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3-Hydroxy 3-Methylglutaryl Coenzyme A Reductase: a New Biomarker of Fish Exposure to Water Pollution

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Abstract The aim of this study was to identify a new putative biomarker in *Salmo trutta* exposed to water pollution. Variations in the levels of hepatic 3-hydroxy 3-methylglutaryl Coenzyme A reductase (HMG-CoAR), the rate-limiting enzyme of cholesterol biosynthesis, were compared to heat shock protein 70 and hypoxia inducible factor α, biomarkers of pollution exposure and lowered O_2, respectively. The results confirm that HMG-CoAR levels increase in polluted water irrespective of water temperature or O_2 content, indicating that HMG-CoAR could be used as a specific biomarker for water pollution.

Keywords HMG-CoA reductase · HIF-1α · *Salmo trutta* · Water pollution

Anthropogenic activities strongly influence aquatic environments through the release of large quantities of urban, industrial, and agricultural chemicals. The resulting aquatic environmental damage is correspondingly detrimental to human health. In particular, inland waters are currently considered to be among the systems most affected by human activities (Dynesius and Nilsson 1994). The anthropogenic results of land use and urban development have been responsible for altering species composition (Jansson et al. 2000), food web structure (Wootton et al. 1996), nutrient cycling (Johnes 1996), and ecosystem functioning (Vörösmarty et al. 2000). Although the disposal of polluting substances and the draining of wastewaters could mitigate the environmental impact of anthropogenic activities, harmful environmental exposures must be able to be determined and prevented. Indeed, monitoring and prevention of water pollution are currently considered two of the hot topics in environmental research. Thus, there is a great need for biomarkers and bioindicators to increase monitoring efficacy and to advance our understanding and prevention of damaging exposures.

Animal fitness is commonly used to monitor quality and functional status of riverine habitats, although the behavioural features and biotic indexes widely used in many studies may be not sufficient to determine animal fitness under chronic stress (Bogé and Roche 1996). Therefore, in addition to traditional markers (biochemical, histological, morphological and physiological), it may be important to seek out alternative parameters such as molecular biomarkers. The search for potential molecular markers should commence with those genes whose expression is modified under different conditions, as well as those genes coding for proteins related to stress (Gornati et al. 2004). Using this approach, the researcher attention has been focused on heat shock proteins 70 and 90 (HSP-70, -90), metallothioneins and cytochrome P4501A, which have each been shown to be transcriptional upregulated in stress conditions (Gornati et al. 2004). In addition, a search performed by Gornati et al. (2005) identified genes whose expression were affected by changes in rearing densities. Gornati et al. (2005) initially observed six bands differentially expressed following farming of fish at various population densities and identified one band as hepatic 3-hydroxy 3-methylglutaryl Coenzyme A reductase (HMG-CoAR), a key enzyme in cholesterol synthesis. It is unknown if

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HMG-CoAR expression is altered in other environmental challenges besides rearing densities.

HMG-CoAR is an endoplasmic reticulum membrane-spanning enzyme that catalyses the four-electron reduction of HMG-CoA to CoA and mevalonate (MVA), which is then converted to isopentenyl pyrophosphate, the building block of cholesterol and nonsterol isoprenoids (Goldstein and Brown 1990). HMG-CoAR has a pivotal role in many cellular functions and its expression is tightly regulated. For example, cholesterol is an essential membrane component in most cells, and it is critical that all cells have a continuous supply of cholesterol for the synthesis of new membranes, the turnover of lipids in existing membranes, and the biosynthesis of other molecules such as steroid hormones and bile acids.

The aim of this work was to determine the variations in hepatic HMG-CoAR expression as well as changes in expression of hypoxia inducible factor-1α (HIF-1α) transcriptional factor, which is very sensitive to O_2 variation. The ability to maintain O_2 homeostasis is essential for the survival of all invertebrate and vertebrate species. In hypoxic conditions, stabilisation of HIF-1α induces expression of hypoxia-responsive genes necessary to alleviate or mitigate decreased O_2 availability (Pallottini et al. 2008). In addition to HMG-CoAR and HIF-1α, HSP-70 has been identified as a biomarker in *Salmo trutta* Linnaeus, 1758, which was isolated from two sites with differing pollution levels along the Aniene River (Latium, central Italy).

Materials and Methods

All materials were obtained from commercial sources and were of the highest quality available. Materials with no specified source were obtained from Sigma–Aldrich (St. Louis, MO).

Adult *S. trutta* (total length > 18 cm – Gandolfi et al. 1991) specimens were isolated from two different sites (Table 1) along the Aniene River (Rome, central Italy). Specimens were obtained by electrofishing under authorisation of the provincial administration of Rome (Section 3, Department V, Hunting and Fishing), and then sacrificed to sex each animal and collect liver samples. The samples were rapidly frozen in dry ice for later analysis in the laboratory.

Table 1 Number of fish collected at each sampling site

Site	Total no.	km	EBI
Ienne	20	19	12–11
Vicovaro	20	58	6–6

Total no. number of pooled males and females, *km* distance from the river source, *EBI* extended biotic index values (winter and spring, respectively)

Samplings were performed in two seasons: June (far from the reproductive period) and January (during the reproductive period). The unique legal standard measure of biological water quality in Italy based on the macroinvertebrate community structure (Extended Biotic Index, EBI – D.L. 99/152) indicates different symptoms of water deterioration (Ghetti 2001) at each sampling site. All invertebrates were collected by kicking and scraping the river bottom and then identified using a guide (Sansoni 1998). Our sampling sites were assigned a water quality class from I to V, increasing in degree of alteration: I, excellent; II, good; III sufficient; IV, poor; V, bad. The Ienne site showed symptoms of an undeteriorated environment (class I), whereas the Vicovaro site was classified as a polluted or deteriorated environment (III). In the Ienne site, chemical components involving phosphate, nitrogen and biochemical oxygen demand showed low values; the pH was 8.0 ± 0.5; and the dissolved oxygen was never below 94%. Conversely, the Vicovaro site showed high levels of phosphate and nitrogen throughout the summer. Although the dissolved oxygen was low at the Vicavaro site, the O_2 saturation of the site increased to 83% during the autumn (Tancioni et al. 2009).

Hepatic protein expression levels were determined by western blotting following solubilisation of proteins in lysis buffer according to Pallottini et al. (2008) and separation by SDS–PAGE. Briefly, the protein concentration of each sample was determined using the Lowry method (Lowry et al. 1951) and twenty micrograms of protein lysate were resolved by 7% SDS–PAGE at 100 V for 60 min. The proteins were subsequently transferred electrophoretically onto nitrocellulose for 80 min at 100 V. The nitrocellulose was treated with 3% BSA in a buffer containing 138 mM NaCl, 27 mM KCl, 25 mM Tris–HCl, 0.05% Tween-20 (pH 6.8), and probed overnight at 4°C with primary antibodies and corresponding secondary antibodies for 1 h. HIF-1α and HSP-70 protein expression levels were determined from total lysate, whereas HMG-CoAR levels were assessed on microsomes prepared as described previously by Pallottini et al. (2008).

The nitrocellulose was stripped by Restore Western Blot Stripping Buffer (Pierce Chemical, Rockford, IL) for 10 min at room temperature and then probed with an anti-tubulin antibody (MP biomedicals, Solon, OH) to ensure equal protein loading. Bound antibodies were visualised using enhanced chemiluminescence detection (ECL, GE Healthcare). The antibodies used were: anti-HMG-CoAR (Upstate, Lake Placid, NY, USA), anti-HSP-70 (Abcam, Cambridge, UK), anti-HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-tubulin (MP Biomedicals, Solon, OH, USA). The secondary antibodies (anti-mouse

and anti-rabbit conjugated to HRP) were obtained from UCS Diagnostic (Rome, Italy).

All images derived from western blots were densitometrically quantified by ImageJ software for Windows. Each reported value is the ratio of arbitrary units obtained from the density of the protein band to the density of the respective tubulin band.

The results are expressed as mean ± standard deviation. To analyse the quantitative differences among the experimental groups the data were subjected to analysis of variance (ANOVA) using the Graphpad Instat (version 3.0) statistical programme. Post-hoc comparisons were made using the Tukey–Kramer multiple comparisons test. The confidence level (α) was set to 0.05 for all calculations ($p < 0.05$ was considered statistically significant).

Results and Discussion

Although both male and female fish were collected and analysed, no difference in the level of hepatic proteins (i.e., HMG-CoAR, HIF-1α, HSP-70) were observed between the sexes. Thus, all the data obtained from male and female were mediated within the same group (site).

The degree of HMG-CoAR expression was quantified for each specimen and compared between groups.

As illustrated in Fig. 1, HMG-CoAR enzyme levels were twice as high in fish from the polluted water (EBI III) compared to fish from the unpolluted site regardless of collection month (January or June). These data indicate that the modulation of the protein is related directly to pollution and that seasonal or temperature variations do not affect the expression of HMG-CoAR. However, experiments performed in parallel to assess HIF-1α protein levels (Fig. 2) showed seasonal differences. HIF-1α protein levels increased significantly both in June in unpolluted water and June and January in EBI III water, suggesting that temperature, which affects water oxygen concentration, could influence HIF-1α protein expression.

To better assess HMG-CoAR and HIF-1α as biomarkers of pollution exposure in fish, we compared the changes of HMG-CoAR and HIF-1α protein expression to HSP-70, a well-known and commonly used biomarker of water pollution.

As seen in Fig. 3 the levels of HSP-70 are increased in specimens from the class I water collected in June compared to those collected in January, demonstrating a temperature-dependence of HSP-70 expression in unpolluted water. However, the levels of HSP-70 are significantly higher in the livers of the fish collected in class III water in both June and January. Thus, the expression of HSP-70

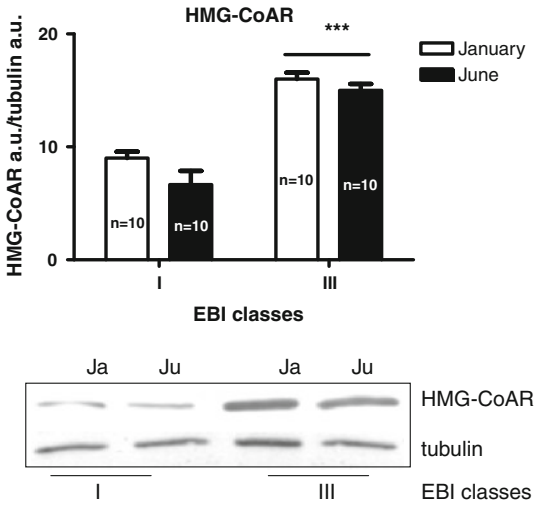

Fig. 1 Hepatic HMG-CoAR expression in *Salmo trutta* is increased in fish collected from a polluted water site. *Top*: Densitometric analysis representing ten different animal samples performed in duplicate. *Bottom*: Western blot of HMG-CoAR and tubulin (loading control) taken from total liver lysates of *Salmo trutta* collected from two sites during January (Ja) and June (Ju) (for details see the main text). *** $p < 0.0001$

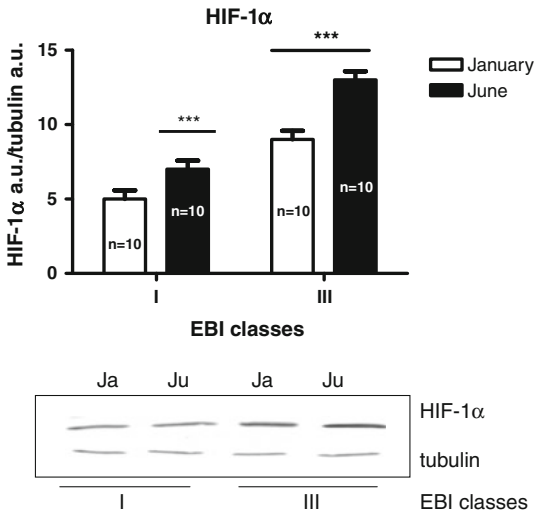

Fig. 2 Hepatic HIF-1α expression in *Salmo trutta* shows a temperature-dependent increase in fish collected from an unpolluted and polluted water site. *Top*: Densitometric analysis representing ten different animal samples performed in duplicate. *Bottom*: Western blot of HIF-1α and tubulin (loading control) taken from total liver lysates of *Salmo trutta* collected from two sites during January (Ja) and June (Ju). *** $p < 0.0001$

seems to be related to changes in temperature, season, and pollution.

Given our results that show an increase in the HMG-CoAR enzyme from samples collected from polluted water in June and in January irrespective of seasonal or temperature changes, HMG-CoAR may be a very useful water pollution biomarker. In fact, we observed no correlation

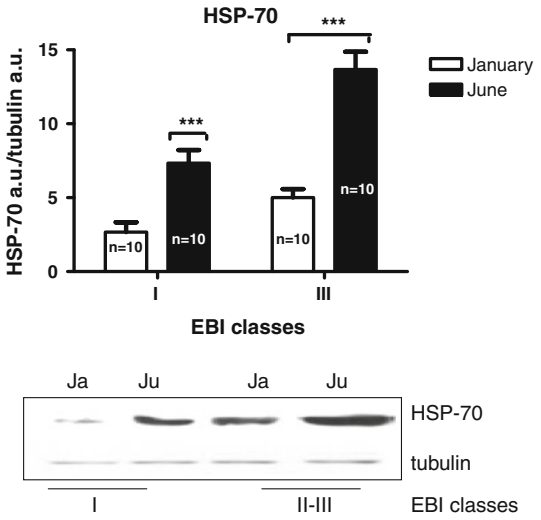

Fig. 3 Hepatic HSP-70 expression in *Salmo trutta* shows a temperature-, seasonal- and pollution-dependence in fish collected from an unpolluted and polluted water site. *Top*: Densitometric analysis representing ten different animal samples performed in duplicate. *Bottom*: Western blot of HSP-70 and tubulin (loading control) taken from total liver lysates of *Salmo trutta* collected from two sites during January (Ja) and June (Ju). *** $p < 0.0001$ versus the respective sampling in I IBE class water

between the increases of HMG-CoAR protein and water temperature in any of the samples collected from polluted water. The observation that HMG-CoAR levels change in stress situations should not be surprising, as this enzyme produces very crucial compounds necessary for proper cell physiology and organismal homeostasis (i.e., cholesterol is the precursor of cortisol). It has previously been suggested that HMG-CoAR mRNA increases in stress situations such as high-density fish rearing and it has been suggested as a biomarker of fish welfare (Gornati et al. 2005).

The significant increase in HIF-1α levels in June observed in unpolluted water is probably due to the lower dissolved oxygen concentration and may not be a direct biomarker of pollution despite the use of HIF-1α as a biomarker of exposure to low oxygen conditions. Moreover, because there is a low degree of eutrophication in the sampling areas, it would be reasonable to argue that HIF-1α is an indirect biomarker of lowered oxygen solubility due to high temperature.

Conversely, HSP-70 displayed not only its intrinsic sensitivity to temperature but also to the degree of pollution.

We have shown that HSP-70 and HIF-1α correlate with both temperature and pollution and, as such, can be used as biomarkers of water pollution and decreased O_2 and/or temperature, although distinguishing between the three parameters is difficult. Conversely, expression of HMG-CoAR seems to be independent of temperature and O_2, indicating that expression of this enzyme in the liver of fish could be used as a specific biomarker of water pollution.

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