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Seasonal Effect on Heat Shock Proteins in Fish from Kuwait Bay

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Abstract Heat shock proteins (HSP70) play a significant role in adaptation to temperature and have been proposed as an indicator of cellular stress. Since the water temperature in Kuwait's marine area varies from 13 to 35°C from winter to summer, HSP70 could be a valuable tool in aquaculture in Kuwait. HSP70 levels were quantified by Western blotting in liver, muscle and gill tissues of two varieties of native fish species captured during the winter and summer months from both inside and outside the highly stressed Kuwait Bay area. The HSP70 levels did not differ statistically between fish captured from the two sampling areas. The most common response in both species was higher median levels of HSP70 in winter months. This inverse relation between HSP70 levels in the fish and the water temperature may be due to either genetic adaptation in the fish to the hot climatic conditions of the region or other stressors, such as changes in pollutant levels in the surrounding water.

Keywords HSP70 · Gills · Liver · Muscle · Seabream · Tonguesole · Kuwait Bay

Kuwait Bay is considered to be a highly stressed area. It is one of Kuwait's most important areas, and has witnessed the rapid urban and industrial development since the discovery of oil in the region. As a result, Kuwait Bay has received significant assaults on its natural environment; therefore, its environment has always been a cause of concern for regulatory authorities.

The average water temperature in the Kuwait marine area varies from 13 to 35°C during the year. Accordingly the concentration of dissolved oxygen, salinity and conductivity varies (EPA 1999). These climatic variations in the environment also cause stress on marine biota, causing them to undergo metabolic adjustments in order to perform normal physiological functions. Given the difference of around 20°C in the water temperature between the summer and winter seasons, it was considered important to study the levels of heat stress-inducible proteins in native fish species. Heat shock protein (HSP70) induction is a universal response of activated protein synthesis after exposure to various physical or chemical stimuli-especially heat stress (Werner et al. 2006). Heat shock proteins play a role in the repair of cellular damage and provide cells with protection from further damage, and thus have been proposed as indicators of cellular stress (Sanders 1993; Scofield et al. 1999; Varo et al. 2002). A correlation between exposure to environmental toxicants and stress protein synthesis has been established in several studies (Hassanein et al. 1999; Ait-Aissa et al. 2003; Padmini and Usha Rani 2008; Eder et al. 2009). The present study was conducted to determine seasonal effects on the expression of HSP70 in the liver, muscle and gill tissues of native fish species collected from the Kuwait marine area during the winter and summer months.

Materials and Methods

Two different types of fish, the predatory sea bream (locally known as shaem) (*Acanthopagrus latus*), and the

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demersal tonguesole (locally known as lassan) (*Lesan althour*) were chosen for this study. Sample fish were collected from inside the Kuwait Bay area and from outside the Bay from the southwestern Auha area. The Kuwait Bay area is influenced by local municipal and industrial discharges with resuspension of sediment deposited pollutants, whereas, the Auha area is located outside Kuwait Bay near the southern tip of Failaka Island, where it is surrounded by depositions from the plume of Iraq's Third River (Fig. 1). Fish were collected by trawling (i.e., using a fish trawl net) in the winter months of December (18.2°C), January (13.6°C) and March (16.5°C) and in the summer months of June (30.4°C), August (34.2°C) and September (31.5°C). Approximately 15 fish of each variety from each of the two locations in each season were placed in liquid nitrogen immediately after capture for storage. In the laboratory, the fish samples were stored at −80°C before analysis. The determination of heat shock protein HSP70 was based on the original method of Varo et al. (2002) with some alteration required for better separation and transfer of the HSP70 protein for the samples.

The proteins were extracted from the liver, muscle and gill tissues of fish by mechanical homogenization of the tissue in extraction buffer containing 20 mM HEPES, 500 mM NaCl, and 12.5 mM KCI, freshly complemented with 1 mM dithiothreitol (DTT), 1 mM phenymethysulfonyfluoride (PMSF), 0.1 g/ml trypsin inhibitor, and Igepal (1%), in a relation of 1:6 w/v. The homogenates were centrifuged for 90 min (39,500×*g* at 4°C), and the supernatants containing the proteins were separated and stored in 100-µl aliquots at −80°C until analysis.

Total protein was determined in the tissue homogenates using the Lowry method (Lowry et al. 1951) with bovine serum albumin as the standard. An aliquot of each sample was diluted to 5 mg/ml total protein with a buffer containing 50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.1% bromophenol blue. Samples were then denatured by boiling for 5 min and allowed to cool before being used for protein detection. Proteins were separated by one-dimensional SDS–polyacrylamide gel electrophoresis (PAGE) on 12% polyacrylamide gel. Each 10-well plate was loaded (20 µl) with a pre-stained molecular weight marker (i.e., a low range, pre-stained, SDS–PAGE standard), a commercial HSP70 standard, and an equal amount of protein from each sample. Gels were run on the electrophoresis system for 2–2.5 h at 60 V (constant) using a power supply. For transfer to a 0.45-µm nitrocellulose membrane, a sandwich was made and kept in a trans-blot electrophoresis transfer system with the required buffer, and run overnight at 25 V at 4°C. After transfer, the membrane was carefully removed and quickly kept in blocking solution (i.e., 5% nonfat powdered milk in Tris-buffered saline, 100 mM Tris, 1.5 M NaCl [pH 8]) for 1 h with shaking. After blocking, membranes were probed for 3 h at 25°C, with a mouse monoclonal anti-HSP70 primary antibody (with dilution at 1:500) in 3% nonfat powdered milk in Tween 20 in TBS (T-TBS). Membranes were then washed three times with

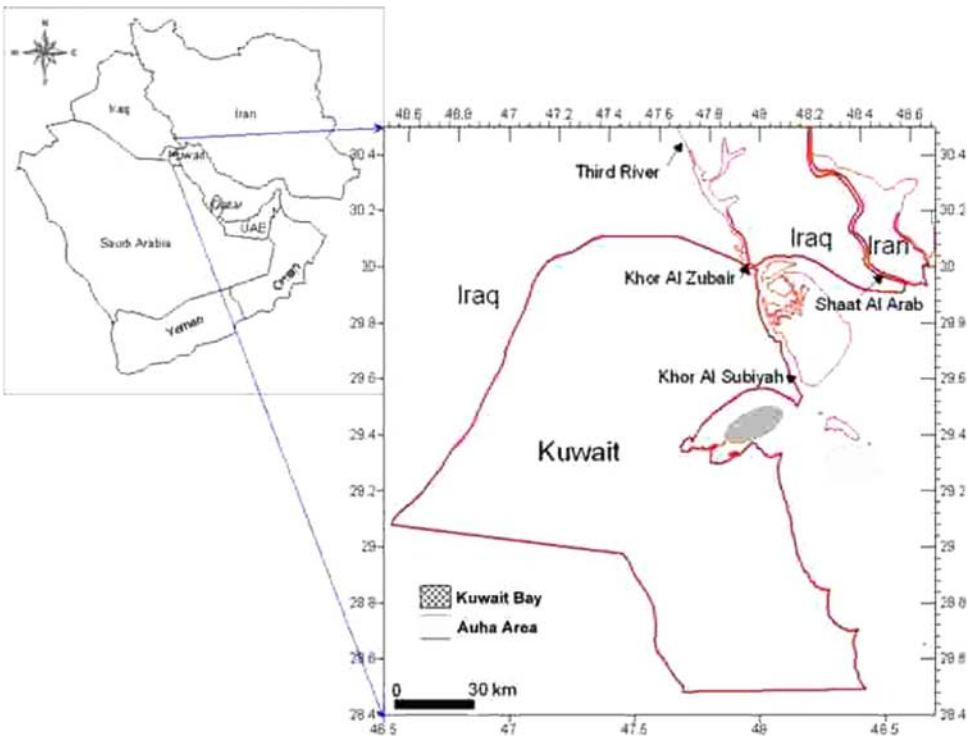

Fig. 1 Sampling stations in Kuwait Bay and the Auha area

T-TBS and one time with TBS for 10 min each, and incubated for 2 h at 25°C with an anti-mouse IgG secondary antibody conjugated with peroxidase diluted at 1:200 in 3% nonfat powdered milk in T-TBS. Blots were washed again with T-TBS three times and once in TBS for 10 min each. The required number of 3,3′-diaminobenzidine tetra hydrochloride (DAB) (Sigma, D4418) and urea hydrogen peroxidase tablets were taken out of the freezer and allowed to reach room temperature. Then, 15 ml of distilled water was added to the tablets and the solution was vortexed until the tablets had dissolved. The solution was used within 1 h. The membrane was then put in the solution, and bound peroxidase was revealed after incubation for 5–10 min in the dark.

HSP70 was quantified using a Total Lab Ultra Lum V2.00 (Nonlinear Dynamics Ltd., UK) image analysis system that consists of an Ultracam digital gel imaging system equipped with a UV–Vis Transilluminator and a high- resolution digital camera with a hood fitted with a white light. The Ultracam package also included a software package for the identification of electrophoretic gels. The HSP70 concentrations in the tissue samples were determined by analyzing a digital image of the immunoblot against the intensity of a blot of a known concentration of HSP standard procured from Sigma Chemicals. To validate the detection accuracy, standard HSP70 was applied at six concentrations (i.e., 1.3, 2.6, 3.9, 5.2, 6.5, and 7.8 µg) for electrophoresis and immunoblotting. Images of the immunoblots were captured and analyzed using the intensity of the blot for each concentration as the standard, allowing the concentrations of the other applied bands to be calculated. The calculated values were within an accuracy level of ±15% compared to the applied concentrations, except the lowest applied concentration, which was detected as being around 30% higher than the actual applied concentration. The HSP70 concentrations reported for the liver, muscle and gill tissues were not corrected since appropriate standards were run along with tissue samples.

Results and Discussion

The present analysis revealed wide variations in the HSP70 levels in samples of two fish species captured from two sampling locations in Kuwait's marine areas during the summer and winter months. The data were subjected to the Kruskal–Wallis test, and between the two sampling areas, i.e., Kuwait Bay and Auha, the difference in the HSP70 levels in the fish tissues were statistically insignificant. Therefore, the data for the same types of tissue from fish from both sampling areas were combined and are presented in box plots to determine both the central tendency as median values and the spread of values in the summer and winter months.

For sea bream, the box plots demonstrated higher median values in the winter months in all of the tissues examined. However, the difference in HSP70 levels between winter and summer months was statistically significant in liver and muscle tissues ($p < 0.05$) whereas the difference in gill tissues was statistically insignificant (Fig. 2).

For tonguesole, the median HSP70 values in the winter were also always higher than in the summer, but the difference was not statistically significant for any of the tissues analyzed. As with seabream, in tonguesole the spread of the HSP70 levels of liver and gill tissues was also higher in the winter samples, as is evident by the box length (Fig. 3).

In this study, large variations in the range of HSP70 levels were observed in both fish species; however, the median values for the summer and winter were 4.4 and 10.3 µg/mg proteins in the liver, 4.0 and 9.9 µg/mg proteins in the muscle and 7.3 and 11.6 µg/mg proteins in the gill tissues of sea bream. In tonguesole, the median values for summer and winter were 5.4 and 8.0 µg/mg proteins in liver, 6.6 and 10.3 µg/mg proteins in muscle, and 5.9 and 8.2 µg/mg protein in gill tissues respectively. The values obtained in this study were close to the reported HSP70 concentration in the gill tissue of the Pacific halibut (*Hippoglossus stenolepsis*) which was averaged 4.6 µg/mg proteins with levels ranging from 2.2 to 14.5 µg/mg proteins (Scofield et al. 1999).

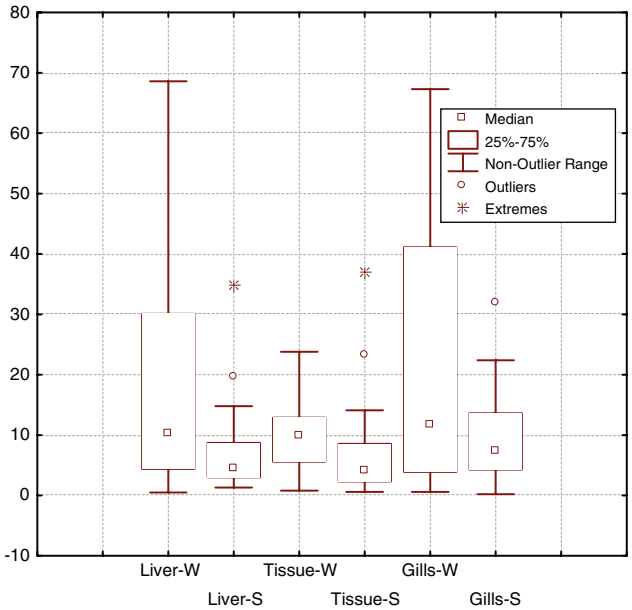

Fig. 2 Box plots of HSP70 levels (µg/mg protein) in liver, muscle and gill of sea bream collected during the winter and summer seasons

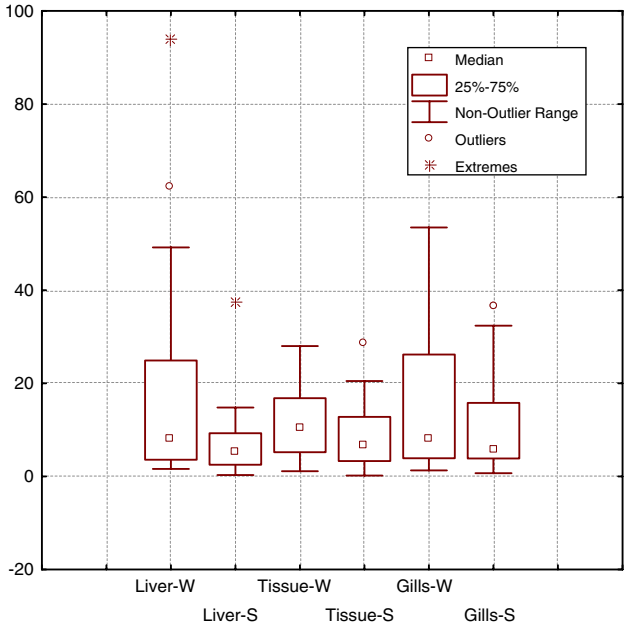

Fig. 3 Box plots of HSP70 levels (μg/mg protein) in liver, muscle and gill of tongue sole collected during the winter and summer seasons

The most common finding in the current study was higher HSP70 levels in winter months, which was contrary to expectations and needs further explanation. Stress biomarkers represent a response to sublethal exposure to stressors that is quantifiable but very difficult to interpret, especially in field situations. In fact, the stress response entails the rapid synthesis of proteins, referred to as stress proteins, which are heat inducible, and for this reason, were initially referred to as heat shock proteins (Sanders 1993). Subsequent studies revealed that HSP 70 consists of an array of stress proteins with similar molecular sizes, some of which are heat inducible and others of which are constitutively expressed. Dunlap and Matsumura (1997) observed significant increase in HSP70 levels in fathead minnows upon experimental exposure to low temperatures. In addition, HSP70 is markedly induced under various other stresses including UV irradiation, and treatment with heavy metals and other pollutants.

The inverse relation between the HSP levels in fish and water temperature obtained in the current study may be due to genetic adaptation of the fish to the hot climatic conditions of this region. Fish adapted to long period of high water temperatures possibly exhibited sensitivity to the relatively low water temperatures occurring in the short winter months. It has been reported that the tide-pool sculpin, which is exposed to wider fluctuations in water temperature in upper tide pools, has higher constitutive liver HSP70 levels that were only slightly influenced by changes in water temperature, whereas, the fluffy sculpin, which prefers lower tide pools with smaller fluctuations in water temperature, showed greater changes in liver HSP70 levels in response to thermal stress at lower temperatures (Iwama et al. 2003). Seasonal differences in the HSP70 levels detected in centipedes collected from the same area but at different time of the year led Pyza et al. (1997) to hypothesize that their HSP70 levels might be significant for adaptation to a periodically changing environment; however, it was not known whether seasonal changes in the HSP70 level appeared as the result of direct exposure to cold or heat, or were regulated by an endogenous clock in the circannual rhythm. Seasonal changes were also found in stream fish, with high levels of HSP70 being measured in the spring and low levels in the fall (Hoffmann and Somero 1995).

The wide variation in the HSP threshold-induction temperature among different marine species, and the wide variation in constitutive HSP levels among and within species may reflect not only recent thermal exposure but also a thermal history of the species during its evolution, and the occurrence of other stressors in the individual's habitat that are capable of activating the heat shock (stress) response (Dietz and Somero 1993; Hamer et al. 2004). Increased synthesis of the HSP70 group of proteins has been shown to be induced by a wide variety of stressors and a correlation between toxic exposure and stress protein synthesis has been established in several studies. Therefore, the higher HSP synthesis in the winter months in the current study might also have been a function of other stressors such as changes in pollutant and dissolved oxygen levels, and the conductivity and the salinity of the surrounding water. Werner (2004) showed that the ability to raise cellular HSP70 levels in response to heat shock was significantly impaired in bivalves collected from a low-salinity field site. In the current study, salinity was found to be slightly lower in the summer than in the winter months, possibly because increased evaporation at high temperature may cause an increased influx of low-salinity water through the Strait of Hormuz to the northern region of the Gulf resulting in decreased salinity in the summer months. The salinity changes are reported to interfere with HSP synthesis, as observed by Werner (2004) with bivalves.

HSP70 is also known to be induced by a number of other stimuli such as the trace elements cadmium, copper, and zinc (Urani et al. 2001), BaP; PCBs; PCP; HCH (Kohler et al. 1999); and PAHs (Cruz-Rodriguez and Chu 2002). Higher levels of PAHs have been observed in seabream collected during the winter months than in summer months (Beg et al. 2009), which may have some bearing on the higher HSP70 level found in the current study in the winter. In some fish small or undetectable levels of PAHs have been observed, whereas in others very high levels have been detected, which is analogous with the variation observed in the HSP70 levels. Further studies are required

to investigate a correlation between HSP expression and exposure to individual toxic substances or thermal stress under local environmental conditions. However, the current study provided a range of HSP70 levels in a demersal and a predatory fish collected from Kuwait's marine area which is influenced by mixed pollution and variable thermal stress in the winter and summer seasons.

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