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Short communication

## Baseline levels of Hsp 70, a stress protein and biomarker, in halibut from the Cook Inlet region of Alaska

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### Abstract

The levels of Hsp 70, a heat shock protein, was quantitatively determined in Pacific halibut, *Hippoglossus stenolepis*, from the Cook Inlet region in south central Alaska. A dot blot analysis using a monoclonal antibody for Hsp 70 was combined with a standard protein analysis to determine Hsp 70 levels in 26 samples from gills. The average Hsp 70 concentration was 4.6  $\mu\text{g}/\text{mg}$ , with levels ranging from 2.2 to 14.5  $\mu\text{g}/\text{mg}$  total protein. Mercury in gill tissue also was measured and, in the 26 samples, only three samples had concentrations of mercury ( $\bar{X} = 0.10$  mg/kg, range = 0.09–0.11) above the minimum detection level. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Alaska; Biomarker; Halibut; Hsp 70; Mercury; *Hippoglossus stenolepis*

### 1. Introduction

Biomarkers are measurements that can be used to monitor the relationship between exposure to a chemical or toxin and impaired health. Any biochemical that is quantitatively correlated with potential impairment of health resulting from exposure to an organic contaminant or metal would

be useful as a biomarker (Fox et al., 1988; Stegeman et al., 1992). Exposure to mercury (Hg) may result in enzyme inhibition or denaturation of proteins. Heat shock proteins (Hsps) function to maintain the proper folding of cellular proteins, that is, to act as chaperones (Frydman and Hartl, 1996). The concentration of Hsps in the cell changes when the cell is challenged by either physical or chemical stressors (Kohler et al., 1996). Because Hg may alter the conformation of the proteins in a tissue, such as fish (*Pices*) gills, Hsps may have potential as a biomarker of effect.

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Gills are a practical and readily available tissue for studying biomarkers and can be useful in evaluating chemical stress due to mercury (Fossi et al., 1997). In ecotoxicology studies, some species of fish, such as Pacific halibut (*Hippoglossus stenolepis*), have an advantage over others in that they remain in a confined area and feed upon benthic species of invertebrates and vertebrates. For example, in Puget Sound, WA, USA, liver lesions and neoplasms in bottom-dwelling fish have been associated with aromatic hydrocarbons in sediments (Krahn et al., 1984). In Alaskan coastal waters, halibut are a major component of northern marine ecosystems and also have both sport and commercial value. Because they are routinely harvested in Alaska, halibut samples can be obtained for analysis without additional damage to the population.

Most studies on fish have focused on the P450 mixed function oxidase system because of their role in detoxifying xenobiotic compounds (Spies et al., 1990). Only a few studies have evaluated Hsp as biomarkers in fish (Stegeman et al., 1992; Sanders and Martin, 1993). As part of a Benthic Surveillance Program with 11 sites on the east and west coasts of the US, Sanders and Martin (1993) reported that Hsp 70 levels in all the field samples was higher than those in control fish. The Hsp 60 levels in many of the sites also were higher than levels in the control fish. Additionally, mercury was reported in white flounder from Boston Harbor, MA and turbot from Santa Monica Bay, CA (Sanders and Martin, 1993).

Ryan and Hightower (1994) showed similar results indicating induction of Hsp 70 after stress from heavy metals in a kidney cell culture system of white flounder. More recently, Williams et al. (1996) reported that Hsp 70 levels increased in gills of both juvenile and adult rainbow trout, *Oncorhynchus mykiss*, after the fish were exposed to metals in either water or their diet. In other studies, the heat shock response also has been reported in eurythermal fishes, such as the killifish, *Fundulus heteroclitus* (Koban et al., 1991; Yu et al., 1994) and the goby fish, *Gillichthys* (Dietz and Somero, 1992). In our study, we report the presence of Hsp 70 in Pacific halibut from Alaska.

## 2. Materials and methods

### 2.1. Sample preparation

Samples were collected by hook and line in early July 1997, and gills removed in the field. Gills were cut with scissors before homogenizing them for  $3 \times 1$  min with a homogenizer at a 1:10 w/v tissue to buffer (50 mM Tris; 1 mM PMSF; 1% nonidet P-40). The homogenates were centrifuged for 1 h at 8°C at  $26\,000 \times g$  on a Sorvall RC5C centrifuge with a SA-600 motor. The supernatant was removed and filtered with a 0.5- $\mu$ m filter and then stored at  $-20^\circ\text{C}$  until analyzed.

### 2.2. Protein concentration of sample

The total protein of each sample was determined by the Bradford dye method (BioRad). Protein concentrations were based on a bovine albumin standard curve. Sample concentrations were occasionally examined using the dot Metric Protein assay (Chemcon).

### 2.3. Hsp analysis

Gel electrophoresis was performed using 15 well minigels from BioRad under the running conditions of 200 V for approximately 40 min. Once this procedure was complete, the gel was then blotted onto a PVDF membrane in Towbin buffer and transferred at 250 mA for approximately 60 min (Bodenstein and Duffy, 1998). The PVDF membrane was developed using an alkaline phosphatase anti-mouse system with the primary antibodies at the concentration suggested by the supplier (StressGen). The Hsp 70 antibodies epitope matches the carboxyl terminus region (502–540). After development, the PVDF membranes were then analyzed using under a densitometer to determine the density of the protein bands. Dot blot analysis was performed on samples after it was demonstrated that only a single band was immunoreactive on the western blots (Bodenstein and Duffy, 1998). The density information was then divided by the protein concen-

tration for each sample; the intraassay variation was 9.8% and the inter-assay variation was 21%.

#### 2.4. Mercury analysis

Mercury analysis was conducted at Northern Testing Laboratories (Fairbanks, AK) using Perkin–Elmer 2380 atomic absorption spectro photometer (EPA Method 7471). Each sample, 4.2 g in 5 ml of distilled H<sub>2</sub>O, was digested in 5 ml aqua regia (3 vols HCL: 1 vol. HNO<sub>3</sub>) by heating the sample for 2 min at 95°C. After cooling, 50 ml H<sub>2</sub>O and 15 ml KMnO<sub>4</sub> was added to sample and heated for 30 min. After cooling again to room temperature, 6 ml of sodium chloride-hydroxylamine solution was added; this was followed by dilution with H<sub>2</sub>O and addition of 5 ml of SnCl. Hg is reduced to the elemental state and absorbance read at 253.7 nm.

### 3. Results

The expression of the stress protein Hsp 70 could be detected in samples from the gills of halibut. A single immunoreactive band was observed on the western blots of each sample at the expected molecular weight. Based on the standards, total Hsp protein in each sample was calculated and normalized for the total protein in sample to give an Hsp 70 concentration in  $\mu\text{g}$  Hsp/mg protein (Table 1). Heat-shock protein (Hsp 70) was expressed in all 26 samples from halibut gills ( $\bar{X} = 4.6 \pm 2.61$  S.D.  $\mu\text{g}/\text{mg}$  protein; range = 2.2–14.5  $\mu\text{g}/\text{mg}$  protein); the coefficient of variation for Hsp was 57.1%. Only 11.5% of 26 gills, however, possessed detectable levels of Hg ( $\bar{X} = 0.10 \pm 0.01$  S.D. mg/kg; range = 0.09–0.11); the remaining 23 samples had Hg levels that were below minimum detectable levels (MDL). Mean levels for Hsp from gills with detectable levels of Hg ( $\bar{X} + 4.4 \pm 1.4$  S.D.  $\mu\text{g}/\text{mg}$  protein) were nearly identical ( $\bar{X} = 4.6 \pm 2.7$  S.D.  $\mu\text{g}/\text{mg}$  protein) to samples in which Hg could not be detected. Table 1 lists these as well as the one-half MDL values for these samples.

Table 1  
Heat shock protein and mercury levels in Alaskan Cook Inlet halibut

Sample	Hsp 70 ( $\mu\text{g}/\text{mg}$ protein)	Hg <sup>a</sup> (mg/kg)
1	7.8	0.05
2	4.2	0.06
3	6.9	0.04
4	3.4	0.10
5	5.5	0.05
6	2.6	0.03
7	7.4	0.04
8	4.8	0.03
9	2.2	0.04
10	2.4	0.04
11	5.5	0.04
12	2.3	0.03
13	3.2	0.04
14	2.6	0.04
15	6.1	0.15
16	2.4	0.06
17	3.3	0.03
18	3.4	0.09
19	3.9	0.03
20	3.9	0.03
21	3.7	0.09
22	2.6	0.05
23	14.5	0.04
24	4.7	0.04
25	3.4	0.04
26	6.0	0.11

<sup>a</sup> 1/2 MDL is reported, except in samples 18, 21 and 26.

### 4. Discussion

Studies in the laboratory (Stegeman et al., 1992; Ryan and Hightower, 1994) have shown that fish can be sensitive to pollutants. Also, Williams et al. (1996) demonstrated that the levels of Hsp were significantly increased in gill but not in livers of juvenile rainbow trout fed a metal-contaminated diet. In our study, gills showed Hsp 70 levels in each sample, but no trend toward increased accumulation of Hsp 70 with increased levels of mercury in the gills in the three samples that had detectable Hg levels.

Because the antibody used measured the inducible form of Hsp 70, our results report the inducible form (Hsp 72). There is a single report

that in fish, Hsp may be present in large amounts (Yu et al., 1994). Studies with invertebrates also support the concept of constitutive forms being present during normal activity (Sanders et al., 1992). Because levels of mercury were relatively low (< 0.11 ppm), our Hsp 70 concentrations may represent either a cross-reacting constitutive form of Hsp 70 or baseline levels of the inducible form. As development continues in the Cook Inlet region, Hsp 70 can be monitored to observe any increase in stress in this fish population. The samples in this study were all collected in the mid summer so seasonal variation, which has been reported to occur in some biomarkers (Fader et al., 1994), did not affect our results. Future baseline studies, however, will characterize the Hsp 70 levels during different seasons.

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### References

- Bodenstein Y, Duffy LK. Expression of Hsp 60, a stress protein, in human nasal septa cells after exposure to MTBE. *Environ Toxicol Pharmacol* 1998;5:79–83.
- Dietz TJ, Somero GN. The threshold induction temperature of the 90-kd heat shock protein is subject to acclimatization in eurythermal goby fishes (genus *Gillichthys*). *Proc Natl Acad Sci USA* 1992;89:3389–3393.
- Fader SC, YU Z, Spotelia JR. Seasonal variation in heat shock proteins (hsp 70) in stream fish under natural conditions. *J Therm Biol* 1994;19:335–341.
- Fossi MC, Savelli C, Casini S, Francini E, Mattei N, Corsi I. Multi-response biomarker approach in the crab *Carcinus aestuarii* experimentally exposed to benzo[*a*]pyrene, polychlorobiphenyls and methyl-mercury. *Biomarkers* 1997;2: 311–319.
- Fox GA, Kennedy SW, Norstrom RJ, Wigfield DD. Porphyria in Herring Gulls: A biochemical response to chemical contamination of Great Lakes food chains. *Environ Toxicol Chem* 1988;7:831–839.
- Frydman J, Hartl FU. Principles of chaperone-assisted protein folding: differences between in vitro and in vivo mechanisms. *Science* 1996;272:1497–1502.
- Koban M, Yup AA, Agellon LB, Powers DA. Molecular adaptation to environmental temperature: heat-shock response of the eurythermal teleost *Fundulus heteroclitus*. *Mol Mar Biol Biotech* 1991;1:1–17.
- Kohler HR, Rahman B, Graff S, Berkus M, Triebkoin R. Expression of the stress 70 protein family (hsp 70) due to heavy metal contamination in the slug, *Deroceras reticulatum*: an approach to monitor sublethal stress conditions. *Chemosphere* 1996;33:1327–1340.
- Krahn MM, Myers MS, Burrows DG, Malins DC. Determination of metabolites of xenobiotics in the bile of fish from polluted waterways. *Xenobiotica* 1984;14:633–646.
- Ryan JA, Hightower LE. Evaluation of heavy-metal ion toxicity in fish cells using a combined stress-protein and cytotoxicity assay. *Environ Toxicol Chem* 1994;13:1231–1240.
- Sanders BM, Pascoe VM, Nakagawa PA, Martin LS. Persistence of the heat-shock response over time in a common *Mytilus* mussel. *Mol Mar Biol Biotech* 1992;1:147154.
- Sanders BM, Martin LS. Stress proteins as biomarkers of contaminant exposure in archived environmental samples. *Sci Total Environ* 1992;139/140:459–470.
- Spies RB, Stegeman JJ, Hinton DE et al. Biomarkers of hydrocarbon exposure and sublethal effects in embiotocid fishes from a natural petroleum seep in the Santa Barbara Channel. *Aquatic Toxicology* 1990;34:195219.
- Stegeman JJ, Brousier M, DiGuilio RT, Forlin L, Fowler BA, Sanders BM, VanVeld PA. Molecular responses to environmental contamination: enzyme and protein system as indicators of chemical exposure and effect. In: Huggett RJ, Kimerle RA, Mehrle PM, Bergman HL, editors. *Biomarkers—biochemical, physiological and histological markers of anthropogenic stress*. Boca Raton, FL: Lewis Publishers, 1992:235–335.
- Williams JH, Farag AM, Stansbury MA, Young PA, Bergman HL, Petersen NS. Accumulation of hsp 70 in juvenile and adult rainbow trout gill exposed to metal contaminated water and/or diet. *Environ Toxicol Chem* 1996;15: 1324–1328.
- Yu Z, Magee WE, Spotelia JR. Monoclonal antibody ELISA test indicates that large amounts of constitutive hsp-70 are present in salamanders, turtle and fish. *J Therm Biol* 1994;19:41–53.