Arch. Environ. Contam. Toxicol. 41, 22–29 (2001)
DOI: 10.1007/s002440010217

ARCHIVES OF
Environmental
Contamination
and Toxicology

Induction of Marine Mollusc Stress Proteins by Chemical or Physical Stress

M. J. Snyder, E. Girvetz, E. P. Mulder

Bodega Marine Laboratory, University of California, Davis, P.O. Box 247, Bodega Bay, California, 94923, USA

Received: 19 September 2000/Accepted: 1 February 2001

Abstract. The cellular stress responses of most organisms in part involve the induction of a class of proteins called heat shock or stress proteins (HSPs) as a result of damage to existing proteins. Cellular proteins can be damaged by chemical exposures known to induce various HSPs. In these experiments, we examine the HSP responses of mussel (*Mytilus galloprovincialis*) and abalone (*Haliotis rufescens*) tissues to both thermal and chemical exposures. HSP70 isoforms, HSP60, and HSP90 all show varying induction capabilities. The results demonstrate that the extent of stress exposure as both a time- and dose-dependent phenomena can be ascertained by examining changes in mollusc HSP protein levels. We also examined the relationship between HSP induction and levels of a mussel cytochrome P450 (CYP4Y1) mRNA in dose-response experiments with the products of biologically degraded weathered crude oil. The increases in HSP70 isoforms and HSP90 were correlated with decreases in CYP4Y1 expression levels in a dose-dependent manner. HSP responses may therefore be a valuable part of a suite of biomarkers in biomonitoring for hydrocarbon exposures in nearshore environments.

The major responses of organisms to acute heat shock (and other physical stressors) are well known to involve changes in gene transcription and translation of heat shock or stress proteins (HSPs). HSPs are coded for by a small set of heat shock genes that show elevated transcription during periods of stress in organisms, ranging from bacteria to humans. Many organisms exhibit increased production of multiple classes of stress proteins: HSP90 (85–90 kDa), HSP70 (68–72 kDa), HSP60 (GroEL, 60 kDa), and a low molecular weight series of HSPs 20–30 (20–30 kDa) (Feder and Hofmann 1999).

HSPs act as molecular chaperones, promoting the initial folding of other proteins at the ribosome and the refolding of unfolded proteins when they are partially denatured (see recent review in Nover and Scharf 1997). Environmental stresses, such as changes in temperature (Hofmann and Somero 1996), hypoxia (Ropp *et al.* 1983), salinity (Gonzalez and Bradley 1994), and metal ion concentration (Steinert and Pickwell 1988; Ryan and Hightower 1994), can all induce the synthesis of HSPs that act to prevent protein aggregation and to maintain functional conformations. A wide range of organic compounds, purportedly causing cellular oxidative stresses, may also induce HSP level changes in many organisms (for review see Feder and Hofmann 1999). The recovery from heat shock and other stress events requires the protein folding abilities of HSPs in all eukaryotes (Feder and Hofmann 1999).

Members of the HSP70 and HSP90 stress protein families (and possibly others) are also involved in the stabilization of different types of intracellular receptors. For instance, both HSP70 and HSP90 are required for stable steroid hormone receptor complexes (Hutchinson *et al.* 1994). HSP90 also binds to and stabilizes the aryl hydrocarbon receptor protein in a state capable of interacting with its substrates (Whitelaw *et al.* 1995).

In this study, we examine the effects of temperature stress and various chemical treatments on the stress protein responses of juvenile abalone (*Haliotis rufescens*). We also examine adult mussel (*Mytilus galloprovincialis*) stress protein and cytochrome P450 mRNA levels in response to both thermal stress and exposure to the microbial degradation products of weathered crude oil. Products of such microbial processes appear to be more toxic to a diverse array of aquatic organisms including mysid larvae and shrimp embryos (*Mysidopsis bahia* and *Palaemonetes pugio*; Middaugh *et al.* 1998), embryonic and larval herring (*Clupea pallasi*; Shelton *et al.* 1999), and inland silversides (*Menidia beryllina*; Middaugh *et al.* 1996).

Materials and Methods

Organisms and Chemicals

Adult mussels (15 g wet weight; 93 ± 7 mm length), *M. galloprovincialis*, were obtained from Hog Island Oyster Company (Tomales Bay, CA). Juvenile abalone (2 g wet weight; 19 ± 2 mm length), *H. rufescens*, were obtained from Bodega Sea Farms (Bodega Bay, CA) but were no longer available for the studies on microbially degraded oil products. All animals were held in ambient sea water (13–15°C) at the Bodega Marine Laboratory and fed either on raw seawater organisms (mussels) or fresh kelp (abalone). The sea water from the Bodega Bay area is considered "clean" (McCain *et al.* 1988), and animals in these experiments are considered to have minimal contact with other anthropogenic chemicals.

Correspondence to: M. J. Snyder; *email:* mjsnyder@ucdavis.edu

All chemicals used were of the highest grade available. Phenobarbital was from RBI (Natick, MA), heptachlor and β-naphthoflavone (βNF) were from Chem Service (West Chester, PA), and pentachlorophenol was from Aldrich. Phenobarbital was chosen as a reference detoxification enzyme inducer in many species (Waxman and Azaroff 1992). βNF is a model hydrocarbon inducer of detoxification responses in many organisms (*e.g.*, Snyder 1998b). The cyclodiene pesticide heptachlor is a persistent organic pollutant present in nearshore marine environments and is known to induce aquatic invertebrate detoxification responses (Snyder 1998a; Masters and Inman 2000).

Pentachlorophenol has been part of wood-bleaching operations on the U.S. West Coast and is a known toxicant to aquatic species such as abalone (*e.g.*, Tjeerdema and Crosby 1992). For all exposure experiments, control animals received only the carrier solution (0.025% 2-methoxyethanol) without the added toxicant. All exposures were done in the laboratory in static 1.5-L aerated seawater jars each with one animal. Water was changed once per day (with toxicant if included). Digestive glands and muscle tissue were individually frozen in liquid nitrogen and stored at −70°C until analysis.

In the last few years, investigators have shown that microbial communities inhabiting sediments near natural oil seeps degrade crude oil to products that are more toxic to aquatic species than the parent compounds (Middaugh *et al.* 1996, 1998; Shelton *et al.* 1999). Metabolites from microbial degradation of oil were obtained by the methods of Middaugh *et al.* (1998) using artificially weathered Santa Barbara crude oil instead of Alaska North Slope crude (weathering was accomplished by autoclaving the oil for 30 min). Eight liters of 1 μm-filtered sea water including 3.27 g/L of Bushnell-Haas broth (Difco) with 4% inoculum of a mixture of microbial mat organisms from Coal Oil Point Seep sites and 0.2% weathered oil (w/v). The culture was maintained at 18°C for 2 weeks with constant stirring with a Teflon-coated stir bar and a magnetic plate. Prior to use in assays, bacteria were removed by centrifugation and the supernatant (degraded oil, DO) was filtered to 0.45 μm. Mussels were individually exposed to different DO concentrations in static aerated 1.5-L jars, and digestive glands removed, frozen in liquid nitrogen, and stored at −70°C until analysis. Water-soluble components of the degraded oil have not been well characterized. They include 10–50- and 200-fold increases in neutral and acidic fractions, respectively (Middaugh *et al.* 1996) and the total water-soluble fraction is 20-fold higher in degraded versus sterile cultures (Middaugh *et al.* 1998).

Alterations in Stress Protein Levels

Levels of stress proteins were examined in response to various stress exposures. The following physical and chemical stress conditions were used: (1) One- hour heat-shock from ambient (14°C) sea water to 30°C for juvenile abalone or adult mussels, followed by return to ambient temperature for recovery. (2) Static sea water exposures (with constant aeration and daily water with chemical or carrier changes) to individual chemicals or carrier alone.

Tissue samples (mussel digestive gland, abalone digestive gland, abalone foot muscle) were homogenized in 0.5 ml (abalone tissues) or 1 ml (mussel digestive glands) of buffer K containing 5 mmol/L $NaHPO_4$, 40 mmol/L HEPES, pH 7.4, 5 mmol/L $MgCl_2$, 70 mmol/L potassium gluconate, and 150 mmol/L sorbitol). Homogenates of each individual were centrifuged 10 min at $10{,}000 \times g$, and supernatants were combined with equal volumes of 2× SDS sample buffer (Laemmli 1970) and boiled for 5 min. Determination of individual homogenate protein content was by BioRad assay. Equal amounts of supernatant protein from each individual (and tissue; 25 μg of abalone muscle, 50 μg abalone digestive gland, and 50 μg mussel digestive gland protein) were analyzed by the following Western blotting procedures.

Discontinuous SDS gels (1 mm) were 6.2% for the stacking gel and 12% for the resolving gel. After running for 2 h at 150 V (BioRad Miniprotean II), SDS gels were electroblotted (BioRad Mini Trans-Blot) onto nitrocellulose membranes (for 1 h at 100 V). The protein band quality of each Western blot was checked by visualization of proteins by ponceau S staining.

The following antibodies from StressGen (Victoria, B.C.) were used to detect HSPs by Western blotting: mouse monoclonal anti-HSP60 (SPA-805), mouse monoclonal anti-HSP70 (SPA-822), and mouse monoclonal anti-HSP90 (SPA-830). The secondary antibody was goat anti-mouse IgG, conjugated to peroxidase (Sigma), and visualization was performed using ECL reagents (Amersham) and exposure of blots to X-ray film. Blot band intensities were compared by laser-scanning densitometry and NIH image software. For each blot, known amounts of standard HSP60 (human HSP60, StressGen), HSP70 (human HSP70, StressGen), and HSP90 (bovine HSP90, StressGen) were included, and scanned intensities of all HSP bands were compared against the intensities of the standards for each blot. The results are compared as HSP70 or HSP90 ng equivalents. Statistical comparisons of each treatment against respective controls were done using *t* tests or ANOVA followed by Student-Newman-Keuls multiple comparison tests.

Mussel Cytochrome P450 mRNA Levels

Additional *M. galloprovincialis* were individually exposed to DO concentrations (n = 4 for each) of 0, 0.1, 1.0, or 5% (16.7 ppm) or a model hydrocarbon βN F (0.06 ppm or 0.33 ppm) for 24 h as indicated above. Digestive glands were dissected, and total RNA was prepared from each individual tissue using the Totally RNA procedure (Ambion). Thirty micrograms of each digestive gland RNA preparation (determined by A_{260} absorbance measurements) was run on 1% denaturing agarose gels and blotted on to nylon membranes before fixation by UV cross linking (Stratagene). Mussel CYP4Y1 cDNA (Snyder 1998b) was ^{32}P-labeled (Stratagene), and blots were all probed together at 42°C in 50% formamide, 5x Denhardt's, 2x SSPE, 0.5% SDS, and 100 μg/ml denatured herring sperm DNA. Blots were washed last in 0.1x SSPE, 0.1% SDS at 60°C, and exposed to X-ray film for 2 days. Films were scanned and analyzed as indicated for Western blots above. All mRNA levels are compared to those of control (untreated) mussel digestive glands. Statistical comparisons were made by ANOVA and Student-Newman-Keuls tests as indicated previously.

Results

Abalone

The effects of various chemical treatments on abalone digestive gland HSP70 levels are shown in Figure 1. As in mussels, three bands of 67, 70, and 74 kDa were recognized in digestive gland with the HSP70 antibody. Treatment with a variety of toxicants for 24 h resulted in elevation of all three HSP70 bands.

Time course experiments with the chlorinated pesticide heptachlor (Hc) are shown in Figure 2. HSP67 levels mirrored those of HSP70 in digestive glands and are not shown. In digestive glands (Figure 2A), there was a significant elevation in HSP70 and HSP90 in the first day of exposure. HSP70 levels did not increase further during the second day of exposure and steadily declined to control levels by day 5, 3 days of recovery. HSP74 and HSP90 levels increased dramatically on the second

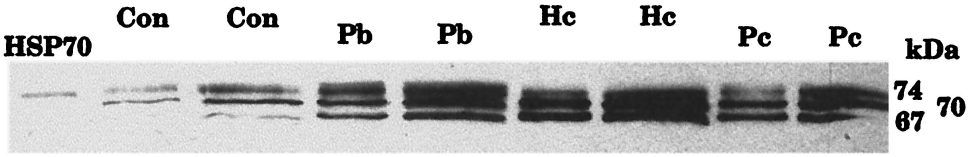

Fig. 1. Western immunoblot of HSP70 protein levels in digestive glands of *H. rufescens* following 24-h xenobiotic exposures. The position of human HSP70 protein and the calculated sizes of abalone HSP70 proteins are shown. Con = control vehicle treated abalone; Pb = phenobarbital (1.4 ppm); Hc = heptachlor (0.7 ppm); Pc = pentachlorophenol (0.1 ppm)

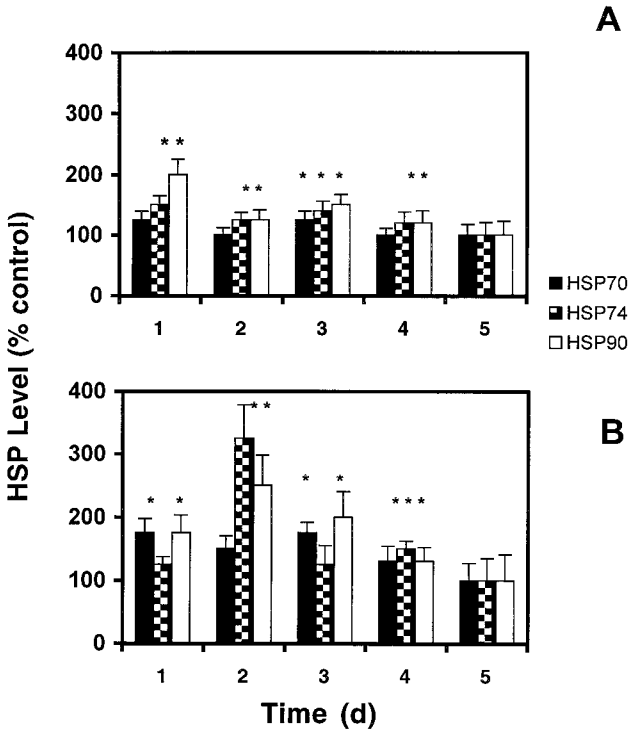

Fig. 2. HSP70 isoforms (HSP70 and HSP74) and HSP90 protein expression levels following 1 day of heptachlor exposure (0.7 ppm) and 4 additional days of recovery in (A) muscle and (B) digestive gland of juvenile *H. rufescens*. Values represent the mean of four determinations plus one standard deviation (as percentage of control, vehicle-treated abalone); asterisks indicate significant differences from controls

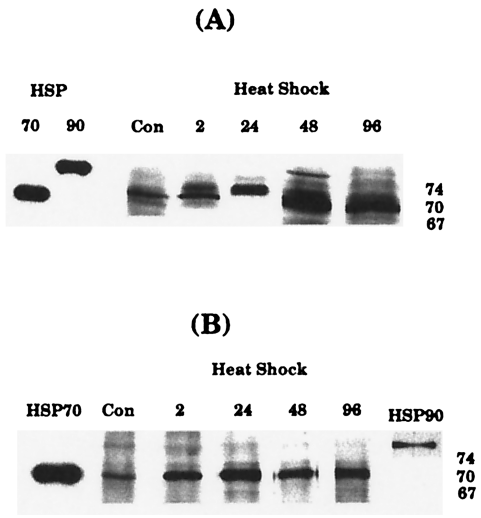

Fig. 3. Representative Western immunoblots of HSP70 and HSP90 protein levels in (A) muscle and (B) digestive gland of juvenile *H. rufescens* following 1-h heat shock (31°C) and the indicated recovery times at 15°C. The positions of human HSP70 and HSP90 proteins are shown

day of Hc exposure and remained significantly elevated until day 5.

In abalone muscle, HSP70 was not significantly affected by Hc exposure except on day 3, 1 day of recovery from exposure (Figure 2B). HSP74 and HSP90 were elevated by Hc and remained high until day 5.

Time course studies on the effects of single 31°C heat shock on abalone digestive gland and muscle are shown in Figures 3–4. In digestive gland, at 2 h of recovery following the heat shock, levels of HSP70 and HSP90 were significantly reduced compared to untreated control abalone. At 24 h of recovery, both HSP67 and HSP70 were nearly absent, whereas HSP74 was significantly elevated. At 48 h of recovery, HSP67 was still reduced, but HSP70 and HSP74 remained high and HSP90 returned to control levels. HSP70 and HSP74 remained high at 4 days of recovery.

A single HSP60 band was also found in all abalone digestive gland treatments (data not shown). The effects of DO concentration were less dramatic in abalone digestive gland than in mussel. DO concentrations of 1–5% and heat shock gave HSP60 levels of 120% and 140% of control values, respectively.

Mussel

Three protein bands of 67, 70, and 74 kDa were recognized in mussel digestive gland by the HSP70 antibody (Figure 5A,B). Two sets of DO exposure experiments were performed. Figure 6A shows the results of DO concentration and heat shock on digestive gland HSP70 levels. There was no significant effect on levels of the 67-kDa protein in the 24-h exposures. Levels of HSP70 and HSP74 were significantly reduced at 2 h of recovery following the heat shock (Figure 6A). Levels of both the 70- and 74-kDa proteins were significantly elevated by concentrations of 0.01–5% DO and by 15 h after recovery

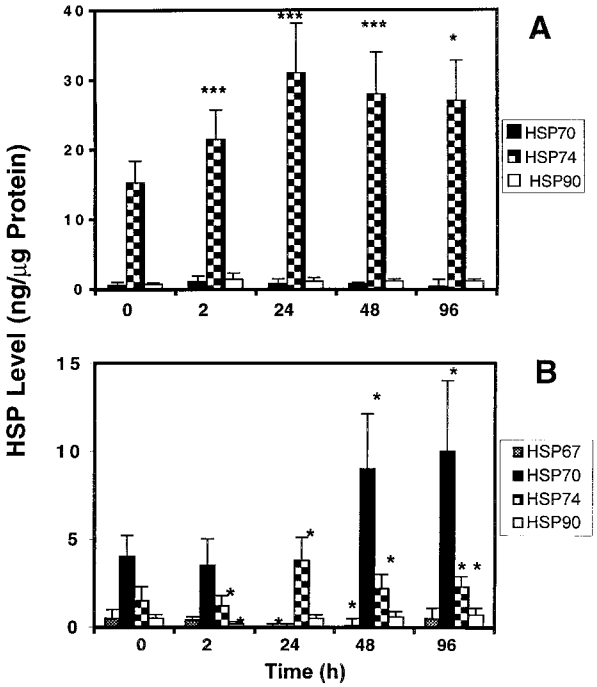

Fig. 4. *H. rufescens* (A) muscle and (B) digestive glands HSP70 and HSP90 levels following 1-h heat shock (37°C) and the indicated recovery times at 15°C. HSP levels (ng/μg tissue protein) represent the mean of four determinations, bars represent one standard deviation from the mean, and asterisks indicate significant differences from control abalone

following 1 h of heat shock. The 1% DO concentration was chosen to perform the time-dependence of HSP70 induction (Figure 6B). Significant elevations (versus control levels) in all HSP70 protein bands were noted within 4–8 h of exposure to 1% DO. HSP70 and HSP74 were not further elevated after 8 h of exposure, although there seemed to be a greater increase in HSP67 between 24 and 36h.

A single band of 60 kDa was found for HSP60 in all mussel treatments (data not shown). There was a dose-dependent increase in HSP60 levels with DO, from 125% of control levels at 1% DO to 225% at 5% DO. Heat shock (1 h at 37°C, followed by 15 h recovery at ambient temperature) resulted in a 200% increase in HSP60 in mussel digestive tissue.

The effects of increasing DO concentrations on mRNA levels of the cytochrome P450 CYP4Y1 are shown in Figure 7A,B.. A dose-dependent significant reduction in CYP4Y1 levels (Figure 7b) were found in *M. galloprovincialis* digestive gland with DO concentrations from 0–5%, similar to that found for βNF (Figure 7A).

Discussion

HSP Responses

Both natural and artificial toxicants affect the expression of HSPs in *M. galloprovincialis* and *H. rufescens* tissues. One goal of this work was to examine HSP levels in tissues that have been largely ignored by most investigators. Generally, mollusc HSP levels have been examined in mollusc gills (Hofmann 1999; Feder and Hofmann 1999) known to be thermally labile (*e.g.*, Hofmann and Someno 1996). Some workers have examined mussel mantle or adductor muscle (Sanders *et al.* 1992; Sanders and Martin 1993; Chapple *et al.* 1997), or oyster hemocytes (Tirard *et al.* 1995). Neither digestive tissues nor foot muscle have been yet examined in any mollusc prior to this study.

The magnitudes of HSP inductions in these experiments were variable depending on HSP isoform. In *M. galloprovincialis*, the major constitutive HSP70 isoform appears to be HSP74 (Figure 5). HSP74 was inducible by heat shock but only several fold versus control samples. HSP67 was little changed by heat shock, but elevated by several hundred fold within 1 h of DO exposure. HSP70 was induced by heat shock or DO exposure to 5–15 times control values. For *H. rufescens*, HSP70 appears to be the major constitutive isoform in both muscle and digestive glands, although HSP74 was present in many control digestive gland samples. Abalone tissues proved less inducible (in the magnitude of HSP inductions with various stresses). Reported HSP induction values for each of these protein families (HSP60, HSP70, HSP90) in aquatic invertebrates range from zero to several hundred fold higher than found in controls (reviewed in Hofmann 1999; Feder and Hofmann 1999).

The HSP60 family of stress proteins is less well understood in terms of stress responses, especially in aquatic organisms. Members of this family in eukaryotes are required for normal mitochondrial functions in terms of importing proteins and folding them into their proper functional conformations (Ryan *et al.* 1997). Results with *M. galloprovincialis* demonstrate that HSP60 may prove useful as an additional marker of stress induced by exposure to hydrocarbons. HSP60 is increased in mussel gills by heat stress (Sanders *et al.* 1992; Sanders and Martin 1993), copper exposures (gill and mantle; Sanders *et al.* 1991; Sanders and Martin 1993), and long-term exposure in contaminated field sites (Sanders and Martin 1993; Lundebye *et al.* 1997). Elevated HSP60 was correlated with decreased survival of amphipods (*Ampelisca abdita*) in sediments contaminated by high levels of polyaromatic hydrocarbons (Werner *et al.* 1998). The alga *Isochrysis galbana* also displayed significantly elevated HSP60 levels on exposure to crude oil fractions and individual hydrocarbons (Wolfe *et al.* 1999).

Another aspect of HSP dynamics is the dramatic reduction in HSP70 isoforms and HSP90 in digestive tissues of both molluscs within several hours of heat shock (Figure 2, 5, 6). Recovery of normal or significantly elevated HSP levels occurred by 15 h in *M. galloprovincialis*. In contrast, *H. rufescens* digestive tissues required 24–96 h to restore or increase HSP levels. These results are similar to that found for lobsters, *Homarus americanus*, midgut gland (hepatopancreas) following heat shock (Spees *et al.* 2001). Similarly, Krebs and Feder (1997) found that *Drosophila* digestive tissues were particularly vulnerable to the effects of heat shock and required much longer to recover than other tissues. It is likely that heat shock disrupts cellular compartments and liberates digestive enzymes that result in greater damage to these tissues, hence longer recovery times.

Mollusc stress protein responses can persist for long periods

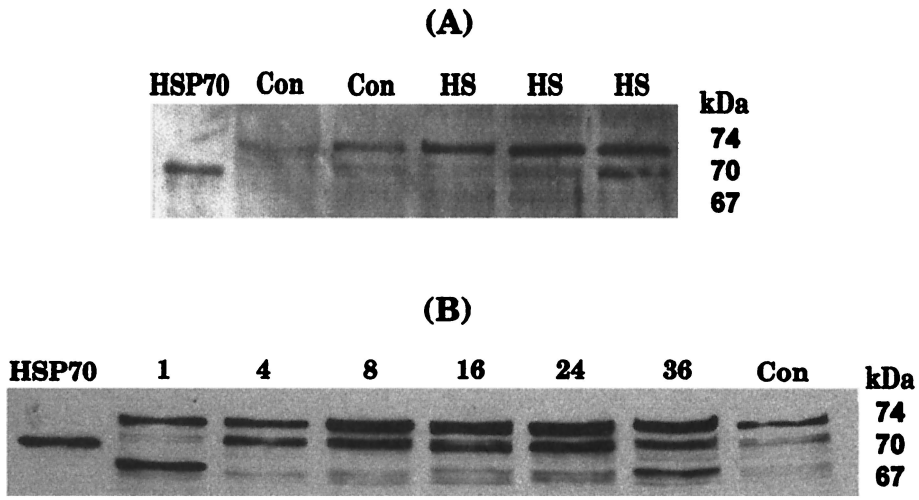

Fig. 5. Western immunoblots of individual adult mussel *Mytilus galloprovincialis* digestive glands in (A) control or heat-shocked (1 h at 28°C, followed by 15 h recovery at 15°C), or (B) control (Con) or following exposure (1–36 h) to 1% biologically degraded oil (DO) for the times indicated. The position of human HSP70 protein is shown in the first lane, and the calculated sizes of mussel HSP70 proteins are listed on the right

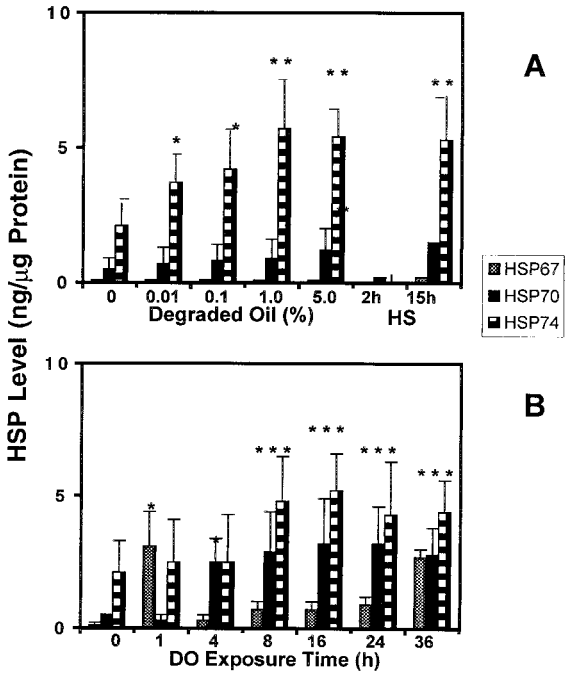

Fig. 6. Adult *M. galloprovincialis* digestive gland HSP70 isoform levels following (A) 24-h exposure to increasing DO concentrations or 1-h heat shock at 28°C followed by either 2 or 15 h of recovery at 15°C, and (B) time course exposure to 1% DO. Values represent the mean of four determinations (ng HSP/μg tissue protein), bars represent one standard deviation from the mean, and asterisks indicate significant differences from controls for each HSP

after the initial stress exposure (Sanders and Martin 1993). In the experiments reported here, abalone required 2–3 days to recover HSP levels to control values after heptachlor exposure. Clegg *et al.* (1998) showed that HSP70 isoforms were induced in oyster gills by heat shock and levels remained higher than controls for at least 2 weeks in gills. In short-term experiments, mussel gills exhibited elevated HSP70 for 5 days following heat shock (Bradley *et al.* 1998; Tedengren *et al.* 1999).

HSPs and Degraded Oil Metabolites

Biologically degraded oil experiments provided an example of the effects of natural toxicants on mussel HSP protein levels in time course and concentration regimes. Water-soluble components of oil from natural nearshore seep sites, such as the Santa Barbara channel, contain hundreds to thousands of different hydrocarbons and potential microbial toxins as well. The mixture is known to inhibit sea star embryo growth in the parts per million range (Spies and Davis 1982). There is evidence that much of the oil toxicity can be explained by the large amounts of lower molecular weight hydrocarbons (Spies 1987). For oil spill emergencies, *in situ* bioremediation is increasingly recommended as a useful response strategy (Lee and de Mora 1999). The techniques involve fertilization with added nitrogen and phosphorous and/or seeding with microbes capable of metabolizing oil compounds. Recent work has indicated that this approach may lead to the production of microbial metabolites that have increased toxicity versus the parent oil in both fish and invertebrates (Middaugh *et al.* 1996, 1998; Shelton *et al.* 1999). The identities of the toxic microbial products of oil are currently unknown, as are their biochemical effects on wild species. This information warrants further study of the biochemical and molecular responses of aquatic species for the future prediction of the effects of *in situ* oil spill responses on local environments.

The identification of a mussel cytochrome P450, CYP4Y1 (Snyder 1998b), allowed the comparison of this P450 mRNA and HSP protein levels following exposure to the DO metabolites in *M. galloprovincialis* digestive gland. The significant reduction in CYP4Y1 mRNA with DO was similar to that found previously with βNF (Snyder 1998b). High concentrations of either the pure hydrocarbon (0.3 ppm) or the degraded mixture (16.7 ppm) inhibit CYP4Y1 expression by nearly 100%. Corresponding increases in mussel digestive gland HSP70 and HSP74 with DO exposures occur in a dose-dependent manner (Figure 6A). Hydrocarbon inhibitions of P450 enzyme activities and specific P450 protein levels have been reported in *M. edulis* (Stegeman 1985) and other molluscs, including *Chryptochiton stelleri* and *Octopus pallidus* and in mammals (Schlenk and Buhler

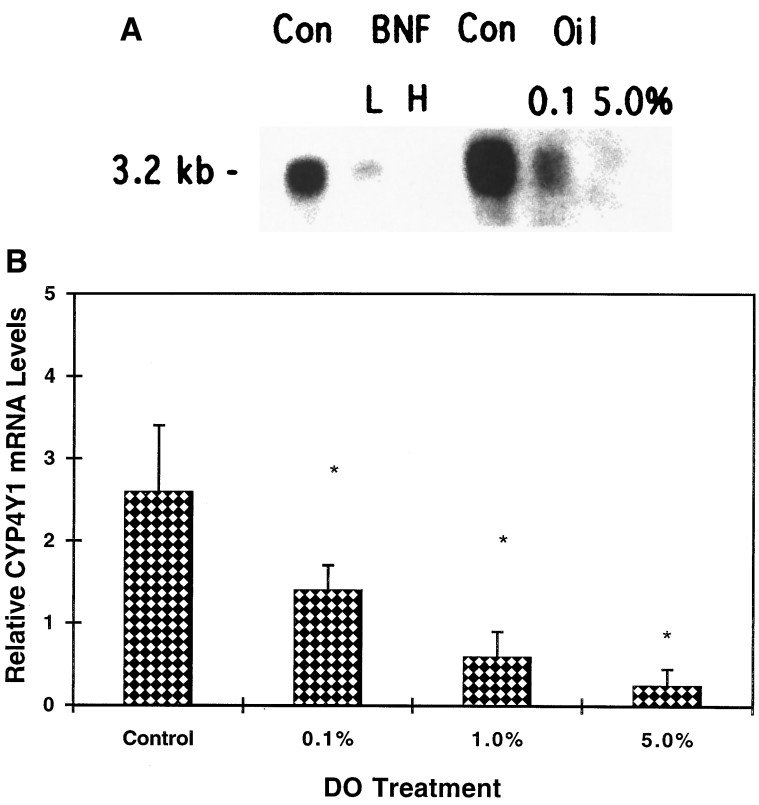

Fig. 7. Adult *M. galloprovincialis* digestive gland CYP4Y1 mRNA levels following 24-h exposure to the indicated βNF or DO concentrations. (A) Representative Northern blot of βNF (Snyder 1998b) and DO exposures; (B) CYP4Y1 mRNA values representing the mean of four determinations at the indicated DO concentrations plus one standard deviation (as percentage of control, untreated mussels), and asterisks indicate significant differences from controls

1989; Cheah *et al.* 1995; Jones and Riddick 1996). It is not known whether this inhibition is maintained over longer exposures. Also, it is unclear if the inhibition is mediated by a specific hydrocarbon receptor mechanism yet to be discovered in aquatic invertebrates.

HSPs as Biomarkers

HSPs have proven useful as part of a suite of biochemical markers of xenobiotic exposure in molluscs. Other such protein markers include, for example, cytochrome P450, esterase, multixenobiotic resistance protein, glutathione S-transferase, and glutathione peroxidase (Darby *et al.* 1993; Dauberschmidt *et al.* 1997; Fitzpatrick *et al.* 1997; Epel 1998; Snyder 1998a, 1998b, 2000). By themselves, HSP inductions are markers of multiple stress exposures, whereas specific proteins such as P450s are generally responsive to a limited group of xenobiotic exposures (Dogra *et al.* 1998; Snyder 1998a). HSPs therefore cannot indicate exposure to any specific stressor without direct observation under carefully controlled conditions (Ryan and Hightower 1994; Feder and Hofmann 1999). When combined with additional physiological observations, HSPs can, however, be indicative of the severity of the stress exposure. For example, Steinert and Pickwell (1993) demonstrated that *M. edulis* gill tissue showed 12-fold induction of HSP70 with tributyltin exposure, and these results correlated directly with a reduction in mussel filtration rates.

The potential of toxicants to interact with lipophilic constituents of membranes and proteins is also related to its ability to induce HSP production. Mechanisms of HSP induction following stress exposures have been studied. Elevated HSP90 expression is found in rat brain, liver, and lung after oral dosing with polycyclic halogenated hydrocarbons and chlorinated or organophosphate pesticides (Bagchi *et al.* 1996). The results were attributed to tissue differences in oxidative stress resulting from these exposures. Ansamycin antibiotics bind directly to HSP90 and change its function from protein refolding to enhancing protein degradation in mammalian carcinoma cells (Schneider *et al.* 1996). HSP induction correlates with the degree of cytotoxicity of a substance (Neuhaus-Steinmetz and Rensing 1997). Also stressors that act via disruption of normal protein folding and transit within cells, the so-called unfolded protein response, also elevate HSP expression (Welihinda *et al.* 1999).

In aquatic invertebrates, a few studies have shown that xenobiotic toxicity may also result from its metabolism to more reactive products. For example, oyster and abalone can produce reactive pentachlorophenol metabolites capable of damaging cellular components (Tjeerdema and Crosby 1992; Shofer and Tjeerdema 1993). Reactive products of hydrocarbons bound to DNA and cellular proteins were produced by cultured digestive gland cells from *M. edulis* (Mitchelmore *et al.* 1998) and cell fractions from a variety of marine invertebrates (Marsh *et al.* 1992). Such compounds have also been reported in mussel tissues following the Aegean Sea oil spill (Solé *et al.* 1996). Further study of xenobiotics, such as DO, and their effects on mussels and other aquatic invertebrates should be directed toward the examination of tissue and cellular changes that may lead to long-term changes in organismal health and reproductive impairment.

Acknowledgments. We thank A. Hamdoun and Dr. G. N. Cherr for help in obtaining the microbial oil metabolites. This work was supported by NSF grant 96-31128 and by the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, under grant number NA66RG0477, project number R/A-108, through the California Sea Grant College Program. The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its subagencies. The U.S. government is authorized to reproduce and distribute this publication for governmental purposes. Contribution 2137 from the Bodega Marine Laboratory, University of California at Davis.

References

Bagchi D, Battacharya G, Stohs SJ (1996) *In vitro* and *in vivo* induction of heat shock (stress) protein (Hsp) gene expression by selected pesticides. Toxicology 112:57–68

Bradley BP, Olsson B, Brown DC, Tedengren M (1998) HSP70 levels in physiologically stressed Baltic Sea mussels. Mar Environ Res 46:397–400

Chapple JP, Smerdon GR, Hawkins AJS (1997) Stress-70 protein induction in *Mytilus edulis*: tissue-specific responses to elevated temperature reflect relative vulnerability and physiological function. J Exp Mar Biol Ecol 217:225–235

Cheah DMY, Wright PFA, Holdway DA, Ahokas JT (1995) *Octopus pallidus* cytochrome P-450: characterisation and induction studies with β-naphthoflavone and Aroclor 1254. Aquat Toxicol 33:201–214

Clegg JS, Uhlinger KR, Jackson, SA, Cherr GN, Rifkin E, Friedman CS (1998) Induced thermotolerance and the heat shock protein-70 family in the Pacific oyster, *Crassostrea gigas*. Mol Mar Biol Biotechnol 7:21–30

Darby PC, Gallgher EP, Di Giulio RT (1993) Glutathione enzymes, glutathione content and *t*-butyl hydroperoxide induced lipid peroxidation in the gill and digestive gland of the estuarine clam, *Rangia cuneata*. Comp Biochem Physiol 106C:809–814

Dauberschmidt C, Dietrich DR, Schlatter C (1997) Esterases in the zebra mussel *Dreissena polymorpha*: activities, inhibition, and binding to organophosphates. Aquat Toxicol 37:295–305

Dogra SC, Whitelaw ML, May BK (1998) Transcriptional regulation of cytochrome P450 genes by different classes of chemical inducers. Clin Exp Pharmacol Physiol 25:1–9

Epel D (1998) Use of multidrug transporters as first lines of defense against toxins in aquatic organisms. Comp Biochem Physiol 120A:23–28

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61:243–282

Fitzpatrick PJ, O'Halloran J, Sheehan D, Walsh AR (1997) Assessment of a glutathione S-transferase and related proteins in the gill and digestive gland of *Mytilus edulis* (L.), as potential organic pollution biomarkers. Biomarkers 2:51–56

Gonzalez CRM, Bradley BP (1994) Are there salinity stress proteins? Mar Environ Res 35:79–83

Hofmann GE (1999) Ecologically relevant variation in induction and function of heat shock proteins in marine organisms. Am Zool 39:889–900

Hofmann GE, Somero GN (1996) Interspecific variation in thermal denaturation of proteins in the congeneric mussels *Mytilus trossulus* and *M. galloprovincialis*: evidence from the heat-shock response and protein ubiquitination. Mar Biol 126:65–75

Hutchinson KA, Dittmar KD, Czar MJ, Pratt WB (1994) Proof that HSp70 is required for assembly of the glucocorticoid receptor into a heterocomplex with HSP90. J Biol Chem 269:5043–5049

Jones EJ, Riddick DS (1996) Regulation of constitutive rat hepatic cytochromes P450 by 3-methylcholanthrene. Xenobiotica 26:995–1012

Krebs RA, Feder ME (1997) Tissue-specific variation in Hsp70 expression and thermal damage in *Drosophila melanogaster* larvae. J Exp Biol 200:2007–2015

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lee K, de Mora S (1999) *In situ* bioremediation strategies for oiled shoreline environments. Environ Tech 20:783–794

Lundebye A-K, Langston WJ, Depledge MH (1997) Stress proteins and condition index as biomarkers of tributyltin exposure and effect in mussels. Ecotoxicology 6:127–136

Marsh JW, Chipman JK, Livingstone DR (1992) Activation of xenobiotics to reactive and mutagenic products by the marine invertebrates *Mytilus edulis*, *Carcinus maenas* and *Asterias rubens*. Aquat Toxicol 22: 115–127

Masters PM, Inman DL (2000) Transport and fate of organochlorines discharged to the salt marsh at upper Newport Bay, California, USA. Environ Toxicol Chem 19:2076–2084

McCain BB, Brown DW, Krahn MM, Myers MS, Clark RC, Chan S-L, Malins DC (1988) Marine pollution problems, North American West Coast. Aquat Toxicol 11:143–162

Middaugh DP, Chapman PJ, Shelton ME (1996) Responses of embryonic and larval inland silversides, *Menidia beryllina*, to a water-soluble fraction formed during biodegradation of artificially weathered Alaska North Slope crude oil. Arch Environ Contam Toxicol 31:410–419

Middaugh DP, Shelton ME, McKenney CL, Cherr G, Chapman PJ, Courtney LA (1998) Preliminary observations on responses of embryonic and larval pacific herring, *Clupea pallasi*, to neutral fraction biodegradation products of weathered Alaska North Slope oil. Arch Environ Contam Toxicol 34:188–196

Mitchelmore CL, Birmelin C, Chipman JK, Livingstone DR (1998) Evidence for cytochrome P-450 catalysis and free radical involvement in the production of DNA strand breaks by benzo[a]pyrene and nitroaromatics in mussel (*Mytilus edulis* L.) digestive gland cells. Aquat Toxicol 41:193–212

Neuhaus-Steinmetz U, Rensing L (1997) Heat shock protein induction by certain chemical stressors is correlated with their cytotoxicity, lipophilicity and protein-denaturing capacity. Toxicology 123: 185–195

Nover L, Scharf KD (1997) Heat stress proteins and transcription factors. Cell Mol Life Sci 53:80–103

Ropp M, Courgeon AM, Calvayrac R, Best-Belpomme M (1983) Possible role of the super oxide ion in the induction of heat shock and specific proteins in aerobic *Drosophila melanogaster* cells during return to normoxia after a period of anaerobiosis. Can J Biochem Cell 61:456–461

Ryan JA, Hightower LE (1994) Evaluation of heavy-metal ion toxicity in fish cells using a combined stress protein and cytotoxicity assay. Environ Toxicol Chem 13:1231–1240

Ryan MT, Naylor DJ, Hoj PB, Clark MS, Hoogenraad NJ (1997) The role of molecular chaperones in mitochondrial protein import and folding. Int Rev Cytol 174:127–193

Sanders BM, Martin LS (1993) Stress proteins as biomarkers of contaminant exposure in archived environmental samples. Sci Total Environ 139/140:459–470

Sanders BM, Pascoe VM, Nakagawa PA, Martin LS (1992) Persistence of the heat-shock response over time in a common *Mytilus* mussel. Mol Mar Biol Biotechnol 1:147–154

Sanders BM, Martin LS, Nelson WG, Phelps DK, Welch WJ (1991) Relationships between accumulation of a 60 kDa stress protein and scope-for-growth in *Mytilus edulis* exposed to a range of copper concentrations. Mar Environ Res 31:81–97

Schlenk D, Buhler DR (1989) Determination of multiple forms of cytochrome P-450 in microsomes from the digestive gland of *Cryptochiton stelleri*. Biochem Biophys Res Commun 163:476–480

Schneider C, Sepp-Lorenzino L, Nimmesgern E, Ouerfelli O, Danishefsky S, Rosen, N, Hartl FU (1996) Pharmacologic shifting of a balance between protein folding and degradation mediated by Hsp90. Proc Natl Acad Sci USA 93:14536–14541

Shelton ME, Chapman PJ, Foss SS, Fisher WS (1999) Degradation of weathered oil by mixed marine bacteria and the toxicity of accumulated water-soluble material to two marine crustacea. Arch Environ Contam Toxicol 36:13–20

Shofer SL, Tjeerdema RS (1993) Comparative disposition and biotransformation of pentachlorophenol in the oyster (*Crassostrea gigas*) and abalone (*Haliotis fulgens*). Pest Biochem Physiol 46: 85–95

Snyder MJ (1998a) Identification of a new cytochrome P450 family, CYP45, from the lobster, *Homarus americanus*, and expression following hormone and xenobiotic exposures. Arch Biochem Biophys 249:187–190

Snyder MJ (1998b) Cytochrome P450 enzymes belonging to the CYP4 family from marine invertebrates. Biochem Biophys Res Commun 249:187–190

Snyder MJ (2000) Cytochrome P450 enzymes in aquatic invertebrates: background, recent advances, and future directions. Aquat Toxicol 48:529–547

Solé M, Porte C, Biosca X, Mitchelmore CL, Chipman JK, Livingstone DR, Albaigés J (1996) Effects of the "Aegean Sea" oil spill on biotransformation enzymes, oxidative stress and DNA-adducts in digestive gland of the mussel (*Mytilus edulis* L.). Comp Biochem Physiol 113C:257–265

Spees JL, Snyder MJ, Chang SA, Chang ES (2001) Thermal acclimation and stress in the American lobster, *Homarus americanus*: equivalent temperature shifts elicit unique gene expression patterns for molecular chaperones and polyubiquitin. Physiol Biochem Zool (in press)

Spies RB (1987) The biological effects of petroleum hydrocarbons in the sea: Assessments from the field and microcosms. In: Boesch E, Rabalais N (eds) Long-term environmental effects of offshore oil and gas development. Elsevier, London, pp 411–466

Spies RB, Davis PH (1982) Toxicity of Santa Barbara Seep oil to starfish embryos. Part 3. Influence of parental exposure and the effects of other crude oils. Mar Environ Res 6:3–12

Stegeman JJ (1985) Benzo[a]pyrene oxidation and microsomal enzyme activity in the mussel (*Mytilus edulis*) and other bivalve mollusc species from the western North Atlantic. Mar Biol 89: 21–30

Steinert SA, Pickwell GV (1988) Expression of heat shock proteins and metallothionein in mussels exposed to heat stress and metal ion challenge. Mar Environ Res 24:211–214

Steinert SA, Pickwell GV (1993) Induction of HSP70 proteins in mussels by ingestion of tributyltin. Mar Environ Res 35:89–93

Tedengren M, Olsson B, Reimer O, Brown DC, Bradley BP (1999) Heat pretreatment increases cadmium resistance and HSP 70 levels in Baltic Sea mussels. Aquat Toxicol 48:1–12

Tirard CT, Grossfeld RM, Levine JF, Kennedy-Stoskopf S (1995) Effect of hyperthermia *in vitro* on stress protein synthesis and accumulation in oyster hemocytes. Fish Shellfish Immunol 5:9–25

Tjeerdema RS, Crosby DG (1992) Disposition and biotransformation of pentachlorophenol in the red abalone (*Haliotis rufescens*). Xenobiotica 22:681–690

Waxman DJ, Azaroff L (1992) Phenobarbital induction of cytochrome P-450 gene expression. Biochem J 281:577–592

Welihinda AA, Tirasophon W, Kaufman RJ (1999) The cellular response to protein misfolding in the endoplasmic reticulum. Gene Expr 7:293–300

Werner I, Kline KF, Hollibaugh JT (1998) Stress protein expression in *Ampelisca abdita* (Amphipoda) exposed to sediments from San Francisco Bay. Mar Environ Res 45:417–430

Whitelaw ML, McGuire J, Picard D, Gustafsson J-A, Poellinger L (1995) Heat shock protein HSP90 regulates dioxin receptor function *in vivo*. Proc Natl Acad Sci USA 92:4437–4441

Wolfe MF, Olsen HE, Gasaud KA, Tjeerdema RS, Sowby ML (1999) Induction of heat shock protein (hsp)60 in *Isochrysis galbana* exposed to sublethal preparations of dispersant and Prudhoe Bay crude oil. Mar Environ Res 47:473–489