae were soaked with 0 or 55 μg ml^{-1} ZSOD for 2-h at room temperature, whereupon they were transferred to a concentration of

100-ppm PQ. Results showed that The SOD activity was increased to 1.8 times that of the untreated group. The larvae with higher SOD activity were then moved to a 100-ppm PQ solution for 24-h. The survival rate increased significantly, demonstrating that SOD does indeed protect fish against the oxidative stress of PQ toxicity.

Field studies that have involved SOD evaluations have also been conducted. Pedrajas et al. (1993) performed liver cell extracts of fish (*Mugil cephalus*) collected from polluted environments that were subject to agricultural and industrial discharges. Their work disclosed new Cu/Zn-superoxide dismutases that had high levels of Cu ions and organic compounds. These metalloenzymes have also been observed to exist in fish muscle tissue (Diaconescu et al. 2008) and in blood (Velkova-Jordanoska et al. 2008).

It has been demonstrated in laboratory studies that several proteins, including metalloproteins, are up- and/or down-regulated in response to different environmental contaminant exposures. In one such study, Mezhoud et al. (2008) reported upregulation of a selenium-binding protein and ferritin H in medaka fish liver after exposure to mycrocistin. In a similar study, Malecot et al. (2009) used this same species model and the same compound to demonstrate upregulation of transferrin. Kling et al. (2008) observed gender-specific proteomic responses in zebrafish liver following exposure to a selected mixture of brominated flame retardants, and reported the downregulation of iron regulatory protein 1 and transferrin in female fish. Wang et al. (2008) analyzed goldfish liver from fish exposed to effluent wastewater in situ, and observed up- and down-regulation of ferritin H, while Smith et al. (2009) observed that superoxide dismutase was upregulated in goldfish suffering from anoxic conditions.

6 Conclusions

The presence of environmental stressors is known to modify the proteome of organisms in specific ways. Such stressor effects have proven that proteomic studies may be quite useful as means to augment the information collected when environmental contamination studies are performed. Even in the brief time that fish protein biomarkers have been successfully used in helping to monitor for environmental contamination, they have become useful tools. Metallomics is a much newer field, and though only in its infancy, promises to become quite useful in addressing the characteristics and behaviors of metal-bound proteins, i.e., metalloproteins. Some metalloproteins are now being successfully used as biomarkers of environmental contamination, and the potential for discovering many more of these metal-bound proteins in fish is high. More research studies are needed in both field and laboratory, and these often complement each other. More work is needed in the topic areas addressed by this review, and we specifically recommend research attention to

- Identify new forms of known metalloproteins and to study their mechanisms of action.
- Search for new, previously unknown, metalloproteins.

– Further investigate whether certain metalloproteins respond to one or several types of environmental pollutants, and, if they do respond, to determine in what way.
– Conduct analyses to determine whether metalloproteins can be successfully used to address key biochemical questions when they are used as biomarkers, such as do metalloproteins vary interspecifically and/or seasonally? Does sex play a role in their expression? Does the same metalloprotein play a different role in different species? Do different maturation and life stages influence metalloprotein expression and function? Are certain biomarkers useful only when applied to some fish species, or are they applicable to fish in general?

Finally, by applying established proteomic protocols and techniques, such as 2D electrophoresis, in tandem with the new analytical techniques that are now available in the metallomic field (e.g., improved mass spectrometry and liquid chromatography), further studies in environmental contamination contexts are now possible, and will foment extensive discoveries in years to come.

7 Summary

Fish are well-recognized bioindicators of environmental contamination. Several recent proteomic studies have demonstrated the validity and value of using fish in the search and discovery of new biomarkers. Certain analytical tools, such as comparative protein expression analyses, both in field and lab exposure studies, have been used to improve the understanding of the potential for chemical pollutants to cause harmful effects. The metallomic approach is in its early stages of development, but has already shown great potential for use in ecological and environmental monitoring contexts. Besides discovering new metalloproteins that may be used as biomarkers for environmental contamination, metallomics can be used to more comprehensively elucidate existing biomarkers, which may enhance their effectiveness. Unfortunately, metallomic profiling for fish has not been explored, because only a few fish metalloproteins have thus far been discovered and studied. Of those that have, some have shown ecological importance, and are now successfully used as biomarkers of environmental contamination. These biomarkers have been shown to respond to several types of environmental contamination, such as cyanotoxins, metals, and sewage effluents, although many do not yet possess any known function. Examples of successes include MMPs, superoxide dismutases, selenoproteins, and iron-bound proteins. Unfortunately, none of these have, as yet, been extensively studied. As data are developed for them, valuable new information on their roles in fish physiology and in inducing environmental effects should become available.

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