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SYMPOSIUM

Proteomics to Assess the Role of Phenotypic Plasticity in Aquatic Organisms Exposed to Pollution and Global Warming

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Synopsis Nowadays, the unprecedented rates of anthropogenic changes in ecosystems suggest that organisms have to migrate to new distributional ranges or to adapt commensurately quickly to new conditions to avoid becoming extinct. Pollution and global warming are two of the most important threats aquatic organisms will have to face in the near future. If genetic changes in a population in response to natural selection are extensively studied, the role of acclimation through phenotypic plasticity (the property of a given genotype to produce different phenotypes in response to particular environmental conditions) in a species to deal with new environmental conditions remains largely unknown. Proteomics is the extensive study of the protein complement of a genome. It is dynamic and depends on the specific tissue, developmental stage, and environmental conditions. As the final product of gene expression, it is subjected to several regulatory steps from gene transcription to the functional protein. Consequently, there is a discrepancy between the abundance of mRNA and the abundance of the corresponding protein. Moreover, proteomics is closer to physiology and gives a more functional knowledge of the regulation of gene expression than does transcriptomics. The study of protein-expression profiles, however, gives a better portrayal of the cellular phenotype and is considered as a key link between the genotype and the organismal phenotype. Under new environmental conditions, we can observe a shift of the protein-expression pattern defining a new cellular phenotype that can possibly improve the fitness of the organism. It is now necessary to define a proteomic norm of reaction for organisms acclimating to environmental stressors. Its link to fitness will give new insights into how organisms can evolve in a changing environment. The proteomic literature bearing on chronic exposure to pollutants and on acclimation to heat stress in aquatic organisms, as well as potential application of proteomics in evolutionary issues, are outlined. While the transcriptome responses are commonly investigated, proteomics approaches now need to be intensified, with the new perspective of integrating the cellular phenotype with the organismal phenotype and with the mechanisms of the regulation of gene expression, such as epigenetics.

Introduction

Since the first appearance of life, organisms have lived, developed, and evolved in a changing environment. Changes in global temperature, pH of the ocean, chemical composition of the atmosphere, and radiation are some of the major events that organisms have to deal with during the course of evolution. Nowadays, human activities subject species to new abiotic conditions. Ongoing releases of chemicals into water, atmosphere, and soil generate a rise in the level of background pollutants as well as cause acute pollution events (Doney 2010). Metals, organochlorines, polychlorobiphenyls, nanoparticules, endocrine disruptors, and brominated compounds are common or emerging stressors found in the environment (Lowry et al. 2010; Murray et al. 2010). Moreover, global warming is one of the first challenging events that organisms will have to face in the near future (Brierley and Kingsford 2009). Together with acidification of the ocean, they are compounding the threats to marine ecosystems (Pandolfi et al. 2011). In some cases,

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unprecedented rates of physical change suggest that biological change is likely to be commensurately quick, although the resistance and resilience of organisms and ecosystems is highly variable.

Fuller et al. (2010) recognized four possible outcomes for a species under the influence of new environmental conditions (Fig. 1). Species may (1) become extinct or extirpated, (2) migrate or shift their current distributional range, (3) adapt through a change in the genetic composition of the population, or (4) become phenotypically plastic. Physiological studies can help predict the effects of environmental stress through determining which species approach their tolerance limits and which physiological systems set those limits (Somero 2010). If a species is close to its limits, an additional change in the stressful condition can force populations to shift their distributional range by migrating to a less stressful environment (Parmesan and Yohe 2003). However, migrating to a new suitable habitat is a challenging or even impossible task for some species (Fuller et al. 2010). For them, genetic changes and phenotypic plasticity are the only outcomes that can prevent local extinction. The rapid rate of environmental change, either pollution or climatic warming, will diminish the chances of observing the evolution of adaptive heritable traits, at least in species with long generation time, through classical Darwinian selection (Bradshaw and Holzapfel 2008). Consequently, phenotypic plasticity, which can be defined as the property of a given genotype to produce different phenotypes in response to different environmental conditions (Pigliucci 2001), is of crucial importance for understanding and predicting the fate of organisms in a changing environment (Somero 2010).

Beyond classical approaches, including physiological, behavioral, or biochemical levels, omics methods are among the most powerful tools for investigating the mode of action of environmental stressors (Lemos et al. 2010; Tomanek 2011). Over the past decade, the rapid development of new technologies—those disciplines focused on the large-scale study of genomes, transcriptomes, proteomes, and metabolomes—has had an impressive impact in diverse fields, and has been responsible for a number of novel discoveries in ecotoxicology. These techniques have received a great deal of attention because they offer the potential to unravel novel physiological mechanisms of the action of toxicants and to discover new biomarkers of exposure and effects. The term ''proteome'' was first defined in 1995 to describe the protein complement of a genome (Wasinger et al. 1995). So far, 2D gel electrophoresis (2-DE) and techniques based on mass spectrometry have been widely used to separate mixtures of complex proteins, while mass spectrometry and bioinformatics enable identification of proteins. Understanding the mechanisms of molecular and subcellular interactions with pollutants using proteomics is viewed as one of the crucial challenges for environmental toxicologists (Moore 2002). The present report aims to review the potential of proteomics as a tool for assessing the effects of chronic environmental stress on aquatic organisms and to help predicting the fate of species subjected to global warming and to chronic levels of pollution.

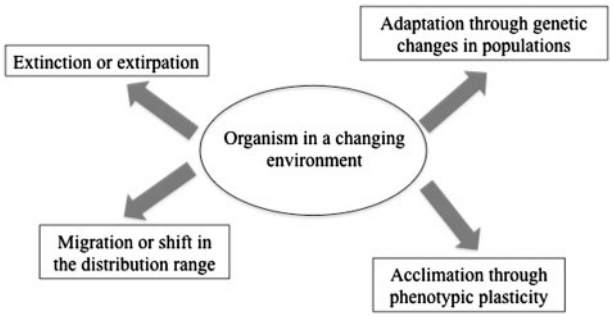

Fig. 1 Four options for an organism in a changing environment (Fuller et al. 2010).

Positioning proteomics in systems toxicology

Ideker et al. (2001) defined ''systems biology'' as the integrated study of biological systems at the molecular level, and involving perturbation of systems, monitoring of molecular expression, integrating molecular data, and modeling systems' molecular structure and network function. It incorporates a top–down approach that complements the traditional, reductionist bottom–up approach. The objective is to define genetic, protein, and biochemical reactions as integrated and interacting networks of an organism and to characterize the flow of information that links these elements, and their networks, to an emergent biological process (Hood and Perlmutter 2004). Likewise, Waters and Fostel (2004) described the toxicogenomics evaluation of biological systems as ''systems toxicology.'' It involves perturbation by toxicants as well as other stressors, monitors molecular expression and conventional toxicological parameters, and iteratively integrates data on responses to model the toxicological system. Nowadays, ecotoxicological studies are moving towards the integration of genomic, transcriptomic, proteomic, and metabolomic datasets to better understand underlying physiology and how animals interact with their environments (Garcia-Reyero and Perkins 2011).

Recent studies demonstrated that transcriptomics and proteomics technologies are complementary to

each other and provide insight into the molecular mechanisms of the action of xenobiotics (Lemos et al. 2010). However, the challenge remains to integrate molecular datasets to be able to consider temporal effects and complex regulation at the level of the transcriptome and proteome (Martyniuk and Denslow 2009). A good example of integrative multidisciplinary studies is described by Ankley et al. (2009). They used a systems-based approach to assess the toxic effects of 12 model endocrine-disrupting compounds on two model species, the fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*). The study employed a combination of transcriptomics, proteomics, metabolomics, bioinformatics, and modeling approaches to develop response linkages across biological levels of organization. Other groups have combined several omics technologies to achieve a broader understanding. An integrated transcriptomic and proteomic approach has been applied to evaluate the molecular effects of tetrabromobisphenol-A or ethinylestradiol in zebrafish liver (De Wit et al. 2008, 2010). In addition, there are a few recent studies using a combination of transcriptomic and metabolomic approaches to elucidate the mechanisms of action of toxicants, such as polycyclic aromatic hydrocarbons (PAHs) (Williams et al. 2009), ethinylestradiol (Katsiadaki et al. 2010), and copper (Santos et al. 2010) in the three-spined stickleback (*Gasterosteus aculeatus*). Jamers et al. (2009) investigated the effects of cadmium on the green alga *Chlamydomonas reinhardtii* at the transcriptomic and metabolomic levels, as well as at higher levels of biological organization. The authors observed both confirmation and complementation of data acquired at different levels of organization. Each level provided information needed to solve the puzzle of the structure of the whole system, allowing a better insight into the mechanisms of cadmium toxicity.

Even if systems biology aims to integrate several omic datasets, working with relevant organisms in environmental biology implies that most of them are only scarcely characterized at the molecular level. It makes the integration of different molecular data challenging. The most developed and routinely applied high-throughput analysis is transcriptomics that aims to provide data on the profiling of gene expression (Gracey and Cossins 2003). The transcriptome represents all messenger RNA (mRNA) constituting the building blocks for translating DNA into amino acids to form proteins. The entirety of mRNA is a mirror of the genes that are actively expressed at a given time and under a specific condition. This provides a better understanding of how organisms respond to changes in the environment (Schirmer et al. 2010). Even if transcriptomics provides important information about changes of gene expression at the mRNA level, the transcriptional level of a gene only gives a rough estimate of its level of translation into a protein (Feder and Walser 2005; Schwanhäusser et al. 2011). As a result, abundance of protein in a biological system is not necessarily correlated with the abundance of mRNA under the same conditions. Feder and Walser (2005) systematically reviewed the literature and concluded that the proportion of variation in protein abundance that can be explained by the abundance of mRNA is typically less than 0.5. It is, therefore, even more difficult to predict protein activity based on the abundance of mRNA (Glanemann et al. 2003). The main reason for this discrepancy is that several steps in regulation exist between gene transcription and the abundance of protein. These include posttranslational modifications such as acetylation (Witze et al. 2007) or alternative splicing (Schmucker and Chen 2009). Schwanhäusser et al. (2011) observed that the cellular abundance of proteins is predominantly controlled at the level of translation. Moreover, the rates and mechanisms of protein degradation substantially vary from one protein to another and leads to corresponding variation in the abundance of protein compared to that of mRNA.

Studying gene expression only at the mRNA level is an uncertain enterprise if one wants to predict the abundance of protein, but why is it so important to obtain data at the protein level while high-throughput data are easier to obtain at the mRNA level? The subset of genes transcribed in an organism is a dynamic link between the genome, the proteome, and the cellular phenotype (Singh and Nagaraj 2006). A detailed understanding of the formation of the phenotype requires the study of all steps during gene regulation and their final products at the proteomic level (Feder and Walser 2005; Karr 2008). Compared to transcriptomics, proteomics is closer to physiology and gives a more functional knowledge of the regulation of gene expression. Ideally, it has been argued that the cellular phenotype should be assessed by quantitatively studying the proteome (Pan et al. 2009). The fact that the proteome, unlike the transcriptome, more directly influences the phenotype is a strong argument in favor of focusing on the proteomics level in evolutionary biology (Futuyma 2009). Furthermore, effects on fitness, the ability of an organism to survive and reproduce, is, in final analysis, what matters the most for an organism exposed to a stressful situation. During an organism's adaptation to a new environmental situation, natural selection acts directly at the phenotypic level to select

individuals with the highest fitness (Doolittle and Sapienza 1980; Khaitovich et al. 2004). At a biochemical level, fitness is primarily a result of the ability of proteins to correctly function under normal or stressful conditions (Feder and Walser 2005). Published work to date confirms that the abundance of mRNA provides little information on fitness and cannot substitute for proteins in detailing functional and ecological analyses of candidate genes (Feder and Walser 2005).

Using proteomics to link the genotype with the phenotype

Integrating cellular and organismal phenotypes in functional terms can be seen as the greatest revolution in store for evolutionists (Aubin-Horth and Renn 2009). According to Diz et al. (2012), proteomics can bridge the gap between genotype and organismal phenotype. Environmental risk assessment and the prediction of the survival and adaptation of species in a new environment now need the integration of newly available mechanistic information at molecular levels with ecologically relevant phenotypes (Schirmer et al. 2010). Phenotype anchoring is defined as the linking of molecular responses, including those at the transcript, protein, and metabolite levels, to changes of the phenotype observed at the organismal and population levels (Schirmer et al. 2010).

Until now, studies of phenotype anchoring in ecotoxicology mostly have built on transcriptome analysis and its connections to the organism's or population's phenotype related to development and reproduction. Connon et al. (2008) exposed juvenile *Daphnia magna* (24-h old) for 24 h to cadmium (6, 20, and 37 μg/L). Gene expression and phenotypic changes such as moulting and the rate of population growth were assessed and could be linked. The authors classified the affected genes into functional pathways using Gene Ontology and showed that 29% of the responding genes were involved in metabolism or in the production of energy, while another 40% were associated with cellular processes such as growth or response to stress. Similarly, Heckmann et al. (2008) connected the effects of ibuprofen at mRNA levels with impairment of reproduction of *D. magna*. They found a strong relation between transcriptomic and phenotypic responses by integrating data on the molecular modes of action of ibuprofen with physiological effects at higher biological levels. Furthermore, survival, growth, and reproduction of *Caenorhabditis elegans* exposed to silver nanoparticles were linked to the microarray profile of gene expression (Roh et al. 2009). These authors proposed that the up-regulation of the gene sod-3, coding for the superoxide dismutase, indicated an oxidative stress that impaired the reproduction of the organism.

Two studies focused on phenotypic anchoring in fish. Ung et al. (2010) linked histological analyses to the transcriptome data and subsequently validated gene expression using real-time PCR in zebrafish exposed to mercury. They proposed a plausible *in vivo* mechanistic model of mercury-induced hepatotoxicity that could lead to phenotypes related to mitochondrial dysfunction, endocrine disruption, and metabolic disorders. Lam et al. (2011) used zebrafish to capture transcriptomic and phenotypic changes after exposure to bisphenol-A (BPA). They identified a set of endocrine-regulated genes operating during the early life of fish sensitive to BPA. At the same time, they revealed that exposure to BPA during early life caused phenotypic modifications such as cardiac edema, cranio-facial abnormality, failure of the swimbladder to inflate, and poor tactile response. Among robust biomarkers found to be sensitive to exposure to BPA, they suggested a role for the *sp4* gene, which encodes a transcription factor, in BPA perturbation in altering development and function of the brain, as well as behavior in laboratory animals exposed to BPA during early life.

Bridging the gap between genotype and phenotype needs further analysis of the gene products at protein level that closely influence the phenotype. It is, therefore, surprizing that proteomics has so far been almost completely absent from studies of phenotypic anchoring. The only existing example concerns three hepatic-derived, cell-culture systems exposed to cadmium and arsenic under conditions of phenotypic anchoring (5% and 25% in neutral red uptake) (Gottschalg et al. 2006). In this study, phenotypic anchoring based on toxicity level was used to compare the effect of $CdCl_2$ and $NaAsO_2$ on the regulation of stress–protein expression. The toxicants elicited selective up-regulation of the expression of some of the stress proteins, but the profile of this up-regulation varied across experimental conditions, according to differences in concentration of metals, cell-type, and the toxicant. The authors concluded that this fingerprint-profile approach might be more difficult to realize at the protein-expression level than expected.

Towards a proteomic norm of reaction

Acclimation (also called physiological adaptation), in contrast to adaptation (or Darwinian adaptation), can be defined as a ''within lifetime'' process in which persistent stress improves the fitness of an individual population to tolerate that stress (Horowitz 2001). A norm of reaction describes the pattern of phenotypic

expression of a single genotype across a range of environmental conditions. Classically, environmental changes can induce a shift of behavioral, morphological, or physiological traits in an organism; these traits constitute the phenotype (Pigliucci 2001). There is now growing evidence that changes at the phenotypic level, irrespective of the genotype, play an important role in driving ecological diversification and speciation (Pfennig et al. 2010). Understanding how environmental stressors act on the phenotypes of organisms will improve predictions of how organisms will deal with the surrounding conditions in a changing environment. Although phenotypic plasticity has had a long history in evolutionary biology, its real implication in diversification and evolutionary processes remains controversial (Gilbert and Epel 2008). According to Price et al. (2003), moderate levels of phenotypic plasticity are optimal for populations in a new environment and in bringing populations to an adaptive peak (Fig. 2). While low levels of plasticity can reduce organisms' survival in a new environment, a high level of plasticity can place the population close to a new adaptive peak that favors its survival. However, it can also decrease the probability of genetic changes through directional selection and genetic assimilation. Moreover, a high phenotypic plasticity will result in complete reversion to the ancestral phenotype if the organism is returned to the ancestral environment.

As a bridge between genotype and organismal phenotype, and as a key component of the cellular phenotype, the proteome could also be assigned a norm of reactions describing the pattern of protein expression across a range of environments. Bedon et al. (2012) used the term ''proteomic plasticity'' to describe the protein-expression patterns of two ecophysiologically contrasted genotypes of *Eucalyptus* trees. They could highlight adaptive mechanisms to water deficit specific to each genotype. Under new environmental conditions, one can observe a shift of the protein-expression pattern, defining a new cellular phenotype that possibly can improve the fitness of the organism. In Fig. 2, modified from Price et al. (2003), we link the proteome of organisms in a population subjected to new environmental conditions to the phenotypic values depicted along the *x*-axis. The adaptive surface for continuously varying traits is a plot of mean fitness against mean phenotype (Fear and Price 1998). The bold line represents the mean fitness of the phenotypes encountered in a single environment. When environmental conditions are changing, the adaptive surface can be modified and one can observe a shift toward the dashed line. Through stabilizing selection, a population can be held at a peak in the adaptive surface (thin solid line) but when the adaptive surface has changed due to the new environmental conditions, phenotypic plasticity can move the population to a new peak (thin dotted line). It is, therefore, important to associate proteomes of organisms exposed to a changing environment to an adaptive surface (and consequently to fitness). The definition of a proteomic norm of reaction for organisms acclimating to environmental stressors, and its link to fitness, will give new insights into how organisms can evolve in a changing environment.

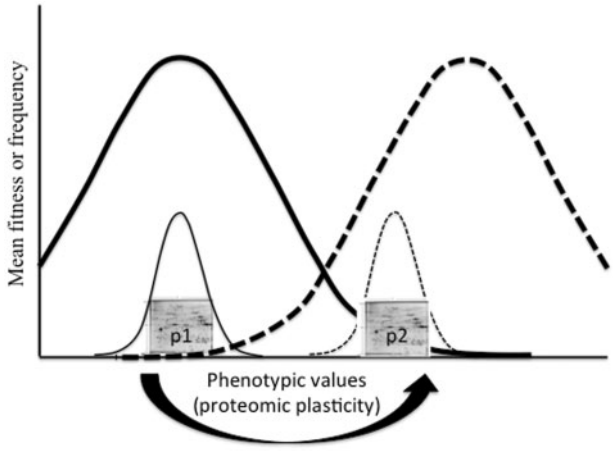

Fig. 2 The contribution of phenotypic plasticity to a peak shift in a changing environment (adapted from Price et al. 2003). Bold and dashed lines represent mean fitness in the old and the new environments, respectively. Thin solid and dotted lines represent the population distribution in the old and the new environments, respectively. The population shift is allowed by phenotypic plasticity. Proteomic plasticity in a changing environment (from proteome p1 to proteome p2) can be interpreted as a change in the cellular phenotype (see more details in the text).

Proteomic plasticity has been demonstrated in different organisms. For example, the proteome of the parasitic nematode worm *Heligmosomoides polygyrus bakeri* showed a considerable plasticity within different host environments, i.e. a ''slow-responder'' mouse host strain that can support a primary parasite infection for months and a ''rapid-responder'' mouse host that can eliminate the nematode infection after some weeks (Morgan et al. 2006). In marine organisms, a comparison of two ecotypes of the snail *Littorina saxatilis* (the RB ecotype resists stresses of desiccation and temperature, while the SU ecotype resists strong physical disturbances) suggests that phenotypic plasticity as well as natural selection maintain these ecotypes in sympatry (Martínez-Fernández et al. 2008). A proteomic analysis of these two ecotypes revealed that 7% of the protein spots showed different expression levels between the two ecotypes. Moreover, the authors assumed that most of the proteomic variation might

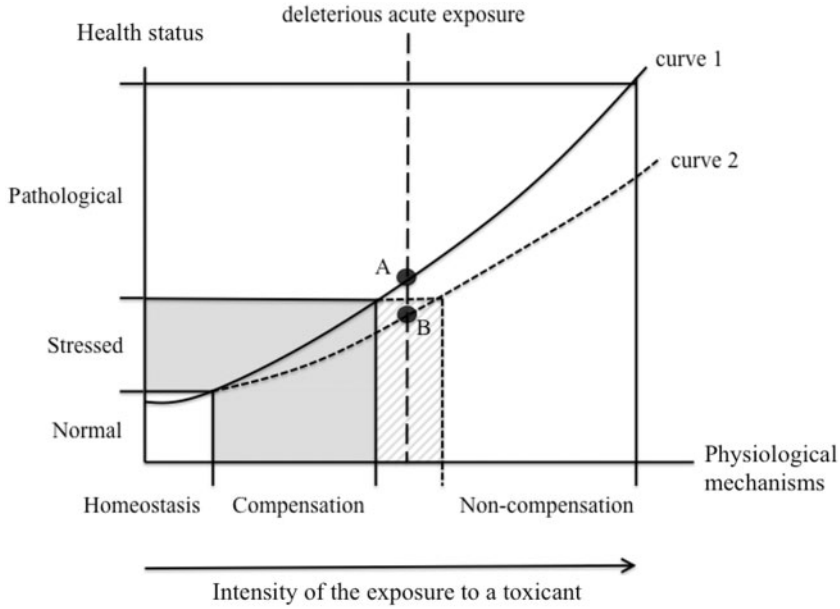

Fig. 3 Evolution of the health status and the physiological mechanisms of an organism when exposed to a pollutant with an increasing intensity (concentration and/or duration) (adapted from Depledge 1994). Curve 1 represents the relation in a normal situation; Curve 2 represents the situation after acclimation. Point A depicts an organism exposed to a deleterious acute exposure to a pollutant when the organism's health status is qualified as pathological; Point B depicts an organism exposed to the same deleterious acute exposure to a pollutant after acclimation. Its health status is then qualified as stressed.

have a genetic origin since these differences are insensitive to environmental changes (Martínez-Fernández et al. 2010).

Acclimation to pollutants

Aquatic organisms seldom encounter acute contamination by toxicants. Usually, they face relatively low concentration over a long duration. Chronic and acute stresses present different ecological challenges. Organisms are likely to exhibit different molecular, biochemical, physiological, or morphological responses to these two kinds of stress. The consequences of chronic exposure to contaminants for survival of populations and species could be severe unless development of phenotypic plasticity within a generation, or adaptation over generations, produce individuals more tolerant of a polluted environment. In ecotoxicology, acclimation is defined as increased tolerance of an elevated, usually lethal, concentration of a toxicant arising from chronic exposure to a sublethal concentration of that toxicant (McDonald and Wood 1993). However, long-term exposure to a pollutant does not systematically induce an increased resistance in the exposed organisms. For example, pre-exposure of yearling trout, *Salvelinus fontinalis*, to sublethal concentrations of lead did not induce acclimation Tang and Garside (1987) and Steadman et al. (1991) even observed decreased survival of *Oncorhynchus mykiss* exposed to N°2 fuel oil after a sublethal pre-exposure. McDonald and Wood (1993) defined the acclimation window to a pollutant as the concentration of that toxicant and the exposure time necessary to observe an increased resistance. Below this threshold, the stress level is not sufficient to trigger acclimation. Tate-Boldt and Kolok (2008) showed that in fathead minnows, enhanced tolerance to copper will only develop in fish that have experienced a pronounced and relatively long-term cycle of branchial damage and repair. On the other hand, above the acclimation window, the sensitizing threshold defines a zone in which the compensatory mechanisms can no longer maintain homeostasis and, if the stress remains, the organisms will die. For an organism exposed to a pollutant, we plotted its physiological mechanisms against its health status (Fig. 3, adapted from Depledge et al. [1994]). During acclimation, the zone in which organisms are stressed (gray in Fig. 3), meaning that their physiological mechanisms compensate for the stressful situation, spreads over the right, pushing away the noncompensatory zone (hatched gray in Fig. 3). This can be explained by a shift downward of the curve when organisms are exposed to a pollutant inside their acclimation window (from Curve 1 to Curve 2 in Fig. 3). Under this new state, a subsequent deleterious exposure to the same toxicant (vertical hatched line in Fig. 3) will no longer induce a pathological state in

the organism (Point A in Fig. 3) and the physiological mechanisms are now compensating (Point B in Fig. 3). This enhanced resistance to a toxicant improves the fitness of the organism in the polluted environment. However, the mechanisms, either physiological, biochemical, or molecular that are triggered during the acclimation process, usually necessitates energy. This metabolic cost explains that when acclimated animals return to a clean environment, their fitness is decreased compared to nonacclimated organisms (Xie and Klerks 2004; Kwok et al. 2009).

We report, here, some recent studies exploring the proteomic response of aquatic organisms to chronic exposure (more than 7 days) of toxicants (Table 1). The blue mussel *Mytilus edulis* is of particular interest in ecotoxicology as it is commonly used for biomonitoring. Proteomes from gills of mussels continuously exposed for 21 days to environmentally relevant concentrations of North Sea oil or to a mixture of North Sea oil, alkylphenols, and extra PAHs, were compared by Manduzio et al. (2005) to protein signatures in unexposed animals. They observed a specific proteomic response for each tested pollutant and they could identify the proteins involved in cellular structure, metabolism, and defense mechanisms. Apraiz et al. (2006) used 2D-DIGE to compare the proteome of a peroxisome-enriched fraction from the digestive gland of mussels exposed for 3 weeks to three marine pollutants (diallyl phthalate, PBDE-47, and bisphenol-A) at a concentration of 1% of the LC_{50}. Specific protein-expression signatures were identified for each pollutant, while a pattern was common for all exposures. Identified proteins were involved in α and β oxidation pathways, xenobiotic degradation, amino acid metabolism, cell signaling, oxyradical metabolism, peroxisomal assembly, respiration, and the cytoskeleton. Mussels were not the only mollusc whose proteome has been studied after chronic exposure to pollutants. The clam *Chamaelea gallina* was exposed for 7 days to Aroclor 1254, copper(II), tributyltin and arsenic(III) at three different concentrations (Rodríguez-Ortega et al. 2003). The authors identified four proteins of the cytoskeleton, such as a putative isoform of tropomyosin, a light chain of myosin, actin, and a truncated form of actin. In another study, the sentinel clam *Ruditapes decussatus* was exposed for 21 days to cadmium at 40 µg/L (Chora et al. 2009). Cd induced changes in the abundance of proteins in the digestive gland and gills. Those proteins were involved in the maintenance of cytoskeletal structure (muscle-type actin, adductor muscle actin, and ß-tubulin), and maintenance (Rab GDP) and metabolism (ALDH and MCAD) of cells, suggesting potential energetic change.

The long-term effects of various pollutants on the proteome were also investigated in fish. The zebrafish, *Danio rerio*, was used as a model species in different studies. The brominated flame-retardant tetrabromobisphenol-A (TBBPA) was applied to adult zebrafish at two concentrations (0.75 and 1.5 mM) for 14 days and proteomic and transcriptomic changes in the liver were assessed (De Wit et al. 2008). Among the differentially expressed proteins, different molecular chaperones elucidated a stress response, while stimulation of phosphoglycerate mutase 1 suggested an interference with the glycolysis pathway. Homocysteine metabolism as well as the cytoskeleton seemed to be affected by the flame retardant. The same approach was used to investigate the effects of 17 α–ethynylestradiol (EE2) at 30 ng/L, a powerful endocrine-disrupting compound, on zebrafish liver during a 4-day and a 28-day exposure (De Wit et al. 2010). Many proteins were identified as belonging to cellular homeostasis, the cytoskeleton, stress responses, cellular transport, and diverse metabolic processes. Wang et al. (2010) investigated the proteomic response of zebrafish liver after a chronic exposure of 30 days to the hepatotoxin microcystin-LR at concentrations of 2 and 20 µg/L. The proteins whose abundances were modified were involved in cytoskeleton assembly, macromolecule metabolism, oxidative stress, and signal transduction, indicating that MCLR toxicity in fish liver is complex and diverse.

Besides zebrafish, other species of fish were also explored. Rainbow trout, *Oncorhynchus mykiss,* were exposed to effluent from a sewage treatment work for 2 weeks (Albertsson et al. 2007). Betaine aldehyde dehydrogenase and lactate dehydrogenase (LDH) were identified to be down-regulated in the livers of exposed fish while mitochondrial ATP synthase alpha-subunit and carbonyl reductase/20b-hydroxysteroid dehydrogenase were up-regulated. A 28-day experiment was carried out in rare minnow, *Gobiocypris rarus,* exposed to perfluorooctanoic acid at three concentrations (Wei et al. 2008). The liver proteome was analyzed and revealed the differential expression of proteins involved in intracellular fatty-acid transport, oxidative stress, macromolecule catabolism, the cell cycle, maintenance of intracellular Ca^{2+} homeostasis, and mitochondrial function. Juvenile beluga (*Huso huso*) was exposed to dietary MeHg for 70 days to evaluate the neurotoxicity of this metal (Keyvanshokooh et al. 2009). Analysis of brain proteome revealed changed expression of eight proteins involved in cell metabolism, protein folding, cell division, and signal transduction. The authors stated that MeHg should induce a metabolic deficiency in the brain, together with oxidative stress and apoptosis. Finally, the proteome of the neuroendocrine region

Table 1 Proteomics analysis in aquatic organisms after chronic exposure (i.e. minimum of 7 days) to environmental toxicants

Species (organs/tissues)	Treatments	Main observed results	References
Rainbow trout *Oncorhynchus mykiss* (liver)	2 weeks exposure to sewage treatment works effluent	Down-regulation of betaine aldehyde dehydrogenase and lactate dehydrogenase; up-regulation of mitochondrial ATP synthase alpha-subunit and carbonyl reductase/ 20b-hydroxysteroid dehydrogenase.	Albertsson et al. (2007)
Blue mussel *Mytilus edulis* (digestive gland/ peroxisome-enriched fraction)	3 weeks exposure to diallyl phthalate, PBDE-47, and bisphenol-A, at a concentration of 1% of the LC50	Specific protein-expression signatures for each pollutant; common signature for all conditions. Proteins involved in α and β oxidation pathways, xenobiotic and amino acid metabolism, cell signaling, oxyradical metabolism, peroxisomal assembly, respiration, and the cytoskeleton.	Apraiz et al. (2006)
Clam *Ruditapes decussatus* (digestive gland and gills)	21 days exposure to cadmium at 40 μg/L	Proteins involved in cytoskeletal structure maintenance, cell maintenance, and metabolism	Chora et al. (2009)
Zebrafish *Danio rerio* (liver)	14 days exposure to tetrabromobisphenol-A (TBBPA) at 0.75 and 1.5 mM	Eight proteins belonging to molecular chaperones or cytoskeleton, or involved in glycolysis pathway or homocysteine metabolism	De Wit et al. (2008)
Zebrafish *Danio rerio* (liver)	4 and 28 days exposure to 17 α–ethynylestradiol (EE2) at 30 ng/L	Proteins belonging to diverse metabolic processes, to cellular homeostasis, to the cytoskeleton, to the stress response, and to cellular transport	De Wit et al. (2010)
Juvenile beluga *Huso huso* (brain)	70 days exposure to dietery methylmercury at 0.8 ppm feed	Eight proteins involved in cell metabolism, protein folding, cell division, and signal transduction	Keyvanshokooh et al. (2009)
Blue mussel *Mytilus edulis* (gills)	21 days exposure to environmentally relevant concentrations of North Sea oil or to a mixture of North Sea oil, alkylphenols and extra PAHs	Specificity of the proteome response Proteins involved in the cellular structure, in metabolism and in defense mechanisms	Manduzio et al. (2005)
Largemouth bass *Micropterus salmoides* (brain/ hypothalamus)	57 days exposure to dietery organochlorine dieldrin at 2.95 mg/kg feed	Identified proteins revealed alteration of oxidative phosphorylation, cell differentiation, proliferation, and survival	Martyniuk et al. (2010)
Clam *Chamaelea gallina* (whole body/ cytosolic fraction)	7 days exposure to Aroclor 1254, copper(II), tributyltin and arsenic(III) at three different concentrations	Four proteins belonging to the cytoskeleton	Rodrìguez-Ortega et al. (2003)***
Zebrafish *Danio rerio* (liver)	30 days exposure to microcystin-LR at 2 and 20 μg/L	Proteins belonging to or involved in cytoskeleton assembly, macromolecule metabolism, oxidative stress, and signal transduction	Wang et al. (2010)
Chinese mitten crab *Eriocheir sinensis* (anterior gills)	30 days exposure to cadmium at 50 μg/L	Protein-expression pattern linked to increased resistance to cadmium (acclimation) Proteins belonging to antioxidative enzymes, molecular chaperones, metabolic pathways, proteolytic enzymes	Silvestre et al. (2006)
Rare minnow *Gobiocypris rarus* (liver)	28 days exposure to perfluorooctanoic acid at 3, 10, and 30 mg/L	Proteins involved in intracellular fatty acid transport, oxidative stress, macromolecule catabolism, the cell cycle, maintenance of intracellular Ca^{2+} homeostasis, and mitochondrial function	Wei et al. (2008)

of the brain of the top-predator largemouth bass, *Micropterus salmoides*, was studied after a subchronic dietary additive of the organochlorine pesticide dieldrin (2.95 mg/kg feed) for 57 days (Martyniuk et al. 2010). Identified proteins revealed alteration of oxidative phosphorylation, cell differentiation, proliferation, and survival.

The only study using proteomics to focus on chronic effects of pollutants in aquatic organisms within their acclimation window was published in 2006 by Silvestre et al. and focused on the Chinese mitten crab, *Eriocheir sinensis*. They previously showed that an acute exposure to cadmium (500 μg/L for 3 days) impaired the osmoregulatory mechanisms of crabs acclimated to freshwater (Silvestre et al. 2005). When crabs were previously exposed chronically to the same metal (50 μg/L for 30 days), they acquired an increased resistance protecting them from subsequent acute exposure. An analysis of the anterior gills proteome revealed changes in protein expression during the acclimation process under a chronic exposure. In acclimated crabs, a strong over-expression of protein disulfide isomerase (PDI) suggested that this enzyme plays an important role in protecting and/or repairing target proteins, thereby reducing the toxicity of the metal. The up-regulation of antioxidative proteins such as glutathione S-transferase or thioredoxin peroxidase during acclimation suggested that cadmium exerts its toxicity through the induction of an oxidative stress and that molecular mechanisms take place in the gills and protect macromolecules against this stress. Finally, it seems that enhanced resistance to cadmium was also linked to an increase of the cells' proteolytic capacity through an over-expression of a proteasome complex subunit and a cathepsin D isoform. This strengthens the hypothesis that a significant part of the toxicity of Cd is due to the formation of abnormal proteins.

These studies revealed that exposure to a toxicant during a period of at least 7 days induced significant changes in the proteome of aquatic organisms. Even if general cellular processes, such as energy metabolism, protein metabolims, cell signaling, and antioxidant defences, are often affected, and even if proteins belonging to the cytoskeleton and to molecular chaperones are commonly observed to be differentially expressed, it is difficult to draw general conclusions. Some of the most commonly identified proteins, like actin, belong to the proteins most often identified in proteomic studies (Monsinjon and Knigge 2007; Petrak et al. 2008). The significance of these proteins in the acclimation process under long-term exposure to an environmental pollutant remains unclear. It is uncertain whether the identified proteins reflect a deleterious effect caused by the toxicant or the opposite, i.e. the proteins belonging to compensatory mechanisms. Such questions should be addressed by generalizing studies within the acclimation window or by linking the proteomic response to changes in the organismal phenotype.

Acclimation to global warming

Temperature affects virtually all levels of biological organization, from the rates of molecular diffusion and biochemical reactions, to membrane permeability, to cellular, tissue and organ function, and to their integration in the whole organism. Given the extent of these thermal effects, it is not surprizing that animals show a variety of strategies, from biochemical to behavioral, that cope with thermal change (Guderley and St-Pierre 2002). The effect of elevated temperature on aerobic performance is thought to be an important factor in setting thermal optima and thermal limits for many animals (Pörtner 2001; Pörtner and Farrell 2008). In the case of temperature-induced hypoxemia, cellular repair mechanisms may be activated and the production of heat-shock proteins (HSPs) and antioxidants increased, thereby enhancing the repair of stress-induced cellular damage (Feder and Hofmann 1999; Kültz 2005). In addition, adjustments of gene expression at the mRNA level are important for re-establishing cellular homeostasis (Schulte 2004).

Acclimatory response to change in body temperature is likely to be an important determinant of the effects of global warming on species. Thermal acclimation, in contrast to adaptation, is a "within lifetime" phenotypic modification involving a suite of adjustments that allow an organism to shift its thermal optimum for numerous physiological activities to new temperature ranges (Horowitz 2001). Ectothermic organisms acclimated to different temperatures frequently exhibit regulatory mechanisms that enable them to minimize some of the temperature-related effects, a process known as temperature compensation of metabolism (Somero 2004). The mechanisms driving this complex process are not fully understood but are thought to include modulation of enzyme activity as well as changes in enzyme levels that can be mediated through transcriptional and translational regulation (Vergauwen et al. 2010). Recently, several transcriptomic studies have investigated the effects of short-term changes in temperature (from hours up to a few days) in various species of fish (Podrabsky and Somero 2004; Buckley et al. 2006; Kassahn et al. 2007), while only a few studies have focused on molecular responses

associated with 4 weeks of acclimation (Logan and Somero 2010; Vergauwen et al. 2010). Small HSPs appeared to play an important role in response to fluctuating temperatures, while chaperones of larger molecular mass, such as HSP70 and HSP90, responded more strongly to chronic high-temperatures stress.

One of the first proteomic attempts to unravel the effects of heat stress on aquatic organisms was reported by Kültz and Somero (1996). Working on long-jawed mudsuckers (*Gillichthys mirabilis*) acclimated for 2 months at 10°C or 25°C and at different salinities, they found five protein spots over-expressed or under-expressed in gill epithelium of animals acclimated to high temperature. Other studies, aimed at focusing on heat-shock proteins' expression, showed a high degree of evolutionary variation of HSP isoforms (Tomanek 2005, 2010). Tomanek and Zuzow (2010) assessed the response of two species of mussel living along the Californian coast, *Mytilus galloprovincialis* and *M. trossulus,* to acute heat stress using a proteomic approach. The latter is a native species with a higher sensitivity to heat stress. It has been displaced to a more northerly range by its invasive and less heat-sensitive congener, *M. galloprovincialis*. After 4 weeks of acclimation at 13°C, mussels were exposed to 24°C, 28°C or 32°C for 1 h before a recovery period of 24 h at the acclimation temperature. The authors observed that in addition to the induction of molecular chaperones, the expression of proteins involved in proteolysis, energy metabolism, oxidative damage, formation of the cytoskeleton, and deacetylation was also found to respond to thermal stress. They hypothesized that small HSPs stabilize cytoskeletal proteins in response to oxidative stress and heat stress and that heat stress produces a switch from a NADH-producing to a NADPH-producing metabolic pathway. Another noteworthy result was the lower abundance at 28°C of the NAD-dependent deacetylase, sirtuin 5. Tomanek and Zuzow (2010) suggested that sirtuins are regulators of the metabolic costs of heat stress and that the acetylation status of proteins would be a key PTM during heat stress.

Effects of heat stress at a proteomic level were also reported for green and white sturgeon larvae (*Acipenser medirostris* and *A. transmontanus*) from the San Francisco Bay/Delta Estuary (Silvestre et al. 2010). These authors tested the combined effects of an increased temperature from 18°C to 26°C and organic selenium micro-injected as Se-L-Methionine until the larvae reached stage D45 (absorbed yolk) (after 8–12 days of exposure). 40S ribosomal protein SA, FK506-binding protein 10, 65-kDa regulatory subunit A of protein phosphatase 2, PDI, stress-induced-phosphoprotein 1, and suppression of tumorigenicity 13 and collagen type II alpha 1, were differentially expressed after high-temperature treatment.

A proteomic comparison between two ecotypes of the marine snail *Littorina saxatilis* revealed specific protein-expression profiles (Martínez-Fernández et al. 2008). While the large-sized, ridged and banded ecotype (RB) is found on the upper shore where it lives among the barnacles and is subjected to water loss and high temperature because of high exposure to sunshine, the small-sized, smooth and unbanded ecotype (SU) occupies the lower shore, lives with mussels, and has a smaller shell with a relatively large aperture, thereby avoiding dislodgement by heavy wave action. A total of 43 protein spots were differentially expressed between the two ecotypes, suggesting that phenotypic plasticity, natural selection, or both, maintain these ecotypes in sympatry. Among these proteins, fructose-bisphosphate aldolase and arginine kinase were up-regulated in the SU ecotype, possibly depicting the need for a rapid supply of energy by increasing the level of glycolysis.

Proteomics analysis of long-term acclimation to elevated temperature in the laboratory has never been approached. However, liver of gilthead sea bream (*Sparus aurata*) was investigated for changes in protein expression after a cold stress of 10 days at 8°C (compared to a control group at 20°C) (Ibarz et al. 2010). Oxidative damages to hepatocytes were induced by exposure to cold as indicated by thiobarbituric acid assay and nitric oxide level. Structural proteins like actin, enzymes of amino-acid metabolism, and enzymes with anti-oxidant capacity such as catalase, were down-regulated by exposure to cold. At the same time, an increase of proteolysis was indicated by the over-expression of proteins like proteasome activator and trypsinogen-like proteins, while the over-expression of peroxiredoxin, thioredoxin, lysozyme, and Raf-kinase inhibitor protein indicated oxidative stress and activation of apoptosis.

On an evolutionary time scale, biochemical compensation for thermal effects has been shown by studies of orthologs of LDH, highlighting the complementary roles of three strategies of adaptation: (1) changes in amino acid sequence that cause adaptive variation in the kinetic properties and stabilities of proteins, (2) shifts in levels of proteins, mediated through changes in gene expression and turnover of protein, and (3) changes in the milieu in which proteins function; these conserve the intrinsic properties of proteins, established by their primary structure, and modulate the activity of protein in response to physiological needs (reviewed by Hochachka and Somero 2002). Depending on

generation time, population size, the level of genetic variation in a population, and other factors, adaptive evolution of proteins potentially may occur rapidly enough to ''keep pace with'' climatic change. Species possessing this potential for rapid evolution could emerge as ''survivors'' in a warming world (Somero 2010). Moreover, while species have historically acclimated or adapted to changes in climate, the rapid rate of current climatic change, coupled with increasingly fragmented and impaired habitats, present unprecedented challenges for modern species. Currently, the consequences of such environmental changes for the dynamics of fish populations are poorly understood and long-term rearing experiments related to the plasticity or adaptability of life-history traits are now needed.

Conclusions and future prospects

Proteomics has gained many improvements during the past few years and its applications in environmental biology are numerous and diverse. In the context of environmental changes brought about by human activities, this approach can bring new answers about the effects these changes have on organisms. Under environmental stress, organisms can develop compensatory mechanisms that help them adapt to new conditions. For many species, whose capacities for migration are limited, phenotypic plasticity is a rapid mechanism that is operative within a single generation and that can increase fitness in a perturbed environment. Proteomics, as the final product of gene expression, is closer to physiology than is transcriptomics and accounts for a great part of the cellular phenotype. It is, therefore, a key element for linking the genotype to the organismal phenotype and future studies on acclimation should focus on the proteome to address questions of evolutionary biology.

Some studies in ecotoxicology are investigating chronic exposure of aquatic organisms to environmental toxicants. Important new hypotheses on the modes of action of pollutants can be deduced from these studies. However, it is tricky to interpret these data in terms of adaptative value, increased fitness, or organismal evolution. The main reason is that usually there is no link between proteomics and phenotypic values at a higher integrative level, e.g. physiology. During a chronic environmental stress, one should search for the acclimation window that corresponds to the conditions under which the fitness has increased through phenotypic plasticity, and associate the observed enhanced resistance to a stressor with a protein-expression pattern. More broadly, phenotypic anchoring conditions should be generalized when investigating proteomic changes in organisms exposed to environmental stress. Likewise, figuring out how organisms can adjust to global warming is an important issue in modern biology. As in ecotoxicology, proteomics has an important role to play but data are still scarce. They are even scarcer in relation to long-term exposure to high temperature and to acclimation.

On the other hand, a better integration of proteomics data with other omics approaches is necessary to have a global view of the perturbations the cellular phenotype endures and to efficiently fulfill the goals of the new field of systems biology. However, besides transcriptomics, we propose that an advance linking the genotype with the phenotype can be achieved by connecting the output of proteomics with epigenetic gene-regulating mechanisms. Epigenetics is the study of heritable changes in gene expression that occur without changes in DNA sequence (Wolffe and Gucshin 2000). According to Bollati and Baccarelli (2010), it holds substantial potential for furthering our understanding of the molecular mechanisms of environmental toxicants. Moreover, linking proteomics with epigenetics opens an opportunity to prospect transgenerational inheritance of an acquired cellular phenotype under an environmental stress. It can thus provide new insights into rapid, adaptative evolution.

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