Biomarkers of Heavy Metal Effects in Two Species of Caddisfly Larvae from Clark Fork River, Montana: Stress Proteins (hsp70) and Lysosomal Membrane Integrity

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ABSTRACT

Sublethal biological effects of heavy metals accumulated in stream macro-invertebrates were examined in larvae of two caddisflies indigenous to the Clark Fork River, Montana, - Hydropsyche spp. and Arctopsyche grandis. Stress proteins, in particular members of the hsp70 family, are involved in cellular protein homeostasis and repair, and are induced by a variety of stressors, which either damage cellular proteins directly or cause cells to synthesize aberrant proteins. Lysosomes are intracellular organelles that play key role in the detoxification of both organic and inorganic xenobiotic compounds. Integrity of the lysosomal membrane is essential for proper function of these structures. Following a heavy metal pollution gradient, larvae of Hydropsyche spp. and A. grandis were collected from 4 or 3 stations, respectively, in the Clark Fork River, and a reference site (Blackfoot River). Samples were immediately frozen in liquid nitrogen for hsp70 analysis, or preserved with Tissue Tek, then frozen in liquid nitrogen for the lysosomal stability assay. Hsp70 was analyzed by western blotting using monoclonal antibodies. Lysosomal integrity was measured in cryosections by acid labilization with acid phosphatase as a marker enzyme. Results show good correlation between elevated tissue concentrations of Cd, Cu, Pb and Zn, significantly increased levels of hsp70, and reduced lysosomal membrane stability in both species collected at the Goldcreek site.

INTRODUCTION

In the Clark Fork River, Montana, bed sediments, flood plain sediments, and benthic insects have been extensively contaminated with Cd, Cu, Pb, and other elements by mining and smelting at Butte and Anaconda, near the river's headwaters. Mine wastes are no longer discharged directly into the river, but contaminated bed sediments extend at least 380 km downstream of the headwaters. To date, sampling of sediments and benthic invertebrates produced an extensive data base on the extent of metal contamination and bioavailability of metals present. Metal concentrations in caddisfly larvae (Hydropsyche spp. and A. grandis) exhibit similar spatial patterns, but differ somewhat in absolute metal concentrations. A. grandis is not found at the station closest to the contamination source, Galen Gage, while Hydropsyche spp. is present in large numbers.

While chronic metal contamination of waterways is a recognized problem, the toxic effects of metal pollution on aquatic organisms are still poorly understood. Our efforts focus on linking two biomarkers considered to indicate exposure and deleterious effect to heavy metal tissue concentrations along this gradient. Here we present our results on stress protein hsp70 and lysosomal membrane stability in Hydropsyche spp. and A. grandis collected from the Clark Fork River and from a reference site on the Blackfoot River, Montana. Stress proteins (or heat shock proteins, hsp), such as members of the hsp70 protein family, are induced by a variety of stressors, including elevated temperature and toxic chemicals which either damage cellular proteins directly or cause cells to synthesize aberrant proteins. Available information indicates that stress proteins confer protection from environmentally induced cellular damage, and accumulation of these stress proteins in organisms has been linked to the intensity of

stress. Lysosomes are cellular organelles, which – among other functions – accumulate and sequester xenobiotic compounds. The integrity of lysosomal membranes is considered an indicator of non-specific acute and chronic toxic effects. Compromised integrity of the lysosomal membrane can lead to leakage of enzymes into the cytoplasm leading to disturbance of cell functions and resulting in cell and tissue degeneration.

METHODS

Sample Collection

Caddisfly larvae were collected during base flow conditions on 10-14 August, 1998, from 4 sites on the Clark Fork River and one reference site on the Blackfoot River, Montana. Hydropsyche spp. was collected from Above Flathead (381 km from source), Turah (189.7 km), Gold Creek (85.6 km), Galen Gage (4.7 km) and Blackfoot River. A. grandis was collected from Above Flathead, Turah, Gold Creek and Blackfoot River. Insects were sampled by kick net from a single riffle at each station, identified and separated, immediately frozen in liquid nitrogen and transported on dry ice. Subsamples were preserved for heavy metal analysis.

Stress Proteins (hsp70)

Hsp70 proteins were detected using Western blotting techniques. Whole animals (10) were homogenized in tris buffer (pH 7.2). Homogenates were centrifuged for 30 min. at 4000 g to remove large particulate material. Resulting pellets were resuspended in homogenization buffer containing 5% SDS and centrifuged at 4000 g. Supernatants (subsequently termed 'fraction 1' and 'fraction 2') were collected, and total protein concentration was determined by the Biorad DC Protein Assay. Subsamples of equal total protein content were separated by SDS-PAGE. Proteins were electroblotted onto Immobilon-P membrane, and a monoclonal hsp70 antibody (1:500; MA3-001, Affinity Bioreagents) was used as a probe. Bound antibody was visualized by chemiluminescent reagents (Tropix Inc.). Hsp70 bands were then quantified by densitometry.

Lysosomal Stability

Only samples from Gold Creek and Blackfoot River were analyzed. Serial cryostat sections (10 mm) of the 'fat body' or hepatopancreas were incubated in 0.1 M citric buffer (pH 4,5) at 37°C for different time periods (0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 35, 40 minutes) to determine the lysosomal destabilization time according to Köhler (1991). Subsequently, sections were incubated for 15 minutes in 3.5 % polypep (Sigma), dissolved in 0.1 M citric buffer, pH 4.5, containing 4 mM naphthol-AS-BI-phosphate as substrate for acid phosphatase. After the slides were rinsed in tap water, an azo-coupling reaction was performed in 0.1 % Fast Violet B salt (Sigma) dissolved in 0.1 M phosphate buffer, pH 7.4. Lysosomal destabilization time, the time period of acid labilization needed to destabilize the membrane, marked by the maximum staining intensity of acid phosphatase in lysosomes, was assessed by image analysis.

Tissue Metal Analysis

Samples of each taxon were analyzed for whole body concentrations of Cd, Cu, Pb, and Zn. Samples were prepared and analyzed by the methods described by Hornberger et al. 1998. Briefly, insect collections were thawed, cleaned, and identified to species. Individuals of each species were

composited into subsamples, dried, weighed, and then digested by nitric acid reflux. The tissue residue was reconstituted in 5% HCl and analyzed by ICAPES. In addition, metal concentrations in different tissue fractions were determined in Hydropsyche spp. These specimens were collected, preserved, and sorted as described by Cain et al. (1998). The method of Wallace et al. (1998) was followed to obtain the intracellular metal fractions divided into heat-stable protein (hsp) and heat-denatured protein (hdp) fractions (also see PMP059, this session). Fractions were freeze-dried, digested by HNO3 reflux, reconstituted in 2% HNO3 and analyzed by ICPAES and GFAA.

RESULTS

The primary antibody recognized two hsp70 isoforms in A. grandis (fraction 1), and one isoform in A. grandis (fraction 2), and Hydropsyche spp. (fraction 2). No hsp70 was detected in fraction 1 of Hydropsyche spp. 'Fraction 1' generally represents the soluble cell proteins including cytosolic and mitochondrial components. 'Fraction 2' primarily represents proteins bound to membranes and small cell organelles which were not broken up in the first homogenization step.

Hsp70 levels were significantly elevated in both Hydropsyche spp. and A. grandis collected at station Gold Creek compared to animals from the reference site on the Blackfoot River. Whereas mean hsp70 levels in A. grandis showed an increasing trend along the Clark Fork River stations with increasing metal pollution, hsp70 in Hydropsyche spp. showed no significant increase above controls at any station other than Gold Creek.

A. grandis generally accumulates higher concentrations of cadmium than Hydropsyche spp. (Tables 1, 2). In addition, although tissue concentrations of Cu, Pb and Zn measured in Hydropsyche spp. at Above Flathead are lower than at all other stations, Cd concentration was higher than at Gold Creek. Enhanced hsp70 expression in animals from this site suggests that cadmium tissue concentrations may disproportionately contribute to stress protein induction. However, the combined effect of the 4 metals is greater than that of Cd alone.

In Hydropsyche spp. and A. grandis from the reference site, lysosomal destabilization time was significantly longer – 33.5±2.7 (SE) and 26.3±6.3 (SE) minutes, respectively - than destabilization times detected in larvae from Gold Creek. Destabilization of the lysosomal membrane at this site occurred after 11.3±1.3 (SE) minutes in tissues of Hydropsyche spp. and 11.0±1.9 (SE) minutes in A. grandis.

Intracellular (cytosolic) distribution of cadmium was similar in both species at the reference site, Blackfoot River. However, at Goldcreek where cadmium concentrations are significantly higher than at Blackfoot River, A. grandis accumulates considerably less cadmium in the metallothionein containing (hsp) fraction than Hydropsyche spp.

CONCLUSIONS

- It is apparent from earlier chemical analyses of sediment that a metal gradient exists in this stretch of the Clark Fork River. Metals bioaccumulated in caddisfly tissue reflect this metal contamination.
- Metal tissue concentrations, in particular cadmium concentrations, were associated with a biological response in the form of increased expression of hsp70 protein. The pattern of

response in these organisms indicates that hsp70 is a good biomarker of exposure to the suite of metals detected in the riverine sediments.

- Intracellular distribution patterns of cadmium could explain species differences in hsp70 levels observed in fraction 1.
- Cadmium contributed disproportionately to hsp70 induction, but the combined effect of Cd, Cu, Pb and Zn was greater than that of Cd alone.
- Induction of hsp70 correlated with a significant decrease in lysosomal membrane stability. Our findings indicate a cause-effect relationship between exposure to heavy metals, enhanced hsp70 expression and deleterious effect at the sub-cellular level.

Table 1. Metal concentrations measured in tissues of A. grandis collected from the Clark Fork and Blackfoot Rivers, Montana, USA, in August 1998.

Station	Cadmium (ug/g d.wt.)	Copper (ug/g d.wt.)	Lead (ug/g d.wt.)	Zinc (ug/g d.wt.)	Total
Blackfoot River	0.12	10.80	0.70	136.00	147.62
Turah	1.67	40.50	5.15	211.00	258.32
Gold Creek	2.29	35.80	3.40	185.00	226.49

Table 2. Metal concentrations measured in tissues of Hydropsyche spp. collected from the Clark Fork and Blackfoot Rivers, Montana, USA, in August 1998.

Station	Cadmium (ug/g d.wt.)	Copper (ug/g d.wt.)	Lead (ug/g d.wt.)	Zinc(ug/g d.wt.)	Total
Blackfoot River	<0.11	13.60	0.84	124.00	138.55
Above Flathead	1.46	31.55	3.40	161.00	197.41
Turah	0.92	47.80	5.39	174.00	228.11
Gold Creek	1.40	63.40	6.30	196.00	267.10
Galen Gage	0.71	51.50	6.20	171.00	229.41

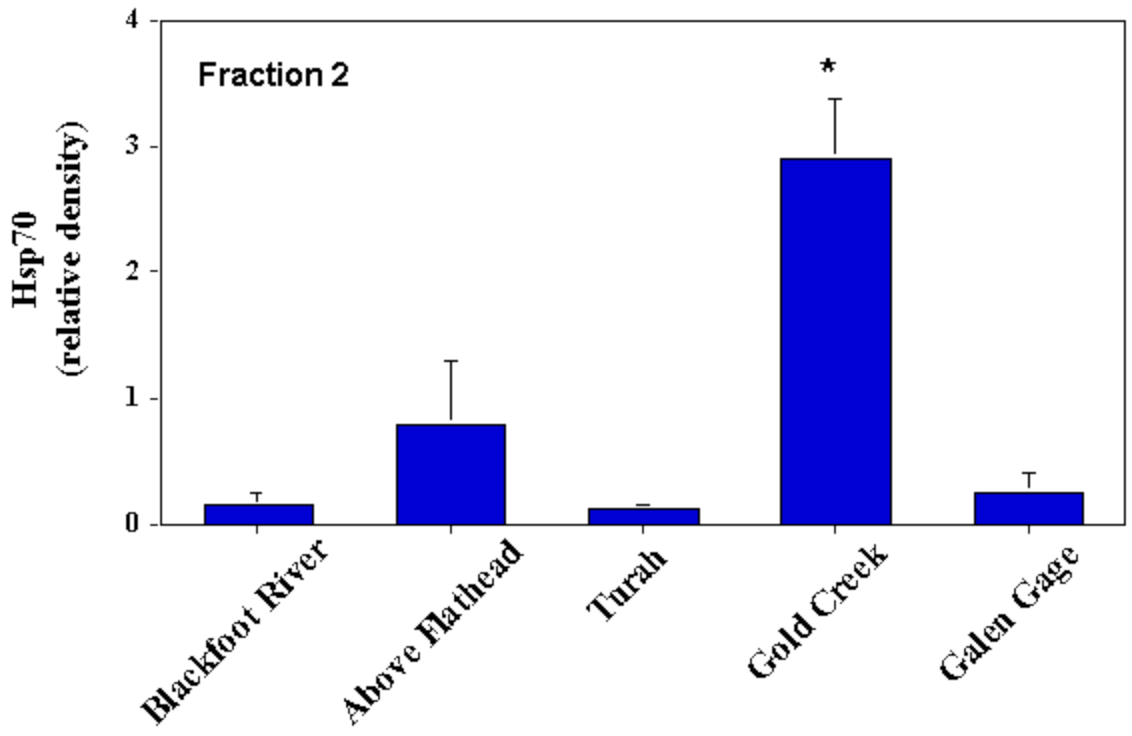

Fig. 1. Levels of stress protein hsp70 in Hydropsyche spp. determined by Western blotting techniques. Graph shows mean hsp70 levels and standard error (n=10). * = significantly different from controls (Blackfoot River)

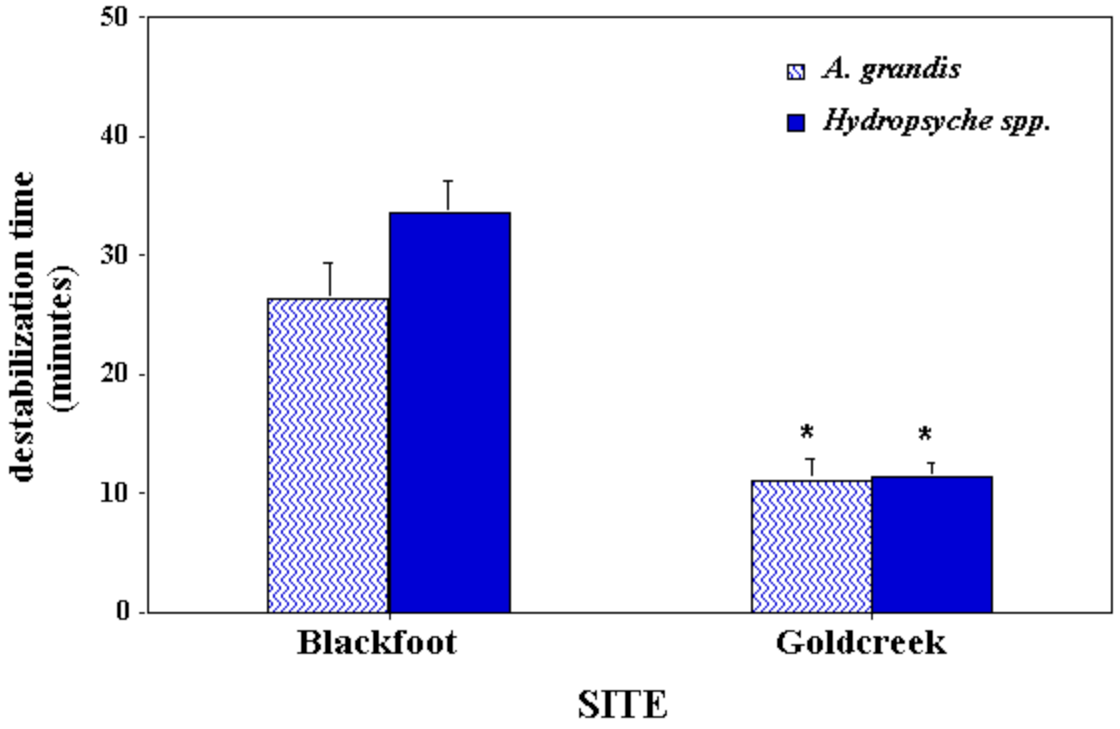

Fig. 2. Destabilization time of lysosomal membranes in A. grandis and Hydropsyche spp. from Blackfoot River (control) and Goldcreek site on the Clark Fork River. Shorter destabilization time indicates reduced lysosomal membrane integrity. * = significantly different from Blackfoot River samples.

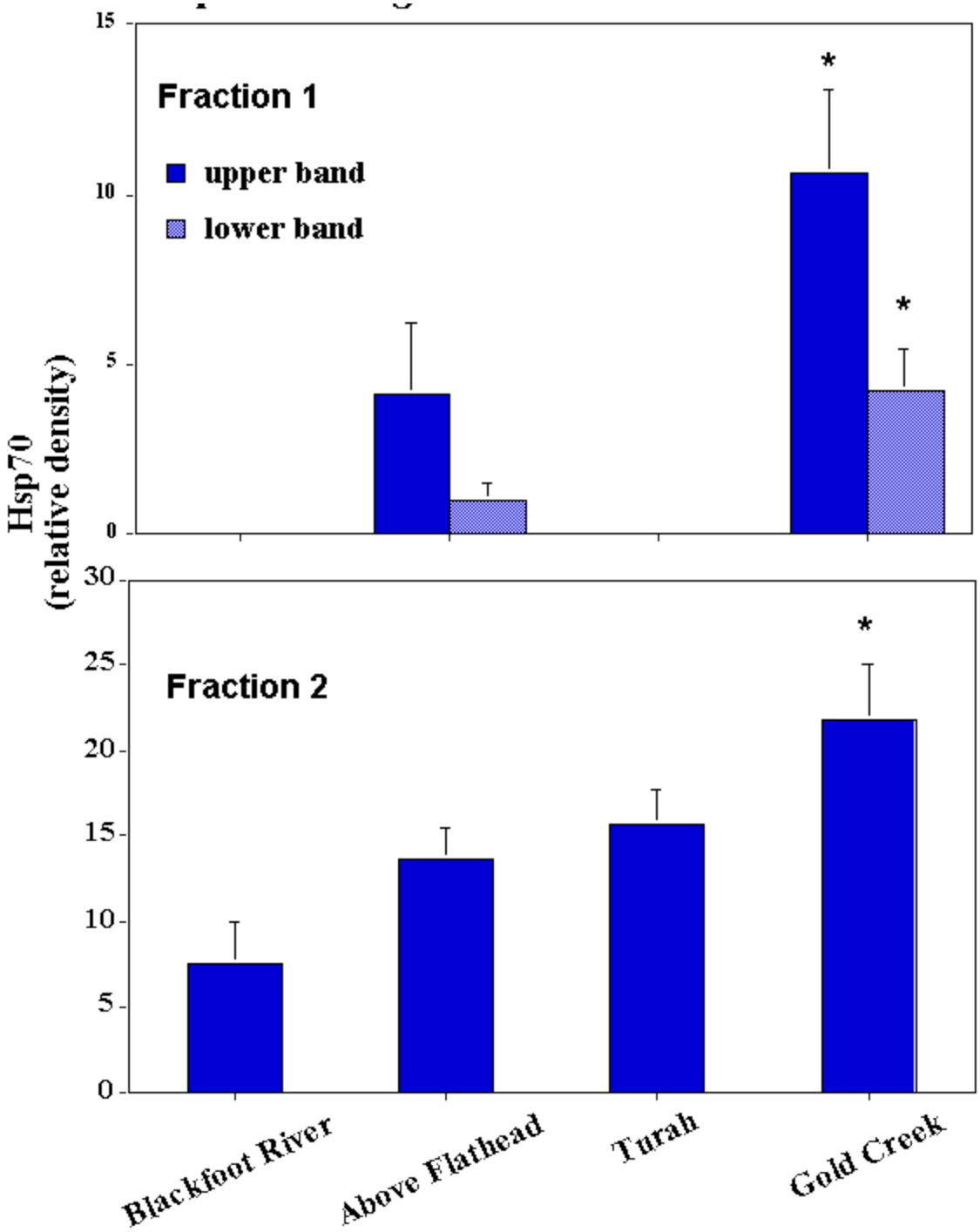

Fig. 3. Levels of stress protein hsp70 in A. grandis. determined by Western blotting techniques. Graph shows mean hsp70 levels and standard error (n=10). * = significantly different from controls (Blackfoot River)