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Field validation of hsp70 stress proteins as biomarkers in Asian clam (*Potamocorbula amurensis*): is downregulation an indicator of stress?

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The focus of this paper is to consider the applicability of the hsp70 stress protein response as a biomarker in field studies. Stress proteins (or heat shock proteins, hsp) of the hsp70 family are induced by sublethal concentrations of a variety of environmental pollutants. However, few studies have applied these proteins as biomarkers of environmental stress under field conditions. Our laboratory is investigating hsp70 proteins and other responses of Asian clam (*Potamocorbula amurensis*) as potential biomarkers in laboratory and field studies. Our efforts include two studies presently being conducted in northern San Francisco Bay: (1) monthly collection of clams from four sites along a cadmium contamination gradient; (2) 7 day *in situ* exposure of clams at two selected sites at Mare Island Naval Shipyard. Here we present results on hsp70 proteins in *P. amurensis* in field-collected and outplanted clams. Both field projects are ongoing, therefore the results presented here do not represent completed studies; rather, they illustrate a portion of our experience. For this workshop, we illustrate weaknesses and strengths of these proteins as biomarkers, and we underscore where additional work is needed. In field-collected clams (study no. 1), site-specific differences in levels of two hsp70 proteins, hsp70 and hsp76, were measured in May and June 1997. Although an inverse correlation exists between cadmium tissue concentrations and hsp70 protein levels, differences detected may be reflective of a salinity gradient. Results from recent laboratory exposures to cadmium and a range of salinities are discussed. After *in situ* exposure for 7 days (study no. 2), both hsp70 and hsp76 levels were significantly reduced in clams from site R. However, given a brief heat-shock in the laboratory, hsp70 protein levels were significantly higher in clams from this site than in controls. Results indicate that downregulation as well as upregulation of hsp70 proteins may be indicators of stress in *P. amurensis*.

Keywords: stress proteins, field validation, molluscs, *Potamocorbula amurensis*, biomarkers, downregulation.

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Introduction

The use of stress proteins (or heat shock proteins, hsp) as molecular biomarkers of adverse but sublethal effect has been proposed and investigated for a number of years (Sanders 1993). Research on mammalian cells showed that certain subgroups of these proteins are induced by a variety of stressors which either damage cellular proteins directly or cause cells to synthesize aberrant proteins (Voellmy 1996). The most abundant and widely studied group of stress proteins is the hsp70 protein family, encompassing constitutive as well as inducible isoforms, and is highly conserved across phyla from bacteria to mammals (Schlesinger *et al*. 1982).

Cellular functions of hsp70 proteins include the stabilization of unfolded protein precursors before assembly, translocation of proteins into organelles, rearrangement of protein oligomers, dissolution of protein aggregates, and refolding or degradation of denatured proteins (Feige *et al*. 1996). Stress proteins

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are thought to confer protection from environmentally-induced cellular damage (Sanders 1993, Feige *et al*. 1996). Numerous studies showed induction of hsp70 proteins after laboratory exposure of cells or organisms to known environmental chemical toxicants (e.g. Cochrane *et al*. 1991, Bauman *et al*. 1993, Sanders 1993, Ryan and Hightower, 1996, Werner and Nagel 1997, Vijayan *et al*. 1998). Sensitivity of the response was up to several orders of magnitude higher than common measures of toxicity such as LC_{50} (the concentration at which mortality reaches 50%; Werner and Nagel 1997) or EC_{20} (the concentration at which 20% of organisms show deleterious effect) for reproduction (Kammenga *et al*. 1998). Linkage between upregulation of stress proteins in organisms and higher level effects, such as histopathologic alterations or scope-for-growth (energy available for growth) have been established (Sanders *et al*. 1991, Goering *et al*. 1993, Clark *et al*. in prep.).

The majority of studies investigating the linkage between toxicant exposure and/or effect and levels of cellular hsp70 protein were performed as controlled laboratory exposures to individual chemicals. Few studies have included multiple stressor exposures such as are encountered in field situations, and available literature on the relevancy of stress proteins for field application is scarce. Moreover, the potential for inhibition of (stress) protein synthesis by environmental contaminants has rarely been addressed (Veldhuizen-Tsoerkan *et al*. 1991a, b, Werner *et al*. 1998).

Results from two field studies will be presented to illustrate the type of information needed for interpreting field data, and to highlight existing gaps in our understanding of hsp70 proteins and their responses to environmental stressors. The results herein are part of an integrated effort to investigate field applicability of a suite of biomarkers, including hsp70 proteins. *P. amurensis* is a euryhaline clam species introduced into San Francisco Bay in the mid-1980s which has spread prolifically, and is now found throughout the Bay in large numbers. Study no. 1 was conducted along a metal contamination gradient (Brown and Luoma 1995; figure 1), where clams were sampled monthly. Lower condition indices, the ratio of tissue weight to shell length, and glycogen levels have been measured in clam populations at the landward stations, and have, in part, been associated with elevated cadmium concentrations in clam tissues (S. Luoma, C. Brown, USGS Menlo Park, CA, personal communication). In study no. 2, clams were outplanted for 7 days at two sites off-shore of Mare Island Naval Shipyard (figure 1), one reference site, and one site where previous sediment bioassays demonstrated toxicity to *Eohaustorius estuarius* (Amphipoda; S. Anderson, UC Davis, Davis, CA, personal communication). Both studies focus on linking biomarkers of exposure and effect at several levels of organization (see also Teh *et al*. 1999). Here we will present and discuss our results on hsp70 stress proteins.

Methods

Study no. 1

Sample collection. Using a Van Veen grab sampler, *P. amurensis* were collected once a month (14 May and 10 June 1997) from four sites (USGS stations 4.1, 6.1, 8.1 and 12.5) in Northern San Francisco Bay (figure 1). In 1991/92, mean concentrations of cadmium measured in *P. amurensis* tissue were approximately 5.6, 4.6, 2.7 and 1.8 mg g^{-1} dry tissue at stations 4.1, 6.1, 8.1 and 12.5 (Brown and Luoma 1995); this pattern still persists (table 1). Clams were sieved from the sediment, and immediately frozen in liquid nitrogen, transported to the laboratory at UC Davis on dry ice, and stored at –80 °C until stress protein analysis.

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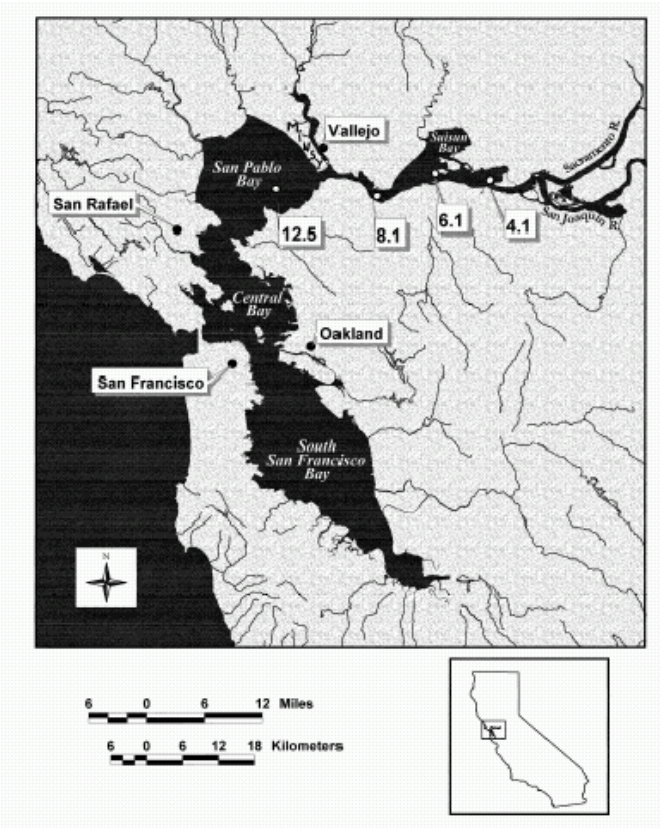

Figure 1. *Potamocorbula amurensis* sampling sites in San Francisco Bay, California, and location of Mare Island Naval Shipyard (MINSY) near Vallejo. Sites 4.1, 6.1, 8.1 and 12.5 follow a heavy metal gradient with highest concentrations at site 4.1 (USGS data).

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Study no. 2

In situ exposure. Clams were collected (30 Sept 1997) from a relatively uncontaminated site west of Martinez Marina (S. Luoma, USGS, Menlo Park, CA, personal communication). They were maintained in the dark for 3 days in aerated 0.2 mm-filtered water from Bodega Bay Marine Laboratory mixed with glass-distilled water at 20 °C, and a salinity of 19 ppt (oxygen concentration: 8.0 mg^{-1}, pH 7.95). During this time clams were not fed. A subsample ($n = 9$) was frozen in liquid nitrogen (zero time controls), just before initiation of the field experiment. Four replicate chambers containing 15 clams each were deployed at each of two field sites, R and PP, approximately 17 m and 10 m off-shore from Mare Island Naval Shipyard (MINSY, figure 1). Sediments collected from site PP were previously shown to be toxic to the amphipod *Eohaustorius estuarius* (S. Anderson, Bodega Marine Laboratory, personal communication). Site R served as a reference site. Initial water quality parameters at both field sites were: salinity 20.6 ppt, temperature 20.6 °C, oxygen concentration 6.86–6.75 mg l^{-1}, pH 7.65–7.60. Laboratory controls consisted of four replicate 1-litre beakers containing 750 ml of control water (see above, maintenance water) and 15 clams each. Water was aerated, changed every 2 days, and maintained at 20 °C and 19–20 ppt salinity. Control clams were not fed.

The experiment was terminated after 7 days, at which time water quality parameters at both sites were: salinity 19.9–20 ppt, temperature 18.4 °C, oxygen concentration 7.4 mg l^{-1} and pH 7.55–7.8. Only one clam at site R died during the experiment. Five clams per container (20 per site) were immediately frozen in liquid nitrogen, transported to the laboratory on dry iced and stored at –80 °C. The remaining clams were transported to the laboratory in water from the deployment sites maintained at approx. 20 °C. Subsamples of laboratory controls were frozen and stored.

Heat shock. A subsample of five clams from each of four replicate containers per treatment (field exposed and laboratory control) received a heat-shock (15 min 37 °C) with a subsequent recovery period of 6.5 h at control conditions. Samples were then frozen in liquid nitrogen and stored at –80 °C until stress protein analysis.

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Sediment chemistry. Sediment samples (5 cm deep) were taken from both field sites. Chemical analyses of heavy metals and organochlorine compounds were performed by CA Veterinary Diagnostic Laboratory System, Davis, CA, by inductively coupled plasma emission spectrometry (ICP) after acid digestion, and gas chromatography (GC) after solvent extraction, respectively. In the vicinity of the sites, heavy metals and organochlorine compounds are considered to be the main chemicals of concern. Sediment samples were screened for the following heavy metals: As, Cd, Fe, Cu, Hg, Mn, Mo, Pb, Zn, Se, Cr and Ni, and organochlorine compounds (detection limits in brackets): aldrin (0.1 ppm), BHC (0.1 ppm), chlordane (0.5 ppm), *p,p´*DDD (0.2 ppm), *o,p´*DDD (0.2 ppm), *p,p´*DDE (0.2 ppm), *o,p´*DDE (0.2 ppm) *p,p´*DDT (0.2 ppm), *o,p´*DDT (0.2 ppm), dicofol (0.2 ppm), dieldren (0.1 ppm), endosulfan I and II (0.1 ppm), endrin (0.1 ppm), gamma chlordane (0.1 ppm), HCB (0.1 ppm), heptachlor (0.1 ppm), heptachlor epoxide (0.1 ppm), lindane (0.1 ppm), methoxychlor (0.1 ppm), mirex (0.1 ppm), toxaphene (4 ppm), arochlor 1016, 1221, 1232, 1242, 1248, 1254, 1260, 1262 (2.5 ppm).

Stress protein (hsp70) analysis

Hsp70 proteins were analysed using Western blotting techniques. Each sample represents the total visceral mass of one clam. Samples were homogenized (1.5 min, glass on glass) on ice in a hypotonic solution containing 66 mM Tris–HCl (pH 7.5), 0.1 %Nonidet, 10 mM EDTA, 10 mM DTT and several protease inhibitors (10 mM benzamidine, 50 mM pepstatin, 0.001 %aprotinin, 1 mg ml^{-1} leupeptin and 0.1 mM PMSF). Homogenates were centrifuged for 30 min at 4000 *g* to remove large particulate material. Resulting pellets were initially resuspended in homogenization buffer containing 5 %SDS, but Western blotting analysis showed that both hsp70 and hsp76 were present in very low concentrations (< 5 %) relative to the first supernatant fraction. Supernatants were collected, and total protein concentration was determined by the Biorad DC Protein Assay (Lowry *et al*. 1951). Sample buffer (Laemmli 1970) was immediately added to each sample, and samples were heated to 95 °C for 2 min. Subsamples of equal total protein content (50 µg) were separated by SDS–PAGE on 10 % polyacrylamide gels with 5 % stacking gels (Blattler *et al*. 1972) using the buffer system described by Laemmli (1970). Hsp70 antigen (50 ng, Stressgen) was applied to one lane to serve as an internal standard for blotting efficiency, and three or four samples per treatment were applied to the same gel. Proteins were separated at 25 mA per gel for approximately 1.5 h, then electroblotted onto Immobilon-P membrane at constant voltage (40 V) overnight. Blotted proteins were stained with Ponceau S solution to check for transfer efficiency and consistent blotting results. In addition, gels were stained with Coomassie blue after transfer to ensure that complete protein transfer had occurred. Membranes were blocked with 5 %skim milk in 20 mM Tris buffer and 0.4 M NaCl (pH 7.5) with 0.05 %Tween-20 for 30 min. A monoclonal antibody for hsp70 (1:500; Affinity Bioreagents MA3-001) was used as a probe. This antibody recognizes two hsp70 isoforms, hsp70 and hsp76, in *P. amurensis*. Blots were incubated for 1 h 30 min with primary anti-hsp70 antibody, then washed three times for 30 min in Tris-buffered saline solution containing 0.05 % Tween-20. Alkaline phosphatase-conjugated goat–anti-rat IgG (1:30000 dilution; Sigma) was used to detect hsp70 probes. Bound antibody was visualized by a chemiluminescent substrate (CDP-Star; Tropix, Bedford, MA) and quantified by densitometry. The hsp70 antigen was used as a marker to ensure that blotting results were comparable between membranes. A standard curve was used to verify that measurements were within the linear range.

Statistical analysis

Data were analysed using Bartlett's test for homogeneity. If data had homogeneous variance, site-specific differences in hsp70 levels were determined using one-way analysis of variance (ANOVA) and Dunnett's mean separation test. If data had heterogeneous variance, they were transformed to relative ranks then analysed by ANOVA and Dunnett's mean separation test. If $p < 0.05$, stress protein levels at test sites were considered significantly different from each other.

Results

The primary hsp70-antibody used in these studies detected two protein bands of approx. 70 and 76 kDa. The hsp70 protein was present in higher concentrations, and was previously observed to be more responsive to chemical stress than the hsp76 protein (Clark *et al*. in prep.).

Study no. 1

Results from hsp70 analyses in field collected clams from May and June 1997 are shown in Figure 2. Stress protein levels were consistently higher at stations 8.1

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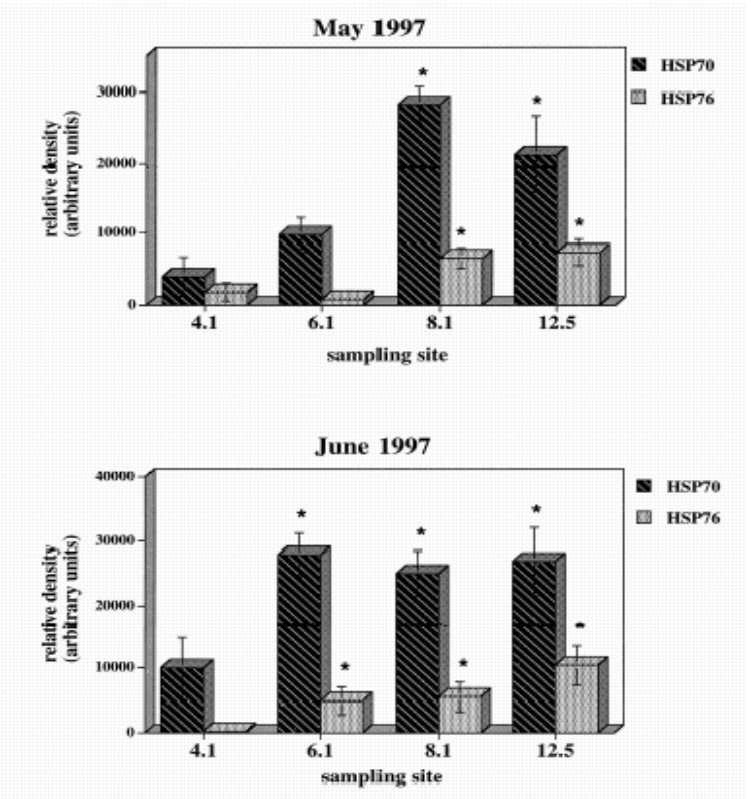

Figure 2. Hsp70 proteins in whole tissue homogenates of *Potamocorbula amurensis* sampled in May and June, 1997, from four sites in Northern San Francisco Bay. Shown are mean densitometer readings and standard errors of protein bands detected by Western blotting; $n = 6$; * significantly different from site 4.1; significance level $p < 0.05$.

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and 12.5 than at station 4.1. Station 6.1 was not statistically different from station 4.1 in May, but significantly different in June. Water temperatures, and oxygen concentrations were similar at all stations (table 1). Salinity did not appear to be correlated with stress protein levels ($r^2 = 0.37$), and daily ranges of specific conductivity (1 ppt roughly equals 1 mhos cm^{-1} in the lower salinity ranges) near station 6.1 were similar on both sampling dates (3.6–16.5 mhos cm^{-1} on 14 May 97, 5.7–16.7 mhos cm^{-1} on 10 June 97; California Dept Water Resources, station RSAC064, continuous monitoring data). However, the time period for which salinities were above 10 mhos cm^{-1} was increased from 13 h in May to 18 h in June when hsp70 levels were higher. Specific conductivity (SC) close to station 4.1 ranged from 0.7 to 2.5 mhos cm^{-1} on May 14, and 1.9 to 7.3 mhos cm^{-1} on June 10 (DWR RSACO77). Levels of hsp70 proteins showed an inverse correlation with cadmium concentrations in clam tissue ($r^2 = 0.68$).

Study no. 2

Tissue levels of hsp70 and hsp76 were significantly reduced in clams exposed for 7 days at site R than both in controls and clams from site PP (figure 3(A)). Control clams showed significantly elevated stress protein levels after 7 days when compared with clams from the starting date of the field exposure. The cause of this

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Table 1. Tissue concentrations of cadmium in *P. amurensis* from field sites in Northern San Francisco Bay, and salinity, oxygen and temperature conditions on collection days in May and June, 1997 (USGS data).

	Site	Cadmium tissue concentration (mg kg^{-1})	Salinity (ppt)	Oxygen saturation (%)	Temperature (°C)
May 1997	4.1	6.09	5	97	19
	6.1	—	7	97	19
	8.1	1.81	17	98	18
	12.5	1.46	22	96	16
June 1997	4.1	7.68	4	102	22
	6.1	3.64	6	98	21
	8.1	1.62	11	95	20
	12.5	1.84	21	92	19

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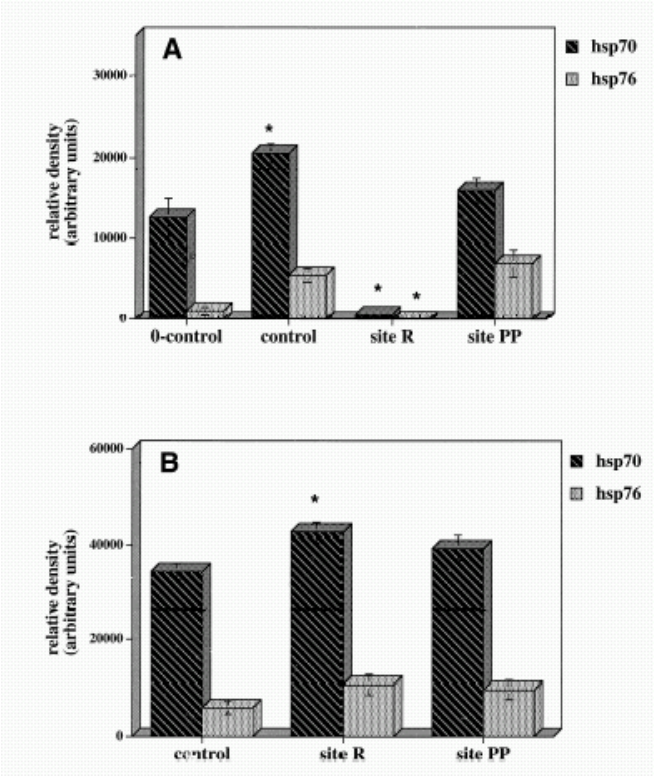

Figure 3. Hsp70 proteins in whole tissue homogenates of *Potamocorbula amurensis* after 7 day *in situ* exposure at two sites (R and PP) at Mare Island Naval Shipyard, Vallejo, CA. (A) Hsp70 in clams before (0-control) and directly after the 7-day exposure; (B) hsp70 in clams after additional heat-shock (37 °C, 15 min) and 6-h recovery period. Graphs show mean densitomer readings and standard errors of protein bands detected by Western blotting; $n = 16$; * significantly different from control; significance level $p < 0.05$.

increase is not known at present. Hsp levels in clams from site PP were not statistically different from the zero time controls. After animals received an additional mild heat-shock, levels of hsp70 in clams exposed at site R were significantly higher than in laboratory controls (figure 3(B)). Chemical analysis of sediment samples revealed higher concentrations of Ni and Cr at site R (59 ppm

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and 57 ppm, respectively) than at site PP (44 ppm and 47 ppm, respectively). No difference between sites was seen regarding the other heavy metals analysed. Concentrations of organochlorine compounds were below detection limits at both sites.

Discussion

Flux in estuarine environmental factors is the norm and seasonal cycles of temperature as well as daily fluctuations (Meury and Kohiyama 1991, Petronini *et al*. 1993, Fader *et al*. 1994, Somero 1995, Roberts *et al*. 1997) must be characterized for their effects on biomarker responses. The two examples presented in this paper indicate the importance of acquiring 'baseline' information on how stress protein expression is influenced by natural variables, as well as a mechanistic understanding of how chemicals, singly or in combination, influence protein expression at the cellular level. Upregulation as well as downregulation of stress proteins needs to be considered as potential indicators of adverse effect in field situations.

We currently lack information on the normal range of values for hsp70 in *P. amurensis*. This important piece of information will become available as laboratory cultures of *P. amurensis* are established, and life cycle studies made possible without the multitude of variables encountered in feral animals. As a consequence, interpretation of data from field studies, such as study no. 1, is difficult without further research which, in part, is discussed below. The series of stations in Northern San Francisco Bay not only follows a cadmium gradient, but also a salinity gradient from almost freshwater (station 4.1) to brackish water (station 12.5) conditions (table 1). Food quality and quantity, and concentrations of other contaminants differ as well, although in a less linear fashion. The data presented in this paper are representative of a trend seen throughout the year, with hsp70/hsp76 concentrations being consistently lower in clams from station 4.1 than in animals from station 12.5 (unpublished data). Cadmium, however, has been shown to induce hsp70 proteins in *P. amurensis* at sublethal concentrations (Clark *et al*. in prep.). Hsp70, but not hsp76, was significantly elevated in clams exposed to 10, 20 and 40 mg l^{-1} Cd for 2 weeks after an additional mild heat-shock. In addition, recently obtained results on the influence of salinity on hsp70 and hsp76 protein expression in *P. amurensis* suggest that salinity is the main variable responsible for lower levels of these proteins seen in clams from station 4.1 and 6.1. Clams collected at 10 ppt were exposed to salinities of 0 ppt, 3 ppt, 6 ppt, 14 ppt and 27 ppt for 24, 48 and 72 h. After 24, 48 and 72 h, hsp70 was twice and hsp76 seven to ten times as high in clams exposed to 27 ppt than to 0 ppt. Research is ongoing.

Mechanisms leading to reduced hsp70 levels may involve disruption of protein synthesis at the transcriptional or translational level, pathological effects and/or reduced energy (ATP) availability. We lack direct information on these processes. However, in study no. 1 low condition indices, low ATP and low glycogen levels were measured in clams from site 4.1 relative to sites 6.1, 8.1 and 12.5 (C. Brown, USGS, Menlo Park, CA, Teresa Fan and R. C. Kaufman, UC Davis, Davis, CA, personal communication). At this site, the low salinities approach the tolerance limit for *P. amurensis*. More energy is probably allocated to osmoregulation, thereby leading to a less favorable energy balance when compared to those clams living at higher salinities. When energy reserves are depleted, organisms may use proteins for additional energy and glucose supplies (Gilbert 1991). Roberts *et al*.

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(1997) and Hofman and Somero (1995) estimate the total cost of protein synthesis constitutes 20–25 % of the energy budget of the bay mussel, *Mytilus edulis*. Increased synthesis of stress proteins would represent an additional energy burden. Furthermore, the function of stress proteins requires ATP; refolding of a protein may consume in excess of 100 ATP molecules (Roberts *et al*. 1997). In conclusion, the depletion of available energy and energy reserves in clams from station 4.1 may result in the observed reduced levels of hsp70 proteins.

Natural stressors such as salinity can be excluded as a cause of the observed effect on hsp70 expression in study no. 2. The two field sites were less than 100 m apart and salinity, pH, oxygen and temperature were almost identical, and well within *P. amurensis*' tolerance range. Clams used in this experiment were collected at the same location and time. However, expression of both hsp70 isoforms was significantly reduced in clams exposed for days at site R. It has been shown that cellular injury resulting from exposure to contaminants can affect cellular processes in target tissues in such a way that biomarker measurements not reflecting the amount of damage can underestimate a real effect (Jimenez *et al*. 1990, Koehler and Pluta 1995). Here, tissue damage is an unlikely cause of the observed effect, since the clams were capable of upregulating stress proteins after receiving an additional heat-shock in the laboratory (in control water). The significantly higher hsp70 levels seen after heat-shock in clams exposed at site R may indicate that the animals were already affected by a stressor, as seen in cadmium-exposed clams after additional heat-shock (Clark *et al*. in prep.). Previous 4-day laboratory exposures of topsmelt (*Atherinops affinis*) embryos to porewater from these sites also resulted in significantly lower hsp70 levels in embryos exposed to site R porewater than in controls and site PP porewater (unpublished data). In addition, average amounts of micronuclei were increased in oyster haemocytes, both after exposure to site R porewater and after *in situ* exposure at R (S. Anderson, UC Davis, Davis, CA, personal communication). An increased number of micronuclei indicates damage at the DNA level (Sadinski *et al*. 1995, Schmid 1975).

Chemical analysis of sediments revealed higher concentrations of Cr and Ni at site R. Chromium and nickel are among seven heavy metals identified as chemicals of ecological concern in the vicinity of both sites (As, Cr, Cu Hg, Ni, Ag, Zn; Tetra Tech EM, Inc. 1998), but the concentrations measured are below concentrations detected in sediment samples from throughout San Francisco Bay (Regional Monitoring Program 1996). In a recent laboratory study, hexavalent chromium was shown to induce expression of hsp70 and hsp76 in *P. amurensis* (Teh *et al*., in prep.). No information is currently available on the combined effects of various metals on stress protein regulation and expression, nor their potential for interaction with other compounds such as PAHs or PCBs.

Little information is available on compounds capable of inhibiting or downregulating hsp70 protein expression. Sanders and Martin (1994) showed that copper inhibits the heat-shock response in sea urchin embryos. Other studies indicate that copper can also act as an inducer (Cochrane *et al*. 1991, Sanders *et al*. 1991, Vedel and Depledge 1996). Edington *et al*. (1989) found that glycerol inhibited stress protein induction in human cell cultures, and several kinds of flavonoids, such as quercetin, genistein and luteorin, inhibited induction of major stress proteins including hsp110, hsp90, hsp70 and hsp40 in human cancer cells, while the synthesis of other proteins was not affected (Hosokawa *et al*. 1990). Even less is known about potential antagonistic, additive or synergistic effects of

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chemicals on the stress protein response in eukaryotes. Koehler and Eckwert (1997) performed laboratory studies with cadmium and lead, cadmium and zinc, or lead and zinc. They concluded that heavy metals in these combinations caused synergistic effects on the stress response of *Oniscus asellus* (Isopoda).

Results from other field studies incorporating stress protein analysis indicate that upregulation as well as downregulation of the response need to be considered as potential indicators of contaminant exposure or effect. Theodorakis *et al*. (1992) performed a study on biomarkers in bluegill sunfish (*Lepomis macrochirus*) exposed to field sediments contaminated with PCBs, PAHs and heavy metals. Hsp70 in gills was above control levels during weeks 1 and 2 of exposure, equal to controls in weeks 4 and 8, and lower than controls by week 16. Triebskorn *et al*. (1997) found in a 2 year field study that hsp70 in liver of brown trout showed marked seasonal differences in controls but not in animals exposed to water from two streams (less polluted, and polluted). In addition, hsp70 levels were consistently lower in trout from the more polluted Koersch river than from the less polluted site. The authors attributed this to pathological effects or energy deficit, but did not show that this was in fact the case. Ultrastructural lesions in liver tissue were increased, and swimming velocity was decreased in trout exposed to water from Koersch river. Veldhuizen-Tsoerkan *et al*. (1991) observed both induction and inhibition of various stress proteins in mussels exposed *in situ* to contaminated sediments for 5 months, after they were given an additional heat shock. Whereas hsp36 was strongly induced, hsp43 and hsp70 were clearly inhibited by this treatment. The authors indicate that inhibition as well as induction of certain stress proteins could serve as indicator of pollutant effect. In amphipods (*Ampelisca abdita*) exposed in the laboratory to sediments from San Francisco Bay, Werner *et al*. (1998) found negative correlations between hsp71 levels and several PAHs, and between hsp64 levels and organotin compounds, whereas hsp64 levels were positively correlated with total PAHs. Inability to link hsp70 levels in field-collected organisms with pollutant concentrations may in part be due to the lack of information on how xenobiotic compounds, singly or in combination, modulate stress protein expression. Therefore, additional carefully controlled experiments are essential.

Furthermore, it is important to remember that the effects of chemicals, and expression of stress proteins are tissue-specific (Dyer *et al*. 1990, Sanders *et al*. 1993, Stringham and Candido 1994, Chapple *et al*. 1997). Vedel and Depledge (1996) concluded that hsp70 levels in gills of shore crabs was not a sensitive indicator of copper exposure, and Pedersen *et al*. (1997) demonstrated in a study on shore crab collected from field sites contaminated with heavy metals, that hsp70 in gill tissue did not reflect the degree of metal exposure. Other tissue, e.g. midgut gland where most of the metals accumulate, were not examined. Ideally, a variety of different organs should be examined in the same individual. Laboratory studies indicate that environmentally relevant metal concentrations cause induction of hsp70 in gill (Williams *et al*. 1996) or liver (Werner I., unpublished data) of rainbow trout. PAHs induced hsp70 in oyster gill tissue (L. Cruz-Rodriguez and F.-L. Chu, College of William and Mary, Gloucester Point, VA, personal communication), and exposure to hexavalent chromium led to increased levels of hsp70 proteins in muscle and gill of *P. amurensis* (Teh *et al*. submitted). If small organisms are studied, whole tissue homogenates may reflect the response, although sensitivity may be diminished. If certain organs and tissues are sites for hsp upregulation, the signal could be diluted by levels from non-responsive tissues

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and organs. Moreover, depending on the species studied, hsp70 proteins may not always be the major responsive group in a particular study organism. Hsp60 was shown to be a more sensitive indicator of chemical stress in several invertebrate species (Bosch *et al*. 1988, Kammenga *et al*. 1988; Werner *et al*. 1998, Cochrane *et al*. 1991).

Biomarker responses are expected to be sublethal, quantifiable, and reliable for interpretation. The latter requirement poses the greatest challenge for potential biomarkers, especially when applied to field situations. There is a need for detailed studies on seasonal and intrapopulation variability of stress protein levels (e.g. Pyza *et al*. 1997). It is obvious that we currently lack thorough knowledge of background levels, endogeneous and exogeneous variation due to seasonal and physiological cycles, and interactions with other cellular processes. In addition, it is important to investigate a variety of organs, and to know which stress protein family is most responsive to stressor effects in the organism studied. The potential for inhibition or downregulation as well as induction or upregulation of stress proteins needs to be included in study designs, and as much information as possible should be collected on all potential stressors, natural and anthropogenic, to which the study organism is exposed. Sensitivity of the stress protein response is high. It indicates deleterious effect at the molecular level, and can be a useful diagnostic tool as part of a suite of biomarkers. Combining biomarkers of exposure and effect at several levels of organization will considerably facilitate interpretation of field data, and help identify deleterious effect caused by xenobiotic compounds.

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