

# Expression of HSP70 in *Mytilus californianus* following exposure to caffeine

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**Abstract** Caffeine, a biologically active drug with many known molecular targets, is recognized as a contaminant of marine systems. Although the concentrations of caffeine reported from aquatic systems are low (ng/l– $\mu$ g/l), harmful ecological effects not detected by traditional toxicity tests could occur as a result of caffeine contamination. We used Hsp70, a molecular biomarker of cellular stress, to investigate the sub-lethal cellular toxicity of environmentally relevant concentrations of caffeine on the mussel *Mytilus californianus*, a dominant species in the rocky intertidal zone along the Oregon Coast. Hsp70 concentrations in the gill and mantle tissue of mussels exposed to 0.05, 0.2, and 0.5  $\mu$ g/l of caffeine for 10, 20, and 30 days were compared to basal levels in control mussels. Hsp70 in the gill tissue of *M. californianus* had an initial attenuation of the stress protein followed by a significant up-regulation relative to controls in all but the 0.5  $\mu$ g/l treatment. Hsp70 in the mantle tissue of mussels exposed to caffeine did not differ from control mussels. This study provides laboratory evidence that environmentally relevant concentrations of caffeine can exert an effect on *M. californianus* gill tissue at the molecular-level.

**Keywords** Mussel · Ecophysiology · Sublethal toxicity · Gill tissue · Mantle tissue · Heat shock protein

## Introduction

Caffeine is among the most common organic contaminants of surface waters and has been detected in streams, lakes, estuaries, and oceans (Buerge et al. 2003). The concentrations of caffeine typically reported from aquatic environments are in the low nanogram per liter range. Yet caffeine is a drug with known physiological effects, even at low concentrations, and is constantly released to the aquatic environment via wastewater effluent and other anthropogenic activities. It remains unclear whether such low concentrations of caffeine, 1/1000000th the concentration of a drip-brewed cup of coffee, have any measurable impact on aquatic organisms.

As the world's most consumed non-prescription drug, the majority of research on caffeine has been aimed at understanding its effects on humans (Benowitz 1990). The modes of action of caffeine include: (i) antagonizing adenosine receptors; (ii) inhibiting phosphodiesterases; (iii) sensitizing ryanodine-sensitive channels in the sarcoplasmic, and endoplasmic reticulum to activation by calcium; and (iv) antagonizing GABA<sub>A</sub> receptors at the benzodiazepine-positive modulatory site (Daly 2007). In humans, cytochrome P450 is involved in the metabolism of caffeine (Berthou et al. 1991). Although cytochrome P450 is highly conserved, caffeine is more toxic to other organisms, including horses, dogs, parrots, and spiders due to their underdeveloped capacity to metabolize the drug (Pollack et al. 2009).

Increasing concerns about the prevalence of caffeine in the aquatic environment and the uncertainty of the effects on aquatic organisms have fueled a few studies investigating the effects of caffeine on aquatic organisms. Fraker and Smith (2004) found that environmentally relevant levels of some organic wastewater contaminants, including

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caffeine, had behavioral and physiological effects on northern leopard frog (*Rana pipiens*) tadpoles. Gagné et al. (2006) reported that, although caffeine was not very toxic to trout (*Oncorhynchus mykiss*) hepatocytes, it produced lipid peroxidation at a threshold concentration of 14  $\mu\text{M}$  after 48 h exposure at 15°C. Moreover, in vitro incubation of caffeine with trout microsomes increased both the rate of oxidation of NADPH and the lipid peroxidation in microsomes after a 60 min incubation at 30°C, suggesting that caffeine exposure could lead to oxidative damage at low milligram per liter concentrations.

Other studies suggest that environmentally relevant levels of caffeine are not a threat to aquatic organisms. Smith and Burgett (2005) reported that environmentally relevant concentrations of caffeine (0.6–600  $\mu\text{g/l}$ ) did not affect the survivorship or activity of American toad (*Bufo americanus*) tadpoles. Similarly, Quinn et al. (2008) classified caffeine as non-toxic based on acute (mortality) and chronic (feeding behavior, attachment, and growth) toxicity tests on the freshwater cnidarian *Hydra attenuata*. Although caffeine exposure impaired the reproduction of the water flea *Ceriodaphnia dubia* (IC<sub>50</sub> = 44 mg/l) and inhibited the growth of the fathead minnow *Pimephelas promelas* (IC<sub>50</sub> = 71 mg/l) (Moore et al. 2008), the authors concluded that given the environmental concentrations reported in the literature, caffeine should pose negligible risk for most aquatic vertebrate and invertebrate organisms. The authors did caution that there could be potential effects from long-term exposure to environmental levels of caffeine.

Few studies have examined the effect of caffeine on marine organisms. Cheney (1945) found that caffeine affects oxygen consumption and the normal rate of cleavage division in the fertilized eggs of the sea urchin, *Arbacia punctulata*. Nath and Rebhun (1976) reported inhibition of mitosis in sea urchin eggs exposed to caffeine. Caffeine induced bleaching in the tropical sea anemone *Aiptasia pulchella*, ostensibly through its effect on levels of intracellular protein phosphorylation (Sawyer and Muscatine 2001). Increased duration of exposure to caffeine resulted in a significant increase in the percent of symbiotic algae released from *A. pulchella*. A subsequent study on the effect of caffeine on four species of coral endosymbionts found that algal cultures grown in 60 mg/l caffeine exhibited up or down regulation of a number of proteins associated with glycolysis, photosynthesis, and the physiological stress response (Pollack et al. 2009). Heatshock proteins (HSP) were up-regulated 2 to threefold in the coral endosymbiont *Symbiodinium* sp. and down-regulated up to ninefold in *Symbiodinium goreau*.

The previous studies show that caffeine can have a deleterious effect on aquatic organisms. However, since these studies have overwhelmingly focused on relatively

high concentrations of caffeine that are unlikely to be found in aquatic environments, the risk from exposure to low concentrations of caffeine, including effects from long-term exposure and sublethal effects, remains unclear. Some challenges exist in detecting impacts to organisms by contaminants that are found at low concentrations in the environment. Contaminants can cause changes at all levels of biological organization and subtle or chronic biological effects resulting in irreversible long-term changes could be occurring in apparently healthy ecosystems (Hyne and Maher 2003). These changes may not be initially detected if the focus of ecological risk assessment is on coarse levels of biological organization.

HSPs have been suggested as sensitive biomarkers of the sub-lethal or subtle toxicity of pollutants (Sanders 1990; Depledge 1994; Lewis et al. 1999) because they are involved in protecting and defending cells from environmental offenses (Sanders 1990) and their induction is much more responsive than traditional indices of contaminant effects (Feder and Hofmann 1999). HSPs are proteins that are synthesized in response to cellular stress that induces denaturation of other proteins. The 70 kDa family (Hsp70) is most highly conserved and has been most extensively studied. Four key features of Hsp70 have driven its application in environmental risk assessment: (1) it is highly conserved in a wide variety of organisms from bacteria to humans; (2) it responds to a variety of environmental stresses, including thermal stress, heavy metal exposure, organic pollutants, hypoxia/anoxia, salinity stress, and exposure to ultraviolet radiation; (3) its induction is very sensitive to environmental assaults; and (4) its expression has been correlated to other toxicological end points.

Concerns about the effects of stress history, induction thresholds and timing on expression of Hsp70 can be minimized in laboratory studies that assess the effect of a single contaminant and employ adequate controls for comparison. Collecting organisms from the same environment with adequate laboratory acclimation can help ensure that test organisms, including controls, have a similar stress history. Measuring the response of Hsp70 in different tissues and over several time periods reduces the risk of missing tissue-specific or time-dependent induction. Hsp70 remains a potent and sensitive tool for investigating the toxicity of contaminants of concern (Mukhopadhyay et al. 2003).

This study investigated the toxicity of environmentally relevant concentrations of caffeine on the common intertidal mussel *Mytilus californianus* using Hsp70 as a biomarker of the effects of this ubiquitous aquatic contaminant. Mussels are ideal marine species to investigate the potential effects of contaminants because they are stationary, widespread, easy to collect, filter feeders that have been used extensively in toxicity studies using Hsp70

as a biomarker of effect (Laporte 2005). The study addressed the following questions:

- Is Hsp70 expression in *M. californianus* increased by exposure to environmentally relevant concentrations of caffeine?
- Is there a difference in the expression of Hsp70 in different tissues of *M. californianus* exposed to caffeine?
- Does the expression of Hsp70 change with the duration of exposure to caffeine?

## Methods

### Study organisms and acclimation conditions

*Mytilus californianus* mussels were collected from a study site in the coastal town of Yachats, Oregon (N 44°18'81.3" W 124°06'52.3"). Mussels (10–12 cm) were collected from the same area of the mid-intertidal zone on a single day in September 2008 and transported in a chilled cooler to Portland State University. In the laboratory, mussels were acclimated in 10 gallon aquaria filled with 26 L of UV filtered deionized water, adjusted to a salinity of 32 PSU (Instant Ocean). The aquaria were connected to in-line chillers (Sealine) and to an in-tank filter system. The acclimation temperature was 10–11°C, the temperature at the time of collection. After 3 days of acclimation the experiment was started. Mussels were held under a natural light cycle of approximately 12 h of daylight and 12 h of dark.

A pilot experiment verified that in-tank caffeine concentration did not decrease appreciably in seawater over a 7 day period. To test for caffeine degradation, a tank was set up with twelve mussels and spiked with a known concentration of caffeine. This set up was identical to the experimental set up. After 1 week, 1 L samples of water were removed from the tank and caffeine degradation from initial concentration was verified using solid phase extraction (EnviCarb, Supelco) and GC–MS analysis.

### Experimental design

*Mytilus californianus* were exposed to one of four caffeine concentrations ranging from 0.05 to 0.5 µg/l. These are field relevant concentrations reported from coastal marine systems (Siegener and Chen 2002; Weigel et al. 2004; Peeler et al. 2006; Comeau et al. 2008). Caffeine solution used to spike tanks at the beginning of the experiment and after each water change, was prepared fresh using Caffeine ReagentPlus® powder (Sigma-Aldrich) dissolved in nanopure water. Control mussels were not exposed to caffeine. Twelve randomly selected mussels were placed in

each of the five caffeine treatments (0, 0.05, 0.1, 0.2, or 0.5 µg/l). The tank water was changed every 5 days and spiked with caffeine using a freshly prepared stock solution. After each water change, mussels were fed 5 mL of Instant Algae (Reed Mariculture).

Four mussels from each treatment were sacrificed after 10, 20, and 30 days. Mussels were dissected and a sample of gill lamellae and mantle tissue from each mussel was stored in separate cryovials. Sample tissues were immediately frozen in liquid nitrogen and stored in a –80°C freezer until tissue preparation. No test organisms died during the experiment. Hsp70 concentrations in mussel tissues were subsequently measured using the protocol described by Buckley et al. (2001).

### Tissue preparation

Approximately 100 mg of tissue was ground in a small centrifuge tube using a pestle. The ground tissue was then homogenized in a 1:1 (volume/volume) solution of 2× homogenization buffer consisting of 32 mM Tris–HCl (pH 6.8) and 2% sodium dodecyl sulfate (SDS). The homogenate was heated to 100°C for 5 min and then centrifuged at 12,000×g for 10 min. The supernatant was collected into a new centrifuge tube and the pellet discarded. The protein content of the supernatant was determined by the Bradford assay (Pierce, Rockford, IL, USA). The supernatant was stored in a –80°C freezer until further analysis. Gill lamellae and mantle tissue were prepared using the same procedure.

### Electrophoresis and immunochemical assay of Hsp70

Equal amounts of protein (15 µg) from each sample were separated via SDS-polyacrylamide gel electrophoresis on ten lane 10% polyacrylamide gels. One lane in each gel was loaded with Precision Plus Protein Kaleidoscope standard (Bio-Rad) and one with a standard sample used to calibrate protein expression within and among gels. The standard sample was from one of the experimental mussel samples. Following approximately 1.5 h of electrophoresis at 150 V, the proteins were transferred to nitrocellulose blots using wet electrophoretic transfer at 30 V overnight (approximately 15 h).

Nitrocellulose blots were blocked in 5% non-fat dry milk in 1× PBS for 1 h. The immunodetection was performed using an Hsp70 polyclonal antibody (Hsp70 (K-20)-R, Santa Cruz Biotechnology) that reacts only with the Hsp70 isoform. Blots were then incubated for 1.5 h in the primary antibody diluted 1:5000 in 5% non-fat dry milk in 1× PBS, followed by three 10-min washes in 1× PBS with 0.1% Tween-20. Blots were then incubated for 1 h in peroxidase-conjugated goat-anti-rabbit antibody (Thermo

Scientific) diluted 1:5000 in 5% non-fat dry milk in 1× PBS, followed by six 5-min washes in 1× PBS with 0.1% Tween-20. Blocking and incubation in primary and secondary antibody was done under constant agitation at room temperature.

Western blots were developed using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) and exposed to film (Kodak BioMax MR-1). Scanning densitometry, using ImageJ (<http://rsb.info.nih.gov/ij/>), was used to determine the levels of Hsp70 expression relative to the standard sample.

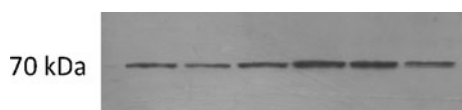
#### Data analysis

Differences in Hsp70 expression were analyzed with a two-way ANOVA with caffeine concentration and exposure duration as the fixed factors and Hsp70 concentration as the dependent variable. Significant fixed factors were further investigated using a one-way ANOVA and post-hoc Tukey tests. Statistical analyses were performed using SPSS 17.0 (2008).

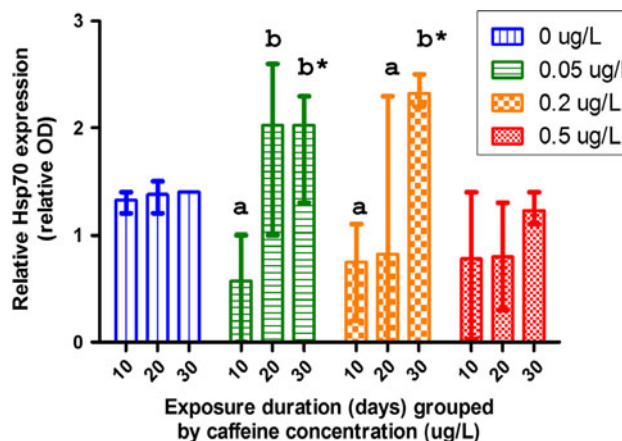
During the first 10 days of the experiment the chiller for the caffeine treatment of 0.1 µg/l experienced a power outage. When the power outage was discovered, the temperature in that tank was 23°C and could have been at that temperature for a maximum of 48 h. Although, mussel samples from that treatment were collected and analyzed for Hsp70 expression, the data are not included since this represented a thermal change that likely affected Hsp70 expression.

#### Results

The primary antibody used against Hsp70 was highly specific producing bands only at approximately 70 kDa (Fig. 1). Hsp70 expression in the gill tissue responded to caffeine exposure. The response was time dependent, but did not exhibit a linear dose–response relationship with increasing concentration, although interaction between dose and time was significant (Fig. 2; Table 1). Some of the trends are not significant due to high variability in Hsp70 concentrations within treatment. However, the gill lamellae and mantle tissue of control mussels exhibited basal levels of Hsp70 that were similar for the duration of the experiment and had low variability.



**Fig. 1** Representative western blot depicting Hsp70 bands in gill tissue from *M. californianus*



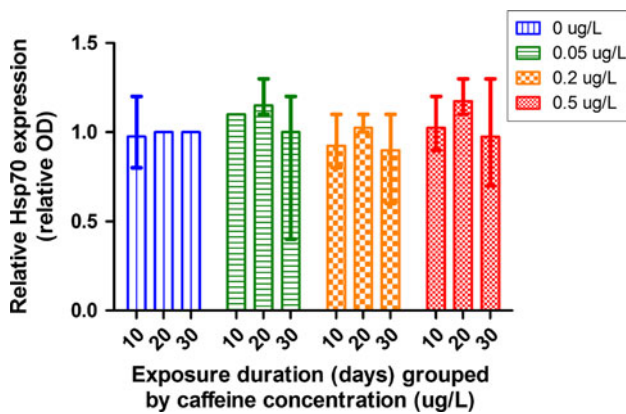
**Fig. 2** Mean Hsp70 expression in the gill lamellae of *M. californianus* exposed to three concentrations of caffeine for 10, 20, and 30 days. Error bars represent the range of Hsp70 concentrations ( $n = 4$ ). Significant differences in Hsp70 concentration for exposure duration within caffeine dose are indicated by the letter convention ( $P$  value  $< 0.05$ ). An asterisk indicates that the Hsp70 expression for that exposure duration was significantly different from the control ( $P$  value  $< 0.05$ )

**Table 1** Summary of two-way ANOVA results for the gill tissue

Source	Sum of squares	df	F	$P$ value
Dose	2.336	3	3.104	0.039
Time	6.296	2	12.548	0.000
Dose × time	6.140	6	4.079	0.003

Caffeine initially produced an inhibitory effect on Hsp70 followed by a time and dose dependent recovery. After 30 days, Hsp70 in the mussels exposed to 0.05 and 0.2 µg/l caffeine was up-regulated relative to controls. At 10 days of exposure to caffeine, mean Hsp70 levels of mussels in all three caffeine treatments were lower than in the control mussels. This trend was not statistically significant because individual mussels exhibited marked variability in the response of Hsp70, but some of the mussels in each of the three treatments had very low or no detectable Hsp70. Similar variability in Hsp70 was not exhibited by the control mussels during the time course of the experiment.

Exposure to caffeine at 0.05 µg/l induced a moderate up-regulation of Hsp70 in the gill lamellae of *M. californianus* after 20 days of exposure. Hsp70 levels remained elevated after 30 days of exposure. In the 0.2 µg/l treatment, the levels of Hsp70 did not exhibit an up-regulation until 30 days of exposure. The maximum Hsp70 levels detected were similar in the 0.05 and 0.2 µg/l treatments. Interestingly, in the highest caffeine treatment (0.5 µg/l), a similar increase in Hsp70 expression was not observed during the duration of this experiment.



**Fig. 3** Relative Hsp70 expression in the mantle tissue of *M. californianus* exposed to three concentrations of caffeine for 10, 20, and 30 days. Error bars represent the range of Hsp70 concentrations ( $n = 4$ )

**Table 2** Summary of the two-way ANOVA results for the mantle tissue

Source	Sum of squares	df	F	<i>P</i> value
Dose	0.181	3	2.308	0.930
Time	0.121	2	2.323	0.112
Dose $\times$ time	0.056	6	0.356	0.902

*M. californianus* exposed to caffeine exhibited a different pattern of Hsp70 expression in the gill lamellae and the mantle tissue. After 30 days, Hsp70 expression in the mantle tissue (Fig. 3) was unresponsive to caffeine exposure (Table 2).

## Discussion

Caffeine, a potent neuroactive drug, is recognized as an ubiquitous contaminant in aquatic systems. Traditional ecotoxicology endpoints suggest that the levels of caffeine currently detected in aquatic systems do not pose a threat to aquatic organisms. There remains a potential for sublethal effects not detected by traditional endpoints and effects from long-term exposure to low levels of caffeine.

This study demonstrates that Hsp70 in the gill tissue of *M. californianus* responds to exposure at environmentally relevant concentrations of caffeine. The response of Hsp70 in the gill tissue exhibited a complex pattern across dose and time. Initially, caffeine appears to have an attenuating effect on Hsp70 expression for all caffeine levels tested. This trend, however, was not significant, likely due to the small sample size and high variability of measured Hsp70 concentrations. High variability in Hsp70 expression in

response to contaminant exposure can mask trends and has been reported in other studies (Staempfli et al. 2002; Laporte 2005).

Increasing the duration of exposure resulted in up-regulations of Hsp70, at low to moderate caffeine concentrations. Hsp70 was up-regulated after 20 days in the 0.05  $\mu\text{g/l}$  caffeine treatment and after 30 days in the 0.2  $\mu\text{g/l}$  treatment, but did not exhibit a similar increase in the 0.5  $\mu\text{g/l}$  treatment over the 30 day duration of this experiment. This type of Hsp70 response to caffeine exposure may indicate a quenching phenomenon (Arts et al. 2004) whereby high levels of stress limit HSP induction.

Only one previous study has investigated the response of Hsp70 to caffeine exposure in aquatic organisms. Pollack et al. (2009) assessed the effect of caffeine on coral algal endosymbionts by identifying proteins sensitive to caffeine exposure. Hsps were up-regulated two to threefold in the coral endosymbiont *Symbiodinium* sp. and down-regulated up to ninefold in *Symbiodinium goreau*. However, the concentration of caffeine used to incubate coral algal endosymbionts (Pollack et al. 2009) falls within the range that would be toxic if found in human blood serum. Therefore, the effect on HSP proteins observed at this high concentration of caffeine is not surprising since it can potentially inhibit many cellular processes. All known stresses, if sufficiently intense, induce HSP expression (Feder and Hoffman 1999).

In contrast, the concentrations of caffeine used in this study were in the ng/l to  $\mu\text{g/l}$  level. Due to the large diversity of Hsp70 inducers, the cellular stress response is thought to be triggered by different mechanisms of toxicity, among which protein damage (e.g., misfolding) would be the common link (Ait-Aissa et al. 2000). There is currently no evidence that the low levels of caffeine tested in this experiment are proteotoxic.

Prolonged oxidative stress from chronic exposure to caffeine could result in an up-regulation of Hsp70 as the organism attempts to cope with cellular damage. Gagné et al. (2006) reported that exposure to caffeine could lead to oxidative damage of trout hepatocytes. However, the concentrations of caffeine at which trout hepatocytes were incubated were in the mg/l range, 1,000 times higher than the concentrations tested in this study. The role of oxidative stress in inducing an Hsp70 response in the gill of *M. californianus* exposed to low levels of caffeine could be investigated further by verifying that oxidative stress is occurring (e.g., quantifying lipid peroxidation).

Other mechanisms not related to proteotoxicity may also induce an Hsp70 response. In humans, caffeine is able to significantly block adenosine receptors at low serum concentrations ( $\mu\text{mol}$ ) and this is considered the most common mode of action of caffeine (Fredholm et al. 1999). Blocking of adenosine receptors by caffeine results in a loss of

the inhibitory effect of adenosine and triggers a catecholaminergic response. Catecholamines have previously been shown to up-regulate intracellular and extracellular Hsp72 in laboratory rats (Johnson et al. 2005).

Several studies have identified neuroendocrine and nervous system functions in molluscs that are analogous to the hypothalamic-pituitary system of vertebrates; similar elements are at the basis of the response and triggering (Stefano et al. 2002; Fabbri et al. 2008). For example, adenosine receptors have been reported from the mussel *Mytilus edulis*. Theophylline, which bears structural and pharmacological similarity to caffeine, blocked the inhibitory effects of a potent adenosine analog on neurotransmitter release in in vitro preparations from the pedal ganglia of *M. edulis* (Barraco and Stefano 1990) In the oyster *Crassostrea gigas*, circulating noradrenaline and dopamine have been shown to increase in response to physiological stress (Lacoste et al. 2001a).

Assuming that the mode of action of caffeine on adenosine receptors is similar between vertebrates and molluscs, exposure to low levels of caffeine could stimulate the release of catecholamines resulting in induction of Hsp70 in *M. californianus*. In at least one study, noradrenaline was shown to induce the Hsp70 gene promoter in the oyster *Crassostrea gigas* and abalone *Haliotis tuberculata* (Lacoste et al. 2001b). The authors postulated that the integrated response to stress is related to the heat shock response.

Cell signal transducers, such as changes in intracellular pH, cyclic AMP, Ca<sup>2+</sup>, Na<sup>+</sup>, inositol trisphosphate, protein kinase C, and protein phosphatases, have also been implicated in the modulation of Hsp70 expression (Kiang and Tsokos 1998). For example, a study of U-937 human promonocytic cells showed that treatment with the cAMP increasing agent isoproterenol plus theophylline decreased basal levels of Hsp70 (Vilaboa et al. 1995). Caffeine can affect many of these cell signal transducers. Caffeine's effect on cell signal transducers could potentially mediate an Hsp70 response.

While the potential mechanisms detailed above could explain the observed up-regulation of Hsp70 in the gill lamellae of *M. californianus* with caffeine exposure, they do not explain the transient and concentration-dependent attenuation of Hsp70 observed here. A quenching phenomenon of Hsp70 has been described in some organisms exposed to some types of contaminants. Arts et al. (2004) proposed that the ability to inhibit HSP induction might indicate a more toxic response than producing elevated levels of these proteins, which indicates that the organism is able to maintain homeostasis under environmental assault.

In discussing this phenomenon, Eckwert et al. (1997) proposed that the dose–response curve for Hsp70 could be divided into three sections: (1) homeostasis: a state of basal

Hsp70 expression, (2) compensation: a state of stress accompanied by Hsp70 induction, and (3) non-compensation: a state of severe stress and pathological damage blocking Hsp70 expression. This type of response was observed in the rotifer *Brachionus plicatillis*. Hsp60 levels of *B. plicatillis* exposed to crude oil in the laboratory were only higher than control rotifers at very low concentrations of crude oil (Wheelock et al. 1999). The model proposed by Eckwert et al. (1997) does not account for the duration of the stress, but the duration of stress can interact with dose to alter the response of Hsp70.

Such a quenching phenomenon could explain the time and concentration-dependent attenuation of Hsp70 observed in this study. In the case of the gill lamellae of *M. californianus*, the attenuation of Hsp70 was transient and concentration-dependent. Gill tissue from mussels exposed to the lowest caffeine concentration exhibited a faster recovery to levels equal to the control mussels. The gill tissue from all but the highest treatment eventually exhibited a time-dependent up-regulation of Hsp70, relative to controls. The actual mechanism(s) involved in quenching (or attenuating) the HSP response is poorly investigated, but may be the result of tissue damage (Eckwert et al. 1997). This is not likely to be the case with caffeine since the attenuation of Hsp70 in the gill lamellae was transient.

Unlike the gill lamellae, the mantle tissue of *M. californianus* did not exhibit an Hsp70 response over the duration of this experiment. Although HSP expression is an ubiquitous molecular mechanism for coping with stress, the HSP response to stresses can be tissue-specific (Feder and Hofmann 1999). Chapple et al. (1997) found tissue-specific inducibility of Hsp70 in *M. edulis* despite all tissues being exposed to the same temperatures. Compared with mantle and adductor muscle tissues, gill tissue showed the greatest increase in levels of Hsp70 proteins. Other tissues of *M. californianus* may exhibit an Hsp70 response to caffeine exposure, but this remains to be tested.

Caffeine is a potent neuroactive drug that is recognized as an ubiquitous contaminant in aquatic systems. *M. californianus* exposed in the laboratory to environmentally relevant concentrations of caffeine exhibited an Hsp70 response. Since Hsp70 affords cellular protection from environmental assaults, both the up-regulation after prolonged exposure and the potential attenuation of the response should be investigated further.

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