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# Comparative baseline levels of mercury, Hsp 70 and Hsp 60 in subsistence fish from the Yukon-Kuskokwim delta region of Alaska

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## Abstract

In subsistence fish; northern pike (*Esox lucius*), burbot (*Lota lota*), whitefish (*Coregonus nelsoni*), grayling (*Thymallus arcticus*) and sheefish (*Stenodus leucichthys*), we determined the Hsp 60 and Hsp 70 levels in 31 samples from adult fish gills. A dot-blot analysis using antibodies to either Hsp 70 or Hsp 60 showed the average Hsp 70 concentration was 9.1 µg/mg protein, while the average Hsp 60 concentration was 147.4 µg/mg protein. Mercury levels in muscle tissue in these fish averaged 0.382 ppm. Using a subset of samples ( $n=24$ ), we determined that the major component in the muscle of Alaskan subsistence fish was methyl mercury. No correlation was observed between Hsp 60 or Hsp 70 expression in gill tissue and mercury concentrations in muscle tissue. Hsp 60 and Hsp 70 protein levels in the gills were correlated. © 1999 Elsevier Science Inc. All rights reserved.

**Keywords:** Alaska; Pike; Grayling; Burbot; Biomarker; Hsp 70; Hsp 60; Mercury; Methyl mercury; Subsistence fish

## 1. Introduction

Biomarkers are measurements that can be used to monitor the relationship between exposure to a chemical and impaired health in an organism [8,18]. Any biochemical response that is correlated quantitatively with potential impairment of health resulting from exposure to an organic contaminant or metal would be useful as a biomarker [8,9,16]. Exposure to metals such as copper and cadmium induces heat shock proteins in invertebrates and fish [11,19]. Heat shock proteins (Hsps) function to maintain the proper folding of cellular proteins, that is, to act as chaperones [10]. The concentration of Hsps in the cell changes when the cell is challenged by either physical or chemical stressors [16]. Because Hg may alter the conformation of the proteins in the tissue of fish, Hsps may have potential as a biomarker of effect for mercury in fish obtained for subsistence uses. Knowledge of actual levels in natural

populations of fish is needed if biomarkers are to be used to indicate future environmental stresses. Because 9% of the fish in Western Alaska have mercury levels >0.5 ppm [5], we questioned what the Hsp levels would be in this population of subsistence fish.

Tissues such as muscle and gills have been proposed for studying biomarkers and in evaluating chemical stress due to mercury [8]. Muscle tissues are used in monitoring mercury and other metal levels [13]. In Western Alaskan rivers, various species of fish, including northern pike (*Esox lucius*), burbot (*Lota lota*), whitefish (*Coregonus nelsoni*), grayling (*Thymallus arcticus*), and sheefish (*Stenodus leucichthys*), are important for subsistence among rural populations and, in some instances have sport-fishery value [14]. Mercury enters the Alaskan environment in two ways: (1) global distribution of industrial emissions through the atmosphere, and (2) from point sources, such as old mining areas and natural erosion of geological deposits [6]. In the Yukon-Kuskokwim Delta, there was community concern that mining activity earlier in this century may have led to high mercury levels in local fish.

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Many studies on fish have focused on the P450 mixed-function oxidase system because of its role in detoxifying xenobiotic compounds [17,18]. Only a few studies have evaluated Hsp 70 and Hsp 60 as biomarkers in fish exposed to metals [7,15,16,19,20]. As part of a Benthic Surveillance Program with multiple sites on the east and west coasts of the United States, Sanders and Martin [16] reported that levels of Hsp 70 in field samples from a polluted area were higher than those in control fish. Ryan and Hightower [15] showed similar results indicating induction of Hsp 70 after stress from heavy metals in a culture system of kidney cells of winter flounder (*Plevronectes americanus*). More recently, Williams et al. [19] reported that Hsp 70 levels increased in gills of juvenile rainbow trout, *Oncorhynchus mykiss*, after these fish were exposed to metals in either water or their diet. This report focuses on adult fish in Western Alaska that may be chronically exposed to high mercury levels, and tests for a relationship between this exposure and Hsp levels.

## 2. Materials and methods

Of 66 fishes collected, 31 were sampled for heat shock proteins. The collection sites were distributed throughout the Yukon-Kuskokwim Delta region as chosen by subsistence users. Fish were collected by multiple collection methods, frozen and transferred within 24 h to Bethel. At the laboratory in Bethel, Alaska, fish were stored at  $-20^{\circ}\text{C}$  until dissection by a trained technician; muscle samples were sent to Frontier Geosciences (Seattle, WA) for analysis. Hg was analyzed by cold vapor atomic fluorescence spectrophotometry (CVAF) after samples were digested with acid [1].

Frozen gill tissue was shipped to Fairbanks for Hsp analysis. Gills were cut with scissors before homogenization at a 1:10 w/v tissue to buffer (50 mM Tris; 0.1 mM PMSF; 1% nonidet P-40) for  $3 \times 1$  min. The homogenates were centrifuged for 1 h at  $8^{\circ}\text{C}$  at  $16\,000 \times g$  on a Sorvall RC5C centrifuge with a SA-600 rotor. The supernatant was removed and filtered with a 0.5 micron filter and then stored at  $-20^{\circ}\text{C}$  until analyzed for either Hsp 70 or Hsp 60.

### 2.1. Protein concentration of gill samples and Hsp analysis

The total protein of each sample was determined by the Bio-Rad dye method. Protein concentrations were based on a standard curve using bovine serum albumin [3]. Total protein loaded into the sample wells for the dot-blot analysis varied from 2 to 40  $\mu\text{g}$  per sample.

### 2.2. Gel electrophoresis

SDS-gel electrophoresis was performed using minigels from BioRad with the running conditions of 100 volts for approximately 90 minutes. Equal amounts of protein samples ( $\sim 20 \mu\text{l}$ ) of known protein concentration were loaded on 10 cm SDS-minigels using a 1:1 dilution with SDS-sample buffer. Twelve percent acrylamide gels without a stacker (Bio-Rad) were used [2].

### 2.3. Western blots

Gels were blotted onto nitrocellulose membranes (Bio-Rad) using a Tris-HCl blotting buffer. Gels were equilibrated for 10 min in the Tris-HCl blotting buffer prior to blotting with a Bio-Rad Transblotter at 0.2 amps for 1 h. Blots were rinsed with distilled water and blocked with 5% dry milk in water. The blots were washed with a 150 mM, 20 mM Tris buffer which contained 0.05% Tween. After three washes for 10 min, the nitrocellulose membrane was incubated with a monoclonal antibody for either Hsp 70 (Stress Gen Nr SPA-810) or Hsp 60 (Stress Gen SPA-806). Both of these antibodies recognize conserved epitopes in vertebrates. The antibody dilution was 1:1000. The western blots were washed as before and incubated with an HRP-conjugated goat antimouse antibody (Bio-Rad, 170-6464). For color development, the western blot was again washed and then stained using DAB as a HRP substrate (Bio-Rad, 170-6535). Positive controls for the western blot were isolated Hsp 60 and 70 proteins (Stress Gen SPP-740; Stress Gen SPP-755). Although the antibodies used in this study detected Hsp 70 and Hsp 60, there could be differences in amounts of immunoreactivity between the proteins that is species specific.

### 2.4. Dot-blots

Because only a single immunoreactive band was observed on the western blots, a dot-blot procedure was used for quantification of samples. A standard curve was developed using a series of known amounts of Hsp 60 (SPP-740), 6–19  $\mu\text{g}$  or for Hsp 70 (SPP-755), 0.2–5  $\mu\text{g}$ . Since species specific Hsp 70 and Hsp 60 proteins were not used as positive controls, the data is reported as microgram equivalents per milligram of total protein. A Bio-Dot microfiltration apparatus (Bio-Rad) provided a reproducible method for binding Hsp proteins onto the nitrocellulose membranes. Immunochemical detection was completed on the membrane in an identical manner to the western blots. Density information was obtained using an Alpha Innotech 1000 and concentrations were calculated by dividing the Hsp amount by the protein concentration for each sample. Total Hsp protein in each sample was calculated to

normalize for the total protein in sample, and the Hsp 70 concentration in  $\mu\text{g}$ -equivalents/mg protein determined. The intra-assay variation was 9.8% and the inter-assay variation was 21%.

### 2.5. Mercury analysis

Mercury analysis was conducted at Frontier Geosciences (Seattle, WA) using cold vapor atomic fluorescence spectroscopy (CVAFS). For tissue samples, 0.5–1.0 g of the homogenized sample was accurately weighed into a Teflon vial. To the vial, 7.0 ml of a 7:3 v/v mixture of concentrated  $\text{HNO}_3 + \text{H}_2\text{SO}_4$  was added. The sample was placed on a  $125^\circ\text{C}$  hotplate for 2 h after the onset of reflux or until all organic matter was dissolved. After cooling, the sample was diluted to about 25 ml with a 10% solution of 0.2N BrCl. Milli. Q water (100 ml) and 0.3 ml  $\text{SnCl}_2$  was added to an aliquot of the digested sample in the bubbler. The gold trap was then placed onto the system to be analyzed [1]. The mercury was released from the gold sand traps using heat and determined using a Tekran detector. After mercuric ions were reduced to Hg with  $\text{SnCl}_2$ , the Hg was then purged onto gold coated sanded traps as a means of preconcentration by using  $\text{N}_2$  and a bubbler. We used Manova and linear regression to test for a relationship between Hg and heat shock proteins, species of fish, and location [21].

### 3. Results

CVAF spectrophotometry, with its increased sensitivity, allowed Hg to be detected in all samples (Table 1). The mean level of total Hg for all species of fish was  $0.382 \text{ ppm} \pm 0.468 \text{ S.D.}$  Methyl mercury (MeHg) was the major component in the muscle tissue (Table 2). There was no relationship between Hg levels and gender, but mercury levels increased with the size of the fish (date not shown).

When species of fish were compared, Hg levels were highest in pike. The mean ( $\pm \text{S.D.}$ ) levels for the different species were: pike,  $0.823 \text{ ppm} \pm 0.567$ ; whitefish,  $0.163 \text{ ppm} \pm 0.076$ ; sheefish,  $0.159 \text{ ppm} \pm 0.022$ ; burbot,  $0.096 \text{ ppm} \pm 0.005$ ; grayling,  $0.101 \text{ ppm} \pm 0.040$ . The Andrefski River and Piamute (near Holy Cross) showed the highest levels. The Andrefski River showed mean levels of  $1.068 \pm 0.803 \text{ ppm}$ , while Piamute was  $0.813 \pm 0.451 \text{ ppm}$  Hg. Lower levels occurred in fish from the Kuskokwim (near Bethel), the Johnston, and the Tuluksak rivers.

The expression of the stress protein Hsp 70 could be detected in samples from the each species of fish. Heat-shock protein 70 (Hsp 70) was expressed in all samples of fish ( $\bar{X} = 9.1 \pm 11.2 \text{ S.D. } \mu\text{g/mg protein}$ ; range = 0.9–40.3  $\mu\text{g/mg protein}$ ). There were individual differ-

ences between species when examined at their mean expression: pike ( $4.3 \pm 3.0 \mu\text{g/mg}$ ); burbot ( $4.2 \pm 0.86 \mu\text{g/mg}$ ); whitefish ( $16.2 \pm 14.5 \mu\text{g/mg}$ ); grayling ( $17.7 \pm 16.0 \mu\text{g/mg}$ ); sheefish ( $1.8 \pm 0.26 \mu\text{g/mg}$ ). Whitefish and grayling had the highest mean Hsp 70 concentrations and also the largest standard deviations. The nine whitefish samples covered the entire range of Hsp 70 levels for all fish analyzed.

Heat-shock protein 60 (Hsp 60) also was expressed in all fish sampled and species differences were detected when their mean levels were compared: pike ( $228.6 \pm 495.5 \mu\text{g/mg}$ ); burbot ( $50.5 \pm 28.8 \mu\text{g/mg}$ ); whitefish ( $113.1 \pm 145.1 \mu\text{g/mg}$ ); grayling ( $211.4 \pm 174.8 \mu\text{g/mg}$ ); sheefish ( $29.7 \pm 25.9 \mu\text{g/mg}$ ). Whitefish and grayling showed relatively high mean levels of Hsp 60, similar to the relative species means for Hsp 70. In contrast to the Hsp 70 means, however, pike showed the highest mean levels. This result was due to sample number 16, which shows the highest Hsp 60 protein levels and the third highest mercury level. The other two pike with high mercury levels had low levels of Hsp 60 (Table 1).

We conducted analysis using MANOVA with Hg, Hsp 60, and Hsp 70 as dependent variables, and species of fish and location as main effects. Overall differences were highly significant for both species ( $F = 140.21$ ,  $df = 9, 44$ ,  $P = 0.0001$ ) and location ( $F = 72.76$ ,  $df = 21, 44$ ,  $P = 0.0001$ ), but we could not estimate a species by location interaction because some species were not captured at all locations. A posteriori analyses for individual dependent variables indicated that Hsp 70 did not vary significantly across species or locations ( $F = 2.04$ ,  $df = 11, 29$ ,  $P = 0.09$ ), but both Hsp 60 ( $F = 4.42$ ,  $df = 11, 29$ ,  $P = 0.01$ ) and Hg ( $F = 17.95$ ,  $df = 11, 29$ ,  $P = 0.0001$ ) did vary. The outcome for Hsp 60 was driven mostly by species ( $P = 0.08$ ) rather than location ( $P = 0.58$ ), but both of these variables were strongly influenced by Hg ( $P = 0.0001$ ).

Direct relationships between the heat shock proteins and Hg were not apparent ( $P > 0.25$ ), but Hsp 60 and Hsp 70 were loosely but significantly related ( $r^2 = 0.35$ ,  $P < 0.05$ ).

### 4. Discussion

Studies in the laboratory have shown that organisms can be sensitive to metals, such as in crabs, where the highest accumulation of metals was in the gills of exposed crabs [12]. Also, Williams et al. [19] demonstrated that the levels of Hsp 70 were significantly increased in gills of fish exposed to a mixture of cadmium, copper, lead and zinc. Although we detected Hsp 70 in each gill sample, a direct relationship between increased levels of Hsp 70 in gills and increased levels of mercury in the muscle tissue was not apparent. Indeed, in Pike, when samples with Hg levels  $> 1 \text{ ppm}$

were compared with those having < 0.3 ppm Hg, the samples with the lower Hg ppm had higher Hsp 70 levels ( $4.5 \mu\text{g}/\text{mg} \pm 1.60$ ). Conversely, the higher Hg exposed pike had lower Hsp70 levels ( $2.4 \mu\text{g}/\text{mg} \pm 1.1$  S.D.). These Hsp 70 concentrations represent either baseline levels of the inducible form or a cross-reacting constitutive form of Hsp 70. The samples in this study were all collected over the year so seasonal variation, which has been reported to occur in some biomarkers and in Hsp 70 [7,16,20], could affect our results by increasing the variation in these baseline levels. Similarly, we observed no statistical relationship between increased levels of Hsp 60 in gills and increased Hg

levels. With the exception of one sample, Hsp levels tended to decrease with increased Hg levels. On the other hand, Hsp 60 and Hsp 70 levels were loosely correlated. In every sample, the level of Hsp 60 was higher than Hsp 70.

There was a statistical relationship between location, Hsp 60, and mercury in fish. Higher mercury levels were not observed in some areas where mining activity occurred in the past. In an earlier survey of Hg, Crayton [4] reported that there was no significant difference in Hg between pacer mined sites and refuge sites in Tuluksak River pike. When converted to wet weight, the Hg values we observed for grayling in 1997 were

Table 1  
Comparison of total Hg and stress protein Hsp 70 and Hsp 60

Fish	Location	Hg total (ppm)	Hsp 70 ( $\mu\text{g equiv./mg protein}$ )	Hsp 70 ( $\mu\text{g equiv./mg protein}$ )
<i>Pike</i>				
03	Piamute	1.482	2.8	16.8
04	Emmonak	0.295	3.7	62.7
07	Gweek R.	0.178	5.8	UND <sup>a</sup>
08	Piamute	0.649	11.8	139.9
09	Piamute	0.259	2.6	58.2
10	Piamute	0.711	2.3	64.3
11	Piamute	0.964	5.3	154.2
16	George R.	1.308	3.8	1,631.8
18	Andrefski R.	0.225	5.8	116.5
19	Andrefski R.	1.156	1.8	35.9
20	Andrefski R.	1.824	1.2	5.8
$\bar{X}$		$0.823 \pm 567$	$4.3 \pm 3.0$	$228.6 \pm 495.5$
<i>Burbot</i>				
05	Bethel	0.094	3.3	18.2
21	Bethel	0.093	5.0	59.4
22	Bethel	0.103	4.4	73.8
$\bar{X}$		$0.096 \pm 0.005$	$4.2 \pm 0.86$	$50.5 \pm 28.8$
<i>Whitefish</i>				
06	Bethel	0.058	11.8	28.2
12	Goodnews R.	0.107	40.3	164.1
13	Kanektok R.	0.160	9.3	102.3
15	George R.	0.326	33.0	402.5
17	George R.	0.137	30.2	288.5
23	Kanektok R.	0.176	0.9	10.7
24	Kanektok R.	0.167	9.7	7.4
25	Kanektok R.	0.121	9.4	8.9
26	Kanektok R.	0.218	0.9	5.8
$\bar{X}$		$0.163 \pm 0.076$	$16.2 \pm 14.5$	$113.1 \pm 145.1$
<i>Grayling</i>				
27	Tuluksak R.	0.160	3.2	87.7
28	Tuluksak R.	0.082	4.7	34.8
29	Tuluksak R.	0.082	33.7	355.4
30	Tuluksak R.	0.079	29.2	367.8
$\bar{X}$		$0.101 \pm 0.040$	$17.7 \pm 16.0$	$211.4 \pm 174.8$
<i>Sheefish</i>				
14	George R.	0.152	2.1	68.5
32	Johnston R.	0.186	1.6	19.9
33	Johnston R.	0.134	1.8	15.9
34	Johnston R.	0.165	1.5	14.6
$\bar{X}$		$0.159 \pm 0.022$	$1.8 \pm 0.26$	$29.7 \pm 25.9$

<sup>a</sup> UND = Undetermined.

Table 2  
Comparison of total Hg and methyl Hg in fish muscle tissue

Fish (Sample no.)	Total Hg (ppm)	Methyl Hg (ppm)
<i>Pike</i>		
(16)	1.308	1.364
(18)	0.225	0.238
(19)	1.156	1.182
(20)	1.824	1.839
$\bar{X}$	1.128 ± 0.667	1.156 ± 0.672
<i>Burbot</i>		
(21)	0.093	0.096
(22)	0.103	0.111
$\bar{X}$	0.098 ± 0.007	0.104 ± 0.011
<i>Whitefish</i>		
(12)	0.107	0.121
(13)	0.160	0.155
(23)	0.176	0.168
(24)	0.167	0.159
(25)	0.121	0.132
(26)	0.218	0.215
(17)	0.137	0.134
$\bar{X}$	0.155 ± 0.037	0.155 ± 0.031
<i>Grayling</i>		
(27)	0.160	0.178
(28)	0.082	0.076
(29)	0.082	0.081
(30)	0.079	0.084
$\bar{X}$	0.101 ± 0.040	0.105 ± 0.049
<i>Sheefish</i>		
(32)	0.186	0.197
(33)	0.134	0.139
(34)	0.165	0.125
$\bar{X}$	0.167 ± 0.026	0.154 ± 0.038

lower than what Crayton [4] reported for grayling. A similar Hg survey was performed by the Tanana Chiefs with pike caught in interior Alaska mining drainages. Their study showed no relationship to mining activity. The range of mercury levels in the Tanana Chiefs study for pike were from 0.091 ppm to 0.832 ppm. Other studies report that the average concentrations in various fish are approximately 0.2 µg/g [6]. In this study, the mean Hg for pike was both above that level as well as the EPA's reference dose [6]. Also, when the whole fish is considered, it is not clear what synergetic effects other compounds such as PCBs might have or the protective effect of selenium or omega-3 fatty acids.

Williams et al. [19] reported the levels of Hsp increased in gills but not in the livers of juvenile rainbow trout raised in a high Cd, Cu, Pb and Zn water environment and fed a metal enriched diet. They did not detect a difference in Hsp 70 that could be related to muscle Hg levels. Unlike Williams et al. [19], we observed no direct statistical relationship between Hsp 70 and increased Hg. A posteriori analysis however, suggested that both Hsp 60 and Hg levels were dependent on species and location. Although the variation of

Hg and Hsp 60 with location and species warrants further investigation, basic studies looking at species specific differential expression of Hsp should be undertaken first in other tissues such as liver or muscle. Other Hsp proteins such as Hsp 27 or Hsp 32 should be studied to determine if other stress proteins would be a more useful biomarker for Hg contamination.

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