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Early detection of changes in the quality of water resources, especially those impacted by anthropogenic contaminants, is of primary concern to individuals involved with managing these resources. The associated fisheries, both commercial and recreational, represent a valuable recources which can be irreparably impacted, as well. Those charged with managing water resources rely heavily on chemical, physical and biomonitoring techniques. A need exists for a rapid means of assessing the "health" of rivers, reservoirs, and estuaries in the nation. A reliable, field applicable method which determines stress levels in fish could provide resource managers with a valuable tool to determine if chemicals from point and non point source pollution are adversely impacting aquatic systems. For example, if the levels of stress in fish were determined upstream and downstream from an industrial and/or municipal discharge, it would be possible to determine the relative health of the two fish populations. Likewise, with proper validation it may be possible to determine if fish are approaching a level of stress nearing a threshold above whjich adverse impacts on growth and reproduction will occur. This report describes the results of the first year of a research project designed to further evaluate the potential of a stress protein

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DEVELOPMENT OF A FISH STRESS PROTEIN ANTIBODY/ANTIGEN-BASED APPROACH FOR BIOMONITORING OF WATER QUALITY

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CONTENTS

INTRODUCTION

Early detection of changes in the quality of water resources, especially those impacted by anthropogenic contaminants, is of primary concern to individuals involved with managing these resources. The associated fisheries, both commercial and recreational, represent a valuable resource which can be irreparably impacted, as well. Those charged with managing water resources rely heavily on chemical, physical and biomonitoring techniques. A need exists for a rapid means of assessing the "health" of rivers, reservoirs, and estuaries in the nation. A reliable, field applicable method which determines stress levels in fish could provide resource managers with a valuable tool to determine if chemicals from point and non point source pollution are adversely impacting aquatic systems. For example, if the levels of stress in fish were determined upstream and downstream from an industrial and/or municipal discharge, it would be possible to determine the relative health of the two fish populations. Likewise, with proper validation it may be possible to determine if fish are approaching a level of stress nearing a threshold above which adverse impacts on growth and reproduction will occur. This report describes the results of the first year of a research project designed to further evaluate the potential of a stress protein antibody/antigen-based biomonitoring method.

Response to environmental stress begins at the molecular/cellular level and then extends to tissues and organs, whose counteractions occur before changes in populations and ecosystems are effected (Bouk, 1984). While revealing, environmental techniques involving whole-animal responses are not based on the mechanisms which underlie the relationship between general stress physiology and toxicity and lack the sensitivity of cellular parameters (Jenkins and Sanders, 1986). Over the past decade, investigations into the use of biochemical parameters as indicators of stress have intensified. Unfortunately, their clinical use is lacking (Bouk, 1984). Clinical use implies that the method has been validated by extensive testing - both to identify the biological significance of the parameter and the limitations in applying the test to the species in question. Few of the existing tests lend themselves to field application (Bouk, 1984). However, acetylcholinesterase (Williams and Sova, 1966), mixed function oxidase enzymes (Melancon et al., 1987), and metallothionein (Jenkins and Sanders, 1986) have excellent possibilities in the clinical arena for identifying specific stressor-mediated abnormalities in environmentally exposed fish. There is a need for sensitive and direct measures for evaluating general stress in laboratory and natural populations. Such a measure would have great predictive value and wide application if it is based on the molecular mechanisms in stress physiology and adaptation (Jenkins and Sanders, 1986).

Stress proteins represent a suite of polypeptides that are synthesized when an organism is exposed to stress. These proteins appear to be ubiquitous, occurring in prokaryotes, protozoans, insects, fish, amphibians, birds, mammals, and plants (Nover, 1984) and are induced by a wide variety of stressors, such as metals (Heikkila, et al., 1982; Kapoor, 1986; Shelton, et al., 1986), amino acid analogues (Duncan and Hershey, 1987), carcinogens (Hiawasa and Sakiyama, 1986), hypoxia (Bultmann, 1986), viruses (Kennedy, et al., 1985), and parasites (Hedstrom, et al., 1987). Recently, Pelham (1986) demonstrated that stress proteins may function as a repair mechanism at the cellular level.

In previous research, we have characterized the stress protein response via laboratory experiments exposing fathead minnows (*Pimephales promelas*) to a variety of compounds having

different modes of action (see below). We verified that there are both qualitative and quantitative differences in the rapid synthesis and accumulation of stress proteins produced by specific stressors.

The purpose of this research was to extend the qualitative and quantitative approach to the application of an antibody/antigen-based method which assess actual levels of stress proteins in fish and other aquatic organisms exposed to both acute and chronic stressor conditions. This report describes the results of our first year of research on the development of a valid stress protein test. Evaluation of the stress protein response as a potential biomonitoring tool consisted of laboratory and *in situ* (field) testing. The laboratory portion included the purification of fish stress proteins. This was followed by the development of polyclonal antibodies specific to each of the stress proteins. Next, we evaluated binding of the antibodies to specific stress proteins (antigens) by exposing various species of fish to heat stress. Stress protein levels were assayed using western blots and densitometric quantitation. The methodology was field tested by exposing another aquatic organism (Asian clams) in an aquatic system impacted by point source pollution. The field study was essential to validate the efficacy of the stress protein antibody assay as a water quality biomonitoring method. Our results are detailed in the following studies.

PURIFICATION OF THE 70- AND 90-KD HEAT SHOCK PROTEINS FROM CATFISH LIVER: IMMUNOLOGICAL COMPARISONS OF THE PROTEINS IN DIFFERENT FISH SPECIES AND THEIR POTENTIAL USE AS STRESS INDICATORS

MATERIALS AND METHODS

For purification of HSP70 and 90, a readily available supply of large, healthy fish was necessary. Channel catfish, *Ictalurus punctatus*, were obtained from a commercial catfish farm and maintained in a flow-through, living stream system. The holding tank temperature remained constant between 22 to 25°C, and fish were fed daily with a high protein fish food.

Expression of the 70- and 90-kD proteins was elicited via *in vivo* heat-shock. Depending on the size of the fish, three to five fish were used. Fish were shocked in 230-liter aquarium with dechlorinated tap water which was well aerated. The water was heated overnight to 34 to 35°C with aquarium heaters prior to treatment. The fish were placed directly from the holding tank into the heat shock tank and maintained at this temperature for 6 to 8 hours, a time period demonstrated by Dyer (1991) to elicit maximal synthesis of heat shock proteins in Fathead minnow tissues. Following heat shock, the fish were sacrificed and immediately placed on ice. Muscle tissues were quickly excised from the fish and kept on ice. Liver tissues were immediately flushed with fish or normal saline to remove excess albumin (Guidon and Hightower, 1986). Flushing was performed until the liver turned from a deep reddish brown to a light pink color.

Purification of HSP70

HSP70 and 90 were purified to apparent homogeneity according to a modification of the method of Welch and Fermisco (1985). Briefly, catfish livers (25 to 30 g) were cut into small pieces and homogenized for 1 min in a Waring blender with 1.5 volumes of 20 mM Tris-Cl, pH 7.5 containing 20 mM NaCl, 15 mM 2-mercaptoethanol, 0.1 mM EDTA and 0.1 mM PMSF (Buffer A). All subsequent steps were conducted at 4°C unless otherwise indicated. The homogenate was then centrifuged at 17,000 *x g* for 1 hour, and the supernatant was filtered through glass wool. The supernatant was centrifuged at 117,000 *x g* for 2 hours, and the supernatant was filtered through glass wool and diluted with cold deionized water until the conductivity was approximately 2 mmho. The sample was then batch loaded onto 80 to 85 ml of DEAE-52, anion exchange resin equilibrated with buffer A. The sample containing resin was incubated for 40 min with occasional swirling. The slurry was poured into a column (55 x 1.5 cm). The column was thoroughly washed with buffer A and then eluted with a linear gradient of 20 to 350 mM NaCl in buffer A in a total volume of 500 ml. Fractions (~2.5 ml) were collected overnight. Samples from every other fraction (40 µl) were loaded on 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted against HSP70 mouse monoclonal IgG. Fractions containing HSP70 which occurred between conductivities of 5 to 12 mmho were pooled, adjusted to 3 mM $MgCl_2$, and applied directly to an ATP-Agarose column (8.0 x 1.0 cm) equilibrated with buffer A containing 3 mM $MgCl_2$ (Buffer B). The column was washed with buffer B containing 0.5 M NaCl to remove unspecifically bound proteins. It was then washed with buffer B until the conductivity returned to the base line (~3.2 mmho). The column was eluted with buffer B containing 1 mM GTP (~25 ml), and then with buffer B containing 3 mM ATP. Fractions (~1.5 ml)

were collected, and the purity of catfish HSP70 and 90 was judged by 12.5% polyacrylamide SDS electrophoresis. With unstressed fish, HSP70 appeared as a single band; whereas with heat shocked fish, two bands were visualized on the gel representing the constitutive HSP70 and the temperature-induced HSP68. HSP90 appeared as a single band on gels. Protein content was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

Antibody Production

HSP70 and 90 purified from catfish were mixed with TiterMax (1:1 v/v) and injected subcutaneously (~0.4 mg) into two New Zealand white rabbits. Subsequent subcutaneous injections with various protein quantities were performed every 2 to 4 weeks for approximately four months. The animals were bled and the blood was allowed to clot at 4°C, then it was centrifuged at 1,000 *x g* for 10 minutes. The pellet was discarded, and the supernatant (serum) was frozen at -80°C until use. Antibodies against HSP70 and 90 in the serum were detected by Western Blot and the cross reactivity experiments were conducted with antiserum titer of 1:1,000.

Electrophoresis

Pure catfish HSP70, HSP90, and the supernatants of ultracentrifuged homogenates of fish liver, gill, and muscle tissues were electrophoresed on 12.5% SDS-PAGE as described by Laemmli (1970). Protein samples (~30 µg) were dissolved in 2% SDS, 75 mM Tris-Cl, 0.001% bromophenol blue, 10% glycerol, 5% 2-mercaptoethanol, and boiled for four minutes. The boiled samples were then loaded onto a 1.5 mm thick 12.5% SDS polyacrylamide gel. The gel was electrophoresed at constant voltage (110 volts) until the dye reached the resolving gel, then the voltage was increased to 150 volts. The protein bands were visualized with either comassie Brilliant Blue R-250 or transferred to a nitrocellulose membrane by Western blotting.

Immunoblotting

Western Blot analyses were performed as previously described by Towbin, et al., 1979. All incubations were conducted at 25°C unless otherwise indicated. Briefly, proteins were transferred to nitrocellulose membranes at 200 mA for two hours in 25 mM Tris-Cl (pH 8.0) buffer containing 192 mM glycine and 20% methanol. Transfers were blocked in 25 mM Tris-Cl (pH 8.0) buffer containing 3% BSA , 137 mM NaCl, and 2.7 mM KCL (TBS). Transfers were then washed with TBS containing 0.05% Tween-20 (TTBS). Following the wash, transfers were incubated with the primary antibody for 2 h at a 1:1,000 dilution. The residual primary antibody was removed by two washes in TTBS. Transfers were then incubated with an alkaline phosphatase conjugated goat anti-rabbit IgG antibody for 2 h. The residual conjugate was remove by two washes in TTBS and a final wash in TBS. Color development of the immunoreactive bands was initiated with 5-bromo 4-chloro 3-indoyl phosphatase p-toluidine salt and p-nitro blue tetrazolium chloride in 100 mM $NaHCO_3$ buffer containing 1 mM $MgCl_2$, pH 9.8. Transfers were incubated until maximum color intensity of the immunoreactive sites and minimum background color were recognized (20 to 40 min.). Color development was halted by rinsing the membrane in distilled water.

Materials

ATP-Agarose (linked through C-8), ATP, Tris, and EDTA were purchased from Sigma Chemical Company (St. Louis, Missouri). Electrophoresis, Western Blot reagents, nitrocellulose membranes, Tween-20, Bradford dye, and goat-anti rabbit IgG alkaline phosphatase conjugate were purchased from Bio-Rad (Richmond, California). DEAE-52 was purchased from Whatman

(Hillsboro, Oregon). HSP70 and 90 mouse monoclonal antibodies were a generous gift of StressGen Biotechnology Corp. (Victoria, B.C., Canada). TiterMax was purchased from CytRx Corp. (Norcross, Georgia). GTP was purchased from Boehringer Mannheim Biochemicals (Indianapolis, Indiana). All solvents used were HPLC grade.

Fish for the cross reactivity study were obtained from several different areas. Fathead minnows, *Pimephales promelas*, were obtained from the University of North Texas stock culture. Red shiners, *Cyprinella lutrensis*, were obtained using a twenty foot seine with a quarter inch mesh size. Black bass, *Micropterus salmoides*, and Bluegill, *Lepomis macrochirus*, were collected from Lake Ray Roberts and the Spring Creek tributary using a backpack electroshocker.

RESULTS AND DISCUSSION

Purification of HSP70 family and HSP90: HSP70 family and HSP90 were purified to apparent homogeneity from catfish liver. In 1985, Welch and Feramisco showed that the amount of HSP70 family obtained from the cytosolic fraction was higher than that extracted from the pellet. To purify the HSP70 family and HSP90 from catfish, livers were flushed with normal saline in order to remove and eliminate albumin which might have interfered with HSP resolution on SDS-PAGE. The crude homogenate was centrifuged at low and high speed. The supernatant obtained from the high spin centrifugation was the source of HSP70 in our experiments. The HSP70 from rat liver is a family of proteins with pI of 5.6 to 5.8 under non-denaturing conditions (Hatayama, *et al.*, 1989), suggesting that these proteins can be anionic at physiological pH. This amphoteric nature of proteins was utilized to eliminate the cationic proteins in the first step of the purification. Consequently, the supernatant obtained from the 117,000 *x g* spin was loaded on an anion chromatography resin (DEAE-52).

The pooled fractions eluted from DEAE-52 chromatography column, contained the HSP70 family (lane 3); whereas, none of HSP70 family was present in the wash, indicating that virtually all the HSP70 have bound to the anion exchange column. Moreover, utilizing the anion exchange chromatography approach as the initial step of the purification procedure proved very important purification step, for a large number of proteins were eliminated.

Craig and Gross, 1989 reported that HSP70 is an ATP-binding protein. Utilizing this HSP70 property, the HSP70-enriched protein pool, obtained from the salt gradient elution of DEAE-52 chromatography, was rechromatographed on a very specific affinity column, ATP-Agarose. All of the HSP70 was retained, while the bulk of the protein was not bound. The 0.5 M NaCl eluted the non-specifically bound proteins but had no effect on releasing HSP70. The application of 1mM GTP in buffer B, a low molecular weight protein was eluted. However, upon application of 3 mM ATP in buffer B, HSP70 was eluted homogeneous as judged by SDS-PAGE. This highly specific affinity chromatography purification step proved very efficient in purifying HSP70, for the protein eluted was homogeneous. From 25 g of livers of heat-shocked catfish, approximately 0.8 mg of HSP68, HSP70, and HSP90 were recovered.

When livers of catfish that had not been heat-stressed were used as a source of HSP70 and HSP90, the final purification product appeared as single bands on SDS-PAGE with molecular weight of 70 and 90 kD. However, when livers of heat-stressed catfish were used, two HSP 70 bands were resolved after the ATP-Agarose affinity chromatography step. These bands had molecular weights of 70 and 68 kD representing the constitutive and the temperature-induced

Table 1. Results of polyclonal antibody to hsp 70 binding for five species of fish representing three families of fish.

Common name	Scientific name	Tissue homogenates[b]		
		Liver	Muscle	Gill
Family Ictaluridae				
Channel Catfish	*Ictalurus punctatus*	++++[a,c]	+++	++++
Family Cyprinidae				
Fathead minnow	*Pimphales promelas*	+	+	+
Red shiners	*Cyprinella lutrensis*	+++	++	+
Family Centrarchidae				
Black bass	*Micropterus salmoides*	+	+	+
Bluegill	*Lepomis macrochirus*	++	+	+

[a] Antibody titer used was 1:1,000.
[b] Approximately 30 µg total protein of each tissue homogenate were used.
[c] + = Lowest cross-reactivity and ++++ = highest cross-reactivity.

HSP respectively.

Immunological studies: To immunize the rabbits, pure HSP70 or 90 were mixed with TiterMax rather than Freund's adjuvant, for the former was shown to elicit antibody production in laboratory animals faster than Freund's adjuvant. However, it took several months to get an acceptable titer of antibody against HSP70 and HSP90.

Western blot analysis of crude homogenates (30 µg) of catfish liver, muscle, and gill with polyclonal antibodies prepared against the purified catfish liver HSP70 family of stress proteins cross-reacted with HSP70 of the three catfish tissues tested, with the highest cross reactivity with liver HSP70. HSP70 from the muscle homogenate was the least cross reactive with the antibody. Cross reactivity of anti-liver antibodies with gill HSP70 was intermediate. Similar tests with HSP90 are ready for analysis.

In order to gain more insight about the homology of HSP70 from different fish families, and to investigate the efficacy of this protein as a potential biomarker of water quality, crude homogenates of liver, muscle, and gill tissues of a number of selected, commercially important fish were Western blotted against polyclonal antibodies raised against HSP70 purified from catfish liver. Table 1 demonstrates the strength of cross-reactivity of HSP70 from crude homogenates of liver, muscle, and gill tissues of Fathead minnows (*Pimphales promelas*), Red shiners (*Cyprinella lutrensis*), Black bass (*Micropterus salmoides*), and Bluegill (*Lepomis macrochirus*) with antibodies raised against HSP70 purified from catfish liver. Western blot analysis with rabbit antiserum prepared against the purified catfish liver HSP70 revealed that tissue homogenates of all the fish species examined were recognized by the anti-HSP70 antibody. However, the highest cross-reactivity was observed with catfish tissue homogenates (Table 1). Red shiners tissue homogenates showed different intensities of cross-reactivity with liver homogenate being the most recognized by the antibody and the gill homogenate being the lowest (Table 1). Black bass tissue

homogenates along with the gill and muscle homogenates of Bluegill were the least recognized by anti-HSP70 antibody: Bluegill liver homogenate showed higher cross-reactivity than the gill and muscle homogenate but significantly lower than catfish tissue homogenates (Table 1).

A number of procedures have been described for the purification of HSP70 stress proteins from various sources (Welch and Feramisco, 1982; Guidon and Hightower, 1986). Hitherto, no attempts have been made to purify HSP70 or HSP90 from fish. In this report, we established a protocol for the purification of HSP70 family and HSP90 from livers of catfish that have been subjected to whole-body hyperthermia.

The purification procedure was very efficient in purifying these stress proteins to apparent homogeneity. Welch and Feramisco, 1982 reported the purification of HSP71/73 from Hela cells. However, two major protein contaminants with relatively low molecular weights were present in their preparations. Likewise, Hatayama, *et al.* in 1989, reported the separation of HSP70 and HSP71 using DEAE-52 anion exchange and ATP-Agarose affinity chromatography, but their preparations contained a main contaminating protein with a molecular weight of 54 kD and minor contaminating proteins with molecular weights of 45 kD, 92 kD, and 30 kD. In our preparations, the addition of protease inhibitors such as PMSF and EDTA to buffers resulted in pure, homogeneous HSP70 and 90 with no detectable contaminating protein as judged by SDS-PAGE. Another reason for obtaining homogeneous HSP70 and 90 without any contamination is that the extreme ends of fractions eluted from DEAE-52 column which contained HSP70 and 90 were eliminated from the pool. When all the fractions that contained HSP70 and 90 were pooled together, the end product contained a major contaminating protein with a molecular weight of approximately 55 kD. One of the major advantages of this purification procedure is that it is rapid and preparative. Approximately 0.8 mg of pure HSP70 or 90 were obtained from 25 g of catfish liver. This amount is comparable to what has been reported by Hatayama, *et al*, 1989.

Studying HSP70 in aquatic organisms is particularly important, for HSP70 have been implicated as a major factor in cellular resistance to physical and chemical stress (Lindquist, 1986). Moreover, it has been suggested that levels of synthesis or accumulation of this protein may be useful in determining whether a particular environmental treatment is perceived by the organism as being stressful (Welch, 1990). To investigate such potential, we utilized the antigen/antibody approach to study the homology of HSP70 from different tissues of a number of fish species. The fact that HSP70 from the sources indicated earlier cross-reacted with HSP70 antibody prepared against pure catfish liver HSP70 indicate that HSP70 from these sources share a common antigenic determinant.

Collectively, affinity chromatography is a rational approach to rapid purification of protein and has been proven to be effective in the purification of HSP70 from catfish liver. We have also shown in this research that substantial quantities of HSP70 can be purified by anion exchange and ATP-Agarose chromatographies unlike the case in which two dimensional PAGE, phenyl-Sepharose, ammonium sulfate precipitation, and gel permeation chromatography are applied (Guidon and Hightower, 1986).

The fact that HSP70 from different muscle tissues of a number of fish species share common epitopes enables us to detect stress protein in ecologically and commercially important fish species such as Fathead minnows (*Pimphales promelas*), Red shiners (*Cyprinella lutrensis*),

Black bass (*Micropterus salmoides*), and Bluegill (*Lepomis macrochirus*) and consequently to determine whether the surrounding aquatic environment is stressful or not.

HEAT SHOCK PROTEIN RESPONSE TO THERMAL STRESS IN THE ASIATIC CLAM, *CORBICULA FLUMINEA*

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 (Kraemer et el., 1986). Factors which have contributed to its successful introduction include adaptation to a wide range of substrates (Sinclair, 1964; White, 1979) and its dispersal ability (Kraemer, 1979). More importantly, Coldiron (1975) determined that *C. fluminea* is preadapted to a broad range of temperature regimes. The lethal temperature for this clam ranges from 38 to 39°C range, 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 into a cellular level response to heat stress.

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 environmental stress can be adapted for biomonitoring.

MATERIALS AND METHODS

Corbicula fluminea specimens, ranging in size from 9 to 11 mm were collected from the Clear Creek, Denton County Texas. This creek has served as a control site for the experiments on environmental assessment by this laboratory. 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-l aquaria whose temperatures had been adjusted with an aquarium heater and maintained at 26°C (control), 29, 32, 35 and 38°C for 96 h. Each aquariam contained filtered creek water and two replicates 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 adductor muscle, and the tissue was removed, homogenized, and sonicated in 150 µl of buffer (150 µM TRIS-CL, pH 7.8, 1 µM phenylmethylsulfonyl fluoride).

Homogenates were centrifuged at 10,000 x g, at 4°C for 30 min. The supernatants were then collected, and protein concentrations were determined (Bradford, 1976).

Proteins were separated on 12.5% polyacrylamide 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 transferred to nitrocellulose by Western blotting using a transfer buffer containing 1% SDS, 0.192 M glycine in 0.025M-TRIS-Cl and 20% methanol, by volume, pH 8.3. The gels were subjected to 25 v for 15 h at 4°C. Transfers were blocked in 25 mM TRIS-Cl (pH 8.0) buffer containing 3% BSA, 137 mM NaCl, and 2.7 mM KCl (TBS). Transfers were then washed with TBS containing 0.05% Tween-20 (TTBS).

The nitrocellulose was probed using a polyclonal antibody developed against HSP 60 from the moth *Heliothis* sp. (StressGen Biotechnology Corp., Victoria, B.C. Canada). Blots were incubated at room temperature with HSP 60 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 antibody 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. The same procedures were followed to detect HSP70, using a monoclonal antibody developed against the laboratory mouse (StressGen Biotechnology Corp., Victoria, B.C. Canada) as secondary antibody, a goat anti-mouse alkaline phosphatase conjugated antibody.

To quantify the immunoreactive bands, gels loaded with a serial dilution of an initial volume of 100 µg of total protein with a range of 100 to 1.56 µg 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 µg 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 (Sokol & Rohlf, 1981)

RESULTS

The bio-blot immunoassay 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

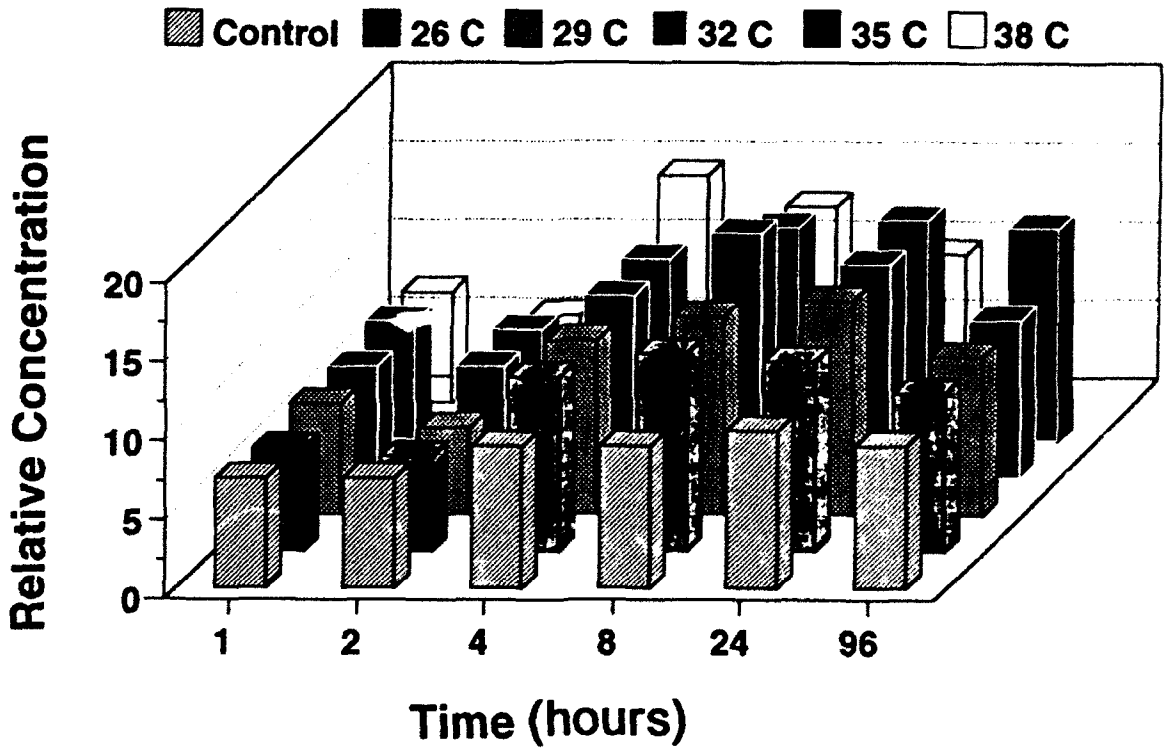

Fig. 1. Heat shock protein 70 accumulation in Asian clams exposed to control conditions and five experimental temperatures for six time periods.

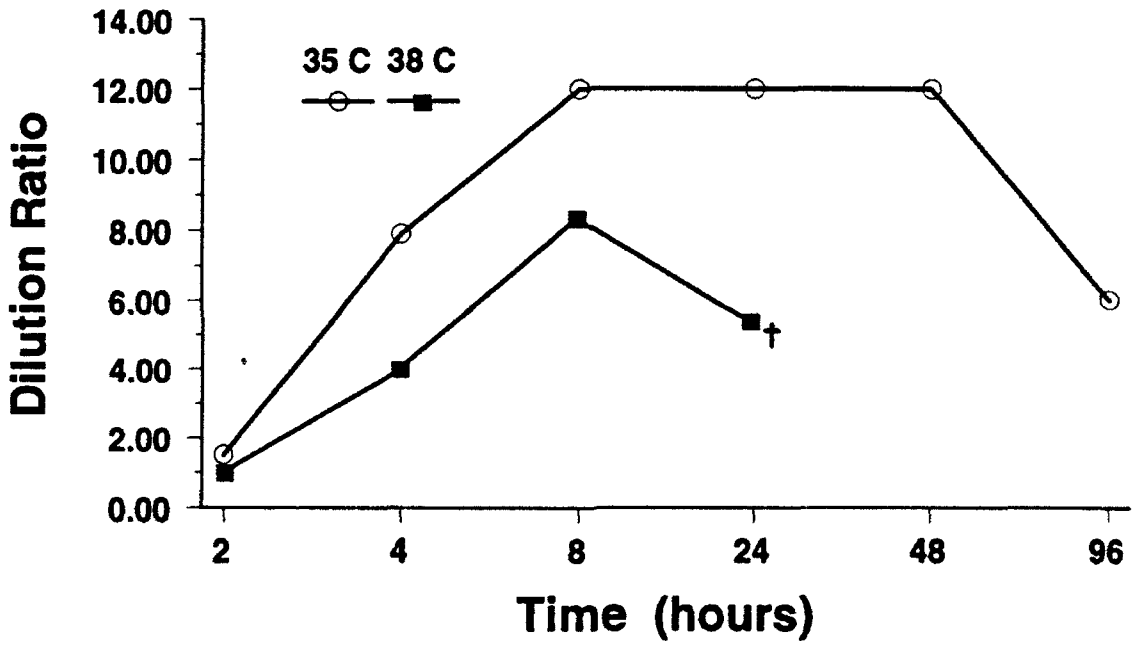

Fig. 2 . Relative accumulation of heat shock protein 70 in Asian clams exposed to 35 and 38°C for 96 hours.

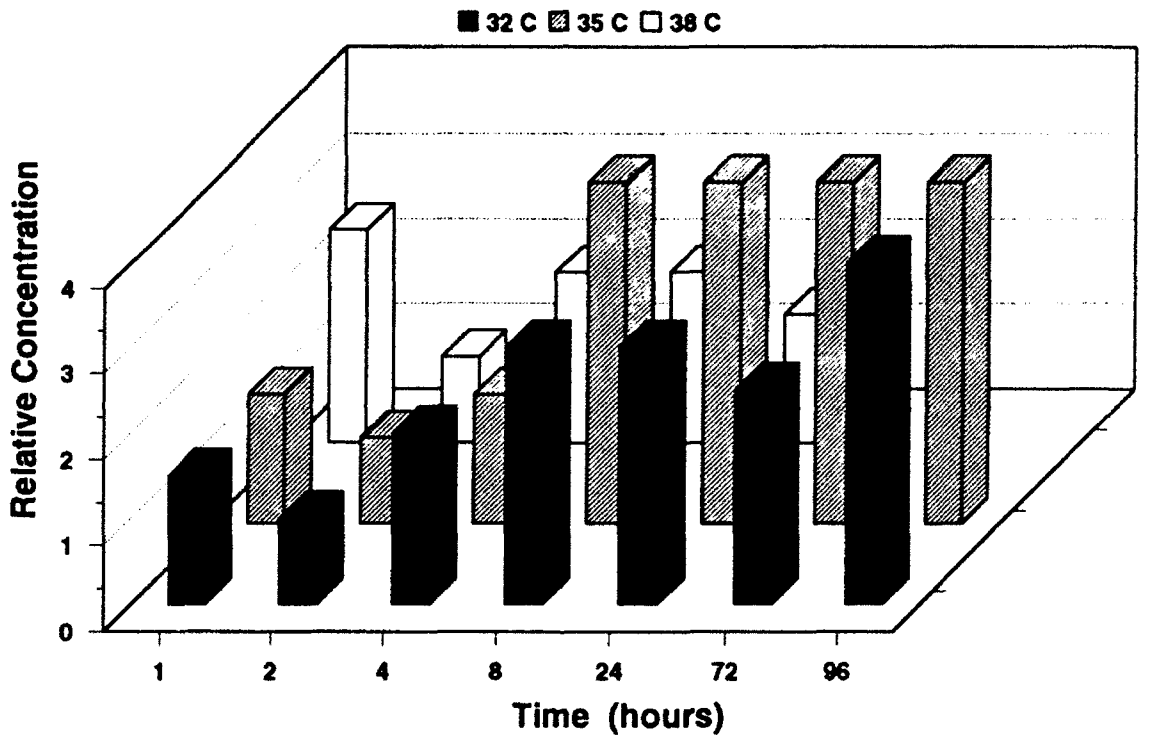

Fig. 3. Accumulation of heat shock protein 60 in Asian clams exposed to three temperatues exposed to control conditions and six experimental temperatures for 96 hours.

and clams maintained at 35°C for 8 to 96 h (Fig.1). Kruskal Wallis ANOVA by ranks, based also on data from the bio-blot showed that clams exposed to 35°C for 96 h had significantly elevated levels of HSP70 relative to the control group maintained at 26°C for the same time period.

Immunoblotting with the monoclonal Ab raised against HSP70 showed specific cross-reactivity in clam tissue (Fig. 2). 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 4 h and 24 h for the clams stressed at their lethal temperature of 38°C. This trend was substantiated by statistical analysis of data from comparative immunoblots (50 µg) 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 than 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.

Immunoblotting with the HSP60 polyclonal antibody showed similar reactivity (Fig 3). 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 levels lower than those observed at 4 h. At 38°C, the level of HSP60 began to decrease at 8 h and continued to decline to 24 h when the clams died. At this temperature, the animals kept their shells closed, and their syphons were retracted from the beginning of 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).

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 this species, 35°C for HSP60 and 38°C for HSP70. Induction of these stress proteins at lower tempertures serves as a protective cellular response, however the clams 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 mildly 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 chaperons, which mediate the correct folding and assembly of other proteins (Rothman, 1989; Ellis, 1990; Nover, 1991). While the HSP70 family appear to be 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 (Osterman 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 environmentally induced damage (Rothman, 1989).

Due to their role in preventing cellular damage under stressful conditions, the HSP60 and 70 families have been associated with physiological adaptation (Sanders et al., 1990, 1992) and organismal adaptation (Sanders et l., 1991), and are 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 (Sanders, 1990; Sanders et al., 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. 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 death results. The effects would already be observed at the organismal level, but the stress protein response would have ceased. To test this hypothesis, Asian clams were in an effluent from a sewage treatment plant on the Trinity River near Dallas, Texas. Their growth over a 30-day period, a valid biomonitoring methodology with this species, and accumulation of heat shock proteins was monitored. These two indices of stress were then compared. Results of this study are provided in the next section.

FIELD TESTS OF ANTIBODY DETECTION OF HEAT SHOCK PROTEIN ACCUMULATION IN ASIAN CLAMS (*CORBICULA FLUMINEA*)

INTRODUCTION

The Trinity River flows through Dallas and Fort Worth, Texas, and is the principal receiving stream for wastewater discharges from the metroplex. During summer months, discharges may compose more than 90% of the total flow in the river. Chlorine is used by wastewater treatment plants as a disinfectant and is a known toxicant impacting the biota of a stream and has been shown to contribute to the formation of chlorinated hydrocarbons. Whole effluent toxicity testing utilizing *Ceriodaphnia dubia* (ceriodaphnia) and *Pimephales promelas* (fathead minnows) as freshwater test organisms has been conducted and predicted biological impact on the Trinity River.

The effects of chlorine on the biota of the West Fork of the Trinity River are being investigated over a 2-year period by personnel of the Institute of Applied Sciences at the University of North Texas. Laboratory toxicity data are available for two stations above and five stations downstream from the Village Creek wastewater treatment plant on the Trinity River, and preliminary results are presented by Guinn et al. (In Press). In short, the effluent at the first two stations downstream from the plant was lethal to fathead minnows, with survivorship increasing to control levels at station 5 downstream from the plant. A similar pattern in growth occurred in fathead minnows, with weight gain equaling control values at stations 3, 4, and 5 downstream from the discharge. Dechlorination resulted in survivorship and growth equal to those of control of the control stations.

The ceriodaphnia tests were even more striking. Survivorship was 100 % at stations 1 and 2 (controls), no animals survived in water collected during summer months from stations 3, 4, 5, and 6 downstream from the effluent. Similarly, reproduction did not occur in water collected at stations 3 and 4 downstream from the effluent, with values at stations 5 and 6 downstream from the discharge that were approximately 15% those of controls. In contrast to the fathead minnow results, dechlorination of the water did not increase the survivorship or reproduction of ceriodaphnia, indicating that other toxicant were present in the effluent which impacted the organisms tested in these water samples.

Toxicity identification evaluations conducted on the effluent samples characterized the toxicant as non-polar organic that included DEBET (benzamide,N,N-dimethyl-3-methyl), phenols (4-1-methyl-1-phylethyl and 2,6-bis 1,1-dimethylethyl)-4-methyl) and 1,2-bezenedicarboxylicacid, butyl-2-methyl.

This background toxicity information on the two sites above and five sites downstream from the Village Creek wastewater treatment plant on the Trinity River provided an excellent opportunity to field test the utility of induction of heat shock proteins as an indicator of stress in another test organism, the Asian clam (*Corbicula fluminea*). This organism has been utilized in toxicity testing with growth being used as an endpoint.

METHODS

Asian clams were collected from an unpolluted site on Clear Creek, Denton Co., Texas, a tributary of the Trinity River. Thirty clams were placed in each of 14 12.5 x 12.5 x 12.5-cm nylon screen cages and transported in creek water to the Village Creek wastewater treatment study site on the Trinity River. Two replicate cages were suspended above the sediment at each site, two sites upstream and five sites downstream from the discharge. The cages remained in the river for 30 days, after which they were removed. The clams were removed from the cages and were frozen immediately in liquid nitrogen for transport back to the laboratory.

In the laboratory, each clam was removed from liquid nitrogen and measured to the nearest 0.1 mm, and all tissue was removed for heat shock protein analyses. The protocol described in the previous section on induction by hyperthermia was followed to determine heat shock protein accumulation in clams from each site.

RESULTS AND DISCUSSION

Considering the two stations above the discharge from the Village Creek Wastewater facility as controls and downstream sites as exposure sites, clearly there was a marked difference in 30-day growth and hsp accumulation among the sites in Asian clams (Figs. 5 and 6). Growth at stations 3 and 4 downstream from the discharge approximated 1/3 (2-3.5% increase) that of clams at the control stations. Growth increased to an average of 4.5% at station 5 and approached control values at sites 6 and 7. This response provides an indication of the gradient in water quality downstream from the discharge and is concordant with previously collected data at these sites for lethality in fathead minnows, and reproduction and lethality measures on ceriodaphnia (see Introduction).

Relative concentrations of heat shock proteins at the seven sites provided surprising results. Relative concentrations of hsp60 were low, <1.0, at site 1, however concentrations at site 2 increased to 2.5. Concentrations at sites 3 and 4 downstream from the discharge were near those of site 1 and increased at sites 5 through 7. While these accumulations appear discordant with other indicators of toxicity at these sites, we believe there is a biologically meaningful explanation.

Growth in Asian clams at the two downstream sites was significantly lower than at control sites. We can assume that toxicity at these sites was extreme and near lethal levels, and that this lack of growth was mirrored our heat shock results. Our laboratory studies on accumulation of heat shock proteins in clams exposed to near lethal temperatures (38°C) indicated that hsp accumulation decreased, perhaps due to a complete shutdown in metabolic processes. If toxicity at stations 2 and 3 was near lethal limits for these clams, then a similar metabolic shutdown and low levels of heat shock proteins would be predicted. At lower levels of toxicity, stations 4-7, a more typical hsp accumulation response was observed. The marked increase in hsp accumulation at site 2 above the discharge cannot be readily explained. However, other indicators of toxicity from this site have provided similar results on occasion. We think this site has some levels of toxicity during certain time periods, but we have been unable to determine the source.

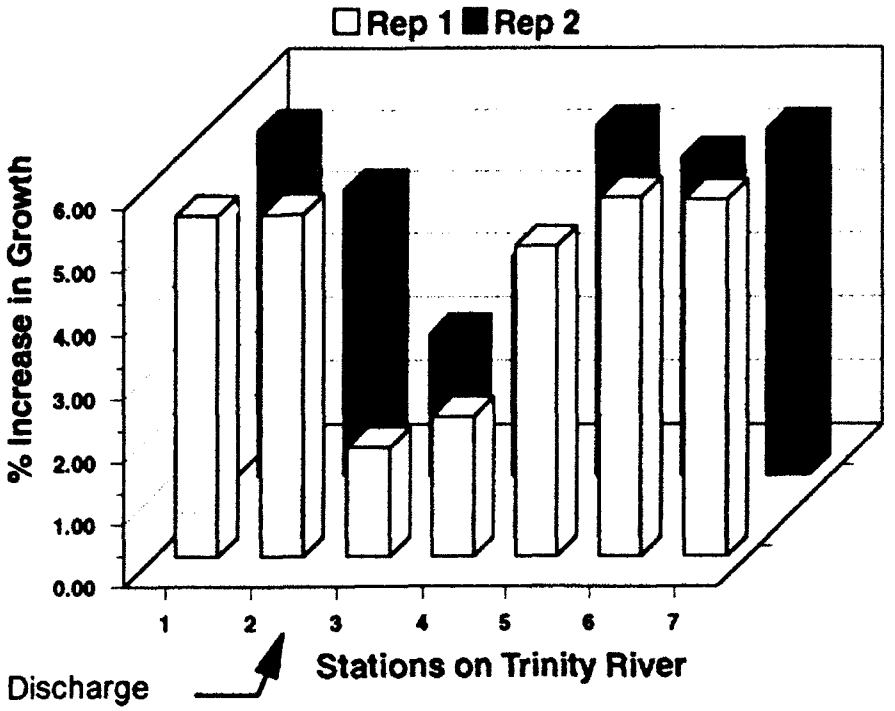

Fig. 4. Change in growth in Asian clams exposed to control (Stations 1 & 2) and effluents downstream from a wastewater treatment plant (Stations 3 - 7) on the Trinity River in northcentral Texas.

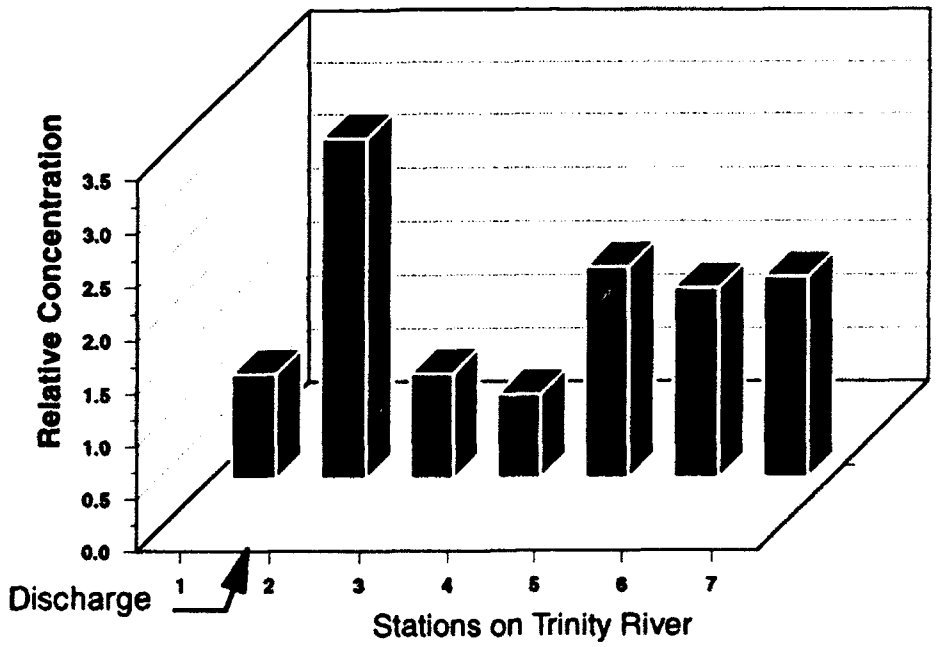

Fig. 5. Accumulation of heat shock protein 60 in Asian clams exposed to control (Stations 1 & 2) and effluents downstream from a wastewater treatment plant (Stations 3 - 7) on the Trinity River in northcentral Texas.

The results of this research suggest the use of stress proteins as biomarkers may not serve as an indicator of environmental perturbation in situations where the response to stress is at or near the lethal tolerance. 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 and the need for additional studies.

ADDITIONAL STUDIES FOR THE SECOND YEAR OF FUNDING

The first year of grant activities has proven to be highly successful, since we have demonstrated the universality of reaction to our hsp 70 polyclonal antibodies among a variety of fish. We have also established sites on the Trinity River which can be used to field-test the applicability of the antibodies to natural systems.

We will continue to test the applicability of these proteins to a greater diversity of fish species to verify their use as indicators of stress in fish. Additionally, we are in the process of isolating both high and low molecular weight heat shock proteins for the production of antibodies. High molecular weight hsp's are indicators of pesticide stress, while low molecular weight hsp's are specific for heavy metal stress. We will evaluate binding of the antibodies to specific stress proteins (antigens) by exposing fish to four different toxicants with different modes of action. Stress protein levels will be assayed using western blots and densitometric quantitation. Resultant supernatant will be subjected to western blot analysis with determination of antibody binding via alkaline phosphatase development and densitometric quantitation to determine antibody specificity and relative binding of antibodies to the stress proteins. Stress protein levels will be compared to toxicity endpoints, such as LC50 and growth. The methods will be field tested by exposing fish in the an aquatic system impacted by point source pollution. A spectrophotometric method, using these antibodies conjugated with a dye to determine levels of stress proteins will be explored and evaluated from fish exposed to various toxicants in the laboratory and field. The field study is essential to validate the efficacy of the stress protein antibody assay as a water quality biomonitoring method.

Field tests will be conducted on caged fathead minnows held for 30 days at the seven sites previously investigated. We will compare survivorship, growth, and hsp accumulation in this toxicity gradient to demonstrate the field applicability of the test for fish. In addition, we will determine if the transient nature of hsp accumulation under near lethal toxicity is typical of fish. This will provide information of the sensitivity of the hsp accumulation test under natural conditions.

STUDENTS AND PERSONNEL SUPPORTED DURING
FIRST YEAR OF STUDY

Students Supported	**Position**
San Covington	M.S. Student (Degree awarded Aug. 1992)
Philip Yeager	PhD Student (in progress)
Imad K. Abukhalaf	Postdoctoral Fellow
Iracema A. Nascimento	Postdocotral Associate

Personnel Supported	**Postion**
Earl G. Zimmerman	Co-Investigator
Kenneth L. Dickson	Co-Investigator

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