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A molecular biomarker system for assessing the health of gastropods (*Ilyanassa obsoleta*) exposed to natural and anthropogenic stressors ☆

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Abstract

We developed a Molecular Biomarker System (MBS) to assess the physiological status of mud snails (*Ilyanassa obsoleta*) challenged by exposure to high temperature, cadmium, atrazine, endosulfan and the water-accommodating fraction of bunker fuel #2. The MBS is used to assay specific cellular parameters of the gastropod cell that are indicative of a non-stressed or stressed condition. The MBS distinguished among responses to each stressor and to non-stressed control conditions. For example, the biomarkers metallothionein and cytochrome *P*450 2E1 homologue distinguished between metal and non-metal stresses. MBS data from this study corroborate toxicological studies of organismal responses to endosulfan, atrazine, fuel and cadmium stresses. The MBS technology aids in the accurate diagnosis of the snail's health condition because the physiological significance of the changes of each biomarker is well known. This technology is particularly relevant for environmental monitoring because gastropods are used as key indicator species in many estuarine, marine, freshwater and terrestrial ecosystems. Finally, the Molecular

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Biomarker System technology is relatively inexpensive, easy to implement, precise and can be quickly adapted to an automated, high-throughput system for large sample analysis. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Environmental and anthropogenic pressures often decrease the health and stability of ecosystems, although the precise effects of these stressors on the many individual components of an ecosystem remain largely unknown. Most attempts to monitor environmental status rely on either determining the abiotic components of an ecosystem (i.e. contaminant analysis) or on assessing the ecological responses (i.e. species richness, population density) (Bro-Rasmussen and Lokke, 1984; Schaeffer et al., 1988; O'Connor, 1996). Although these types of monitoring methods are well developed, they do not readily reveal physiological/mechanistic responses to environmental stressors (e.g. Otte et al., 1993; Wilson et al., 1996). A gap exists in our knowledge of how stressors affect ecosystems. For example, although traditional water and sediment analyses can document the quality and quantity of a contaminant in the environment, these analyses cannot readily describe (and therefore cannot predict) biotic responses to that contaminant. Indeed, the presence of a particular contaminant does not necessarily decrease ecosystem health. Since ecosystems are complex, hierarchical systems, a number of different compensatory mechanisms operating at these levels (e.g. cell, tissue, individual levels) may ameliorate stress before it reduces the fitness of an individual organism or alters its functional role in the community (Allen and Starr, 1982). Traditional methods that measure species diversity or population density monitor the net indirect effects of a stressor on an ecosystem. Under most circumstances, a stressor(s) *indirectly* affects higher levels of the ecosystem hierarchy but *directly* affects molecular and cellular level processes. Therefore, a technology that can assess the effects of different stressors on the cellular and molecular processes that govern organismal health and fitness in complex ecosystems will provide much more relevant information than these other indirect measurements alone (Schaeffer et al., 1988; van Gestel and van Brummelen, 1996).

Using molecular biomarkers to assess ecosystem or organismal health is a popular concept. Already, there is abundant evidence of the effectiveness in specific case studies of single species (e.g. Coleman et al., 1995; De Pomerai, 1996; Heckathorn et al., 1996; Feder and Hoffman, 1999). However, little published work documents systematic use of multiple biomarkers to assess the health condition of a non-human, non-agricultural organism or a complex ecosystem (Adams and Ryon, 1994). For example, abundant literature supports the validity of biomarkers as indicators of organismal responses to a contaminant or stressor exposure (for review, see de Zewart et al., 1999; Shugart et al., 1993; Adams and Ryon, 1994). Many studies also examine a single or small set of physiological parameters in order to assess an overall physiological response to acute and chronic exposures to toxic contaminants (Adams et al., 1992). Surprisingly, few studies attempt to integrate overall physiological status with multiple, specific molecular biomarkers (Adams et al., 1992; Schaeffer et al., 1988; Stegmann et al., 1992).

Therefore, developing a comprehensive biomarker system to assess species or ecosystem health will provide the information required to better understand, manage and monitor compromised habitats.

An integrated assessment system that incorporates the simultaneous use of multiple molecular biomarkers that are designed to quantify known physiological responses to stressors would reveal: (1) whether an organism is physiologically stressed, (2) whether an organism has physiologically acclimated or evolutionarily adapted to a chronic stress, and (3) the physiological impact of the stress. In essence, an integrated assessment system using molecular biomarkers will allow for the diagnosis of an organism's cellular physiological condition when challenged with a real or suspected stress. Molecular biomarkers specific to particular stressors could identify the environmental insults that are physiologically affecting an organism. Thus, an integrated system of relevant molecular biomarkers would be an extremely powerful tool to quantify the health status of an individual in response to a natural or anthropogenic stressor.

We developed a Molecular Biomarker System (MBS) that assays specific parameters of the snail cell that are indicative of a non-stressed or stressed physiological condition. These cellular parameters are: lipid peroxide levels (LPO), total glutathione (GSH), heat-shock protein 60 (Hsp60), heat-shock protein 70 (Hsp70), αB-crystallin homologue, small heat-shock protein 22 (Hsp22), small heat-shock protein 26 (Hsp26), manganese superoxide dismutase (Mn SOD), cytochrome *P*450 2E1 homologue, metallothionein class I and ubiquitin. These parameters were chosen because they represent specific cellular physiological functions ranging from biological membrane integrity to protein renaturation. Measuring these parameters indicates: (1) whether the structural integrity of the cell is challenged, (2) whether there is a response to oxidative stress, and (3) evidence of the etiological agent of the stress (i.e. heat stress, heavy metal, polyaromatic hydrocarbons).

We chose the eastern mud snail, *Ilyanassa obsoleta* (Say), as a model gastropod species because it is a common and conspicuous element of the intertidal fauna from Canada to northern Florida. Mud snails are nonspecific grazers on organic deposits, benthic algae, epiphytes and even scavenge carrion (Feller, 1984; Curtis, 1985; Cranford, 1988; Kinlan et al., 1997). They reach highest densities on open mud flats with low flow, burrowing to escape dislodgement by the tide (Levinton et al., 1995). *Ilyanassa* are perennial, with individual life spans estimated to reach 30–40 years (Curtis, 1995). Egg capsules, laid on shells and other hard substrates, hatch to planktonic larvae that metamorphose and settle after several weeks (Richmond and Woodin, 1996; Froggett and Leise, 1999). The phenomenon of "imposex," the imposition of male characteristics on female gastropods, has made *Ilyanassa* a useful indicator of organotin pollution (Curtis, 1994; Oberdoerster et al., 1998).

In this paper, we report cellular parameter levels in *I. obsoleta* exposed to five different stressors: (1) heat, (2) cadmium, (3) atrazine (an herbicide), (4) endosulfan (a pesticide), and (5) the water-accommodating fraction of bunker fuel #2. We also provide evidence that the MBS can differentiate between *I. obsoleta* that experienced different stressors. Finally, we discuss the data obtained using the MBS and its ramifications for understanding the cellular physiological status of *I. obsoleta* under environmentally relevant conditions.

2. Materials and methods

All chemicals for buffered solutions were obtained from Sigma (St. Louis, MO, USA). Cadmium chloride was obtained from Sigma. Endosulfan and atrazine were obtained from Supelco Chemicals (Bellefonte, PA, USA). Diesel and bunker fuel #2 were generous gifts from Exxon (Charleston, SC, USA). PVDF and nitrocellulose membranes were obtained from Millipore (Bedford, MA, USA). Dot blot and gel electrophoresis equipments were obtained from Bio-Rad (San Diego, CA, USA). GSH-420 glutathione assay kits (Cat. #21023) and LPO-560 assay kits (Cat. #21025) were obtained from Oxis International (Portland, OR, USA). Antibodies against Hsp70 (Cat. #SPA822), Hsp60 (Cat. #SPA805), αB-crystallin (Cat. #SPA224) and ubiquitin (Cat. #SPA200) were obtained from Stressgen Biotechnologies (Victoria, British Columbia, Canada). Metallothionein antibody against MT-Class I (Cat. #18-0133) was obtained from Zymed (South San Francisco, CA, USA). Hsp22 antibody was a generous gift from Dr. Thomas MacRae and Hsp26 antibody was a generous gift from Dr. John Towers. Anti-rabbit- and anti-mouse-conjugated alkaline phosphatase antibodies were obtained from Promega (Madison, WI, USA). Protein standards of Hsp70, Hsp60 and αB-crystallin were obtained from Stressgen Biotechnologies. Protein standards of ubiquitin, Cu/Zn SOD, Mn SOD and metallothionein were obtained from Sigma. Antibody to cytochrome *P*450 2E1 was a generous gift from Oxis International.

2.1. Collection and husbandry of I. obsoleta

I. obsoleta were collected on February 1999 in the Wadmalaw River near Charleston, SC, USA. This site is considered an un-impacted reference site and is a long-term ecologically monitored site by both the South Carolina Department of Natural Resources and US National Oceanic and Atmospheric Administration's Ecotoxicology Program. Water and sediment sample analyses of this site are conducted at least on a yearly basis for a number of pesticides, herbicides, metal, polyaromatic hydrocarbons and eutrophic parameters (US NOAA NOS ORCA 128; SCDNR and US NOAA NMFS Tidal Creek Project).

Preliminary experiments were carried out to determine how to minimize stress responses during laboratory experiments (data not shown). Snails were raised at 22–24°C with a 14-h photoperiod in 80-l tanks filled with filtered, brackish water (20-ppt salinity), aerated with air stones and tank sides were covered with black, light-impermeable cloth. Snails were fed commercially available algal pellets that consisted of a mixture of cyanobacteria and green algae. Snails were acclimated to the laboratory at least 1 month prior to stress challenges. There were no significant differences in MBS parameters between snails immediately caught and frozen at the collection site and snails that had acclimated for 3 weeks under laboratory conditions (unpublished results).

2.2. Stress exposures

Transferring *I. obsoleta* from culture tanks to dosing chambers induced a significant response in many MBS parameters, which is not unusual for many experimental organisms, such as grass shrimp (Oberdörster et al., 1999). Preliminary experiments

demonstrated that the best method for reducing background stress responses was to acclimate the snails in their dosing chambers 6 days before a stress challenge. This was accomplished for each stress challenge by placing one snail (110–140 mg wet weight) in 1-l glass dosing chambers filled with 400 ml of filtered, brackish water (20-ppt salinity). Snails were held in these dosing chambers for 6 days before the beginning of the stress challenges. We exchanged 300 ml of the brackish water in the dosing chamber with fresh brackish water on the 2nd, 4th and 6th day of dosing chamber acclimation. Snails were fed algal pellets on the 1st day of dosing chamber acclimation. Chambers were substrate-free and aerated with air stones while oxygen content and temperature were continuously monitored during the 6-day chamber acclimation. Ammonia content was measured on days 1 and 6. During acclimation, snails were subjected to a 25-μmol m^{-2} s^{-1} photosynthetic-active-radiation 14-h photoperiod at 22°C. Dosing chambers were labeled for the type and dosage concentration of the stress, with five replicates per treatment. Dosing chambers were then arranged in a randomized block design for stress challenging (Sokal and Rohlf, 1995). At the end of each 8-h challenge, snails were immediately collected, frozen in liquid nitrogen and kept at −80°C until sample preparation.

Heat stress—Snails were exposed to water temperatures of either 22°C or 38°C. Temperature was ramped from 22°C to 38°C over a 70-min period.

Cadmium exposure—Snails were exposed to either 0, 5 or 50 μM cadmium chloride. The solvent carrier was brackish water.

Atrazine exposure—Snails were exposed to either 0, 1 and 500 μg/l or 1 mg/l acetone stock of technical grade atrazine (99%). Control animals were exposed to 50 μl of acetone/400 ml brackish water, which was equal to the highest volume of solvent carrier delivered to atrazine-challenged samples.

Endosulfan exposure—Snails were exposed to either 0, 50 and 500 ng/l or 1 μg/l acetone stock of technical grade endosulfan (99%). Control animals were exposed to 50 μl of acetone/400 ml brackish water, which was equal to the highest volume of solvent carrier delivered to atrazine-challenged samples.

Bunker fuel #2 exposure—Snails were exposed to either 0, 0.5, 1 or 3 g/l of the water-accommodating fraction of bunker fuel that was prepared according to Blenkisopp et al. (1996). One liter of brackish water was added to Teflon-coated 1.1-l Nalgene bottles (Nalge, Rochester, NY, USA). The appropriate amount of bunker fuel was added to each bottle and mixed using a Teflon-coated magnetic stirrer for 48 h. Brackish water was used as a control.

2.3. Sample preparation and assay

Snails were cracked, the shell discarded, and soft tissues examined for parasitism (Curtis, 1997) and imposex (Curtis, 1994). No evidence of either was found. The whole soft body was frozen in liquid nitrogen, ground frozen in a mortar and pestle, and then suspended in a solution consisting of 10 mM phosphate buffer (pH 7.8), 5 mM butylhydrotoluene and 0.5% SDS. Samples were vortexed for 30 s, then centrifuged at $10{,}000 \times g$ for 5 min, and the supernatant was divided and placed into two separate tubes: one tube was labeled for spectrophotometric analysis and the other for ELISA

analysis. Protein concentrations of samples were assayed by the method of Ghosh et al. (1988).

Samples were analyzed spectrophotometrically for LPO and total GSH content following the manufacture's instruction. Samples for each assay were analyzed in triplicate. After LPO, GSH and protein concentration assays, 100 μl of a solution containing 20% SDS, 50 mM Tris–HCl (pH 7.8), 100 mM dithiothreitol, 80 mM EDTA, 3% polyvinyl pyrrolidone (w/v), 20 mM phenylmethylsulfonyl fluoride, 20 mM benzamide, 50 μM α-amino-caproic acid and 1 μg pepstatin A was added to 900 μl of sample labeled for ELISA analysis. Samples were then boiled for 3 min, allowed to sit at 25°C for 5 min, and then centrifuged at $10{,}000 \times g$ for 5 min. Supernatant was transferred to a new tube and the pellet was discarded. The sample was then subjected to a protein concentration assay.

2.4. ELISA, gel electrophoresis, immunoblotting and densitometric analysis

Samples were then assayed for Hsp70, Hsp60, αB-crystallin, Hsp22, Hsp26, ubiquitin, cytochrome *P*450 2E1, Mn SOD and metallothionein using immunochemical analysis. All samples were analyzed in triplicate. For ELISA, nitrocellulose membrane was used in the dot blot apparatus. Western blotting on PVDF membrane was done after samples were subjected to SDS-PAGE. Both types of blots were blocked for 1 h in either 5% non-fat-dried milk in 1 × TBS (50 mM Tris/HCl (pH 9.8), 10 mM NaCl) or for ubiquitin blots in 0.1% Tween-20 TBS solution. Blocking solution was decanted and blots were incubated in the appropriate primary antibody solution for 12 h at 4°C. Primary antibody solution was decanted, blots subjected to four 10-min washes in 1 × TBS, and then incubated in the appropriate secondary antibody solution for 1 h. Secondary antibody solution was decanted, blots were again subjected to four 10-min washes in 1 × TBS, then developed in a nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution.

Once developed, blots were scanned into a computer and analyzed using NIH image software (http://rsb.info.nih.gov/nih-image). A serial dilution of purified protein for each cellular parameter was included in each assay to allow sample quantification and assay quality control. For example, titered concentrations of purified metallothionein from rabbit were used as both a qualitative and quantitative standard for the samples assayed with metallothionein. Concentration standards for each assay were determined and a quadratic or polynomial equation was used to determine the concentration of each sample.

Because of the uniqueness of each cellular parameter, specific modifications were made for each ELISA assay. For example, ELISA analyses of metallothionein proteins followed a slightly modified protocol described by Mizzen et al. (1996). Small heat-shock proteins or αB-crystallin analysis employed higher concentrations of SDS and DTT in the buffer and were boiled for 3 min to prevent oligomerization of the sHsps under mild or non-denaturing conditions (Downs et al., 1998, 1999b).

2.5. Antibody validation

To determine that the antibodies bound to their specific targets, preliminary experiments were conducted to ensure the validity of the antibody assays. Snails were exposed

either to high temperature, cadmium, ethanol or acetone, homogenized in an SDS/50 mM dithiothreitol buffered solution, subjected to SDS-PAGE, immunoblotted to PVDF membrane, and assayed with at least three different antibodies per cellular parameter. Antibodies finally chosen as the enzyme immunoassay (EIA) had the best signal-to-noise ratio for each cellular parameter (data not shown). Purified protein of each cellular parameter was run beside control and treated samples.

2.6. Cellular parameters

2.6.1. Lipid peroxide

Lipids, especially those derived from polyunsaturated fatty acids, may react with active oxygen species to form peroxyl adducts, which in turn may react with other lipids in an auto-oxidation chain reaction (Girotti, 1998). Formation of lipid peroxides indicates that the integrity of biological membranes is being assaulted or has been compromised (Duthie, 1993). Thus, LPO levels reflect the structural integrity of one component of the cell superstructure. In addition, production of LPOs indicates that active oxygen species are overwhelming a number of different anti-oxidant defenses, thus also indicating oxidative stress (Rikans and Hornbrook, 1997; Girotti, 1998).

2.6.2. Glutathione

Glutathione is a tripeptide with a single cysteine residue that plays a significant role in xenobiotic detoxification, as an anti-oxidant, and (perhaps to a lesser degree) serves as a pathway for tolerance to metal toxicity (Klaassen et al., 1999; Sies, 1999). During a xenobiotic challenge, glutathione may be conjugated to a xenobiotic by glutathione-*S*-transferase, which represents a major detoxification pathway (Sies, 1999). During oxidative stress, reduced glutathione (GSH) acts as an anti-oxidant in a number of different pathways: (1) it reacts with hydrogen peroxide via glutathione peroxidase to form water and oxidized glutathione (GSSG); (2) in conjunction with ascorbate, it is an essential component of the Asada–Halliwell pathway—a major anti-oxidant cyclic pathway; and (3) it can act independently as a hydroxyl and superoxide quencher (Asada and Chen, 1988; Halliwell, 1999). Glutathione can also bind to cadmium and be excreted from the cell (Klaassen et al., 1999). Within hours, intracellular GSH levels are known to significantly decrease in response to initial exposures to an oxidative or xenotoxic stress (Sagara et al., 1998). Then, as a compensatory action, GSH levels can increase several fold compared to levels prior to the oxidative or xenobiotic stress event (Sies, 1999).

2.6.3. Hsp70 and Hsp60

Hsp70 and Hsp60 are molecular chaperonins that regulate protein structure and function under normal physiological conditions as well as during and following stress. They renature denatured proteins into active states in an ATP-dependent manner (for review, see Hartl, 1996). Both Hsp70 and Hsp60 are ubiquitous chaperones found in all phyla and are essential components for cellular function during both normal and stressed conditions (Hartl, 1996). Furthermore, mitochondrial and chloroplastic Hsp70 and

Hsp60 homologues work in concert—an essential multi-step pathway for correct conformation of protein structure (Hartl, 1996). Hsp70 and Hsp60 levels increase during stress, specifically in response to increased protein synthesis and denaturation (Hartl, 1996). These two chaperonins are indicators that the "house-keeping" proteins in the cell are experiencing denaturing conditions. Thus, these chaperones reflect the structural integrity of the protein component of the cell superstructure.

2.6.4. The small heat-shock proteins

αB-crystallin, Hsp22 and Hsp26 share domains of common homology to one another, but have different cellular functions (de Jong et al., 1993). Small Hsps from all phyla share a common motif near the carboxyl-terminal end of the protein, known as the "heat-shock domain" or α-crystallin domain (de Jong et al., 1993). Other areas of these proteins are not homologous and are specific to the sub-family of sHsps. In most cases, the small heat-shock proteins are not present during optimal growing conditions and are only elicited by stress (de Jong et al., 1993).

αB-crystallin is a small heat-shock protein found only in the cytosol of animals, where it protects cytoskeletal elements during stress (Derham and Harding, 1999). Evidence indicates that Hsp22 localizes to neural-type cells and follicular cells in *Drosophila melanogaster* (Downs et al., unpublished data). Furthermore, this protein localizes to the mitochondria in arthropods and has been suggested to have a functional role similar to that of plant and mammalian mitochondrial sHsps (Tanguay, personal communication; Downs et al., unpublished data; Downs and Heckathorn, 1998; Downs et al., 1999a). Thus, the presence and concentration of different small heat-shock proteins reflect the physiological status of several metabolic and structural pathways in the cell.

2.6.5. Ubiquitin

Ubiquitin is a 76-residue protein found in most phyla and marks proteins for rapid degradation (Hershko and Ciechanover, 1998). Ubiquinated proteins are then degraded by proteolytic enzymes known as proteosomes (Hershko and Ciechanover, 1998). During stress, some proteins are targeted for degradation usually because they have become irreversibly denatured (Iwai, 1999). Increase in ubiquitin levels indicates increased protein degradation and hence, increased protein turnover (Goff et al., 1988). To compensate for decreased functional protein levels caused by stress, cells will increase production of these same proteins (Jennissen, 1995; Iwai, 1999). Thus, measuring ubiquitin level indexes the structural integrity of the protein component of the superstructure of the cell (Mimnaugh et al., 1999). Increased ubiquitin levels indicate: (1) a protein-denaturing stress is occurring, (2) more energy is required to compensate for protein turnover, and (3) individual fitness is reduced (Hawkins, 1991).

2.6.6. Mn superoxide dismutases

Superoxide dismutases catalyze the reaction of superoxide ions and two protons to form hydrogen peroxide and O_2 (Fridovich, 1995). Copper/zinc SOD is found only in the cytosol of animal cells, while homologues are found in both the cytosol and chloroplast in plants and algae (Fridovich, 1995). Manganese SOD is localized only to

the mitochondria in eukaryotic cells (Fridovich, 1995). Superoxide dismutases accumulate in response to oxidative stress and are one of the main anti-oxidant defense pathways. Increased levels of Mn SOD have been linked to increased longevity, increased tolerance to ischemic/reperfusion events, and increased tolerance to factors that induce oxidative stress (Fridovich, 1995). Increased SOD levels indicates that cells are responding to an oxidative stress, and more specifically, Mn SOD can serve as an index for mitochondrial response to oxidative stress (Fridovich, 1995).

2.6.7. Metallothionein

Metallothioneins are cysteine-rich, low-molecular-weight proteins that will bind a variety of metals depending on the class of metallothionein (for review, see Klaassen et al., 1999). In vitro evidence demonstrates that metallothionein may act as an anti-oxidant, a factor to ensure a reservoir of zinc, and can protect against metal toxicity (ibid.). In vivo evidence using transgenic animals shows only strong support for its function as a protectant against metal toxicity. Metallothioneins can be grouped into three classes (MTI, MTII and MTIII) (Klaassen et al., 1999). Preliminary experiments demonstrated that we were able to detect MTI class in snails (Downs et al., unpublished results). Metallothioneins are strongly induced by metal toxicity and are indicators of exposure of toxic levels of some metals.

2.6.8. Cytochrome P450 2E1

Antibody against mammalian cytochrome *P*450 2E1 cross-reacted with a single band of about 40–45 kDa inducible in snails by ethanol exposure (Downs et al., unpublished results). Cytochrome *P*450 2E1 is known to specifically oxidize ethanol to acetaldehyde via a monooxygenase mechanism, as well as other xenobiotics such as imidazole-based derivatives (Lieber, 1997). Cytochrome *P*450 2E1 has both physiologically relevant oxidative and reductive reactions and is known to associate and catalyze as many as 60 xenobiotic-based substrates (Koop, 1992; Lieber, 1997). For example, it causes the demethylation of *N*,*N*-dimethylnitrosamine and the hydroxylation of *p*-nitrophenol and chlorzoxazone (Koop, 1992). Reduction reactions include reduction of a number of different lipid types. One of the primary reasons for using the 2E1 class of cytochrome *P*450s is that it is not induced by heat stress, but can respond to hypoxia/reperfusion events in mammals (Bayanov and Brunt, 1999). In this study, cytochrome *P*450 2E1 is used as an indicator of a xenobiotic response of snails.

2.7. Statistical analysis

In an earlier paper (Downs et al., 2001), we used multivariate analysis of variance (MANOVA) to test the null hypotheses that treatments had no significant effect on mean biomarker levels. When significant differences were found, we used separate univariate analyses (one-way analyses of variance: ANOVA) to interpret MANOVA results. This procedure is commonly used to limit the probability of committing a Type I error (rejecting a true null hypothesis), even when response variables are correlated (e.g. Fauth and Resetarits, 1991). It also provides a more powerful test when differences in many mean biomarker levels are not large enough to be detected with individual

ANOVAs. However, if response variables are uncorrelated, as one expects of biomarkers on independent biochemical pathways, a series of ANOVAs may be more powerful than a single MANOVA (Zar, 1999). We expected experimental treatments to have substantial effects on mean biomarker levels, and therefore analyzed the data using a series of one-way ANOVAs. To hold the experiment-wise Type I error rate at the desired level ($\alpha = 0.05$), we used the Dunn–Śidák method (Sokal and Rohlf, 1995) and set a critical probability of $P' = 0.00465$ for each of the 11 analyses. When significant differences were found among treatment means, we used the Tukey–Kramer Honestly Significant Difference (HSD) method as an exact alpha-level test for all differences between means (Sokal and Rohlf, 1995). These conservative procedures limited the probability of rejecting a true null hypothesis.

When data did not meet the homogeneity of variances requirement for one-way ANOVA, we substituted a Welch ANOVA. For the two-sample case, this is equivalent to a *t*-test with unequal variances (SAS Institute, 1995), and Welch's ANOVA is recommended when variances are heteroscedastic or normally distributed (Sokal and Rohlf, 1995). In one case (Hsp26 in the endosulfan experiment), undetectable levels of biomarker in the controls gave that treatment a mean and variance of zero, preventing use of parametric statistical tests. We therefore used the nonparametric Kruskal–Wallis test, which is less powerful than its parametric counterparts (Sokal and Rohlf, 1995).

We used canonical correlation analysis (CCA) strictly as a heuristic tool to illustrate how biomarkers might discriminate among environmental stressors in a complex environment. CCA is an eigen analysis method that reveals the basic relationships between two matrices (Gauch, 1985), in our case those of stressor treatments and MBS data. This technique required combining data from all five experiments into one matrix, which we did by expressing biomarker responses in a given treatment as a proportion of their mean level in the control. Two assumptions of CCA, that stressor gradients were independent and linear, were constraints of the experimental design. The other main assumption, that biomarker responses were linear, was met with few exceptions (αB-crystallin, Mn SOD and metallothionein responses to atrazine; Mn SOD responses to bunker fuel; ubiquitin, metallothionein and Hsp responses to endosulfan). All statistical analyses were performed using JMP v. 3.2.2 (SAS Institute, Cary, NC, USA).

3. Results

The Hsp22 antibody cross-reacted with a ca. 20–22-kDa protein that was heat-inducible (Fig. 1). The Hsp26 antibody cross-reacted with a 25–28-kDa protein that was also heat-inducible (Fig. 2). Cytochrome *P*450 2E1 cross-reacted with an ethanol- and acetone-inducible protein of 50 kDa (Fig. 3). This protein was detectable in controls and other treatments, but cadmium and heat stress did not elicit significant accumulation of this protein (data not shown). Hsp60 antibodies cross-reacted with one protein band of about 66 kDa that was inducible in all treatments (data not shown). The Hsp70 antibody chosen for this study cross-reacted with two to three proteins between 70 and 73 kDa. The 73-kDa isoform was not present in control samples, but present in response to a

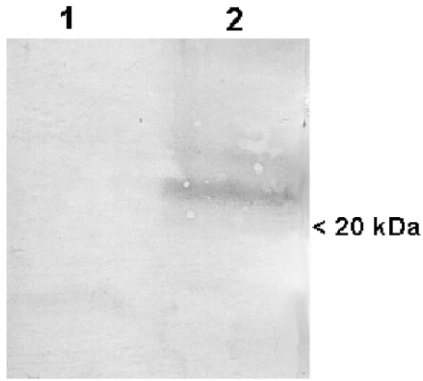

Fig. 1. **Hsp22**. Mud snails were exposed to control conditions (lane 1) or heat stress (38°C) for 8 h (lane 2). Shrimp were homogenized and subjected to SDS-PAGE (40 μg total soluble protein per lane), Western blotting and assayed with antibody to Hsp22.

variety of stressors. The other Hsp70 homologues were present in control individuals and were also inducible by all treatments except ethanol and acetone (data not shown). αB-crystallin antibody cross-reacted with a single, ca. 19–21-kDa protein that was inducible by all treatments but undetectable in the control samples. This antibody does not cross-react with the sHsp22 or sHsp26 (p26) protein homologues of *Artemia* or

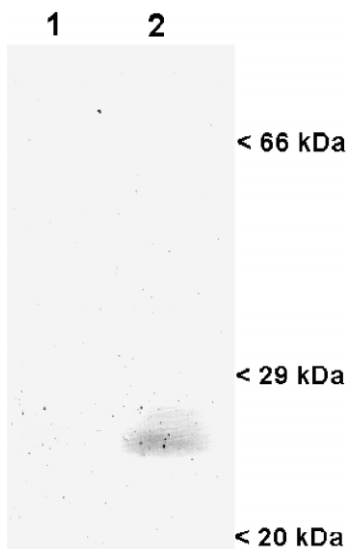

Fig. 2. **Hsp26**. Mud snails were exposed to control conditions (lane 1) or heat stress (38°C) for 8 h (lane 2). Shrimp were homogenized and subjected to SDS-PAGE (40 μg total soluble protein per lane), Western blotting and assayed with antibody to Hsp26.

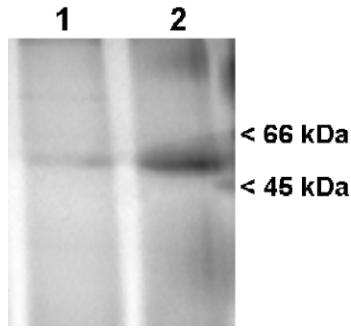

Fig. 3. **Cytochrome *P*450 2E1**. Mud snails were exposed to control conditions (lane 1) or 7% ethanol for 8 h (lane 2). Shrimp were homogenized and subjected to SDS-PAGE (40 μg total soluble protein per lane), Western blotting and assayed with antibody to cytochrome *P*450 2E1.

Drosophila sHsp26 (Downs et al., unpublished data; data not shown). Mn SOD antibody cross-reacted with a single protein of about 26 kDa that was inducible by both heat stress and cadmium, but not by exposure to acetone (data not shown). Metallothionein antibody cross-reacted with several proteins between 6 and 15 kDa that were not detectable in controls, but accumulated in response to 50-μM cadmium chloride exposure (Fig. 4).

Treatments had statistically significant effects on the biomarker responses in all five experiments. Treatment means for each biomarker, their standard errors, and test statistics are reported in Tables 1–5.

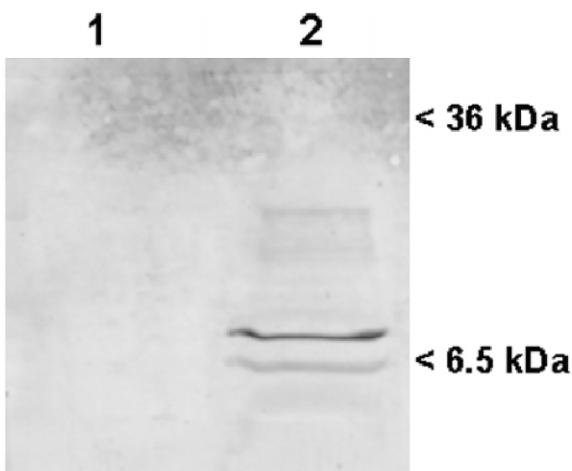

Fig. 4. **Metallothionein**. Mud snails were exposed to control conditions (lane 1) or 50 μM cadmium chloride for 8 h (lane 2). Shrimp were homogenized and subjected to SDS-PAGE (40 μg total soluble protein per lane), Western blotting and assayed with antibody to metallothionein.

3.1. Responses to heat stress

Seven biomarkers responded significantly to heat stress (Table 1). Each was lower in the control (22°C) and higher in the heat-stressed samples (38°C), except for GSH. There were no statistically significant differences between control and heat-stressed samples in levels of Hsp22 and Hsp26. However, this result should be interpreted with caution because unusual variances and the conservative adjusted P' (0.00465) limited statistical power in these comparisons. Levels of metallothionein and cytochrome *P*450 did not vary significantly among treatments (Table 1).

3.2. Responses to cadmium stress

Nine biomarkers responded significantly to cadmium chloride treatments (Table 2). All biomarkers except GSH were lowest in the control and responded in a dose-dependent manner. In contrast, mean GSH levels were highest in the control, lower in the low $CdCl_2$ treatment and lowest in the high $CdCl_2$ treatment (Table 2). Metallothionein was the most sensitive biomarker, with more than a sixfold increase in mean levels at high $CdCl_2$ concentrations compared to the control. Mean levels of cytochrome *P*450, and LPO did not differ significantly among treatments (Table 2).

3.3. Responses to fuel oil stress

Four biomarkers responded significantly to bunker fuel treatments (Table 3). Levels of LPO, Mn SOD, Hsp22 and ubiquitin were significantly higher in fuel-exposed

Table 1
Summary of biomarker responses to heat stress in mud snails (*I. obsoleta*)

Biomarker	Treatments		$F_{1,9}$
	Control	Heat-stressed	
GSH	57 ± 2.0	34 ± 4.3	22.4 *
LPO	0.96 ± 0.051	1.32 ± 0.058	21.6 *
Ubiquitin	39 ± 2.5	105 ± 4.7	153.5 * * *
Hsp22	0 ± 0.2	24 ± 4.7	25.0, ns
Hsp26	0 ± 0	41 ± 7.6	29.4, ns
Hsp60	0.184 ± 0.0117	0.53 ± 0.0226	185.3 * * *
Hsp70	9.2 ± 1.02	36.4 ± 1.96	151.0 * * *
αB-crystallin	12 ± 3.5	35 ± 4.6	16.3 *
Mn SOD	14.2 ± 1.53	28.6 ± 2.98	18.5 *
Metallothionein	19.2 ± 1.40	18.2 ± 2.13	0.2, ns
Cytochrome *P*450	9.6 ± 1.78	13.6 ± 2.54	1.7, ns

Treatments were 22°C (control) and 38°C. Entries in the table give treatment means ± 1 S.E., *F*-statistics from a Welch (Hsp22 and Hsp26) or one-way ANOVA (all other biomarkers) and significance levels; ns = not statistically significant (α adjusted for multiple tests; see text). Units: mM GSH/μg total protein; mM LPO/mg total protein; ng ubiquitin/μg total protein; pg Hsp or αB-crystallin/μg total protein; relative protein concentrations for SOD, and cytochrome *P*450; ng metallothionein/mg total protein.

* $P < 0.00465$.

* * * $P < 0.0001$.

Table 2
Summary of biomarker responses to cadmium chloride stress in mud snails (*I. obsoleta*)

Biomarker	Treatments			$F_{2,12}$
	Control	Low	High	
GSH	$75^{a} \pm 2.9$	$64^{b} \pm 2.4$	$60^{b} \pm 2.8$	8.7^{*}
LPO	0.92 ± 0.058	1.1 ± 0.089	1.16 ± 0.093	2.3, ns
Ubiquitin	$30^{a} \pm 1.9$	$40^{ab} \pm 2.8$	$50^{b} \pm 5.0$	8.1^{**}
Hsp22	$2^{a} \pm 1.3$	$31^{b} \pm 5.9$	$64^{c} \pm 11.0$	18.1^{**}
Hsp26	$14^{a} \pm 3.5$	$32^{b} \pm 4.1$	$58^{c} \pm 5.3$	26.2^{**}
Hsp60	$0.145^{a} \pm 0.0054$	$0.419^{b} \pm 0.0271$	$0.509^{b} \pm 0.0558$	27.8^{***}
Hsp70	$14.0^{a} \pm 2.63$	$66.4^{b} \pm 12.32$	$72.8^{b} \pm 7.24$	14.8^{**}
αB-crystallin	$18^{a} \pm 2.7$	$54^{b} \pm 9.6$	$76^{b} \pm 6.4$	18.3^{**}
Mn SOD	$28.0^{a} \pm 1.00$	$58.2^{b} \pm 5.30$	$61.8^{b} \pm 4.14$	22.3^{***}
Metallothionein	$14.0^{a} \pm 2.00$	$52.0^{b} \pm 4.48$	$70.2^{c} \pm 5.56$	44.8^{***}
Cytochrome *P*450	28.0 ± 2.55	28.6 ± 3.11	27.4 ± 3.14	0.04, ns

Treatments were 0, 5 or 50 μM cadmium chloride. Entries in the table give treatment means ± 1 S.E., *F*-statistics from a Welch (Hsp70 and Mn SOD) or one-way ANOVA (all other biomarkers) and significance levels; ns = not statistically significant (α adjusted for multiple tests; see text). Treatment means with different superscript letters differed significantly. Units as in Table 1.

$^{*}P < 0.00465$.

$^{**}P < 0.001$.

$^{***}P < 0.0001$.

samples than in controls, while GSH had the opposite pattern. Seven biomarkers did not respond significantly to bunker fuel treatments: Hsp26, Hsp60, Hsp70, αB-crystallin, metallothionein and cytochrome *P*450 (Table 3).

Table 3
Summary of biomarker responses to bunker fuel stress in mud snails (*I. obsoleta*)

Biomarker	Treatments			$F_{2,12}$
	Control	Low	High	
GSH	$68^{a} \pm 5.5$	$48^{b} \pm 2.0$	$43^{b} \pm 4.5$	9.3^{*}
LPO	$0.88^{a} \pm 0.073$	$1.34^{b} \pm 0.108$	$1.34^{b} \pm 0.081$	9.0^{*}
Ubiquitin	$20^{a} \pm 2.9$	$33^{b} \pm 2.7$	$35^{b} \pm 3.9$	6.6^{*}
Hsp22	$2^{a} \pm 2.0$	$27^{b} \pm 7.7$	$40^{b} \pm 11.7$	21.5^{***}
Hsp26	7 ± 3.2	26 ± 8.2	41 ± 11.3	4.1, ns
Hsp60	0.144 ± 0.0206	0.286 ± 0.0500	0.358 ± 0.0802	3.8, ns
Hsp70	6.6 ± 1.54	34.6 ± 7.17	35.6 ± 8.43	6.5, ns
αB-crystallin	22 ± 6.2	44 ± 3.2	49 ± 6.6	6.9, ns
Mn SOD	$13.0^{a} \pm 3.30$	$47.4^{b} \pm 2.68$	$36.6^{c} \pm 2.48$	38.3^{***}
Metallothionein	6.6 ± 1.66	8.4 ± 1.83	8.8 ± 1.36	0.5, ns
Cytochrome *P*450	15.6 ± 4.86	35.4 ± 7.30	37.2 ± 4.57	4.4, ns

Treatments were 0, 0.5, 1 or 3 g/l of the water-accommodating fraction of bunker fuel. Values for Hsp22 were $\log_{10}(x+1)$ transformed to meet the normality assumptions of parametric analyses. Table entries and units of measurement are as in Table 2.

$^{*}P < 0.00465$.

$^{***}P < 0.0001$.

Table 4
Summary of biomarker responses to atrazine stress in mud snails (*I. obsoleta*)

Biomarker	Treatments			$F_{2,12}$
	Control	Low	High	
GSH	88 ± 2.8	85 ± 3.6	83 ± 3.1	0.8, ns
LPO	1.10 ± 0.071	1.16 ± 0.040	1.32 ± 0.037	5.5, ns
Ubiquitin	27 ± 2.3	42 ± 3.9	41 ± 3.7	6.2, ns
Hsp22	2 ± 1.5	6 ± 2.0	6 ± 1.9	0.9, ns
Hsp26	9^{a} ± 5.2	35^{b} ± 3.1	35^{b} ± 1.7	12.6 *
Hsp60	0.252 ± 0.0828	0.238 ± 0.0883	0.158 ± 0.0086	0.8, ns
Hsp70	125.0 ± 5.96	123.4 ± 7.05	121.8 ± 6.08	0.02, ns
α B-crystallin	17 ± 2.2	29 ± 1.3	25 ± 2.1	8.7, ns
Mn SOD	6.4 ± 2.50	9.0 ± 1.76	7.8 ± 1.66	0.3, ns
Metallothionein	13.8 ± 4.09	18.2 ± 2.06	8.6 ± 1.72	3.9, ns
Cytochrome *P*450	5.8 ± 2.13	18.0 ± 2.90	17.8 ± 3.07	7.0, ns

Treatments were 0, 1 and 500 μg/l or 1 mg/l acetone stock of technical grade atrazine (99%). Table entries and units of measurement are as in Table 2.

* $P < 0.00465$.

3.4. Responses to atrazine stress

Only Hsp26 responded significantly different among atrazine treatments (Table 4). Levels of Hsp26 were almost four times higher when atrazine was present than under control conditions with the carrier solvent alone.

Table 5
Summary of biomarker responses to endosulfan stress in mud snails (*I. obsoleta*)

Biomarker	Treatments				Test statistic
	Control	Low	Medium	High	
GSH	62^{a} ± 8.0	48^{ab} ± 3.1	37^{bc} ± 2.3	29^{c} ± 2.035	12.0 * *
LPO	0.98^{a} ± 0.049	1.08^{a} ± 0.049	1.60^{b} ± 0.084	1.80^{b} ± 0.089	29.2 * * *
Ubiquitin	44^{a} ± 2.786	44^{a} ± 3.8	50^{a} ± 3.22	26^{b} ± 3.78	9.4 * *
Hsp22	1^{a} ± 1.4	11^{a} ± 3.9	34^{b} ± 8.9	39^{b} ± 5.418	9.0 *
Hsp26	0 ± 0	20 ± 12.673	19 ± 5.9	23 ± 3.3	10.6, ns
Hsp60	0.172^{a} ± 0.0124	0.238^{a} ± 0.0302	0.396^{b} ± 0.0194	0.232^{a} ± 0.0384	11.7 * * *
Hsp70	6.6^{a} ± 1.86	15.4^{b} ± 2.38	23.2^{b} ± 1.20	15.2^{b} ± 2.31	12.7 * * *
α B-crystallin	16 ± 3.2	12 ± 3.1	11 ± 3.9	13 ± 2.7	1.0, ns
Mn SOD	3.2^{a} ± 2.27	26.2^{b} ± 4.22	29.2^{b} ± 6.95	33.8^{b} ± 3.18	7.0 *
Metallothionein	17.8 ± 3.20	19.4 ± 1.99	22.2 ± 3.15	14 ± 3.11	1.3, ns
Cytochrome *P*450	8.2^{a} ± 5.96	8.6^{a} ± 2.36	36.2^{b} ± 8.78	45.6^{b} ± 2.80	29.2 * * *

Treatments were 0, 50 and 500 ng/l or 1 μg/l acetone stock of technical grade endosulfan (99%). Table entries and units of measurement are as in Table 2. Test statistics are $X^2_{(3)}$ from a nonparametric Kruskal–Wallis test (Hsp26) or $F_{3,15}$ from a Welch (Hsp22) or one-way ANOVA (all remaining biomarkers).

* $P < 0.00465$.

* * $P < 0.001$.

* * * $P < 0.0001$.

3.5. Responses to endosulfan stress

Eight biomarkers responded significantly to endosulfan treatments (Table 5). Levels of GSH decreased significantly and in a dose-dependent manner in all three endosulfan treatments. Levels of LPO, Hsp22, Mn SOD and cytochrome *P*450 increased significantly with increasing concentrations of endosulfan, while GSH levels declined. In contrast, levels of ubiquitin, Hsp60 and Hsp70 were highest at intermediate endosulfan concentrations (Table 5). Three biomarkers did not respond significantly to endosulfan treatments: Hsp26, αB-crystallin and metallothionein. However, the nonsignificant variation in Hsp26 levels should be interpreted with caution, because unequal variance and the conservative adjusted P' level limited the statistical power of this test.

4. Discussion

Biomarkers can be defined as biological parameters that reflect changes in physiological systems indicative of health status or condition. Health and environmental studies incorporate three biomarker categories into four monitoring designs: (1) monitoring potential exposure, (2) monitoring biological effect, (3) monitoring physiological effect, and (4) health surveillance (de Zwart et al., 1999). Most non-human environmental studies using molecular biomarkers have employed experimental designs for either monitoring potential exposure (#1), monitoring of biological effect (#2), or in some cases, monitoring both exposure and biological effect (#2 and #3). Health surveillance studies typically use a defined suite or array of biomarkers to identify individuals or populations at risk to adverse health conditions, so preventative measures can be taken. The best example of this form of monitoring is a standard medical blood "work-up" or analysis. Between 17–25 molecular biomarkers are assayed for a standard medical blood analysis; each assay is indicative of a specific physiological condition (Porth, 1994). No single biomarker is a definitive benchmark of health—changes in each biomarker are considered in association with those of all others when evaluating an individual's condition. If levels of some biomarkers differ significantly from nominal levels, additional assays are conducted to more accurately diagnose the health condition and to better identify possible causative agents (Porth, 1994). Using molecular biomarkers in a similar fashion for environmental health surveillance requires:

1. A priori justification for each biomarker used
2. A thorough understanding of each biomarker's contribution to cellular physiology
3. Researchers must be proficient in the techniques used to prepare and assay samples
4. Recognizing the limitations of molecular biomarker data when evaluating the health of organisms and populations

The Molecular Biomarker System we developed meets all four criteria. The MBS distinguished between stressed and non-stressed conditions, and responded differently to different stressors (Fig. 5). The MBS technology could be used to explore the physio-

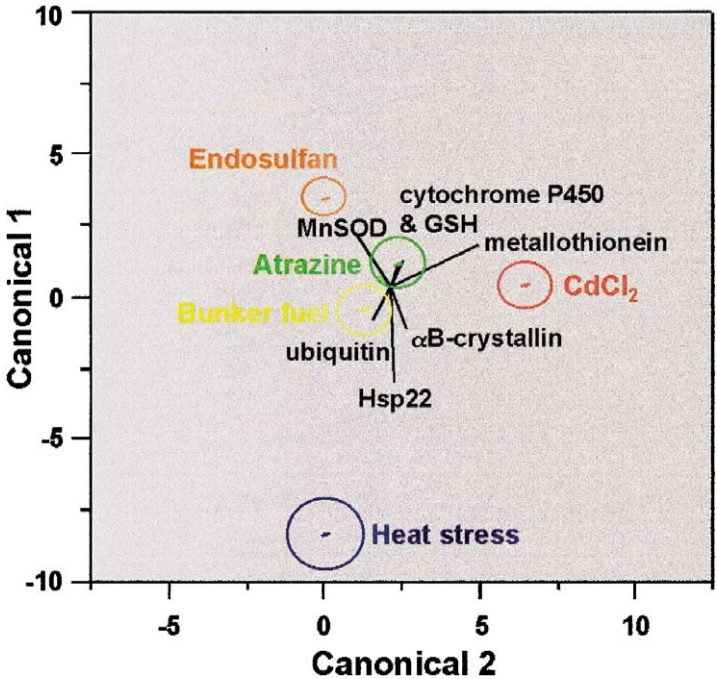

Fig. 5. Canonical centroid plot of standardized biomarker responses. Original variates were biomarker levels expressed as a percentage of the control value in each experiment. Data from all experiments were combined for this figure, which is presented for heuristic purposes (hypothesis tests were performed separately for each experiment). Centroids of the distribution are marked with an *x*; circles around the centroids show 95% confidence intervals. Bi-plot rays show the directions of the original biomarker responses in this canonical space. Thus, the biomarker metallothionein (which assays heavy metal stress) distinguishes cadmium chloride treatments from all others; Mn SOD differentiates endosulfan from the other treatments, etc.

logical condition of snail responding to a particular stressor, which may be important in alleviating the stressed condition.

4.1. High-temperature exposure

Heat stress is known to stimulate a concomitant induction of oxidative stress that can result from heat-induced conformational changes in enzymes that foster increased reactive oxygen species (ROS) production (Halliwell and Gutterudge, 1999). Examples are aconitase's heat-induced ROS generation via Fenton chemistry from its iron–sulfur cluster or heat-induced electron transfer to O_2 instead of ubiquinone reduction by NADH/ubiquinone oxidoreductase (Halliwell and Gutterudge, 1999). In mud snails, LPO levels were significantly higher in heat-stressed samples than controls and GSH levels decreased more than expected, especially after an 8-h heat stress (Downs et al., 2001; Downs et al., unpublished results; Halliwell, 1999). Loss of GSH suggests that other factors that utilize GSH may be enhanced (e.g. GSH-*S*-transferase) or that the GSH synthesis pathway was adversely affected by heat stress. Mn SOD levels were also

significantly elevated, indicating increased superoxide radical generation in the mitochondria or cytoplasm (Fridovich, 1995). Induction of Hsp22 also indicates that mitochondria were responding to insults of oxidative phosphorylation and the citric acid cycle (Tanguay, personal communication; Downs et al., unpublished data).

Increased LPO levels during or following a stress event also indicated a decline in functional lipid levels (Duthie, 1993). Ubiquitin, an indicator of protein turnover, was significantly higher in heat-stressed snails than in controls. These two parameters alone suggest that a major insult to cell structure integrity occurred during heat stress. This interpretation is supported by the significantly higher levels of chaperone accumulation (Hsp60 and Hsp70) in response to heat stress when compared to control conditions, indicating increased protein denaturation (Iwai, 1999; Tomanek and Somero, 2000). Hsp26, related to the p26 protein of *A. franciscana*, plays a role not only in heat tolerance, but also in anoxic tolerance (Clegg et al., 1999). Its behavior in gastropods, if similar to that of p26 in arthropods, suggests that it may protect some aspect of nuclear function such as protection against nuclear protein degradation, especially since it is known to associate in a pH-dependent manner with nuclei during stress (Liang et al., 1997; Clegg et al., 1999). Induction of αB-crystallin, which is a small heat-shock protein found only in the cytosol of animals, protects cytoskeletal elements during stress (for review, see Derham and Harding, 1999). Its induction suggests that cytoskeletal integrity was being compromised.

Metallothionein and cytochrome *P*450 2E1 did not respond to heat stress, indicating that snails were not being stressed by a xenobiotic or heavy metal. More importantly, it indicated that these two markers were unresponsive to heat stress, a situation that does occur for other cytochrome *P*450s (e.g. CYT *P*450 1E1) allowing for better discrimination among multiple causal factors.

4.2. Cadmium exposure

Cadmium is detrimental to cell systems by several mechanisms; the two most prevalent are (1) as a competitive or uncompetitive enzyme inhibitor and (2) generation of oxidative stress via Fenton reaction (Halliwell and Gutterudge, 1999; Klaassen et al., 1999). MBS data indicated that an oxidative stress occurred in mud snails exposed to cadmium chloride. Levels of LPO and Mn SOD were higher in exposed snails than in controls. GSH levels were significantly lower in exposed snails than in controls.

Higher levels of LPO, Hsp60 and Hsp70 indicated that cell structural integrity was under assault in cadmium-exposed shrimp compared to controls. Levels of Hsp26 and αB-crystallin homologue were also significantly higher in cadmium-exposed snails than in controls, indicating a stress response of both the cytoskeleton and nuclear function. Increased Hsp22 and Mn SOD levels in response to cadmium exposure indicated a stress on mitochondrial function.

Induction of metallothionein proteins is a well-described response and adaptation to heavy-metal exposure (Klaassen et al., 1999). Metallothionein levels were significantly higher in exposed snails than in controls, indicating both a specific response to heavy-metal exposure and induction of a compensatory action against heavy-metal

toxicity (Klaassen et al., 1999; Masters et al., 1994). Cytochrome *P*450 2E1 was not induced by cadmium exposure, indicating that it was not elicited either by a protein denaturing stress or an oxidative stress.

4.3. Bunker fuel exposure

The water-accommodating fraction (WAF) of bunker fuel elicited a strong cytochrome *P*450 2E1 response, indicating that this biomarker was an appropriate gauge of bunker fuel exposure. Cell structure integrity was compromised by bunker fuel exposure as demonstrated by the significant accumulation of LPO. Bunker fuel did not induce significant accumulation of Hsp60, but significantly increased levels of Hsp70. This is surprising because diverse species have been demonstrated to accumulate higher levels of Hsp60 and Hsp70 in response to a fuel or fuel-like exposure (Oberdörster et al., 1999; Wolfe et al., 1999; Downs et al., in review). Work done in our labs with diesel and bunker fuel exposures in *Palaeomonetes pugio* (grass shrimp) also showed differential induction of Hsp60 and Hsp70 by bunker and diesel fuel (Downs et al., 2001). Wheelock et al. (1999) observed that copper exposure induced accumulation of Hsp60 in rotifers, but the water-accommodating fraction of Prudhoe Bay crude oil did not elicit induction of Hsp60. This phenomenon was suggested to result from an interference of protein synthesis by the WAF oil. Our data indicate this mechanism is not inclusive of an inhibition of overall protein synthesis, but may result from a number of specific factors, including an inhibition of specific Hsp60 or Hsp70 transcriptional or translational factors. An alternative hypothesis is that the signal-transduction cascade for induction of Hsp60 or Hsp70 was compromised by the WAF of bunker fuel. It also could be that membrane integrity is compromised but not enzyme or protein integrity, thus precluding the need for protein chaperoning.

Exposure to the WAF bunker fuel likely induced an oxidative stress, as indicated by significant induction of LPO and Mn SOD. GSH levels were significantly lower in snails exposed to bunker fuel than in controls, indicating a xenoresistance role. Whether this is a xenobiotic response or an oxidative-stress response is unknown, indicating that a more sophisticated array of biomarkers must be used to identify the underlying mechanism (e.g. glutathione peroxidase vs. glutathione-*S*-transferase activity or content).

Differential expression of Hsp22, Hsp60 and Mn SOD in response to bunker fuel indicates a particular physiological condition for the mitochondria. Expression of Hsp22 and Mn SOD indicates a response to reactive oxygen species. Oxidative stress should increase protein turnover in mitochondria (Halliwell and Gutterudge, 1999). Higher Hsp60 levels are then expected, because Hsp60 is a biomarker for the rate of mitochondrial protein turnover. However, this did not occur. One interpretation is that bunker fuel had a negligible affect upon protein/enzyme condition within the mitochondria. Increased levels of ubiquitin in bunker fuel-exposed snails suggest that protein denaturation and protein import and synthesis increased in the cell. This suggests but does not demonstrate, that protein turnover should also increase in the mitochondria. This discrepancy, based upon assumptions concerning the theory of protein turnover during a cellular stress, especially during an oxidative stress, suggests that Hsp60 may be an insensitive or inappropriate biomarker for mitochondrial protein turnover when the

suspected stressor is a fuel. Further assays (e.g. Grp75) are needed to test this and other possible hypotheses.

4.4. Atrazine exposure

Atrazine is a broad-spectrum herbicide that binds the Qb binding site of Photosystem II, preventing electron transfer from the Qb to quinone, resulting in the generation of several different reactive oxygen species, including singlet oxygen (Halliwell and Gutterudge, 1999). The MBS pattern for snails exposed to atrazine exposure was surprising: only Hsp26 levels differed significantly from controls.

One possibility is that atrazine at the concentrations and exposures we used were non-stressful to snails. This interpretation is supported by the toxicological literature concerning atrazine toxicity in vertebrates and invertebrates (for review, see IARC, 1999). Even if snails exposed to atrazine were not stressed on the cellular superstructure level, one would expect a xenobiotic response. Our data indicate that the GSH/mixed oxygenase xenobiotic response pathway was not elicited. In concordance with a xenobiotic response, GSH levels are usually significantly altered in atrazine-exposed animals and plants (Kramer et al., 1988; Halliwell and Gutterudge, 1999; IARC, 1999). Several studies demonstrate that in mammals, cytochrome *P*450 2E1 is instrumental in the catabolism of atrazine (Hanioka et al., 1998, 1999; IARC, 1999). One possibility is that the dosage and time of exposure were below a threshold level for xenobiotic-response induction that these assays could detect, indicating that other xenobiotic molecular biomarkers are required to detect a mud snail response to atrazine.

Because of the acute exposure design of this experiment, Hsp26 may act as both a diagnostic marker and as a prognostic marker. For example, a chronic exposure experimental design may uncover further symptoms of a stressed condition (e.g. decreased feeding, decreased growth, increased respiration, etc.; IARC, 1999). Further investigation of the function of Hsp26 and its role in atrazine-induced pathology may lay the groundwork for the development of prognostic biomarkers.

4.5. Endosulfan exposure

Endosulfan is a broad-spectrum insecticide used worldwide (US Department of Health and Human Services, 1998). It adversely affects physiological and genetic status, although toxicological studies have focused predominantly on its inhibition of acetylcholinesterase activity (Naqvi and Vaishnavi, 1993). Endosulfan induces oxidative stress while altering GSH redox status. For example, endosulfan induces lipid peroxide and significantly decreases free GSH levels with a concomitant increase in GSH-*S*-transferase activity (Blat et al., 1988; Rafi et al., 1991; Hincal et al., 1995). Endosulfan also adversely affects mitochondrial function, specifically ATP production and the electron transport chain, suggesting that one major mode of action is oxidative stress-inhibition of metabolic pathways (e.g. mitochondrial-linked pathways: TCA cycle, oxidative phosphorylation and fatty-acid metabolism; Yamano and Morita, 1995).

Data generated by the MBS in this experimental system support the oxidative-stress mode of toxicity by endosulfan. Both LPO and Mn SOD levels were elevated signifi-

cantly in response to endosulfan exposure. Both xenobiotic biomarkers (GSH and cytochrome *P*450) were also altered significantly by endosulfan exposure. Both GSH and cytochrome *P*450 play a major role in the detoxification of endosulfan (Naqvi and Vaishnavi, 1993). The effect of endosulfan on protein turnover and protein chaperoning had not been previously described. Ubiquitin levels decreased in the highest exposure to endosulfan, but were unaffected by lower endosulfan concentrations. Both Hsp60 and Hsp70 increased in response to endosulfan exposure, indicating either increased protein denaturation or increased protein synthesis. One interpretation is that endosulfan denatures proteins, but not irreversibly, so proteins do not require degradation.

The small Hsps also reacted unexpectedly; both Hsp22 and Hsp26 increased in response to an endosulfan exposure, but αB-crystallin was unchanged. A meaningful explanation of this response is difficult. This pattern does indicate that the small Hsps may be regulated differentially in invertebrates, as they are in plants and mammals (Downs et al., 1999b). Regardless, MBS data on endosulfan exposures warrant further investigations into its action within the cell.

The primary objectives of the MBS are to (1) determine if an organism is stressed or non-stressed, (2) determine the cellular structural status, and (3) determine the type of cellular physiological response in order to determine the nature of the stressor (Fig. 5). Although the assays we used met these objectives, their depth and sensitivity could be increased significantly. For example, measurement of protein carbonyl and DNA oxidation adducts can be included in the array to measure the extent of oxidative stress, and as additional parameters for cellular structural integrity. Published and unpublished work from our laboratories on corals, shrimp, fish, mammals and plants demonstrate that the MBS array of assays can be expanded and arranged to distinguish between responses to exposures of cadmium or copper (i.e. Dallinger et al., 1997; Downs et al., 2000, 2001) and between certain polyaromatic hydrocarbons (e.g. naphthalene and endosulfan). Increased sophistication demands greater understanding of the cellular physiology and the genetics and biochemistry of cellular stress responses for each focal species (e.g. DNA or protein sequence of the cellular parameters of interest). It also demands that assays be specially designed for each focal species e.g. *I. obsoleta* cytochrome *P*450 4-class and 6-class or sHsp22 and Hsp26 antibodies and protein standards.

A number of issues concerning the valid use and limitations of the MBS to a snail population that were not addressed in this experimental design must be recognized. One issue is the developmental stage or age of the individual. We used only sexually mature, non-gravid adults. The values for many of the biomarkers under both control and the treated conditions used in this project may be significantly different between juvenile and adult stages of snail, and even between gravid and non-gravid females. For example, some isoforms of the small heat-shock protein family in shrimp and *Drosophila* are developmentally regulated and localize only to neural and follicular tissue of adults (King and Towers, 1999; Downs et al., unpublished results). Other physiological parameters are known to be significantly altered due to age and development stage in organisms ranging from marine mussels to humans. Another issue is the lack of baseline information concerning qualitative/quantitative responses of the MBS to a mixture of the stressors used in this project (e.g. heat stress and atrazine or bunker fuel and cadmium chloride), producing an uncertainty as to how the MBS can distinguish

between multiple/simultaneous stressors. Finally, seasonality of sampling must be considered. Several papers demonstrate that Hsp70, ubiquitin and other stress markers alter significantly in response to seasonality, which can affect the interpretation between a stressed organism or an organism with nondisease-associated increased metabolic activity (Hofman and Somero, 1995; Minier et al., 2000; Shaw et al., 2000).

During the assay validation period, considerable resources were spent to optimize and determine which commercially available antibodies and spectrophotometric assays could validly be used with *I. obsoleta*. We are now creating monospecific polyclonal antibodies against the gastropod cellular parameters presented in this paper. Even at best, cross-reactivity was low for most cellular parameters compared to tests against mammalian or vertebrate samples (e.g. human tissue). Production of snail-specific antibodies will enhance the resolution and precision by almost five orders of magnitude and would allow less biological material to be used.

The MBS technology is analogous to human medical diagnostic technology and analysis. Employing MBS to address ecological or environmental questions requires the rigorous experimental designs and statistical methods used by those disciplines (Schaeffer et al., 1988). Often, this requires large sample sizes. The MBS techniques described in this paper are relatively inexpensive and easy to do, although even using an ELISA protocol, MBS is a relatively time-consuming process. To that end, we have developed automated, high-throughput fluorometric immuno-absorption assays and spectrophotometric techniques, and data informatics and manipulation programs to meet the needs of large sampling programs.

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