Immunohistochemical localization of constitutive and inducible Heat Shock Protein 70 in carp (*Cyprinus carpio*) and trout (*Oncorhynchus mykiss*) exposed to transport stress

C. Poltronieri, E. Negrato, D. Bertotto, D. Majolini, C. Simontacchi, G. Radaelli
Department of Experimental Veterinary Sciences, Faculty of Veterinary Medicine,
University of Padua, Italy

In the present work we investigated by immunohistochemistry the cellular localization of constitutive as well as inducible heat shock protein 70 in several tissues of common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) exposed to transport stress. In carp, the constitutive form (HSC70) was detected only in red skeletal muscle of both control and stressed animals. In the same species, the inducible form (HSP70) was evident in the epithelia of renal tubules, gills and skin of stressed animals, whereas in controls only red skeletal muscle exhibited an immunopositivity to HSP70 antibody. In trout, immunostaining to HSC70 antibody was found mainly in the epithelia of intestine, gills and skin of both control and stressed animals although the reactivity was generally higher in animals exposed to transport stress. In the same species immunostaining to HSP70 antibody was observed only in red skeletal muscle and epidermis of control animals.

Key words: HSP70, HSC70, immunohistochemistry, transport stress, fish.

Correspondence: Giuseppe Radaelli,
Department of Experimental Veterinary Sciences
Viale dell'Università, 16/Agripolis,
35020, Legnaro (PD) Italy
Tel.: +39.049.790165.
Fax: +39.049.641174.
E-mail: giuseppe.radaelli@unipd.it

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Fish are exposed to stressors in nature, as well as in artificial conditions such as in aquaculture, or in laboratories. These stressors, such as handling, weighing, crowding, grading, transportation, vaccination determine the activation of the hypothalamic-pituitary-interrenal axis (HPI) and the release of corticosteroid hormones such as cortisol (Wendelaar Bonga 1997, Mommsen *et al.* 1999). The generalized stress response at the cellular level is characterized by a family of proteins referred to as the heat shock proteins (HSPs) (Iwama *et al.* 1998).

Heat shock proteins (HSPs), also called *stress proteins*, are a family of highly conserved cellular proteins that are present in all cells in all life forms (Morimoto *et al.* 1990, Iwama *et al.* 1998, Feder and Hofmann 1999). Studies on different model species have revealed three major families of HSPs: HSP90 (85-90 kDa), HSP70 (68-73 kDa), and low molecular weight HSPs (16-24 kDa). In the unstressed cell, there is a constitutive production of these proteins that are required in various aspects of cellular homeostasis. HSP70 is known to assist the folding of nascent polypeptide chains, act as a molecular chaperone, and mediate the repair and degradation of altered or denatured proteins. HSP90 is active in supporting various components of cell signaling, including the cytoskeleton, enzymes, and steroid hormone receptors. The low molecular weight HSPs have diverse functions and it has been proposed that they function as molecular chaperones, preventing irreversible protein aggregation (Iwama *et al.* 2004). In fish, as in mammals, there are constitutive members (HSC70) of the heat shock proteins, which play important chaperoning role in unstressed cells, and inducible (HSP70) forms, which are expressed in detectable levels after acute stressor insults (Iwama *et al.* 1998).

Heat shock protein 70 expression has been stud-

ied in fish after exposure to pesticides, virus, metals and other toxic compounds (Roy and Bhattacharya 2006, Eder *et al.* 2007, Hansen *et al.* 2007, Maradonna and Carnevali 2007). Podrabsky and Somero (2007) found that an inducible or embryo-specific form of HSP70 is expressed during embryonic development in killifish (*Austrofundulus limnaeus*) and is elevated during diapause II in this species. Gornati *et al.* (2004, 2005) detected a different expression of HSP70 and HSP90 mRNAs in sea bass farmed at different stocking densities. Moreover, handling and stocking density procedures can affect cortisol levels as suggested by several authors (Marino *et al.* 2001, Skjervold *et al.* 2001, Simontacchi *et al.* 2008). Recently, we demonstrated that inducible HSP70 mRNA, examined by Real Time PCR, was significantly higher in skin and skeletal muscle of sea bass exposed to transport stress than in controls (Poltronieri *et al.* 2007); in the same experimental conditions, inducible HSP70 protein, detected by immunohistochemistry, was found only in skeletal muscle of stressed animals (Poltronieri *et al.* 2007) suggesting that HSP70 mRNA and protein could be potential candidates to describe welfare in fish.

The aim of this work was to examine the cellular localization of the constitutive and inducible HSP70 proteins in two aquaculture species, common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) before and after transport, one of the most stressful procedures in aquaculture facilities. The localization of the two proteins (HSC70 and HSP70) was investigated by immunohistochemistry.

Materials and Methods

Animals

Adults of common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) were obtained from a freshwater fish-farm (Durigon, Santa Cristina, Treviso, Italy). Some animals (7 carp and 10 trout) were sampled directly at the farm immediately after capture (control animals). An equal number of fish from each species was submitted to the same sampling procedure after transport to the laboratory in aerated bags, a journey of 1.5 hours for both species. For immunohistochemistry, several tissues (intestine, liver, pancreas, spleen, skeletal muscle, brain, gills, kidney, skin) were removed and fixed in 4% paraformaldehyde prepared in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) at 4°C overnight. All sampled animals were euthanased using an overdose (300 mg/litre) of the anaesthetic MS-222, Sandoz, Italy.

Immunohistochemistry

Fixation and embedding

Samples fixed as described above were washed in PBS, dehydrated through a graded series of ethanol and embedded in paraffin. Sections were cut at a thickness of 4 µm using a microtome.

Antisera

To localize the constitutive protein, a polyclonal anti-HSC70 antibody was used. The antibody was raised in rabbit against a 13 residue synthetic peptide based on the human HSC70 (Stressgen Biotechnologies, SPA-816, USA) and used at a dilution of 1:2000. To localize the inducible protein, a monoclonal anti-HSP70 antibody was used. The antibody was raised in mouse against a purified HSP70 isolated from human Hela cells (Stressgen Biotechnologies, SPA-810, USA) and used at a dilution of 1:200.

Immunohistochemical procedure

Immunohistochemical staining was done using the Envision system (goat anti-rabbit or goat-anti-mouse immunoglobulins conjugated to peroxidase-labeled complex, Dako, Italy). Before applying the primary antibody, endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 in methanol. Non-specific binding sites were blocked by incubating the sections in 1:10 dilution of rabbit or mouse serum (Dako, Italy). The immunoreactive sites were visualized using diaminobenzidine (DAB) (Sigma, Italy) as the chromogen. To ascertain structural details, sections were counterstained with Mayer's hematoxylin.

The specificity of the immunostaining was verified by incubating sections with: (1) PBS instead of the specific primary antibodies (see above); (2) normal rabbit serum instead of the primary antibody; (3) PBS instead of the secondary antibody. The results of these controls were negative.

Results

In general, HSC70 antibody gave an immunostaining in numerous tissues of control as well as stressed trout, whereas HSP70 antibody mainly reacted with tissues from stressed carp (Table 1).

Common carp

Immunoreactivity to HSC70 antibody was detected only in red skeletal muscle which exhibited a moderate positivity in controls and a faint immunostaining in stressed animals (Table 1). In controls, a moderate immunoreactivity to HSP70 antibody was found only in red skeletal muscle, whereas all other tissues were negative (Table 1; Figure 1A, C, E). In stressed animals, HSP70 immunostaining was found in the apical cytoplasm and brush border of epithelial cells of renal tubules (Figure 1B), in the epithelium lining primary lamellae of gills (Figure 1D) and in the epidermis of skin (Figure 1F).

Rainbow trout

Immunoreactivity to HSC70 antibody was detected in both controls and stressed animals at the level of: i) the epithelium of intestine (Figure 2A, B); ii) the epithelium lining both primary and secondary lamellae of gills (Figure 2C, D) and iii) the epithelium of skin (Figure 2E, F). In the epithelia of intestine and gills from stressed animals (Table 1; Figure 2B, D) the immunoreactivity seems to be higher than that observed in controls (Table 1; Figure 2A, C). In controls, a faint immunoreactivity to HSC70 antibody was also observed in red skeletal muscle (Table 1). Immunoreactivity to HSP70 antibody was detected in red skeletal muscle and skin from controls, whereas no immunostaining to inducible HSP70 antibody was detected in all tissues from stressed animals (Table 1).

Discussion

In this study we detected the cellular localization of the constitutive (HSC70) and inducible (HSP70) members of the heat shock protein 70 in adults of two aquaculture species, common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) exposed to transport stress.

In fish the stress response has been categorized into the primary, secondary and tertiary responses (Mazeaud *et al.* 1977, Wedemeyer *et al.* 1990). The primary response represents the perception of an alterated state which involves the rapid release of stress hormones, catecholamines and cortisol, into the circulation (Gamperl *et al.* 1994, Iwama *et al.* 1998). The secondary response is defined as the manyfold immediate actions and effects of stress hormones at blood and tissue level, whereas tertiary response represents whole-animal and population-level changes associated with stress (Wendelaar Bonga 1997, Iwama *et al.* 1998). At the cellular level, fish respond to stressors by an increased synthesis of

Table 1. Immunohistochemical localization of HSC70 and HSP70 in carp and trout exposed to transport stress.

	Carp				*Trout*			
Tissue	*HSC70*		*HSP70*		*HSC70*		*HSP70*	
	Controls	*Stressed*	*Controls*	*Stressed*	*Controls*	*Stressed*	*Controls*	*Stressed*
Skeletal	$+^{R}$	$+/-^{R}$	$+^{R}$	$-^{R}$	$+/-^{R}$	$-^{R}$	$+/-^{R}$	$-^{R}$
muscle	$-^{W}$	$-^{W}$	$-^{W}$	$-^{W}$	$-^{W\backslash}$	$-^{W}$	$-^{W}$	$-^{W}$
Brain	-	-	-	-	-	-	-	-
Intestinal epithelium	-	-	-	-	+	++	-	-
Kidney epithelium	-	-	-	+	*	*	*	*
Gill epithelium	-	-	-	+	++	+++	-	-
Skin	-	-	-	++	++	++	++	-
Spleen	-	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-	-
Pancreas	-	-	-	-	-	-	-	-

*Staining: -, not detectable; +/-, slight but above background levels; + moderate; ++, marked staining; +++, strong. *Tissue not examined in this species. R, W: red and white muscle fibres.*

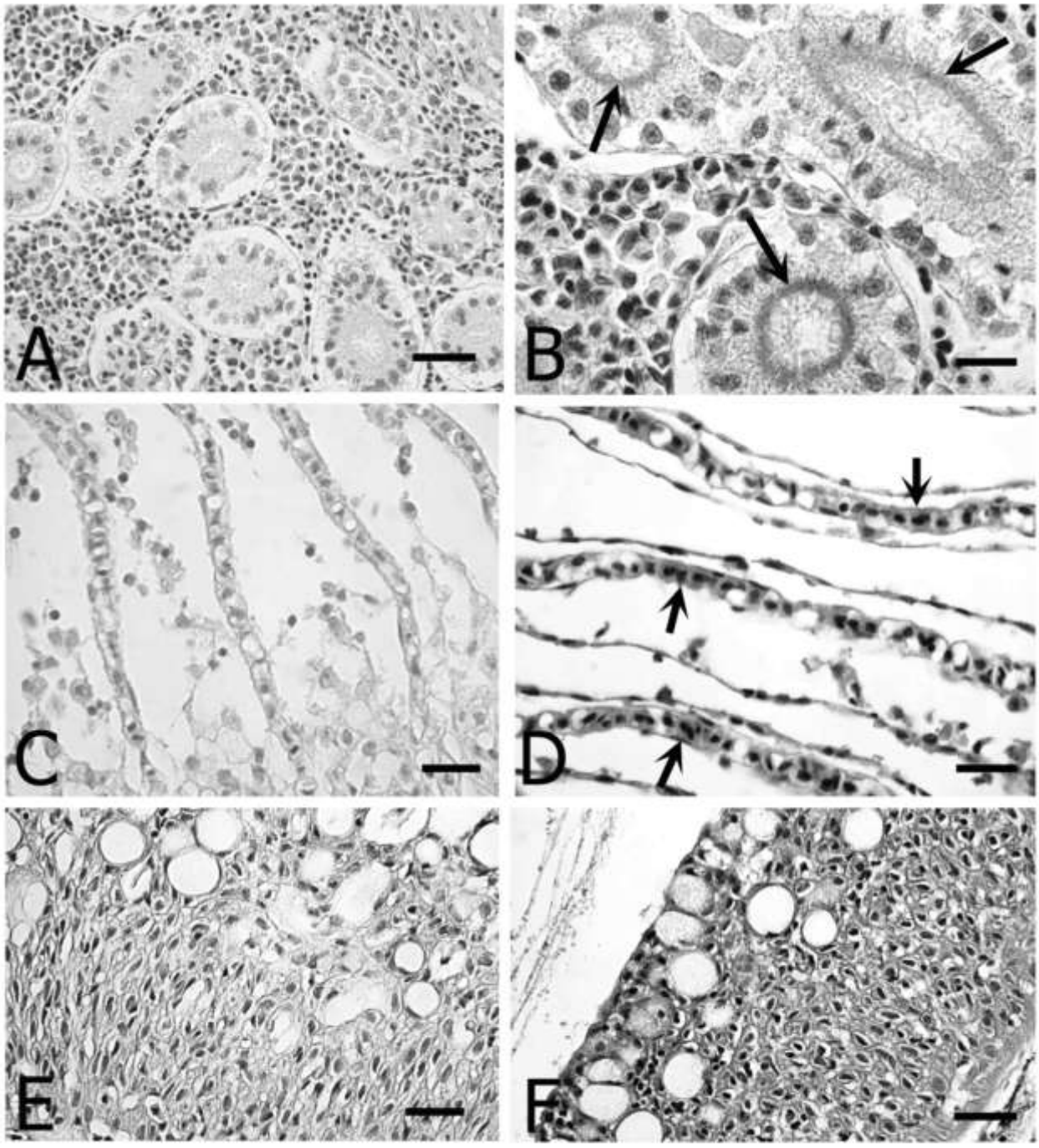

Figure 1. Immunohistochemical localization of inducible HSP70 in adults of carp. A, C, E: Control animals. B, D, F: stressed animals. All panels are counterstained with haematoxylin. A) The parenchyma of the kidney is negative. B) In stressed animals, epithelial cells of renal tubules show immunostaining at the level of the apical cytoplasm and brush border (*arrows*). C) Epithelium of gill filaments is negative. D) In stressed animals, a moderate immunostaining is present in the epithelial cells lining the secondary lamellae of the gills (*arrows*). E) Epidermis is negative. F) In stressed animals, epithelial cells of skin show a marked immunostaining. *Bars*: A, 20 μm; B, 10 μm; C-D, 12.5 μm; E-F, 20 μm.

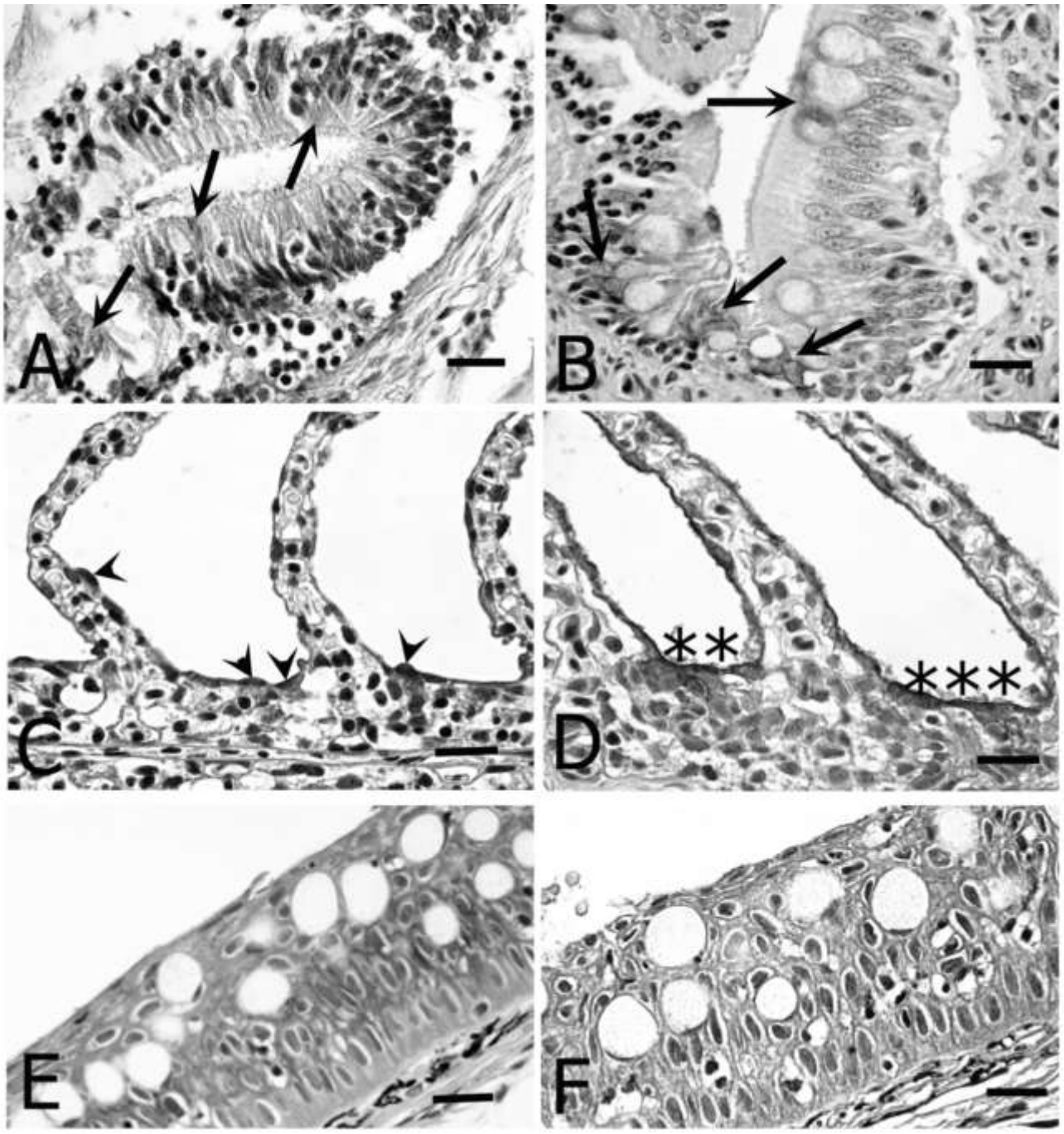

Figure 2. Immunohistochemical localization of constitutive HSC70 in adults of trout. A, C, E: Control animals. B, D, F: stressed animals. All panels are counterstained with haematoxylin. A) The epithelium of the intestine shows rare positive cells (*arrows*). B) In stressed animal, intestinal epithelium exhibits a marked immunoreactivity at the level of goblet cells (*arrows*). C-D) A marked immunostaining is present in the epithelial cells lining both the primary and secondary lamellae of the gills, although in stressed animals (D, *asterisks*) the reactivity is stronger than in controls (C, *arrowheads*). E-F) In skin of both control and stressed animals, epithelial cells exhibit a marked immunostaining. Bars: A-B, 12.5 μm; C-D, 10 μm; E-F, 20 μm.

HSPs (Iwama *et al.* 1998) suggesting the use of these proteins as indicators of stressed states in fish. The aim of this work was to investigate the use of HSP70 family as indicator of transport stress in fish; this family includes several members of proteins, some of which are constitutively (HSC70) expressed under normal growth conditions and others which are inducible (HSP70) under various stress conditions (Morimoto *et al.* 1990). In our experimental condition the stress response to transport has been evaluated by measuring plasma cortisol levels demonstrating a significantly increased of cortisol levels in stressed animals (these results are object of another paper already submitted for publication).

The expression of HSP70 mRNA has been studied in numerous fish species subjected to different stress conditions (Sanders *et al.* 1995, Williams *et al.* 1996, Vijayan *et al.* 1997, 1998, Schmidt *et al.* 1998, Duffy *et al.* 1999, Hassanein *et al.* 1999, Currie *et al.* 2000, Ali *et al.* 2003a, b, Zarate and Bradley 2003, Gornati *et al.* 2004, Deane and Woo 2005, Ojima *et al.* 2005). Our group demonstrated by Real-Time PCR an increased expression of inducible HSP70 mRNA in 40 day larvae and 80 day fry of sea bass exposed to transport stress (Poltronieri *et al.* 2007); moreover, muscle and skin of stressed adults showed an higher significant expression of inducible HSP70 mRNA respect than controls (Poltronieri *et al.*, 2007). In the same species, Gornati *et al.* (2004) detected an increased expression of HSP70 after overcrowding exposure.

Since most inducible HSPs do not contain introns, their mRNA is quickly translated into protein within few minutes of exposure to stressors (Mayer *et al.*, 2000). For this reason, in the present work expression of HSP70 has been examined by immunohistochemistry in order to detect the cellular localization of both constitutive and inducible proteins after a transport stress.

Technical specifications from the company indicate that the two commercial antibodies used for this immunohistochemical investigation distinguish between the constitutive and inducible forms of HSP70; above all the inducible HSP70 antibody detects a protein, corresponding to the apparent molecular mass of inducible HSP70 on SDS-PAGE immunoblots, in samples from different vertebrate species, including carp.

In this work immunopositivity to HSC70 antibody has been found only in red skeletal muscle of both control and stressed carp, although the constitutive member of HSP70 is required in the unstressed cells in various aspects of metabolism to maintain cellular homeostasis (Fink and Goto 1998). These findings are in contrast to the ubiquitous distribution of HSC70 protein found by immunohistochemistry during ontogenesis of controls as well as transported sea bass (Poltronieri *et al.* 2007). Moreover, Ali *et al.* (2003a, b) observed a tissue- and stressor-specific differential expression of two HSC70 genes in carp. It would be interesting to examine the expression of HSC70 by Real-Time PCR in order to verify a possible effect of transport stress on mRNA expression of carp. Interestingly no immunoreactivity for inducible HSP70 protein has been observed in most of tissues from control carp; only red skeletal muscle exhibited a moderate immunostaining. Barton and Iwama (1991) suggested that the presence of HSP70 protein in red skeletal muscle is in agreement with an increased muscular activity. A novel observation in the present study is the immunolocalization of inducible HSP70 protein in the epithelia of renal tubule, gills and skin of stressed carp. In the same tissues from controls the immunostaining was negative suggesting that inducible HSP70 is required for allowing cells to cope with stressor events (Boone and Vijayan 2002). Moreover, the immunopositivity observed in skin epithelium agrees with the expression of inducible HSP70 mRNA observed in sea bass after transport stress (Poltronieri *et al.* 2007).

In trout, the pattern of immunoreactivity for constitutive HSC70 was similar to that observed in sea bass (Poltronieri *et al.* 2007) since several tissues from both control and stressed animals exhibited a positivity. In the same species, Zafarullah *et al.* (1992) observed that HSP70 mRNA is constitutively expressed in different trout tissues. Moreover, in our experiments, in the epithelia of intestine and gills from stressed animals the immunostaining was higher than in controls suggesting an increased expression of constitutive HSC70 after transport. In the present study inducible HSP70 protein has been detected in red skeletal muscle and in skin epithelium from control trout whereas no immunoreactivity was

found in all tissues from stressed animals. Lack of immunoreactivity in all tissues from stressed trout is in contrast to the expression of inducible HSP70 protein observed in the epithelia of renal tubules, gills and skin from stressed carp suggesting that in trout the mRNA has not been translated into a protein. Moreover, our results are in agreement with those observed by Washburn *et al.* (2002) who suggested that the capture and transport of trout for environmental monitoring purposes should not interfere with the use of stress proteins as biomarkers.

In conclusion, the different cellular distribution of both constitutive and inducible HSP70 proteins suggest that transport stress can affect their expression.

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