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# Heat shock protein response to thermal stress in the Asiatic clam, *Corbicula fluminea*

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**Abstract**

A recent approach to evaluate environmenta induced damages has been damages has been suggested, based on the stress response. The approach involves the detection of stress protein induction in organisms to infer about environmental conditions in their surroundings. However, to be an indicator of adverse biological effects in the environment, the elevation of stress proteins should be compared to a response pattern for the experimental species. Juvenile *Corbicula fluminea*, collection from a control site, were submited to heat-shock stress in the laboratory to obtain the stress response pattern under normal and extreme conditions. Acclimated to 26 °C, the specimens were submited to 29, 32, 35 and 38 °C, for 96 h. After 1, 2, 4, 8, 24, 48, 72 and 96 h of exposure, clams were removed from each vial and prepared for stress protein analysis. Animals from the control site were frozen in liquid nitrogen at the time of collection, and prepared for stress protein analysis. Hsp60 and 70 were detected by immunoreactivity after separation on 12.5% polyacrylamide gels and transference to nitrocellulose by western blotting, to determine the stress protein concentrations. The result showed that hsp70 increased at 4 h from the beginning of the experiment and progressed over the 96 h experimental period in animals exposed to 35 °C. However hsp70 levels decreased between 4 h and 24 h for the clams stressed at their lethal temperature of 38 °C. Immunoblotting with hsp60 showed similar reactivity. At 38 °C there was an increase in the amount of hsp60 at 4 h, reaching a maximum eight-fold level at 8 h. By 96 h, the amount decreased to levels lower than those observed at 4 h. At 38 °C the level of hsp60 began to decrease at 8 h and continue to decline to 24 h when the clams died. The data support the hypothesis of increasing concentrations of stress protein until the heat shock approaches the thermal limits for the species. The results of this research suggest the usefulness of using the stress response as a diagnostic in environmental toxicology. They confirm that the sps response may serve as a valid biomonitoring tool under chronic, sublethal exposures when it is still possible to prevent effects at organismal or higher organizational levels.

## 1. Introduction

The Asiatic clam *Corbicula fluminea* is a successful North American invader which has proven to be of major ecological and economic concern in fresh water habitats (Rutledge, 1982). Since its introduction in the western United States (Morton, 1973), *C. fluminea* has dispersed and become adapted to a major portion of fresh water habitats (Britton and Morton, 1979). This species burrows into well-aerated substrates early in the life cycle (Aldridge, 1976), a behavior which provides protection against control agents (Kraemer et al., 1986). Factors which have contributed to its successful introduction include adaptation to a wide range of substrates (Sinclair, 1964; White, 1979) and its dispersal hability (Kraemer, 1979). More importantly, Coldiron (1975) determined that *C. fluminea* is preadapted to a board range of temperature regimes. The lethal temperature for this clam ranges from 38 °C to 39 °C and optimum temperatures for growth is above 24 °C (O'Kane, 1976).

Various strategies for control have been investigated, however environmentally acceptable methods have been unsuccessful (Rutledge, 1982). Use of high temperatures to manage the Asiatic clam in electrical power plant facilities has been tried where heated water or steam is available (Mattice, 1979). The broad range of temperature tolerance in the species necessitates additional investigation onto a cellular level response to heat stress.

Recently, the heat shock protein response of organisms to a variety of stessors, including elevated temperatures, has provided a means to explain tolerance at the biochemical level. The rapid synthesis of a suite of heat shock proteins (hsp's) is a fundamental cellular response to a variety of stressors, not just heat, in prokaryotes and eukaryotes (Schlesinger, 1986), hence they are now referred to as *stress proteins* (Lindquist, 1986). Their accumulation appears to enhance the cell's ability to recover from stress (Schlesinger, 1986), and they are considered to play an important role in physiological adaptation (Sanders, 1988; Sanders et al., 1992) as well as in organismal adaptation (Sanders et al., 1991) and acquired tolerance (Dean Atkinson, 1982; Landry et al., 1989; Stephanou et al., 1983; Dyer et al., 1991). Stress proteins have also been suggested as biological indicatiors of environmental contamination and to predict adverse effects to toxicant exposure (Sanders, 1990).

The purpose of this investigation was to determine the stress protein response to elevated temperatures in the Asiatic clam, *C. fluminea*, including temperatures at its thermal limits. The results will have a bearing on the use of thermal control of the species, but more importantly they can provide baseline information on the stress protein response in this opportunistic species whose responses to environmenta stress can be adapted for biomonitoring.

## 2. Materials and methods

*Corbicula fluminea* specimens, ranging in size from 9 to 11 mm were collected from the Clear Creek, Denton Country Texas. This creek has been served as a control site for experiments on environmental assessment. Clams were collected from the creek after the water temperature at the site had remained constant at 26 °C for 15 days. Therefore the animals were considered to be acclimated to this temperature. Some animals were immediately frozen in liquid nitrogen at the time of collection to serve as controls for the following experiments.

Immediately after the clams were returned to laboratory, they were transferred to open vials which were then submerged in 4–1 containers whose temperatures had been adjusted and maintained at 26 (control), 29, 32, 35 and 38 °C for 96 h with control controlable heaters. Each container contained filtered creek water and two replicated of 16 animals. One aquarium was filled up with dechlorinated tap water and adjusted to 26 °C. After 1, 2, 4, 8, 24, 48, 72 and 96 h of exposure, two replicate clams were removed from each vial and prepared for stress protein analysis.

Sample preparation was performed according to the methods of Dyer (1991). Clams were opened by cutting the aductor muscle, and the tissue was removed, homogenized, and sonicated in 150 ul of buffer (150 mM TRIS-HCL, pH 7.8, 1 $\mu$M phenylmethylsufonyl fluoride). Homogenates were centrifuged at 10,000 × *g*, at 4 °C for 30 min. The supernatents were then collected, and protein concentrations were determined (Bradford, 1976).

Proteins were separated on 12.5% polycrylamide gels with a 5% stacking gel as described by Blattler et al. (1972), using the buffer system described by Laemmli (1970). Prestained, low molecular weight standards were used to identify individual stress proteins. After electrophoresis, the proteins were transferred to nitrocellulose by Western blotting using a transfer buffer containing 1% SDS, 0.192 M glycine in 0.025 M-TRIS and 20% methanol, by volume, pH 8.3. The gels were subjected to 25 v for 15 h at 4 °C. Transfer were blocked in 25 mM TRIS-HCI (pH 8.0) buffer containing 3% BSA, 137 mM NaCl, and 2.7 mM KCI (TBS). Transfers were then washed with TBS containing 0.05% Tween-20 (TTBS).

The nitrocellulose was probed using a polyclonal antibody raised against hsp60 from the moth *Heliothis sp.* (StressGen Biotechnology Corp., Victoria, B.C. Canada). Blots were incubated at room temperature with hsp60 antibody for 90 min and rinsed several times with TBS containing 0.5% Tween-20 (TTBS). The blots were incubated with goat anti-rabbit alkaline phosphatase conjugated for 90 min. The blots were washed several times with TTBS and TBS. Color development of the immunoreactive bands was initiated with 5-bromo 4-chloro 3-indolyl phosphate p-toluidine salt and p-nitro blue tetrazolium chloride in 100 mM $NaHCO_3$ buffer containing 1 mM $MgCl_2$, pH 9.8.

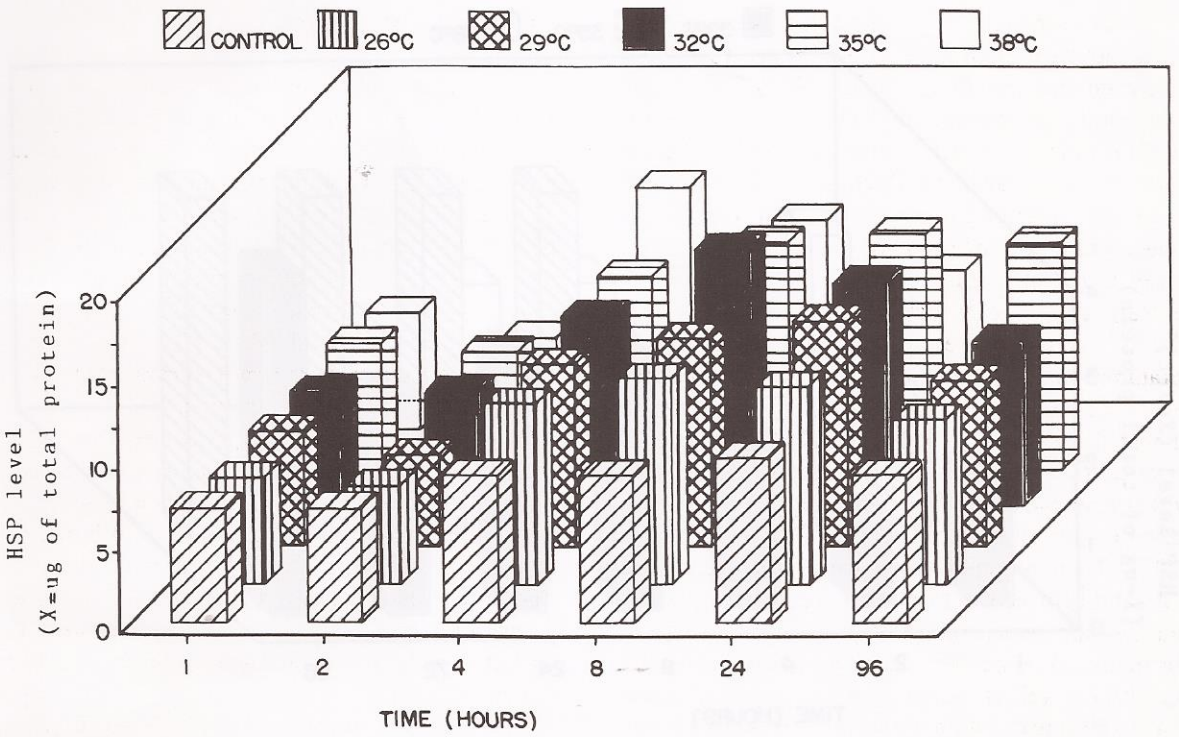


*Figure 1*. Relative hsp70 concentration in *C. fluminea* total tissue based on results from bio-blots. The control represents the response to heat shock of the clams acclimated at 26 °C and immediately frozen when taken from the control area. The hsp levels are expressed as relative values of total protein at detection limit of the antibody.

The same procedures were followed to detect hsp70, using a monoclonal antibody raised against the laboratory mouse (StressGen Biotechnology Corp., Victoria, B.C. Canada) and as secondary antibody, a goat anti-mouse alkaline phosphatase conjugated antibody.

To quantify the immonoreactive bands, gels were loaded with a serial dilution of initial volume of 100 $\mu$g of total protein with a range of 100 to 1.56 $\mu$g distributed in seven concentrations. Stress protein concentrations of each sample relative to control values were expressed as the inverse of the total protein at detection limit of the sample, divided by the total protein at detection limit of the control value (Sanders et al., 1991). To determine time of induction, stress protein intensities were compared visually from western blots of gels loaded with 50 $\mu$g of total protein.

A more rapid method of immunoassay of the stress proteins utilized a 46-well Bioblot with a nitrocellulose filter (Bio-Rad, Richmond, CA). Homogenates of known total protein content were digested in SDS sample buffer, serially diluted in TBS in one well column of the apparatus, and placed onto the nitrocellulose filter under vacuum. After filtration, the nitrocellulose membrane was removed and immunoblotted for hsp70, using the procedures described above. However to amplify the immunoreaction, three antibodies were used in series. After quantitation, total values for each sample for all the dilutions were compared to control values by two-way anova (Sokal & Rohlf, 1981).

## 3. Results

The bio-blot immuno assay for detection of hsp70, revealed there were no significant differences (ANOVA, $P < 0.05$) in the heat stress response between clams maintained at control conditions (26 °C) and those incubated at 29 and 32 °C. However, significant differences (ANOVA, $P < 0.05$) were found between controls and those stressed at 38 °C for 4 h and between controls and clams maintained at 35 °C for 8 to 96 h (Figure 1).

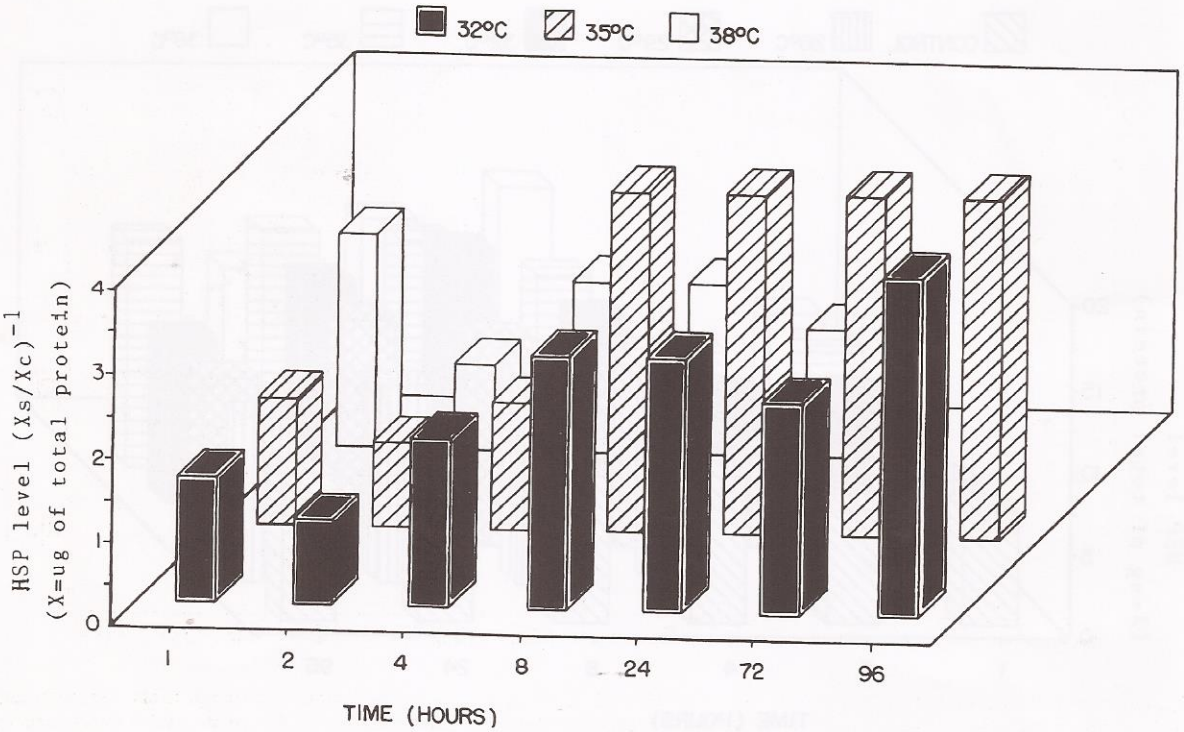


*Figure 2*. Relative hsp70 concentration in *C. fluminea* total tissue based on results from quantitative immunoblots. Bars represent the variation of clams response to heat shock stress at 32, 35 and 38 °C, during 96 h of exposure. The values are expressed as the inverse of the total protein in each sample at detection limit of the antibody, divided by the control value.

Immunoblotting with the monoclonal Ab raised against hsp70 showed similar reactivity. In general, the relative concentrations of hsp70 increased at 4 h from the beginning of the experiment and progressed over the 96-h experimental period in animals exposed to 35 °C. However, hsp70 levels decreased between 8 h and 24 h for the clams stressed at their lethal temperature of 38 °C (Figure 2). This trend is by the statistical analysis of data from comparative immunoblots (50 $\mu$g) of total protein for all of the samples. Hsp70 concentrations were significantly correlated with time of exposure ($R_2$ = 0.79) for clams maintained at 35 °C, while there was a negative correlation ($R_2$ = –0.26) for hsp70 concentrations and temperature at 38 °C. Hsp70 increased to a maximum of five times greater that control values by 96 h of heat shock at 35 °C, and then decreased to about 50% below the 4-h level at 38 °C by 24 h (Figure 2).

Immunoblotting with the hsp60 polyclonal antibody showed specific cross-reactivity in the clam tissue as indicated by standard markers (Figures 3 and 4). At 35 °C, there was an increase in the amount of hsp60 at 4 h, reaching a maximum eight-fold level at 8 h. By 96 h, the amount decreased to lower than those observed at 4 h. At 38 °C, level of hsp60 began to decrease at 8 h and continued to decline to 24 h when the clams died (Figure 5). At this temperature, the animals kept their shells closed, and their syphons were retracted from the beginning of the this heat exposure. Based on comparative immunoblots with hsp60, there was a significant positive correlation between hsp60 concentration and time elapsed only for animals maintained at 32 °C ($R^2$ = 0.64).

## 4. Discussion

The two approaches (semi-quantitative immunoblotting and bio-blot immunoassay) provided similar results, and demonstrated that the concentrations of hsp60 and hsp70 in *Corbicula fluminea* increased initially with heat stress, however the response appears to be transient under temperatures that approach the maximum tolerated by the species (38 °C). Induction of these stress proteins at lower temperatures serves as a protective cellular response, however the clams

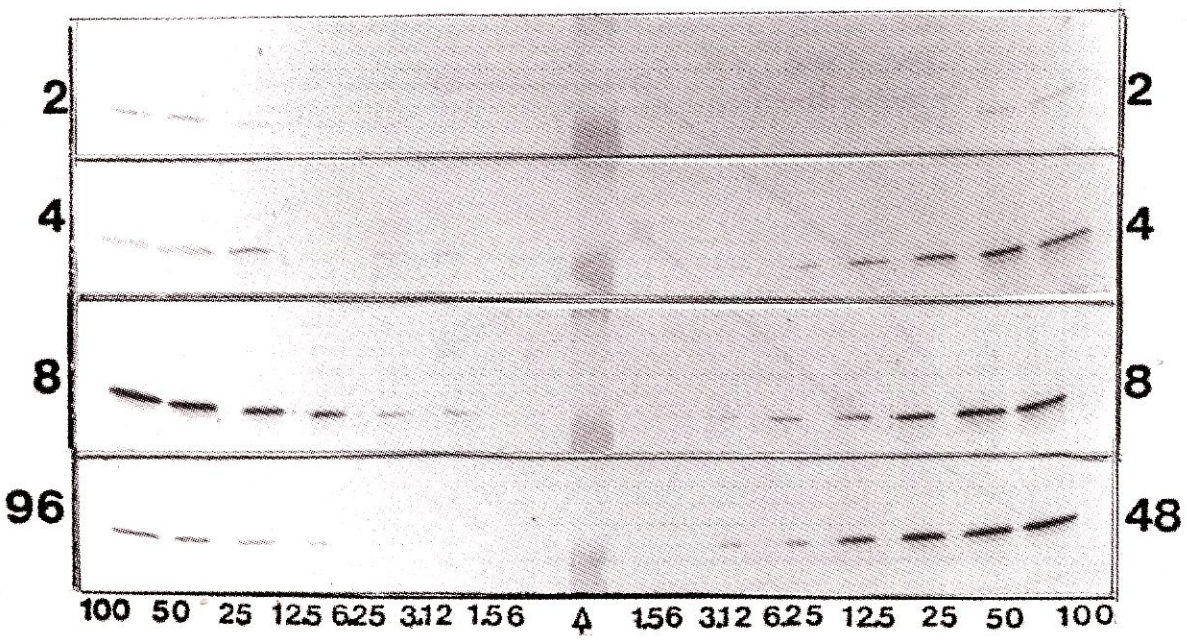


*Figure 3*. Western blot with hsp60 antibody of *C. fluminea* exposed to 35 °C. Numbers at the bottom indicate concentrations of total protein ($\mu$g). Numbers at the sides indicate time elapsed from the beginning of exposure. Arrow indicates prestained molecular standards of approximately 84 an 47 kDa, respectively, for all the gel strips.

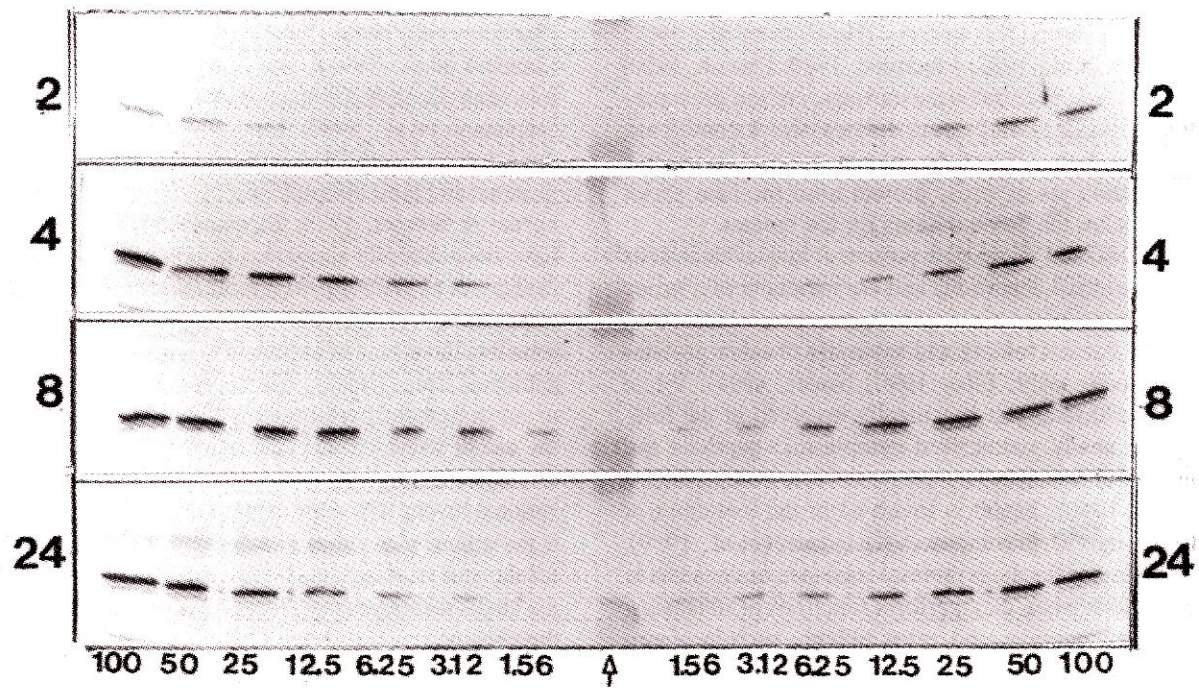


*Figure 4*. Western blot with hsp 60 antibody *C. fluminea* exposed to 38 °C. Numbers at the sides indicate time elapsed from the beginning of exposure. Arrow denotes prestained molecular standards of approximately 84 an 47 kDa, respectively, for all the gel strips.

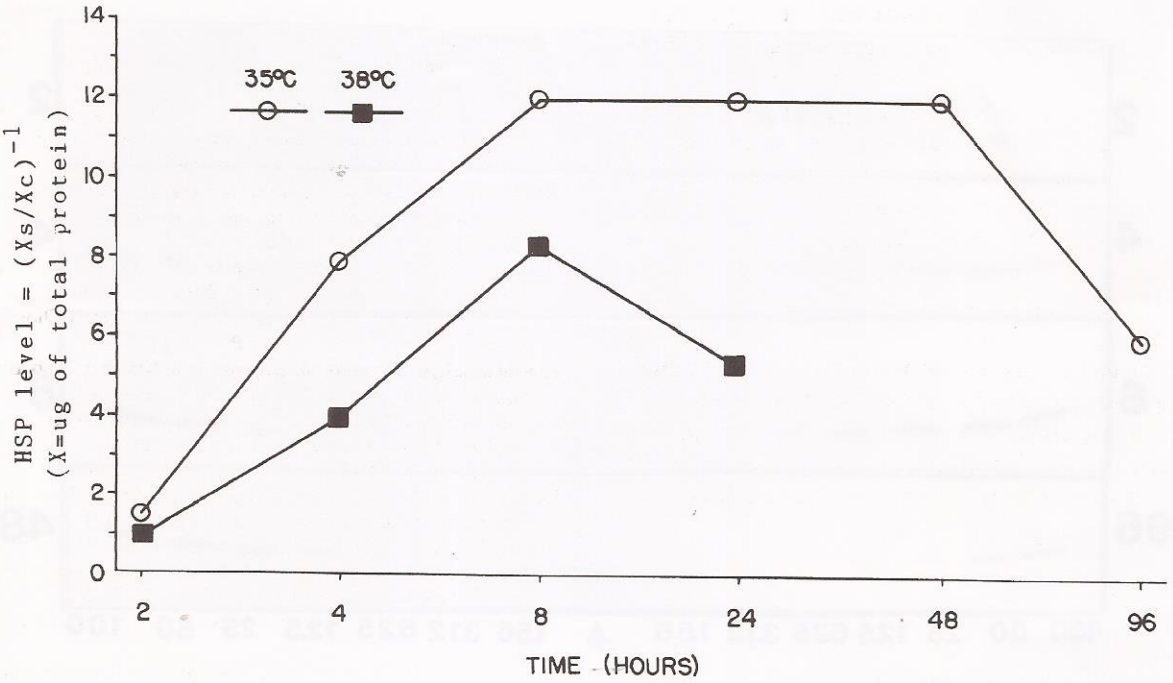


*Figure 5.* Relative hsp60 concentration in *C. fluminea* total tissue. Comparative response to heat shock at 35 and 38 °C during 96 h of exposure. The values are expressed as the inverse of the total protein in each sample at detection limit of the antibody, divided by the control value.

are unable to maintain elevated levels at higher temperatures. Previous workers (Heikkila et al., 1982; Gedamu et al., 1983; Lindquist, 1986; Pascoe, 1990) have also demonstrated the transiency of the stress protein response at midly stressful heat shock conditions, and our data support the maintenance of increasing concentrations of stress protein until the heat shock approaches the thermal limits for the species.

Hsp60 and 70 are both present at low levels in cells under normal conditions and are members of a group of proteins called molecular chaperones, which mediate the correct folding and assembly of other proteins (Rothman, 1989; Ellis, 1990; Nover, 1991). While the hsp70 family is specifically involved in the folding of newly synthesized cytoplasmic peptides and their translocation into other cellular compartments (Ellis, 1990), hsp60 is involved in the assembly of large oligomeric complexes (Ostermann et al., 1989). The demand for those proteins increases under adverse conditions when they perform functions of renaturing damaged peptides and resolubilizing protein aggregates that could be formed as a consequence of environmental induced damage (Rothman, 1989).

Due to their role in preventing cellular damage under stressful conditions, the hsp60 and 70 families have been associated with physilogical adaptation (Sanders et al., 1990, 1992) and organismal adaptation (Sanders et al., 1991), and correlated with acquired tolerance (Dean & Atkinson, 1982; Landry et al., 1989; Stephanou et al., 1983; Lindquist, 1986). Based on the important cellular functions performed by stress proteins and their ubiquity among all organisms (Ashburner & Bonner, 1979; Deshaies et al., 1988), they have been recently suggested as potential biomarkers (Sander et al., 1990, 1991). biomarkers refer typically to physiological or biochemical responses that serve as sensitive indicators of exposure to contaminants and or sublethal stress (Baker, 1988). Whereas the response to environmental perturbations begins at cellular level, the use of stress proteins as indicators would provide an early warning to prevent damage to higher organizational levels. The approach has much support, since if the intensity of stress is not lethal and it is possible for the animal to adapt. In this case, the persistence of the stress response may correlate with the intensity of the stressor.

However, continuous exposure to severe environmental stress may lead to a physiological state from which the animal can no longer maintain the stress protein response and recover and ultimately dye. In this

case the effects would already be observed at organismal level but the stress protein response would have ceased.

The results of this research suggest the use of stress proteins as biomarkers may not serve as an indicator of environmental purturbation in situations where the response to stress is at or near the lethal tolerance and is evident at organismal level. The stress protein response may serve as a valid biomonitoring tool under chronic, sublethal exposures when it is still possible to prevent the biological consequences of exposures which affect organismal or higher organizational levels. Preliminary results obtained in our laboratory on the correlations of the stress protein response, extreme pollution stress, and decreased growth in *C. fluminea* confirm the transient nature of this fundamental response.

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