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Environmental Proteomics: Changes in the Proteome of Marine Organisms in Response to Environmental Stress, Pollutants, Infection, Symbiosis, and Development

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Abstract

Environmental proteomics, the study of changes in the abundance of proteins and their post-translational modifications, has become a powerful tool for generating hypotheses regarding how the environment affects the biology of marine organisms. Proteomics discovers hitherto unknown cellulareffects of environmental stressors such as changes in thermal, osmotic, and anaerobic conditions. Proteomic analyses have advanced the characterization of the biological effects of pollutants and identified comprehensive and pollutant-specific sets of biomarkers, especially those highlighting post-translational modifications. Proteomic analyses of infected organisms have highlighted the broader changes occurring during immune responses and how the same pathways are attenuated during the maintenance of symbiotic relationships. Finally, proteomic changes occurring during the early life stages of marine organisms emphasize the importance of signaling events during development in a rapidly changing environment. Changes in proteins functioning in energy metabolism, cytoskeleton, protein stabilization and turnover, oxidative stress, and signaling are common responses to environmental change.

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1. PROTEOMICS AS A FIRST STEP TOWARD SYSTEMS BIOLOGY

Post-translational modifications (PTM): chemical modification (phosphorylation, acetylation, ubiquitination, and many others) of a protein following its synthesis (translation)

Mass spectrometry (MS): use of a mass spectrometer to measure the mass of a molecule in Daltons (1 Da = 1 atomic mass unit)

Across a number of disciplines within marine science, there is interest in understanding the cellular mechanisms of organism-level responses to changes in the physical and chemical environment, including changes in temperature, osmolality, oxygen concentration, and pollutants, as well as the mechanisms of developmental pathways, infections, and symbioses. Proteomics, with its rapidly expanding analytical tools, provides a means to study the changes occurring at the level of the proteome—the entire protein pool—including the processes of protein synthesis, post-translational modifications, and degradation, in response to both the external environment and ontogenetic events in marine animals, plants, and bacteria. In this review, I first provide a brief overview of the basic approach and the methodological tools available to study different aspects of the proteome. Then I survey the cellular processes that are shifting in response to environmental change and ontogenetic processes and highlight how a proteomics approach is a powerful tool for hypothesis-driven investigations on a number of exciting fronts in basic and applied environmental science.

Co-expression patterns of proteins are indicative of association through a common cellular pathway, e.g., glycolysis, or of direct interaction through protein–protein interactions (PPI). Although one step away from directly describing networks of interacting proteins (Yamada & Bork 2009), such co-expression patterns are a step toward a more comprehensive understanding of the systems dynamics of cells in response to perturbations by the environment (Souchelnytskyi 2005). Although proteomic analyses are complementary to transcriptomic analyses, which quantify transcript (mRNA) abundance by using microarrays or sequencing of all mRNA molecules (Gracey & Cossins 2003), variation in transcript levels in general explains less than half of the variation in protein abundances (Feder & Walser 2005). Another reason to focus on proteomics is that proteins represent the molecular phenotype of cells and as such have a direct effect on organismal physiology and thus fitness (Feder & Walser 2005).

Although the stated goal of proteomics is to characterize the entirety of all protein forms, including their post-translational modifications (PTM) and PPI, technical limitations restrict our access to the entire proteome (Aebersold & Mann 2003; Mann et al. 2001). Despite limitations, proteomics based on mass spectrometry (MS) has been extremely successful in discovering new insights into the complexities of biological systems and thus has proved the importance of a hypothesis-generating discovery approach (Cravatt et al. 2007). A particularly powerful research program utilizes the less biased comprehensive survey of such a discovery approach to generate hypotheses, and then follows up with hypothesis-testing experiments (Boogerd et al. 2007).

In this review, I present the results of proteomics studies that focused on cellular responses of organisms to a changing environment during various life stages, with an emphasis on marine systems. Reviews of the emerging field of marine microbial proteomics (including meta-proteomics) can be found elsewhere (Schneider & Riedel 2010, Schweder et al. 2008). Although several marine algae proteomics studies exist, the majority have focused on the freshwater genus *Chlamydomonas* (Rolland et al. 2009).

2. MASS SPECTROMETRY–BASED PROTEOMICS

The main idea behind recent advances in proteomics is that we match peptides to protein identities through the application of MS (Aebersold & Mann 2003, Mann et al. 2001, Nesvizhskii et al. 2007). Although proteomic work flows (**Figure 1**) vary depending on protein isolation protocols and the type of mass spectrometry and database searches, at some point in the work flow, proteins are digested to peptides by proteases that cut at predetermined sites, most commonly using trypsin.

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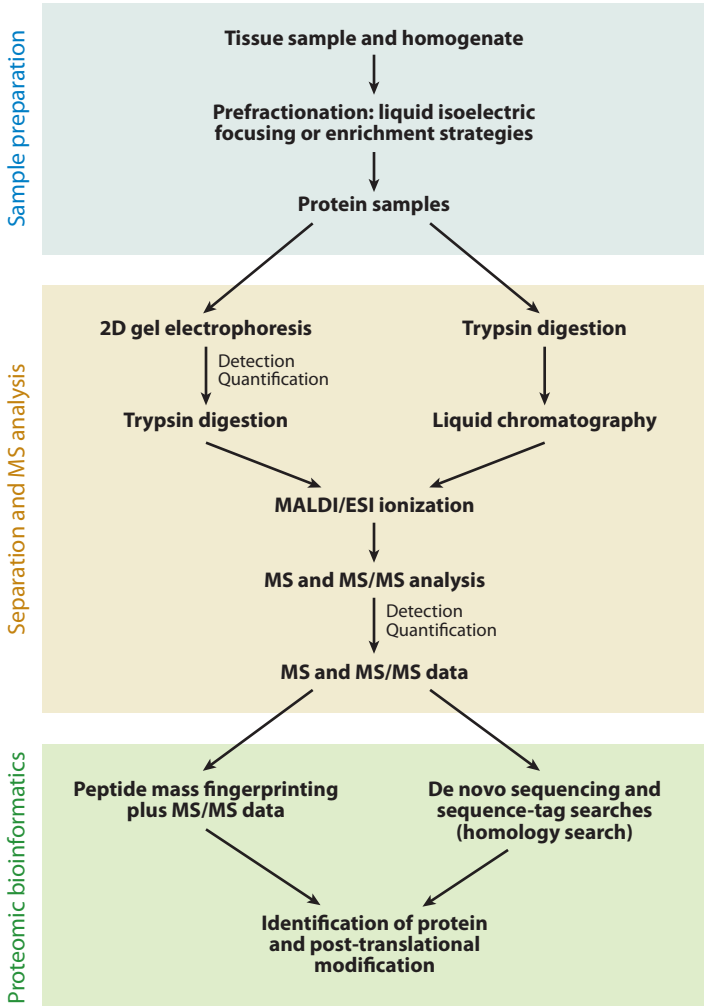

Figure 1

Overview of different proteomic work flows. Abbreviations: 2D (two-dimensional)-gel electrophoresis; MALDI, matrix-assisted laser desorption ionization; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry.

During the first step of the MS analysis, the masses of the peptides, measured as mass (in Da) over charge (m/z), provide a unique peptide mass fingerprint (PMF) (**Figure 2*b***). Using a protein database, the PMF can be matched to a theoretical digest extracted from all the proteins that are included in the database. In order to confirm the match, a number of peptides are selected and further fragmented into ions, creating a peptide fragment fingerprint (PFF) that contains information about the amino acid sequence ((**Figure 2*c***). This process is called tandem mass spectrometry (MS/MS). If the peptides originated from a single protein, e.g., by in-gel digestion of proteins that were separated by two-dimensional gel electrophoresis (2DE) (**Figure 2*a***), the peptide fragments can be linked and combined with the PMF to undergo a single search that contains MS and MS/MS data. In other cases, a number of proteins are digested by trypsin before separation, and every resulting peptide is fragmented by MS/MS and analyzed as to whether it

Peptide mass fingerprint (PMF): distribution of peptide masses resulting from the digestion of a protein by proteases (expressed as mass over charge, or m/z)

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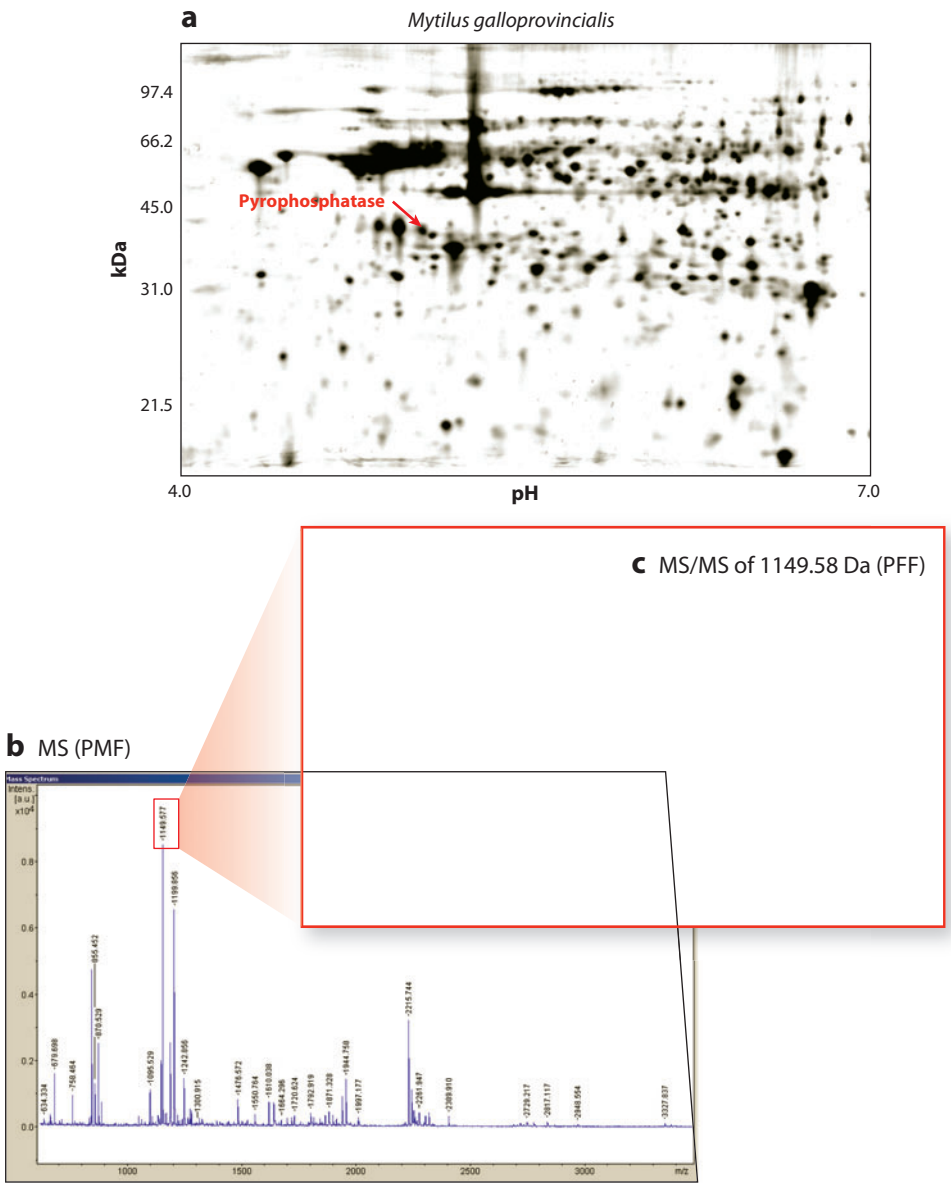

Figure 2

(*a*) Two-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel of *Mytilus galloprovincialis* gill tissue proteins. Immobilized pH gradient (IPG) gel strips (11 cm, pH range 4–7) were used to separate proteins according to their isoelectric point (pI). 400 μg of total protein was loaded. Gels were stained with colloidal Commassie blue. A gel-plug including one protein spot was treated with the protease trypsin, and the resulting peptides were embedded into a matrix. (*b*) The peptide mass fingerprint (PMF) of pyrophosphatase was obtained with matrix-assisted laser desorption ionization (MALDI) mass spectrometry. (*c*, *inset*) A peptide (M_r = 1149.58 Da) selected from the PMF of (*b*) was further fragmented (using tandem mass spectrometry, or MS/MS) into a peptide fragment fingerprint (PFF), which produces ions that contain information about the amino acid composition and sequence of the peptide and can be matched to theoretical fragments obtained from candidates originating from a database.

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matches a protein; all the peptides matching the same protein are later combined. The former path is more typically applied with 2DE separation followed by matrix-assisted laser desorption ionization (MALDI) MS (**Figure 2**). The second path is applied when cell homogenates are digested and then separated by chromatographic methods followed by electrospray ionization (ESI) MS (**Figure 1**). Both forms of MS are soft ionization techniques that leave peptides intact during the ionization process and are at the center of the application of MS to biological questions (Siuzdak 2006).

Given recent advances in mass spectrometry, obtaining MS and MS/MS data is relatively straightforward. However, identifying peptides by interpreting the MS spectra is often challenging because of the limited information contained in the available databases, especially for nonmodel organisms, those whose genomes have not been fully sequenced. Three types of databases are available: protein databases (Entrez, Swiss-Prot, and TrEMBL), genomic databases, and translated expressed sequence tag (EST) databases (Nesvizhskii et al. 2007). Very few protein sequences of marine organisms are found in protein databases. Genomic databases are available for the puffer fish *Takifugu rubripes* and *Tetraodon nigroviridis*, the sea squirts *Ciona intestinalis* and *C. savignyi*, and the purple sea urchin *Strongylocentrotus purpuratus*. Partial genomic information exists for a number of organisms, e.g., the intertidal gastropod *Lottia gigantia*. Finally, there are an even greater number of EST databases available, e.g., for the mussel *Mytilus*, that can be used to translate transcripts into proteins. The use of each of these is often dictated by availability, completeness, and the purpose of the peptide search (Nesvizhskii et al. 2007).

Peptide fragment fingerprint (PFF): distribution of peptide fragment masses (from tandem mass spectrometry), which contain amino acid information, resulting from the dissociation (fragmentation) of peptides

MS/MS: tandem mass spectrometry

2DE: two-dimensional gel electrophoresis

Matrix-assisted laser desorption ionization (MALDI): a peptide ionization process activated through the vibration of a matrix-embedded peptide by a laser

Electrospray ionization (ESI): a peptide ionization (positive charge) process activated by the evaporation of the liquid-dissolved peptide in a vacuum

ESI: electrospray ionization

3. THE PROTEOMIC WORKFLOW

In this review, I will outline only the major proteomic work flow strategies and refer to a number of excellent introductions to proteomic techniques (Kraj & Silberring 2008, Link & LaBaer 2009, Simpson 2003, Siuzdak 2006, Westermeier et al. 2008). A proteomics project consists of three major steps: (*a*) sample preparation, (*b*) protein separation and analysis with mass spectrometry, and (*c*) data analysis using bioinformatics. The two major work flows are as follows (**Figure 1**): Either proteins are separated by 2DE and then identified by either MALDI or ESI MS after trypsin digestion, or they are first digested and then separated by liquid chromatography before identificaton with ESI (or sometimes MALDI) MS.

Before any work flow is chosen, it is important to consider the origin of the biological sample. In general, the more targeted the sample collection, e.g., erythrocytes and mitochondria, the more powerful the analysis. The decision of which work flow to use also depends on the preferred method for quantification and the availability of a mass spectrometer. Quantification is either gel-based and dependent on protein spot detection (Berth et al. 2007), or gel-free, using either stable isotope labeling or label-free spectral counting (Nesvizhskii et al. 2007; Vaudel et al. 2010).

Mass spectrometers consist of a (peptide) ionization chamber, mass analyzer, and detector. The two ionization techniques are MALDI and ESI, which differ both in their spectra and the method of analysis (Siuzdak 2006). Although various mass analyzers with important differences exist, for most proteomic analyses today, it is crucial that the instrument be capable of MS/MS to obtain PFFs (Nesvizhskii et al. 2007, Siuzdak 2006).

Success of either work flow is highly dependent on sample preparation: A general protocol for most tissues employs a urea-based homogenization buffer, a detergent, a reducing agent, and in some cases, protease inhibitors (Shaw & Riederer 2003). For marine organisms, it is advisable to perform a precipitation of the sample to eliminate salts that could interfere with gel-based and chromatographic separation methods (Link & LaBaer 2009). Prefractionation can reduce the protein species and simplify analysis downstream: Examples include liquid isoelectric focusing

REVIEWS IN ADVANCE

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(Horth et al. 2006) and enrichment strategies, e.g., antibody-based affinity columns, to detect PTM (Zhao & Jensen 2009).

Reactive oxygen species (ROS): oxygen species with an unpaired electron (H_2O_2, $OH^{\bullet}$, $O2^{\bullet-}$) that react with and damage biological macromolecules (proteins, lipids, DNA)

The next steps in 2DE involve the separation of proteins, first with immobilized pH-gradient (IPG) gel-strips according to their isoelectric point and then with vertical sodium dodecyl sulfate (SDS)-gel electrophoresis according to their mass (**Figure 2**) (Görg et al. 2004, Link & LaBaer 2009). Methods for spot detection, two-dimensional (2D)-gel image analysis, and in-gel digestion for MS are routine and depend on the investigator's interest in detecting total protein spot intensities or PTM (Berth et al. 2007, Görg et al. 2004, Link & LaBaer 2009, Patton 2002). Alternatively, several liquid chromatographic (LC) methods can be employed to separate peptides according to size, charge, and hydrophobicity, followed by ESI MS/MS (Link & LaBaer 2009; Simpson 2003). This method has greater dynamic range of detection than 2DE but can be more prone to technical problems through the LC separation. In addition, the data analysis associated with this method is more complex because of multiple charged peptide ions.

Peptide identification using tandem mass spectra (**Figure 2**) can be achieved through two methods. A possible candidate match using PMF restricts search parameters, and then peptide fragments can be compared with theoretical PFF originating from the sequence of the potential match. A fit of two peptides to the candidate match is generally considered to be sufficient for a positive identification. This approach relies on the existence of an organism-specific protein, genomic database, or EST database. If none exists, it is necessary to assign an amino acid sequence to the peptide through de novo sequencing (Seidler et al. 2010). The resulting sequence tag can be used to search for homology with proteins from other organisms. Finally, PTM can be accounted for by MS/MS, greatly expanding the potential of MS to a plethora of new biological questions (Ahrne et al. 2010, Witze et al. 2007).

Given the emphasis on proteomic responses to a changing environment, this review focuses mainly on marine nonmodel organisms that are of interest to physiologists because of their ecology but for which there is limited genomic information available. Since protein identification relies on available sequence information, the success of proteomics projects focusing on nonmodel organisms is largely dependent on these resources. Thus, many of the studies presented in this review have been limited by a paucity of genomic information and thus identified only a few significant (or changing) proteins. However, some studies have been able to identify a large percentage (>50%) of the proteins that were found to vary in response to a changing environment by utilizing EST libraries generated from transcriptomics studies or through searches for homologous proteins among existing genomic information of closely related species.

4. ENVIRONMENTAL STRESS

Recent reviews on the cellular stress response (Kültz 2005) and on the functional classification of proteins that increase in response to cellular stress in proteomic studies (Petrak et al. 2008, Wang et al. 2009) have shown that there is a convergence toward a common set of stress-induced proteins in widely diverse taxa. These include molecular chaperones that stabilize denaturing proteins during cellular stress and proteases that regulate protein turnover. Furthermore, proteins that sense and repair DNA and RNA damage and that are involved in fatty acid metabolism are also activated. Together, these proteins indicate that cells sense and respond primarily to macromolecular damage to proteins, DNA, and lipids during acute stress (Kültz 2005). Proteins involved in energy metabolism are also represented, as are proteins involved in redox regulation, emphasizing detoxification of reactive oxygen species (ROS) and production of reducing equivalents such as NADPH (Kültz 2005). It is also hypothesized that there is a trade-off between the energetic demands of cell cycle progression (and therefore growth) and the synthesis of proteins that repair

REVIEWS IN ADVANCE

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macromolecular damage, essentially leading to cell cycle arrest during acute stress. Severe stress can lead to apoptosis or controlled (programmed) cell death. Although these studies highlight the unity of the acute cellular stress response, cells adjust to a stressor in specific ways over longer time periods to reestablish cellular homeostasis (Kültz 2005). During the cellular homeostasis response, cells adjust in response to the novel environment in order to maintain basic cellular functions over the long term, requiring compensatory responses that are specific to the stress and that limit cellular damage. A number of cytoskeletal proteins change in response to stress (Wang et al. 2009). Although this could indicate protein damage, the cytoskeleton may be not only a passive but potentially an active player during stress, especially in response to ROS (Dalle-Donne et al. 2001).

Protein expression signature (PES): a unique protein spot pattern specific to a treatment detected through two-dimensional gel electrophoresis and subsequent protein detection

Together, the following reviews of how thermal, osmotic, and hypoxic stress change the proteome provide a framework of the common pathways that affect organisms under various environmental stresses.

Thermal Stress

The effect of thermal stress on the proteome of marine organisms has long focused on the expression of heat-shock proteins (Hsp), using 1DE or 2DE (Hochachka & Somero 2002; Tomanek 2008, 2010). Studies using 2DE have assessed the variation of heat-induced proteins by using metabolic labeling with ^{35}S-labeled amino acids. In particular, early studies on intertidal limpets (Sanders et al. 1991), the mudsucker *Gillichthys mirabilis* (Kültz 1996), and desert pupfish (*Poecilipis*) (Norris et al. 1997, White et al. 1994) showed that there is great variation in the number of Hsp that can be induced in response to stress. Using metabolic labeling and 2DE, a multispecies comparison of the response of an intertidal gastropod genus (*Chlorostoma*, formerly *Tegula*) to acute heat stress and chronic warm-acclimation showed a high degree of evolutionary variation of Hsp isoforms (Tomanek 2005). It also showed a greater shift in induction temperature of Hsp isoforms from lower to higher temperatures with increasing acclimation temperature in the more stenothermal in comparison to the more eurythermal congeners, in contrast to what was predicted (Tomanek 2005; Tomanek & Somero 1999, 2002). Protein expression signatures (PES) were also used to assess the number of proteins detected during heat stress under normoxic (21% oxygen) and hyperoxic (40% oxygen) conditions in an intertidal gastropod, the dogwhelk (*Nucella lapillus*) (Gardeström et al. 2007). Protein spot numbers were more similar between dogwhelks exposed to 16°C and 26.5°C normoxia and 30°C hyperoxia in comparison to 30°C normoxia. This paralleled a reduction in heat-induced mortality in the 30°C hyperoxic in comparison to the 30°C normoxic conditions, supporting the importance of oxygen limitation in setting thermal tolerance limits (Pörtner 2010).

A comparison of the proteomic responses to acute heat shock between the warm-adapted blue mussel *M. galloprovincialis* and the cold-adapted *M. trossulus* showed that proteins functioning in molecular chaperoning, proteolysis, energy metabolism, oxidative damage, cytoskeleton, and lifespan changed abundance (Tomanek & Zuzow 2010). Specifically, changes in a number of small Hsp isoforms, which are known to stabilize cytoskeletal elements, and cytoskeletal proteins suggested that the cytoskeleton is a major target of Hsp activity. Due to parallel changes in abundance of oxidative stress proteins (thioredoxin, aldehyde dehydrogenase, and superoxide dismutase), the authors hypothesized that heat-induced oxidative stress is a costress that may affect cytoskeletal elements. Increasing levels of proteins involved in the production of NADPH, including enzymes belonging to the Krebs cycle (isocitrate dehydrogenase) and the pentose phosphate pathway (transketolase and lactonase), suggested an increase in the production of reducing equivalents that can scavenge reactive oxygen species (Go & Jones 2008). In parallel, proteins of the electron

REVIEWS IN ADVANCE

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transport chain (ETC; i.e., NADH dehydrogenase and cytochrome c reductase) and of the Krebs cycle (i.e., malate dehydrogenase), which feed NADH into the ETC, showed lower abundances with heat stress. This could indicate a downregulation of parts of the ETC and thus reduced production of ROS. These changes suggest a switch from ROS-generating, NADH-producing pathways to ROS-scavenging, NADPH-producing pathways, possibly reducing the production of ATP. In addition, a NAD-dependent deactylase (called sirtuin), which has been shown to correlate positively with lifespan upon caloric restriction (Finkel et al. 2009), showed lower levels at elevated temperatures, suggesting that sirtuin is a possible indicator of the metabolic costs of heat stress.

The changes elucidated by the proteomics approach described above were more pronounced in the cold-adapted *M. trossulus* than in the warm-adapted *M. galloprovincialis*, especially at the highest temperature (32°C) (Tomanek & Zuzow 2010). Specifically, *M. trossulus* showed lower levels of oxidative stress proteins and sirtuin at the highest temperature while it switched from NADH- to NADPH-producing pathways, suggesting that heat-induced oxidative stress is a major costress and possibly plays an important role in setting thermal tolerance limits. Wide-ranging changes in cytoskeletal proteins occurred in both species, indicating that cytoskeletal modifications may be another important, albeit not well understood, cellular process instrumental in setting thermal tolerance limits. A comparison of proteomic differences between the warm-adapted blue mussel *M. galloprovincialis* and the cold-adapted *M. edulis* collected from their native environments showed that interspecific differences in Hsp70 and calreticulin, both molecular chaperones, exist that could contribute to the greater heat tolerance of the warm-adapted congener (López et al. 2002).

A proteomic comparison of two snail (*Littorina saxatilis*) ecotypes (high and upper intertidal zone) showed qualitative (1.4% of the proteome) and quantitative (16% of the proteome) variation in field-collected animals (Martinez-Fernandez et al. 2008). Two metabolic enzymes were identified (fructose-bisphosphate aldolase and arginine kinase) that showed higher levels in the upper-zone ecotype, which is exposed to a greater range of physical extremes.

Changes in the proteome in response to long-term thermal variation due to (laboratory) acclimation have been investigated in liver tissue of the gilthead sea bream (*Sparus aurata*), a fish native to the Mediterranean Sea. Fish acclimated to 8°C versus 22°C differed in proteins representing the cytoskeleton, carbohydrate metabolism, proteolysis, amino acid interconversion and catabolism, cellular defense, stress response, and protein synthesis (Ibarz et al. 2010). In this species, temperatures below 13°C lead to growth arrest and metabolic depression. Cold-acclimation induced higher levels of lipid peroxidation and nitric oxide production, both indicative of higher levels of oxidative stress. These changes were accompanied by decreasing abundances of two antioxidative stress proteins (glutathione transferase and catalase). Cold acclimation also induced higher levels of proteins representing protein degradation and proteolysis (peptidases, proteasome activator factor, and lysosomal trypsin-like protein) as well as proteins of the cellular stress response (natural killer–enhancing factor and protein disulfide isomerase (PDI), both proteins with peroxiredoxin and thioredoxin protection activities, possibly linking higher levels of ROS with greater rates of protein damage. Several proteins representing carbohydrate metabolism (glycogen phosphorylase, fructose bis-phosphate aldolase, and enolase) showed higher abundance under cold conditions. Three isoforms of elongation factor 1α (EF-1α), a protein that functions in peptide chain elongation during protein synthesis, showed higher levels in cold-acclimated fish. At the same time, proteins functioning in amino acid catabolism (glutamate dehydrogenase, aspartate aminotransferase, and homogentisate 1,2-dioxygenase) showed lower levels. In a similar study of skeletal muscle tissue, cold acclimation (10°C versus 30°C) in the common carp (*Cyprinus carpio*) showed only subtle changes in PES but an accumulation of fragments of creatine kinase consistent with a cold-induced increase in protein degradation (McLean et al. 2007).

REVIEWS IN ADVANCE

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In summary, acute and chronic temperature stress induces wide-ranging proteomic changes in protein synthesis and degradation that are accompanied by increasing levels of chaperones. Oxidative stress levels vary greatly in parallel with major changes in energy metabolism. Targeted proteomic studies, ideally coupled with measurements of stress-related changes in metabolic flux, are now required to elucidate the cellular costs of temperature stress.

Osmotic Stress

Marine organisms may encounter changing salinity (osmotic) conditions near shore due to proximity to estuaries and major rivers (Evans 2009). Osmotic stress may also become more severe with climate change due to more severe rain events and subsequent runoff into marine or brackish waters. A number of teleost fish and elasmobranchs, e.g., some sharks, are known to live over a wide range of salinities because they move between brackish estuaries and the sea, either during adulthood or early development. Proteomics offers an excellent tool to characterize the cellular changes that accompany osmoregulatory challenges.

A proteomic comparison between osmoregulatory organs (kidney, intestine, gill, and rectal gland) and nonosmoregulatory organs (heart and brain) in the spiny dogfish shark (*Squalus acanthias*) discovered similarities in the former tissues that could provide insights into the general cellular processes involved in osmoregulation (Lee et al. 2006). This species is a partially euryhaline (from full-strength to 50–70% seawater) shark that keeps its blood plasma either iso- or slightly hyperosmotic relative to the external environment. The analysis showed that osmoregulatory organs are enriched for proteins whose molecular functions include lysis, phosphorylation, and hydrolysis. The two lyases that were identified represent an important glycolytic enzyme (fructose-bisphosphate aldolase) and an enzyme involved in membrane biogenesis and reorganization (phosphatidylserine decarboxylase). Among the kinases were creatine kinase and an enzyme with an adenylsulfate kinase activity [3′-phosphoadenosine 5-phosphosulfate (PAPS) synthase], indicating a high capacity for converting ATP to alternative energy-rich metabolites. A hydrolase, i.e., pyrophosphatase, also plays a role in transferring energy-rich phosphoryl groups. Another hydrolase specifically expressed in osmoregulatory organs, NG,NG-dimethylarginine dimethylaminohydrolase 1, suggests NO synthase–mediated cell signaling in osmoregulation. Its presence also suggests that the production of the important shark osmolyte urea is not limited to the liver. Finally, enzymes involved in amino acid metabolism (glutamate dehydrogenase and 4-aminobutyrate aminotransferase) were also dominant in osmoregulatory organs. A pathway analysis showed that cytoskeletal modifications by Rho-GTPase (a small G-protein) are overrepresented.

A study that followed up on these results showed that feeding, which is often associated with high salt loads and the requirement for osmoregulation, caused increasing levels of enzymes representing Krebs cycle reactions [malate dehydrogenase (MDH) and isocitrate dehydrogenase (IDH)] in rectal gland tissue of the spiny dogfish shark (Dowd et al. 2008).

A proteomic analysis of the osmoregulatory organs (rectal gland and gill) of the partially euryhaline leopard shark (*Triakis semifasciata*) in response to hyposaline conditions showed a decrease in several enzymes representing glycolysis [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglyerate mutase], Krebs cycle (IDH), malate-aspartate shuttle (MDH), oxidative phsophorylation (H^+-transporting ATP synthase and ATP synthase), and mRNA processing and transport (heterogenous nuclear ribonucleoprotein) in the rectal gland (Dowd et al. 2010a). In addition, 26S proteasome subunits responsible for protein degradation were found to change in response to hyposaline conditions. Similar changes were found in gill tissue, indicating that energy metabolism is reduced overall.

REVIEWS IN ADVANCE

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European sea bass (*Dicentrarchus labrax*) reared under seawater and freshwater conditions showed an overabundance of cytoskeletal proteins and aromatase cytochrome P450 (which converts androgens into estrogens in vertebrates) in gills under seawater conditions (Ky et al. 2007). Under freshwater conditions, the prolactin receptor and major histocompatability complex class II β-antigen were higher in gill tissue, as was the Iroquois homeobox protein Ziro5, a protein regulating cell development in the intestine. Prolactin is the major fish hormone known to respond to freshwater conditions during acclimation. Thus, unlike sharks that stay either iso- or slightly hyperosmotic to the external environment in response to widely varying salinities, changes in developmental proteins and hormone pathways suggest that marine teleosts restructure their osmoregulatory organs in response to extreme salinities.

In the case of a freshwater teleost with life stages in brackish water, the juvenile ayu (*Pleocoglossus altivelis*) showed lower abundances of several enzymes representing a link between glycolysis and the Krebs cycle (pyruvate dehydrogenase), the Krebs cycle itself (IDH), ATP synthesis (ATP synthase), and an iron-binding protein (ferritin) in response to brackish conditions. Iron homeostasis is linked to the expression of Krebs cycle enzymes, and thus these changes are consistent with lower energy requirements in response to brackish conditions that are more similar to the blood plasma than freshwater, reducing the osmotic gradient that has to be maintained (Cheng et al. 2009). However, proteins involved in methylation (S-adenosylhomocysteine hydrolase) and amino-acid metabolism [Cys/Met metabolism pyridoxal phosphate (PLP)-dependent enzyme] were higher under brackish conditions. These changes were interpreted as representing modifications of DNA methylation, and thus expression patterns, as well as the biosynthesis of amino acids (Cheng et al. 2009).

Analyses of the soluble and the membrane-associated proteome of the halotolerant alga *Dunaliella salina* showed that high-salinity conditions increase metabolic pathways that accumulate glycerol, its major compatible osmolyte (Katz et al. 2007, Liska et al. 2004). Membrane stabilization may be achieved by peptidoglycans that are typically found in bacteria. Furthermore, several membrane-associated changes seem to be regulated by small G-proteins of the Ras-superfamily. Finally, high salinity induced several molecular chaperones and proteases, suggesting higher requirements for protein stabilization and degradation.

In summary, proteomic studies have characterized physiological similarities among osmoregulatory organs in elasmobranchs, e.g., higher levels of enzymes involved in energy, urea, and amino acid metabolism (Kültz et al. 2007). They also showed that cytoskeletal modifications are occurring that may be regulated by small G-proteins. Importantly, levels of enzymes involved in energy metabolism seem to change in response to differences in osmolality between blood plasma and the external environment. Differences in the proteomic responses between marine teleosts that are osmoregulators and elasmobranchs that are (delayed) osmoconformers suggest that changing salinity conditions show hormone-induced and developmental changes in the former but not the latter. It will be interesting to see if a broader phylogenetic comparison between these groups confirms these differences and specifies the pathways that are activated.

Anaerobic Stress

Given that many freshwater lakes, rivers, and entire ocean basins can become hypoxic or even anoxic, proteomic changes in response to low-oxygen levels may provide important insights into the physiological effects and defense strategies of organisms in response to exposure to anaerobic conditions (Childress & Seibel 1998). In general, animals respond to low oxygen with two strategies: they downregulate metabolism and thus decrease their energy requirement by reducing ion pumping and protein synthesis, and/or they upregulate anaerobic pathways, e.g., glycolysis,

REVIEWS IN ADVANCE

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that are inefficient relative to oxidative phosphorylation (Hochachka & Lutz 2001, Hochachka & Somero 2002).

Metabolic depression has been shown to occur during hypoxia in muscle tissue of the kuruma prawn (*Marsupenaeus japonicus*) through the downregulation of fructose-bisphosphate aldolase, a glycolytic protein (Abe et al. 2007). Hypoxic stress lowered levels of a number of proteins involved in energy metabolism, antioxidant processes, chaperoning, and cytoskeleton while increasing abundances of proteins involved in the immune response in hepatopancreas of the Chinese shrimp (*Fenneropenaeus chinensis*) (Jiang et al. 2009). Few proteomic changes were observed in zebrafish skeletal muscle in response to hypoxic conditions, in contrast to widespread transcriptomic changes (Bosworth et al. 2005, Ton et al. 2003).

Several fish species are extremely anoxia-tolerant, mainly due to adaptation protecting the brain from the metabolic consequences of hypoxic or anoxic conditions (Hochachka & Lutz 2001). A proteomic analysis of brain tissue of the anoxia-tolerant crucian carp (*Carassius carassius*) in response to anoxic conditions showed reductions in the levels of proteins functioning in glycolysis (five proteins), neuronal apoptosis (voltage-dependent anion channel), and neural degeneration (dihydropyrimidinase-like protein 3 and vesicle amine transport protein 1), all responses suggesting that the brain becomes more protected against cellular injury (Smith et al. 2009). The epaulette shark (*Hemiscyllium ocellatum*), which tolerates episodes of hypoxia in its tropical reef environment, showed decreases in proteins (e.g., glutaminase) involved in glutamate production and release and changes of proteins associated with the regulation of internal calcium stores in the cerebellum in response to hypoxia (Dowd et al. 2010b). These changes were interpreted as illustrating proteomic responses to compensate for changes in internal calcium stores and excitotoxicity due to increased glutamate release. In species that do not tolerate hypoxic or anoxic conditions, the proteins representing similar cellular functions will increase instead of decrease. However, the hypoxia-tolerant medaka (*Oryzias latipes*) showed an increase in aldolase, a glycolytic enzyme, and succinate dehydrogenase, a Krebs cycle enzyme, in brain tissue in response to hypoxia (Oehlers et al. 2007). In addition, increasing levels of hemoglobin and carbonic anhydrase suggestenhanced blood flow and vascularization, possibly increasing the delivery of existing levels of blood oxygen.

One critical question is whether the cellular response depends on how anoxic conditions are applied to the organism. A proteomic comparison between rainbow trout hypodermal fibroblast cells experiencing temporary anoxic conditions, either due to chemical poisoning of the ETC with sodium azide (NaN_3) or nitrogen-purged air, showed that only six out of forty proteins changed their abundance in response to both treatments (Wulff et al. 2008); these included ribosomal proteins that were decreasing and represent protein synthesis.

Proteomic studies on anaerobic stress are providing evidence for a global reduction of enzymes involved in energy metabolism in organisms that are hypoxia-tolerant. These changes occur in a number of tissues simultaneously. Future studies may focus on PTM to elucidate the regulatory mechanisms of the signaling events that activate the cellular response to hypoxia.

5. ECOTOXICOPROTEOMICS

Risk assessment of marine pollution levels can be achieved by analyzing the routes by which pollutants enter ecosystems and, then, accumulate along trophic networks (Newman & Clements 2008). However, this mode of analysis assumes that the biological effects of chemicals of interest are established. In reality, the biological effects of emerging pollutants are often poorly understood. The application of systems biology approaches has been proposed as an ideal tool to assess effects of pollutants at the cellular level because this approach could provide a more comprehensive risk assessment of the cellular effects than do the current, generally more narrowly focused methods

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(Calzolai et al. 2007, Lemos et al. 2010, Lopez-Barea & Gomez-Ariza 2006, Waters & Fostel 2004). However, specific protein biomarkers, which are the focus of current methods, may indicate a mode of action on cells that may be specific to the pollutant and thus provide direct insights into the chemical mechanism of toxicity (Sarkar et al. 2006). Although the mechanisms of toxicity may not be known, if we assess changes at the systems level, previously unknown effects are likely to be discovered. Transcriptomics, e.g., microarray studies, have led the way in this effort, but several proteomics studies have now established the feasibility of a proteomic approach (Lemos et al. 2010). These studies can be divided into three categories. Early studies used the unique PES obtained through 2DE in response to pollutant exposure as a signal without identifying proteins. The second group of studies included protein identifications after establishing PES for specific tissues, cells, or subcellular locations involved in the detoxification of xenobiotics. A third group of studies focused on PTM of proteins as a signal for toxicity.

Protein Expression Signatures

A number of early studies applied 2DE to generate unique PES in response to pollutant exposure. The sentinel mussel genus *Mytilus* was used to identify PES for copper and polychlorinated biphenyls (PCBs) (Olsson et al. 2004, Shepard & Bradley 2000, Shepard et al. 2000). Eastern oysters (*Crassostrea virginica*) were used to detect changes in response to zinc exposure (Meiller & Bradley 2002). When the method was used to compare zebrafish (*Danio rerio*) embryos exposed to 17β-estradiol and the estrogen mimic 4-nonylphenol, it was possible to show differences in their PES suggesting that the physiological actions of these pollutants may be more distinct than previously assumed (Shrader et al. 2003).

This conceptual approach of focusing on signatures has found another application through surface-enhanced laser desorption/ionization (SELDI) MS (Tang et al. 2004). SELDI-MS first separates proteins by chromatographic methods using a protein chip and then obtains a mass spectrum through time-of-flight mass spectrometry. Differences in mass fingerprints can represent unique signatures that can distinguish different disease states in organisms and be used as biomarkers. When used to analyze digestive glands of blue mussels (*Mytilus*) between control locations and sites contaminated with polyaromatic hydrocarbons (PAH) and heavy metals or mussels exposed to oil or oil spiked with alkylphenols, it was possible to correctly classify samples from different treatments with 90% accuracy (Bjornstad et al. 2006, Knigge et al. 2004).

Mass Spectrometry–Based Proteomics with Peptide Identification

Taking advantage of advances in mass spectrometry and an expanding protein database, a number of studies identified the proteins in their PES. Using MS/MS, a study on the clam *Chamaelea gallina* showed that several cytoskeletal proteins (actin, myosin, and tropomyosin) changed in response to exposure to PCBs, copper, tributyltin (TBT), and arsenic (Rodriguez-Ortega et al. 2003). The identifications were based on homologies with sequences from other organisms, and thus it can be assumed that only the most evolutionarily conserved proteins were identified; however, this was evidence for the potential role of the cytoskeleton in pollutant stress. Another study on TBT-exposed *M. galloprovincialis* showed similar changes in cytoskeletal proteins, e.g., tubulin (Magi et al. 2008). Changes in the proteome of the hepatopancreas of mussels (*M. edulis*) in response to azaspiracid, a dinoflagellate-derived toxin found in shellfish, included proteins involved in eliminating xenobiotics (Nzoughet et al. 2009).

Several other studies have focused on proteomic changes in response to heavy metal exposure: One study on gill tissue of Chinese mitten crabs (*Eriocheir sinensis*) showed that acute and chronic

REVIEWS IN ADVANCE

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exposure to cadmium causes changes in the abundance of cytoskeletal proteins, oxidative stress proteins [glutathione S-transferase (GST), thioredoxin peroxidase], molecular chaperones (PDI), proteases (proteasome and cathepsin D), and a number of proteins involved in energy metabolism (arginine kinase, GAPDH, MDH, and ATP synthase) (Silvestre et al. 2006). Similar changes were observed in gill tissues of blue mussels (*Mytilus*) in response to (heavy metal–containing) crude oil (Manduzio et al. 2005) and clams (*Scrobicularia plana*) contaminated with heavy metals (Romero-Ruiz et al. 2006). In a sturgeon species (*Huso huso*), methylmercury caused proteomic changes in brain tissue representing energy metabolism, protein folding, cell division, and signal transduction (Keyvanshokooh et al. 2009). In brain tissue of the fish *Paralichthys olivaceus*, cadmium exposure caused a synchronous PES that included transferrin, an iron-binding protein (Zhu et al. 2006). Exposure to oil and oil spiked with alkylphenols as well as PAH identified plasma proteins of juvenile cod (*Gadus morhua*) functioning in fibrinolysis and the complement cascade, immune function, fertility, calcium homeostasis, fatty acid metabolism, oxidative stress, and apoptosis as changing abundance (Bohne-Kjersem et al. 2009). Copper induced a number of proteins involved in buffering oxidative stress in the marine brown alga *Scytosiphon gracilis* (Contreras et al. 2010).

Estrogen and androgen mimics are now common in the environment (Norris & Carr 2006), and proteomic analyses are characterizing their biological effects. A comprehensive study on the proteomic effects of exposure to the androgenic chemical 17β-trenbolone, used as a cattle growth hormone, and its antagonist, flutamide, in livers of fathead minnows (*Pimephales promelas*) showed that a number of proteins functioning in energy metabolism, oxidative and cellular stress, and translation changed their abundances (Martyniuk et al. 2009). Treatment with gonadotropin-releasing hormone of fertility-impaired F1 Senegalese sole (*Solea senegalensis*) showed changes in the abundance of testis proteins involved in energy metabolism, cytoskeleton, and oxidative stress (Forne et al. 2009).

In an attempt to enhance detection of low-abundance proteins involved in the detoxification of xenobiotics, several studies first enriched samples from the digestive gland of *Mytilus* for peroxisomes, organelles that use molecular oxygen to produce hydrogen peroxide (H_2O_2), which in turn is used to oxidize a number of substrates, including fatty acids, phenols, and alcohol (Amelina et al. 2007, Apraiz et al. 2006, Mi et al. 2005). Several spots were identified as peroxisomal proteins involved in the degradation of xenobiotics (epoxide hydrolase) and oxidation of thiol groups (Mi et al. 2005). Peroxisomal preparations were also used to detect PES in *Mytilus* for three different pollutants: a phthalate, polybrominated diphenyl ether (PBDE), and bisphenol A (Apraiz et al. 2006). Each pollutant caused a unique PES, although there was some overlap between all three (14% of protein spots changing). Several spots that showed increasing levels in response to all three pollutants represented catalase, an abundant peroxisomal protein that converts H_2O_2 to water. Another protein that showed higher levels in response to two pollutants was superoxide dismutase (SOD), which converts superoxide anions to H_2O_2. Abundances of other proteins involved in xenobiotic metabolism (cytochrome P450), β-oxidation of fatty acids (enoyl-CoA hydratase), and cell signaling (phospholipase A_2) were also higher in response to two of the pollutants. Levels of other proteins that represent the cytoskeleton, energy metabolism, oxidation reactions (alcohol and aldehyde dehydrogenases), and detoxification of xenobiotics (GST) decreased. Using principal component analysis, a related study of *Mytilus* from polluted and control sites near Gothenburg harbor, Sweden, showed that peroxisomal PES clustered more polluted sites closer to each other than to the control site (Amelina et al. 2007). Proteins that showed higher abundance in the polluted sites were involved in protein degradation (cathepsin), β-oxidation (acyl-CoA dehydrogenase), and detoxification (GST). Proteins that showed lower abundances included constitutive isoforms of Hsp70, an actin-binding protein associated with the cytoskeleton (fascin), and an

REVIEWS IN ADVANCE

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oxidative stress protein (aldehyde dehydrogenase). The same approach was used to assess the effect of the *Prestige*'s oil spill on mussels from the Iberian Peninsula (Apraiz et al. 2009).

A proteome analysis was also applied to assess ecosystem health along the Shenandoah River, Virginia, which has seen increasing levels of fish death since 2004, using the smallmouth bass (*Micropterus dolomieu*) (Ripley et al. 2008) as study speies. A similar study was performed on Gaobeidian Lake in Beijing, China, using the goldfish (*Carassius auratus*) (Wang et al. 2008). The latter study found changes in the goldfish liver proteome representing energy metabolism (fatty-acid binding protein and betaine homocysteine methyltransferase), antioxidative damage proteins (glutathione peroxidase and ferritin), and protein degradation (proteasome) when animals were exposed to water from either the polluted lake or a reservoir used for drinking water, which contained much lower levels of pollutants.

Post-Translational Modifications

An alternative assessment of the toxic action of pollutants focuses on various PTM. Although the types of PTM number in the hundreds (Walsh 2006), so far only changing patterns of carbonylation, glutathionylation, thiol-modifications, and ubiquitination have been studied in response to pollutants (Sheehan 2006). The former three are modifications caused by the increased production of ROS and can occur due to a change in the oxidative environment of the cell. Protein ubiquitination is a process regulated by a number of substrate-specific and nonspecific ubiquitin-ligases and leads to the degradation of proteins by the proteasome, constituting the main protein degradation pathway in eukaryotic cells (Glickman & Ciechanover 2002).

A number of amino acids (lysine, arginine, proline, and threonine) can be modified by ROS to reactive aldehyde and ketone groups (called carbonylation), leading to the inactivation of proteins and their breakdown (Dalle-Donne et al. 2003). In contrast to carbonylation, glutathionylation is a reversible process, which can, under conditions of oxidative stress, protect cysteine residues from oxidation (Schafer & Buettner 2001). Another common modification of proteins by ROS is the oxidation of methionine residues (Levine et al. 1999). This modification can be reversed by the abundant methionine sulfoxide reductases and thus allows the pool of methionines to serve as a buffer against a surge in ROS. Finally, all these modifications can lead to the denaturation of proteins and their degradation along the ubiquitin-proteasome pathway (Davies 2001). Thus, given that a great number of pollutants are known to increase production of ROS, PTMs caused by ROS and subsequent changes in levels of protein degradation have the potential to be sensitive global markers of pollutant stress.

For example, blue mussels (*M. edulis*) from polluted sites in Ireland showed greater levels of carbonylated proteins but few changes in protein abundance in gill and digestive tissues in comparison to control sites (McDonagh et al. 2005). Subsequent exposure of mussels to H_2O_2 followed by 2DE showed that oxidative stress increases levels of carbonylated proteins (McDonagh et al. 2005). Because the formation of aldehyde and ketone groups (carbonylation) among amino acid side chains has been shown to cause protein degradation, it may be assumed that carbonylation due to oxidative stress leads to corresponding patterns of protein ubiquitination, and thus degradation. A study comparing PES among the three pro-oxidants H_2O_2, $CdCl_2$, and menadione found few qualitative differences in protein abundance but showed increased levels of carbonylation and ubiquitination of proteins on 2D immunoblots (McDonagh & Sheehan 2006). Carbonylation was also used as a biomarker to discover that *p,p′*-dichlorodiphenyldichloroethylene causes oxidative stress in the clam *Ruditapes decussatus* (Dowling et al. 2006a).

Another PTM whose occurrence is linked to oxidative stress is glutathionylation. Unlike carbonylation, glutathionylation is a reversible modification that is catalyzed by the enzyme

REVIEWS IN ADVANCE

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glutathione S-transferase (GST). GST conjugates glutathione to sufhydryl groups on proteins during oxidative stress. Upon return to nonstressful conditions, these glutathione–protein disulfide bonds can be oxidized. Thus, glutathionylation protects chemical groups that are sensitive to oxidation. A study separating glutathione affinity–purified proteins using 2DE showed that gill, digestive, and mantle tissues of the clam *Tapes semidecussatus* differ in the number and abundance of GST isoforms, suggesting widely varying redox conditions in these tissues (Dowling et al. 2006b).

Amino acids that contain sulfur groups, e.g., cysteines and methionine, are oxidized by ROS and thus are major targets of oxidative stress. For example, oxidations of protein-thiol to disulphides (-SH HS- to -SS-) may include cysteines of the same protein (intramolecular) or those of glutathione (intermolecular). These oxidations may buffer the effects of ROS, serve as signals that modify protein function, or at higher levels may lead to protein degradation. There is evidence that a number of abundant proteins in *Mytilus* are targets of ROS (McDonagh & Sheehan 2007). Specifically, actin, PDI, and other chaperones (heat shock protein gp96 and calreticulin) have been shown to form intramolecular disulphide bonds in response to exposure to menadione, a pro-oxidant, possibly sequestering ROS before they can cause uncontrollable damage to other proteins, lipids, or ribonucleotides.

Challenges for Ecotoxicoproteomics

Proteomics applied to assess the biological effects of pollutants in marine organisms is beginning to reveal some of the systemic changes that occur on the cellular level. First, even a qualitative description of cellular changes through PES shows that the systemic changes occurring during exposure are pollutant-specific. Second, proteins common to many pollutant-stress responses include oxidative stress proteins, cytoskeletal proteins, chaperones, proteases, and proteins involved in the detoxification of xenobiotics as well as β-oxidation. Together, these changes suggest that the production of ROS leads to the denaturation of proteins as well as wide-ranging modifications of cytoskeletal elements. Finally, PTMs present a novel frontier to assess the biological effects of pollutants. Among the PTMs that are likely to be the most important are those that increase with oxidative stress, e.g., carbonylation and glutathionylation, and that are indicators of protein denaturation, e.g., ubiquitination.

Ecotoxicoproteomics has the potential to become the assessment method of choice for emerging pollutants but has to overcome several shortcomings: First, none of the proteomics studies presented here has been fully quantitative, in part because investigators have relied on silver staining, which has a limited linear range. In addition, sample sizes have been low and provide little statistical power to detect a small but relevant change in protein abundance. Protein identification has been limited, but with more EST libraries available, it is possible to identify more than 50% of the proteins from such sentinel nonmodel species as *Mytilus*. Finally, acute and long-term exposures as well as exposures to a range of pollutant concentrations are needed to detect possible nonlinear low-dose responses (Calabrese et al. 2007). Despite these current shortcomings, proteomics provides a powerful approach for marine ecotoxicologists to assess the biological effects of emerging pollutants and to develop comprehensive biomarker profiles.

6. PROTEOMIC RESPONSES TO INFECTION

Increasing reliance of human food consumption on fish and shellfish raised in aquaculture facilities has increased concerns about infections, in part to protect humans but also to minimize economic losses (FAO 2009). Comparisons of infected, vaccinated, or otherwise treated animals to boost immune defense (e.g., probiotic bacteria), have already provided insights into the proteomic

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changes accompanying immune responses. For example, a study on the proteomic changes in the Pacific white shrimp (*Penaeus vannamei*) 24 h after an infection with the Taura syndrome virus, an important shrimp pathogen, showed changes in the abundance of several proteins involved in host defense (Chongsatja et al. 2007, Havanapan et al. 2009). Among the proteins that decrease in abundances were several involved in defense mechanisms, e.g., hemocyanin, transglutaminase, and GST. Interestingly, proteins that increased were involved in a number of cellular functions but not immune defense. In another proteomic analysis of shrimp exposed to the White spot syndrome virus, a completely different set of proteins was shown to be differentially expressed (Robalino et al. 2009).

A number of fish studies addressed various aspects of proteomic changes in response to infection. A study on differently expressed proteins in liver and kidney in diseased versus healthy Atlantic salmon (*Salmo salar*) showed that exposure to Infectious hematopoietic necrosis virus (IHNV; genus *Rhabdoviridae*) and *Renibacterium salmoninarum*, which causes bacterial kidney disease, changes the abundances of a number of proteins related to the infectious process (Booy et al. 2005). Among those are natural killer cell enhancement factor (NKEF), which was shown to be downregulated by IHNV exposure. NKEF is a peroxidase and augments the activity of natural killer cytotoxicity against tumor cells and also enhances interleukin-2 induction of lymphokine-activated killer cell function and proliferation (Kim et al. 1997). Thus, NKEF may be important in host resistance against IHNV, and its downregulation may support the progression of the infection (Booy et al. 2005). Other proteins found to have differential expression included procathepsin L, superoxide-producing NADPH oxidase, and interferon-induced viral resistance protein Mx (Booy et al. 2005).

One of the primary immune organs in fish is the head kidney. An analysis of head kidney proteins changing in response to a virulent strain of the bacterium *Edwardsiella ictaluri* between families of the channel catfish (*Ictalurus punctatus*) with high and low susceptibility to infection showed proteins functioning in macrophage activity and cellular stress response as well as energy metabolism (Booth & Bilodeau-Bourgeois 2009). A proteomic study on tilapia (*Oreochromis mossambicus*) provided evidence for a reduction in phagocytic activity of head kidney and spleen leukocytes exposed to blood serum from fish treated with an acute osmotic stress, suggesting that hypersalinity may affect immune function (Kumar et al. 2009). Among the proteins found to be upregulated in serum from fish exposed to seawater, and which may thus have a modulating effect on phagocytic activity, were complement C3, sphingomyelinase, semaphorins, and caspase 3. Limited immune function in cod larvae (*G. morhua*) may require alternative strategies to vaccination for successful rearing conditions in commercial hatcheries. A proteomic analysis of cod larvae showed that treatment with probiotic bacteria, whose benefit is to enhance host defense function, actually lowered the abundance of proteins that function in stress and immune responses, suggesting a potentially decreased challenge by pathogenic bacteria (Sveinsdottir et al. 2009). Using a proteomics approach, vaccination of the commercially important large yellow croaker (*Pseudosciaena crocea*) was shown to increase β_2-microglobulin, a small serum protein that is the noncovalently bound light chain of major histocompatibility complex (MHC) class I proteins (Chen et al. 2010), and a role of peroxiredoxin in the immune response was discovered (Yu et al. 2010). Interferons (IFN) are cytokines that affect a wide range of immune activities, among them activation of macrophages as part of the innate immune response to invading pathogens and the production of antigenic peptides through the MHC as part of the adaptive immune response. Using a cell line enriched for macrophages and derived from the head kidney of the Atlantic salmon, stimulation by IFN-γ activated elongation factor 2, suggesting increased levels of protein synthesis (Martin et al. 2007). Mx, an antiviral protein with a GTP binding domain and a leucine zipper motif as well

REVIEWS IN ADVANCE

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as C-type lectin, a cell surface receptor that binds to viral and bacterial pathogens, also increased upon IFN-γ stimulation.

In summary, proteomic analysis can be used to generate a systems-wide perspective of the cellular and physiological changes that occur during infection but can also be used to characterize the cellular pathways of more targeted manipulations (e.g., vaccination or exposure to immune-stimulating treatments).

7. PROTEOMIC MODIFICATIONS IN RESPONSE TO SYMBIOTIC RELATIONSHIPS

Symbiotic relationships are numerous in the marine environment and support the energy flow through entire ecosystems, e.g., coral reefs (Hoegh-Guldberg et al. 2007). The close, intracellular relationships that exist between hosts and their symbionts are characterized by an attenuated immune response (Weis 2008). Analysis of PES using 2DE between symbiotic and aposymbiotic individuals of the intertidal cnidarian *Anthopleura elegantissima* (Weis & Levine 1996) and the symbiont-colonized and uncolonized light organ of the Hawaiian squid *Euprymna scolopes* showed that establishment and maintenance of the relationship requires changes in the host's proteome (Doino Lemus & McFall-Ngai 2000). In the former case, two of the proteins were further characterized and identified as carbonic anhydrase, an enzyme involved in the transport of inorganic carbon between the algal-cnidarian symbiosis (Weis & Reynolds 1999), and sym32, a fascilin I homolog that may play a role in communication between the host-symbiont organisms (Reynolds et al. 2000). However, two additional proteomic studies of cnidarians failed to detect any changes in protein expression, possibly because the protein isolation protocols were not directly targeted to the organs or cells of the symbiosis (deBoer et al. 2007, Schwarz & Weis 2003). An approach targeting the membrane envelope that engulfs the symbiont, the so-called symbiosome, identified a number of proteins that may be involved in maintaining the symbiotic relationship between the sea anemone *Aiptasia pulchella* and the dinoflagellate symbiont (Peng et al. 2010). Proteins tentatively functioning in ATP synthesis (ATP synthase), cross-membrane transport of proteins and small molecules (PDI, Hsp70, ABC transporter), stress response and antiapoptotic pathways (PDI, Hsp70, α-crystallines), membrane signaling (G-protein-coupled receptor), and cytoskelatal remodeling (symbiont and host actin, glial fibrillary acidic protein) were identified as being part of the symbiosome.

Although it is assumed that temperature plays a major role in causing coral bleaching, it is also acknowledged that a multitude of other factors, e.g., pollution by wastewater, may overlap and exert synergistic effects (Hoegh-Guldberg et al. 2007). Caffeine, a psychoactive stimulant serving human sanity worldwide, is by now recognized as one of the most commonly found organic chemicals in surface waters and is thus a part of the ubiquitous human influence on reef health. A proteomic survey of changes of various strains of the dinoflagellate symbiont of corals (cnidarians) showed that a number of proteins functioning in the cellular stress response (Hsp70) and energy metabolism (glycolysis and photosynthesis-associated proteins) changed in response to caffeine exposure (Pollack et al. 2009).

A proteomic analysis of the bacterial symbiont of the hydrothermal vent worm *Riftia pachytila* showed that three proteins involved in sufide oxidation (dissimilatory sulfite reductase, adensine phosphosulfate reducatse, and ATP sulfurylase) together constitute 12% of the detected proteome and thus illustrated the importance of the chemolithoautotrophic energy metabolism of the symbiont (Markert et al. 2007). The analysis also discovered high levels of four enzymes that could drive a reverse or reductive CO_2-fixing Krebs cycle in the symbiont, suggesting that the cycle may

REVIEWS IN ADVANCE
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augment or replace the Calvin cycle for CO_2 fixation under certain environmental circumstances, e.g., varying H_2S concentrations (Markert et al. 2007).

Together, these studies illustrate the power of a proteomics approach but also the challenges one faces when the process of interest is isolated in small subsets of cells. Specifically targeted organ, cellular, or subcellular separations to enrich for proteins of interest can greatly improve the discovery of pathways involved in establishing and maintaining a symbiotic relationship.

8. ECOLOGICAL DEVELOPMENTAL PROTEOMICS

Early life stages of marine organisms undergo complex developmental pathways that include fertilization, early cleavage, organogenesis, larval development, and metamorphosis to the adult stage (Gilbert & Epel 2009). These life stages are greatly affected by environmental stresses and rely on sensing a rapidly changing environment, e.g., from a pelagic (planktonic larvae) to a benthic (settled larvae) one. Thus, studying proteomic changes in early developmental stages should provide insights into how the environment affects cellular pathways that establish the adult form.

A proteomic analysis of barnacle (*Balanus amphitrite*) larval development, attachment, and metamorphosis showed that the swimming larval form (cyprid) was characterized by a much higher number of proteins than the attached or metamorphosed form (Thiyagarajan & Qian 2008). In general, naupli and swimming cyprid larvae shared a closer PES than both the attached and the metamorphosed cyprid larvae. These observations supported the hypothesis that completion of larval attachment and metamorphosis does not require de novo protein synthesis in marine invertebrates (Carpizo-Ituarte & Hadfield 2003). However, the results also emphasize the role of PTM and protein degradation. A proteomic study on barnacle (*B. amphitrite*) and bryozoan (*Bugula neritina*) larval development showed that metamorphosis requires substantial changes in the phosphorylation status rather than the de novo synthesis of proteins (Thiyagarajan et al. 2009). Although rapid changes in protein abundance exist during early developmental stages, the importance of signaling events, e.g., calcium signaling cascades, and of the phosphorylation status of proteins in particular was also illustrated for the early stages of purple sea urchin (*Strongylocentrotus purpuratus*) development, using a proteomics approach (Roux et al. 2006, 2008; Su et al. 2005). For example, the number of phosphorylated proteins doubles in the 2 min following fertilization (Roux et al. 2006).

A comprehensive proteomic analysis of the changes in PES from unfertilized egg, to the 16-cell-stage embryo, to the tadepole larvae of *Ciona intestinalis* illustrated the importance of proteins functioning in energy metabolism, translation, protein degradation, cytoskeleton, and chaperoning, as well as small G-protein signaling during development (Nomura et al. 2009). An analysis of the *Ciona* sperm proteome complements this study (Hozumi et al. 2004).

A study investigating the proteomic changes within zebrafish (*Danio rerio*) and gilthead seabream (*Sparus aurata*) oocytes identified several proteins functioning as egg-yolk proteins (vitellogenin-derived lipovitellin and phosvitin), chaperones (TCP-1 chaperonin), and protease-inhibitors (serpin) as changing levels during in vivo oocyte maturation (Ziv et al. 2008). The correct occurrence of vitellogenin cleavage products during embryogenesis has been shown to serve as a potential indicator for toxic stress in zebrafish (Gundel et al. 2007).

The early events of development depend in large part on egg characters, e.g., transcripts and proteins, because of the delay in translation and transcription after fertilization. Thus, variation in the egg proteome may greatly affect life history traits, e.g., egg size and fitness. To investigate the proteomic variation in eggs and its underlying genetic heritability, PES of eggs from the blue mussel (*M. edulis*) were compared (Diz et al. 2009). Egg proteomes of different female mussels

REVIEWS IN ADVANCE

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showed significant variation and moderate to high heritability, suggesting that abundant genetic variation exists for natural selection to act on. A functional characterization of the egg proteome showed that proteins functioning in stress response and protein folding pathways as well as cellular motility and cytoskeleton were overrepresented in comparison to other mussel tissues (Diz et al. 2009).

Protein expression may also provide clues about reproductive barriers between species. A proteomic comparison of PES in foot tissue of the blue mussels *M. edulis* and *M. galloprovincialis* and their hybrids showed that the latter one has much more variable PES than the nonhybrid animals, possibly suggesting the existence of postzygotic isolation mechanisms (Diz & Skibinski 2007).

In summary, proteomic studies have confirmed that larval attachment and metamorphosis are largely independent of de novo protein synthesis and that there is a plethora of signaling events. The protein content of eggs suggests that the maternal investment equips the future organism with a number of stress proteins in expectation of a stressful environment.

9. CONCLUSION

Environmental proteomics has established itself as a powerful approach to investigate the cellular effects of environmental stresses. In future, MS-based proteomics will be at the center of analyzing PTM that accompany the signaling events that occur in response to a changing environment. Although studies of global patterns of PPI have not yet been conducted in environmental proteomics, more targeted approaches (e.g., immuno-precipitation) may become more common and establish PPI. Finally, the broad possibilities that proteomics offers will be more fully realized once we apply proteomics to biological questions in innovative ways and increase technical expertise by biologists.

SUMMARY POINTS

1. MS-based proteomics. Peptides can be ionized in a vacuum with MALDI and ESI. The mass of peptides and their fragments can be analyzed, providing a unique signal that can lead to the identification of a protein.
2. Environmental stress–Thermal stress. Thermal stress triggers changes in protein synthesis and degradation, requiring the activity of molecular chaperones and proteases. Oxidative stress levels accompany cold and heat stress in conjunction with changes in energy metabolism.
3. Environmental stress–Osmotic stress. Osmoregulatory organs of vertebrates have higher levels of proteins functioning in energy, urea, and amino acid metabolism. Differences in the proteomic responses between marine teleosts that are osmoregulators and elasmobranchs that are (delayed) osmoconformers suggest that changing salinity conditions induce proteomic changes consistent with a greater degree of cell remodeling in the former in comparison to the latter group.
4. Environmental stress–Anaerobic stress. Proteomic studies on anaerobic stress are providing evidence for a global reduction of enzymes involved in energy metabolism in organisms that are hypoxia-tolerant. These changes occur in a number of tissues simultaneously.

REVIEWS IN ADVANCE
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5. Ecotoxicoproteomics. Proteins commonly induced with pollutant-stress responses include oxidative stress proteins, cytoskeletal proteins, chaperones, proteases, and proteins involved in the detoxification of xenobiotics as well as breakdown of fatty acids. A commonly observed phenomenon is that the production of ROS leads to protein denaturation and wide-ranging modifications of cytoskeletal elements. Post-translational modifications associated with oxidative stress (carbonylation and glutathionylation) and protein denaturation (ubiquitination) increase in response to pollutant-stress.
6. Symbiotic relationships. Proteomic analyses of coral symbioses indicate that enzymes involved in the transport of inorganic carbon are needed for the maintenance of the host–symbiont relationship. The membrane envelope that surrounds the symbiont is characterized by proteins involved in ATP synthesis, membrane transport of proteins and small molecules, cellular stress, anti-apoptosis, signaling, and cytoskeletal modifications.
7. Ecological development. Proteomic studies have confirmed that larval attachment and metamorphic development involve a plethora of signaling events but are mostly independent of de novo protein synthesis. The protein content of eggs suggests that the maternal investment equips the future organism with a number of stress proteins in anticipation of a stressful environment.

FUTURE ISSUES

1. Post-translational modifications. Stress-induced signaling cascades involving such PTM as phosphorylation, glycosylation, acetylation, etc., translate signals from a rapidly changing environment into cellular action. MS-based proteomics is one of the most effective tools to detect those PTM.
2. Protein-protein interactions. With the help of antibodies, it is possible to co-immunoprecipitate interacting proteins. MS-based proteomics can identify these interacting proteins and thus characterize PPI.
3. Enrichment procedures. Targeted (versus global) approaches will enable proteomics to focus on low-abundance proteins due to enrichment procedures, e.g., ubiquitin-tagged protein affinity columns. These approaches will provide increasing depth in our analysis and thereby discover new stress-induced cellular pathways.
4. Cytoskeleton. The cytoskeleton is emerging as being actively responsive to stresses of widely different sorts. Investigating the interplay between cytoskeleton-attached enzymes and cytoskeletal dynamics will provide insights into how the cytoskeleton is impacted by stress and confers stress resistance.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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