Quantitative RT-PCR analysis and immunohistochemical localization of HSP70 in sea bass *Dicentrarchus labrax* exposed to transport stress

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In aquaculture, fish are exposed to stressful conditions, which cause an increased synthesis of heat shock proteins (HSPs) at the cellular level. In this work we considered the expression of the constitutive and inducible forms of HSP70 as an indicator of stress caused by transport, during development of the sea bass (*Dicentrarchus labrax*), a teleost fish of high value for aquaculture. Qualitative RT-PCR analysis revealed expression of inducible HSP70 gene in larvae and fry (25, 40 and 80 days) as well as in adult tissues (liver, brain, muscle, gills, kidney, gonads, heart, spleen and skin) of both control and stressed animals. Expression of inducible HSP70 mRNA examined in different adult tissues by Real-Time PCR, was significantly higher in skin and skeletal muscle of stressed animals than in controls. Immunolocalization of inducible and constitutive forms of heat shock protein 70 (HSP70 and HSC70), reported here for the first time, demonstrated an ubiquitous distribution of HSC70 protein in several tissues of both stressed and control animals (at all stages), while inducible HSP70 protein was found only in skeletal muscle of stressed animals. In all stressed animals, regardless of their developmental stage, cortisol levels were higher than in control animals.

Key words: HSP70, immunohistochemistry, RT-PCR, Real-Time PCR, stress fish.

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In aquaculture, fish are exposed to stressful conditions, which 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). At the cellular level, fish can respond to stressors by an increased synthesis of heat shock proteins (Iwama *et al.* 1998).

Heat shock proteins (HSPs) represent a family of highly conserved cellular proteins, which are distributed in all organisms that have been examined, including fish (Morimoto *et al.* 1990, Iwama *et al.* 1998, Feder and Hofmann 1999). Three major families of HSPs have been described: HSP90 (85-90 kDa), HSP70 (68-73 kDa) and low molecular weight HSPs (16-47 kDa). 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 (Morimoto *et al.* 1990, Yamashita *et al.* 2004).

In fish culture, stress conditions can be due to routine procedures (handling, selection, weighing), management factors (high stocking density, feeding, temperature and water quality or disease), transport conditions and slaughter procedures. The expression of HSP70 and HSP90 has been examined in sea bass maintained at different population densities (Gornati *et al.* 2004, Gornati *et al.* 2005). It has been demonstrated that handling and stocking density procedures can affect cortisol levels (Marino et al. 2001, Skjervold *et al.* 2001) whereas handling stress did not influence the levels of hepatic HSP70 (Vijayan *et al.* 1997). However, no data is currently available in the literature regarding the expression of HSP70 in relation to transport stress, which represents one of the most stressful procedures in aquaculture facilities.

In this work, the expression of the HSP70 has been examined in larvae, fry and adults of sea bass

(*Dicentrarchus labrax*) before and after transport. In particular, we looked for the expression of both constitutive and inducible members of the HSP70. HSC70 is known to assist the folding of nascent polypeptide chains, acts as a molecular chaperone and mediates the repair and degradation of altered or denatured proteins, whereas expression of inducible HSP70 is influenced by stress conditions (Iwama *et al.* 2004). The expression of mRNA for inducible HSP70 was determined by qualitative RT-PCR and quantitative Real-Time PCR analysis, whereas the localization of the two proteins (HSC70 and HSP70) was investigated by immunohistochemistry. In addition, whole body cortisol as well as circulatory cortisol levels have been examined in larvae/fry and adults, respectively, by radioimmunoassay (RIA) in order to confirm the HPI axis activation in fish subjected to transport stress.

Materials and Methods

Animals

Larvae/fry and adult of sea bass were obtained from the Pellestrina (VE, Italy) fish hatchery and from the Bonello (VE, Italy) fish farm, respectively. Some animals were sampled directly at the farms immediately after capture (control animals). Others were sampled in our laboratory after a transport (stressed animals); during the three hour travel, animals were kept in tanks filled with sea water. Biomass density was about 10 kg/m^3 in both control and stressed animals. For immunohistochemistry, 10 larvae (5 controls and 5 stressed animals) and 10 fry (5 controls and 5 stressed animals) as well as several tissues (intestine, liver, skeletal muscle, heart, gills, kidney, skin) removed from 6 adult fish (3 controls and 3 stressed animals) were fixed in 4% paraformaldehyde prepared in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) at 4°C overnight. For RNA expression analysis of larvae and fry, one pool for control (5 individuals) and one for stressed animals (5 individuals) were stocked in RNA Later Reagent (Ambion, USA) at –20°C until required. The same storage protocol was applied for adult tissues sampled from 3 controls and 3 stressed animals. For cortisol analysis (refer to Table 2), larvae and fry were stored at –20°C until extracted for subsequent whole body cortisol assay; adults were bled (2 mL of blood volume) from gills or heart immediately prior to euthanasia and blood was placed in heparinized tubes on ice, centrifuged (1,500 g for 15 min at 4°C) and plasma stored at –20°C until cortisol RIA was performed. All sampled animals were euthanased using an overdose of the anaesthetic MS-222, Sandoz, Italy.

Qualitative RT-PCR

RNAs were extracted using TRIZOL® Reagent (Gibco-BRL, Gaithersburg, MD, USA) from the following stages: 25 and 40 day larvae, 80 day fry as well as from 100 mg of the following adult tissues: liver, brain, muscle, gills, kidney, gonads, heart, spleen and skin. 1.5 μg of total RNA were retrotranscribed into cDNA. The first-strand cDNAs were synthesised using Superscript II RNase H$^-$ reverse transcriptase protocols (Invitrogen, Life technologies, UK) and a mixture of random hexamers as primer (synthetized by MWG-Biotech, Ebersberg, Germany). The cDNAs obtained were diluted 1:10 and 2 μL were used as template for expression analysis by RT-PCR. PCR reactions (35 cycles at: 95°C for 45 sec, 55°C for 45 sec and 72°C for 45 sec) were performed to amplify a 176 bp fragment using inducible HSP70 specific primers (forward 5'GTCTGGACAAAGGCAAGAGC and reverse 5'CACAAAGTGGTTGACCATGC) designed on the basis of inducible HSP70 mRNA sequence from sea bass (Accession number AY423555; Gornati *et al.*, 2004) which discriminate between HSP70 and HSC70. To assess the quality of RNA and the efficiency of RT reaction, a fragment of 165 bp of the β-actin cDNA (Accession number AJ493428) was amplified for each sample (Figure 1) with the following primer: for 5'ACCCAGTCCTGCTCACAGAG and rev 5'CGGAGTCCATGACAATACCAGTG. PCR products were electrophoresed on a 2% agarose gel and visualized under UV light.

Real-Time PCR and statistical analysis

Relative quantification assays were performed to detect the relative expression of inducible HSP70 mRNA between control and stressed larvae and between control and different tissues from stressed adult sea bass (muscle, skin, brain and kidney). The expression analysis was performed by SYBR Green I dye chemistry detection with an ABI 7500 Real-Time PCR System (Applied Biosystems) and data were collected with ABI's 7500 System SDS Software. The experiments were conducted in sin-

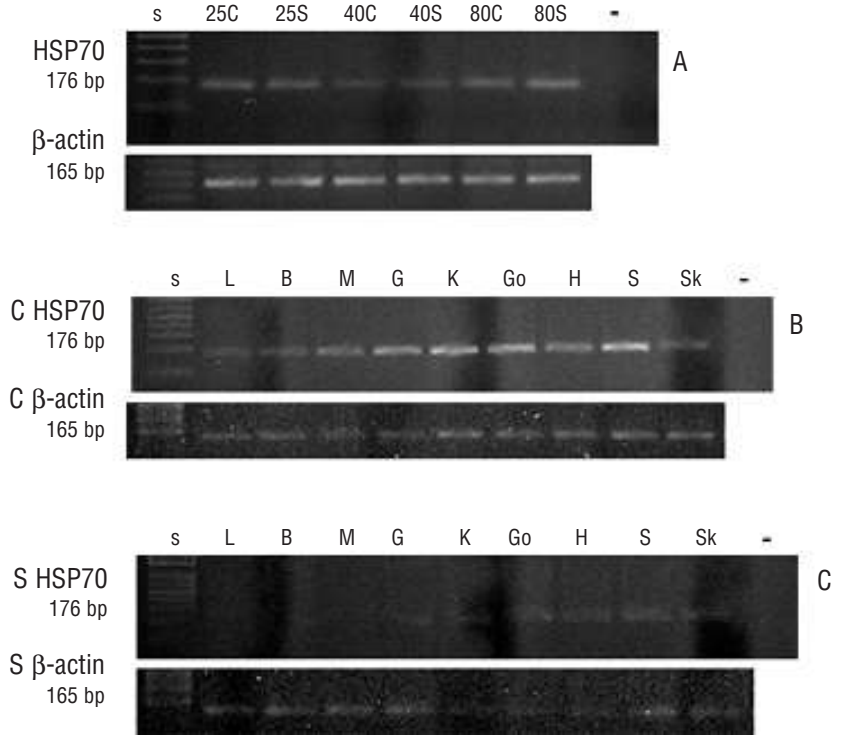

Figure 1. A-B-C. Qualitative expression analysis of inducible HSP70 mRNA (176 bp) investigated by RT-PCR in sea bass samples. A fragment of β-actin (165 bp) was amplified for each stage considered. Different developmental stages were analysed from control (C) and stressed (S) larvae aged 25 and 40 days as well as from fry aged 80 days. The following tissues removed from adults were analysed in both stressed (S) and control (C) animals: liver (L), brain (B), muscle (M), gills (G), kidney (K), gonads (Go), heart (H), spleen (S) and skin (Sk). (s) indicates standard size and (–) indicates negative control. A) In larvae and fry mRNA expression of inducible HSP70 is evident in both control and stressed animals although it seems stronger in the latter. B-C) In adults the levels of inducible HSP 70 mRNA is higher in stressed animals (B) than in controls (C).

gleplex as described in Patruno *et al.* (2006) and Matsakas *et al*. (2006), made from SYBR Green PCR Master Mix (Applied Biosystem), with the inducible HSP70 specific oligonucleotides (as described above) using the following PCR conditions: an activation stage at 95°C for 10 min and 40 cycles at: 95°C for 15 sec, 60°C for 1 min. β-Actin gene was amplified in separate tubes as an active endogenous reference to normalize quantification of a mRNA target since its regular expression in different experimental conditions. Fluorescence signal baseline and threshold were set manually for each detector generating a threshold cycle (Ct) for each sample. Three replicates of each sample and endogenous control were amplified. Data are generated by running stressed and control pool of five larvae and tissues from three stressed animals as well as three controls. For inducible HSP70 and β-actin assays, a 5-fold serial dilution (12 wells) of cDNA sample was amplified to assess standard curve and PCR efficiency. Data were collected with ABI's 7500 System SDS Software. To demonstrate that efficiencies of targets and reference gene were approximately equal, a validation experiment was performed calculating the ΔCt = Ct (target gene) – Ct (reference gene) value. The ΔCt was plotted against the logarithm of the dilution factors; the absolute value of the slope from the efficiency plot should be less than 0.1. Relative quantification was calculated using the Ct method (comparative Ct method). The $\Delta\Delta Ct$ calculation (ΔCt Sample – ΔCt Calibrator) was performed to generate the expression plot of inducible HSP70 gene. The amount of target, normalised to β-actin gene and relative to the calibrator sample (muscle of an unstressed animal), is given by the formula $2^{-\Delta\Delta Ct}$. The calibrator tissue is expressed in arbitrary units (1 unit). Dissociation curves confirmed the specific amplification of the cDNA target and the absence of nonspecific products. Data analysis was performed by means of non-parametric (U Mann/Whitney and Kruscal-Wallis) tests (Fowler *et al.* 1998) in order to compare multiple groups (several samples) using the SigmaStat version 2.03 (SPSS Inc., CA, USA).

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 inducible 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 PBS. Non-specific binding sites were blocked by incubating the sections in 1:5 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. Moreover, a positive control was performed exposing larvae of 30 and 45 days to a heat shock of 10°C for 2 hours. Dewaxed sections from these animals showed immunopositivity to inducible HSP70 antibody in skin, gut and muscle demonstrating the specificity of the antiserum in this species. Unshocked animals did not exhibit any positive staining.

Radioimmunoassay procedure

Larvae and fry

For larvae and fry, whole body cortisol levels were determined by RIA using the following procedure: all samples were thawed out, frozen with liquid nitrogen, pulverized with a pestle, after which 0.5 mL of phosphate buffer (PBS, pH 7.2) were added to each sample. The aqueous suspension was then extracted with 8 mL of diethyl ether and the supernatant was evaporated to dryness. The dry extracts were dissolved in 0.5 ml of PBS and 10, 20 or 50 µL were used for RIA. In order to collect enough sample material for cortisol detection, the smallest larvae were pooled (5 and 2 individuals for 25 and 40 day larvae, respectively) and weighed, whereas the largest fry (80 days) were weighed and individually processed (Table 2).

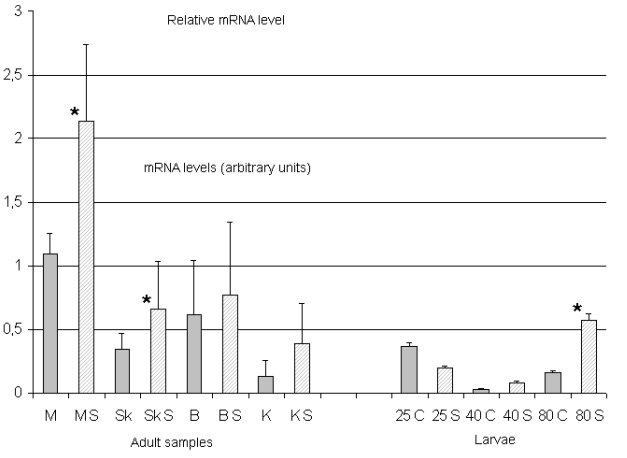

Figure 2. A-B-C. Inducible HSP70 mRNA levels measured with SYBR Green Real-Time PCR assay in different control and stressed (S) tissues: muscle (M), skin (Sk), brain (B), and kidney (K); and in different control (C) and stressed (S) larval stages: 25, 40 and 80 days old. The muscle has been used as a calibrator sample. Results are presented as fold stimulation of inducible HSP70 expression compared to muscle and normalised to β-actin expression. Bars represent mRNA level measured by 7500 System Software and relative quantification is expressed in raw data. Values are means ± SD (n=3). *$p<0.05$ has been measured by means of non-parametric statistical tests.

Diethyl ether extraction efficiency was already validated in our laboratory on gilthead sea bream (*Sparus aurata*) fry: whole-body homogenates were added with 4000 cpm of 1,2,6,7-[^{3}H] cortisol. Recovery rate of cortisol resulted 75-80% (n=3). To validate the whole-body determination, three dose-response competitive binding curves were performed by serial dilutions of extracts of sea bass (*Dicentrarchus labrax*) fry. The displacement curve for whole-body fry extracts was parallel to that of cortisol standard, demonstrating the validity of the extraction and RIA method used.

Adults

Cortisol was extracted from 100 µL of plasma with 8 mL of diethyl ether and the supernatant was evaporated to dryness. The dry extracts were dissolved in 1 mL of phosphate buffer (PBS, pH 7.2) and different aliquots, according to cortisol concentration, were used for RIA.

Cortisol was measured using a specific microtitre RIA as described by Simontacchi *et al.* (1999); dif-

ferences between control and stressed groups were analyzed using a two-way t-test for independent samples. Data are expressed as mean ± standard deviation (m ± S.D.)

Results

Inducible HSP70 expression

Qualitative RT-PCR analysis revealed expression of inducible HSP70 gene in larvae and fry (25, 40 and 80 days) as well as in adult tissues (liver, brain, muscle, gills, kidney, gonads, heart, spleen and skin) of both control and stressed animals although the cDNA seems more expressed in the latter (Figure 1A, B, C). A Real-Time PCR approach followed the standard PCR assay since the latter did not show a quantitative differences between gel-band intensity. Relative quantifications were calculated using the ΔΔCt method comparing the concentration of the samples to the concentration of the calibrator (muscles sampled at birth) that is expressed in arbitrary units (1 unit) and normalised to the endogenous genes. The highest levels of inducible HSP70 mRNA were detected in the muscle (Figure 2). The general tendency of inducible HSP70 expression revealed an increased mRNA level in all stressed samples, both larvae and adults, although a significant increase was detected only in whole 80 day fry as well as in muscle and skin of adults ($p<0.05$; Figure 2).

HSC70 immunostaining

In general, HSC70 antibody gave an immunopositivity in several tissues of both control and stressed animals examined throughout larval, postlarval and adult stages (Table 1).

Larval stages

During larval life, immunoreactivity to HSC70 antibody was found in heart musculature as well as in the epithelia of skin, pharynx, oesophagus, intestine and gills of both control (Figure 3A-C and Figure 4A) and stressed (Figure 4B-C) animals. Already during early developmental stages HSC70 immunostaining was present in the epithelium layers of skin (Figure 3A), intestine (Figure 3B) and oesophagus (Figure 3C). In the intestine, immunoreactivity was observed in the apical cytoplasm and brush border of the enterocytes (Figure 3B and Figure 4B-C). In the gills, immunoreactivity appeared in scattered cells of the epithelium at the level of secondary lamellae (Figure 4A and inset). No immunoreactivity was observed in the skeletal muscle of either control or stressed animals.

Fry

During postlarval life, immunoreactivity to HSC70 antibody was found in heart musculature as well as in the epithelia of skin (Figure 5A), intestine (Figure 5A-B), stomach (Figure 5C) and gills of both control and stressed animals. In the intestine, immunostaining was present again in the apical cytoplasm and brush border of enterocytes (Figure 5A-B). Pancreas and liver did not exhibit any immunoreactivity. Also the skeletal muscle did not exhibit any immunoreactivity in either control or stressed animals.

Adults

A strong immunostaining was evident in the heart musculature of both stressed (Figure 6A) and control (inset of Figure 6A) animals but also in the epithelia of skin and gut (Table 1). In the intestine of both control and stressed animals, immunostain-

Table 1. Immunohistochemical localization of HSC70 in control and stressed sea bass.

Tissue	*Larvae*		*Fry*		*Adult*	
	C	*S*	*C*	*S*	*C*	*S*
Gut epithelium	++	++	+++	++	++	++
Liver	-	-		-	+	-
Pancreas	-	-		-	-	-
Skeletal muscle	-	-	-	-	-	-
Heart	+	+	+	+	++	++
Gill epithelium	+	+	+	+	++	++
Kidney epithel.	-	-	-	-	-	-
Skin	+++	+++	+++	+++	+++	+++

C, control animals; S, stressed animals; +, slight but above background levels; +++, strong staining; -, no staining.

Table 2. Cortisol levels from larvae\fry whole body and adult plasma in control and stressed sea bass.

		Body weight (g)	*n*	*Cortisol (ng\g)*	*p*
25 days old larvae	Control	0.029±0.002	2*	8.24±1.3	--
	Stressed	0.028±0.007	2*	44.1±18.1	
40 days old larvae	Control	0.05±0.018	6**	35.8±12.9	$p<0.001$
	Stressed	0.039±0.01	6**	131.8±17.1	
80 days old fry	Control	0.27±0.05	7	12.9±6.3	$p<0.001$
	Stressed	0.25±0.04	8	60.2±22.6	
		Body weight (g)	*n*	*Cortisol (ng/mL)*	*p*
Adult	Control	223.6±47.0	7	67.5±30.8	$p<0.001$
	Stressed	214±20.1	7	179.2±44.1	

** pools of 6 larvae; ** pools of 2 larvae.*

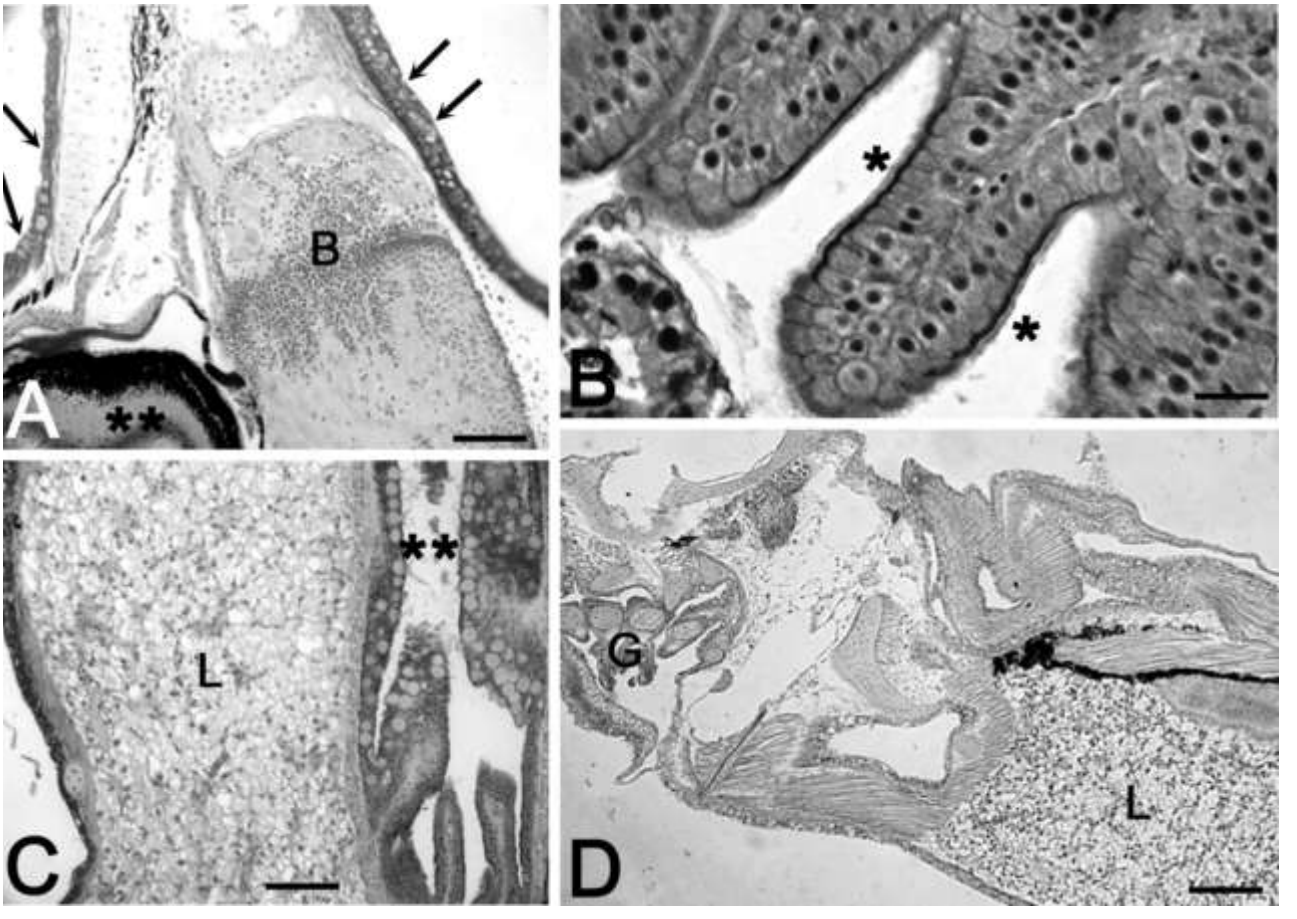

Figure 3. A-C. Immunohistochemical localization of HSC70 in 25-day larvae (controls) of sea bass. All panels are counterstained with haematoxylin. A) Immunostaining is detectable in skin (arrows), whereas brain (B) and eye (*asterisks*) are negative. B) The apical cytoplasm and brush border of intestinal epithelium show HSC70 immunoreactivity (*asterisks*). C) Oesophagus showing HSC70 immunoreactivity in the epithelium (*asterisks*). Superficial mucous cells are negative. The parenchyma of the liver (L) is negative. D) Immunohistochemical localization of HSP70 in 25-day larva (stressed) of sea bass. The panel is counterstained with haematoxylin. All tissues are immunonegative to HSP70 antibody. L: liver; G: gills. *Bars* 40 μm (A-C), 20 μm (B), 100 μm (D).

ing was observed in the apical cytoplasm and brush border of enterocytes, whereas in gills immunostaining was observed in the epithelium at the level of secondary lamellae (Figure 6B-C).

Inducible HSP70 immunostaining

In general, during larval stages (Figure 3D, Figure 4D) no immunostaining for inducible HSP70 antibody was detected. A moderate reactivity was detected only in skeletal musculature of fry (Figure 5D) and adults (Figure 6D) of stressed animals. In adults, immunolabelling was detected in red muscle (Figure 6D), whereas white muscle was negative (Figure 6E).

Cortisol RIA

Larvae and fry

The results are reported in Table 2. As the sampling material from 25 day larvae was insufficient to make any statistical analysis, only mean ± standard deviation is reported. Cortisol levels in 40 and 80 day larvae\fry, expressed as ng/g of body weight, increased significantly after transport, indicating a stress response.

Adult

In plasma of adults, cortisol concentration was significantly higher in stressed animals than in controls, again indicating a stress response to transport.

Discussion

This study examines the expression of the constitutive (HSC70) and inducible (HSP70) members of the heat shock protein 70 in larvae, fry and adults of the sea bass, a teleost fish of interest to

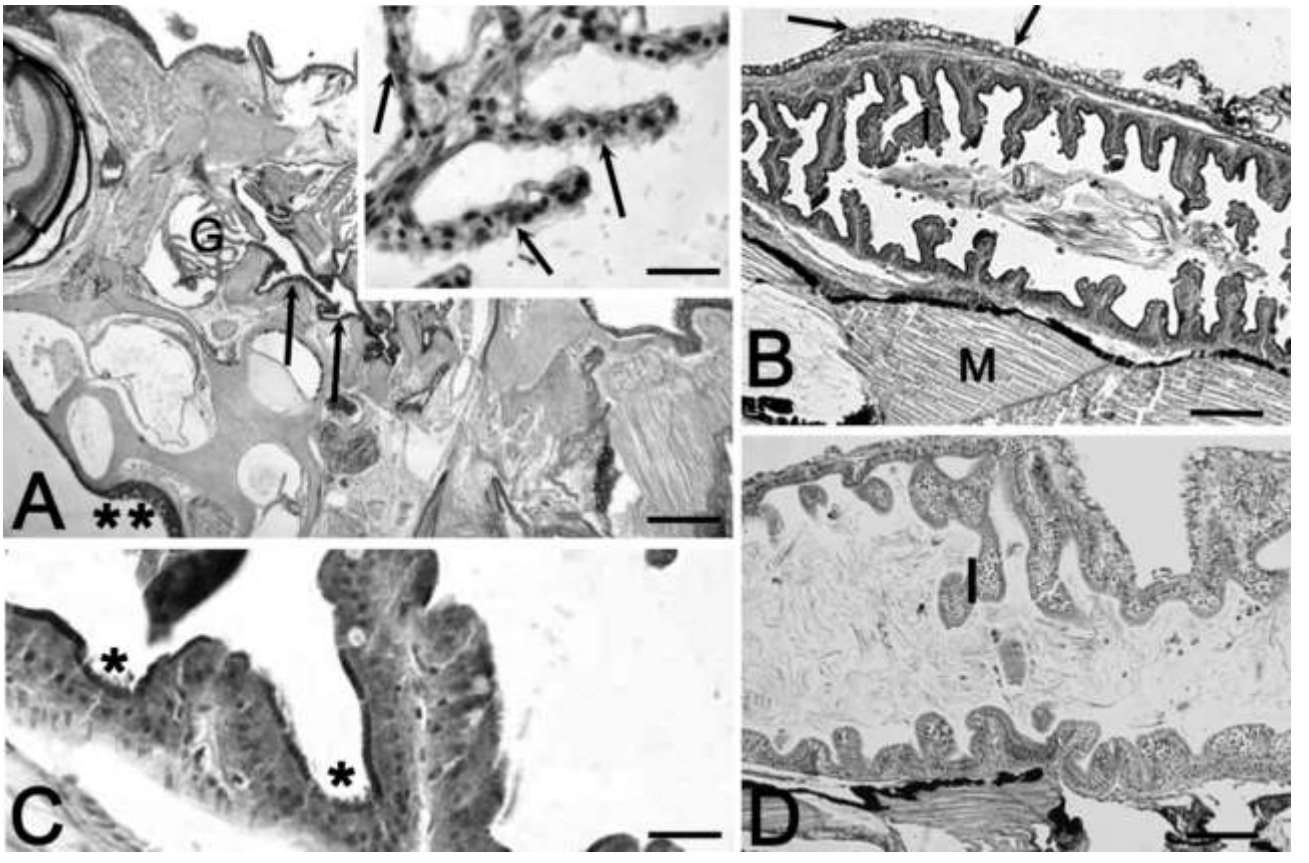

Figure 4. A-C. Immunohistochemical localization of HSC70 in 40-day larvae of sea bass. All panels are counterstained with haematoxylin. A) Control animal. HSC70 immunoreactivity is present in the skin surface epithelium (*asterisks*) and in the pharynx epithelium (*arrows*). G: gills. Inset: at higher magnification, immunoreactivity is visible in the epithelium of the gill filaments (*arrows*). B) Stressed animal. Immunoreactivity is evident in skin (*arrows*) and intestine (I). Lateral muscle (M) is immunonegative. C) Stressed animal. In the intestine, the apical cytoplasm and brush border of enterocytes show HSC70 immunoreactivity (*asterisks*). D) Immunohistochemical localization of HSP70 in 40-day larva (stressed) of sea bass. The panel is counterstained with haematoxylin. All tissues are immunonegative to HSP70 antibody I: intestine. *Bars* 200 μm (A), 100 μm (B-D), 10 μm (C, inset in A).

aquaculture. The aim was to investigate if transport practice (although handling could minimally influence stress condition) can modify the expression of the two members of the heat shock proteins 70 examined by two approaches: qualitative RT-PCR and quantitative Real-Time PCR analysis to determine the expression of mRNA for inducible HSP70, and immunohistochemistry to localize the cellular distribution of HSC70 and inducible HSP70 proteins. Whole body as well as circulatory cortisol levels were examined in larvae/fry and adults, respectively, in order to verify that the fish were indeed stressed after a short transport.

Exposure of fish to aquaculture practice such as hypoxia, netting, handling, transport and crowding (Airaksinen *et al.*; Dini et al. 2006) involves a stress response which also includes increases in plasma catecholamines, cortisol and glucose levels, branchial blood flow and muscular activity (Barton and Iwama 1991).

The response of fish to stressors may also involve an increased synthesis of heat shock proteins at the cellular level (Iwama *et al.* 1998). The heat shock proteins 70 family includes several members of proteins, some of which are inducible (HSP70) under various stress conditions and others which are constitutively (HSC70) expressed under normal growth conditions (Morimoto *et al.* 1990).

Concerning HSC70, Deane and Woo (2005) showed that its mRNA varies after acute stress, although it increased less than inducible HSP70 mRNA. In our work, expression of inducible HSP70 mRNA examined by RT-PCR during development and growth of sea bass did not exhibit any qualitative differences between control and stressed larvae and fry whereas by Real-Time PCR

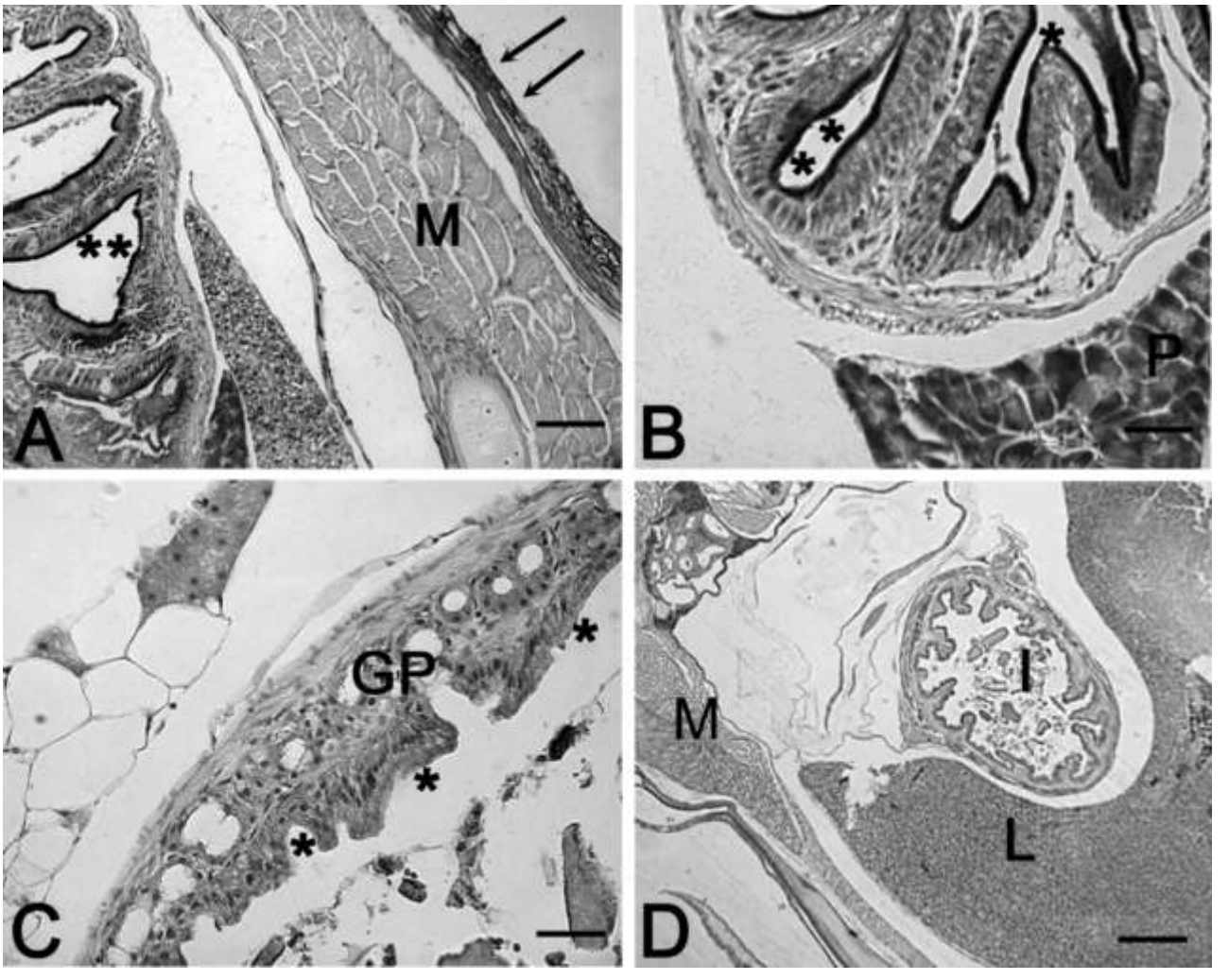

Figure 5. A-B-C. Immunohistochemical localization of HSC70 in 80-day fry of sea bass. A-B: Control animals. C: stressed animal. All panels are counterstained with haematoxylin. A) In the intestine the reactivity is detectable in the brush border of the enterocytes (*asterisks*), whereas the trunk musculature (M) is negative. *Arrows* indicate the immunopositivity in the skin. B) Enterocytes of the intestine show immunostaining at the level of the apical cytoplasm and brush border (*asterisks*). Pancreas parenchyma (P) is negative. C) The epithelium of the stomach shows immunoreactivity to HSC70 antibody (*asterisks*), whereas gastric pits (GP) are immunonegative. D) Immunohistochemical localization of HSP70 in 80-day fry of sea bass. Stressed animal. The panel is counterstained with haematoxylin. All tissues are immunonegative to HSP70 antibody, although skeletal musculature exhibits a faint immunolabelling. L: liver; I: intestine; M: skeletal musculature. *Bars* 40 μm (A), 10 μm (B), 20 μm (C), 2 μm (D).

an increased expression of inducible HSP70 was evident in 40 day stressed larvae and was significantly higher in 80 day stressed fry than in controls. In adults, the overall inducible HSP70 mRNA expression measured by Real-Time PCR was higher in stressed animals than in controls; in particular, muscle and skin of stressed animals showed an higher significant expression respect than controls. Similarly, a high expression of HSP70 mRNA has been reported in sea bass after high population density exposure, while apparently no induction was detectable for HSP90 (Gornati *et al.* 2004). Vijayan *et al.* (1997) observed that handling stress did not affect the expression of hepatic HSP70 and conjugation HSC71 gene from rainbow trout (*Oncorhynchus mykiss*). In the same species, Washburn et al. (2002) found that handling stress did not alter expression levels of muscle, gill, heart and hepatic HSP70. Although rearing density (Gornati *et al.* 2004) and heat shock (Enes *et al.* 2006) cause a modification of HSP70 expression in liver of sea bass, transport stress (our data) does not influence the expression of HSP70 mRNA in the same tissue.

Although the expression of HSP70 has been reported in fish subjected to different stress condi-

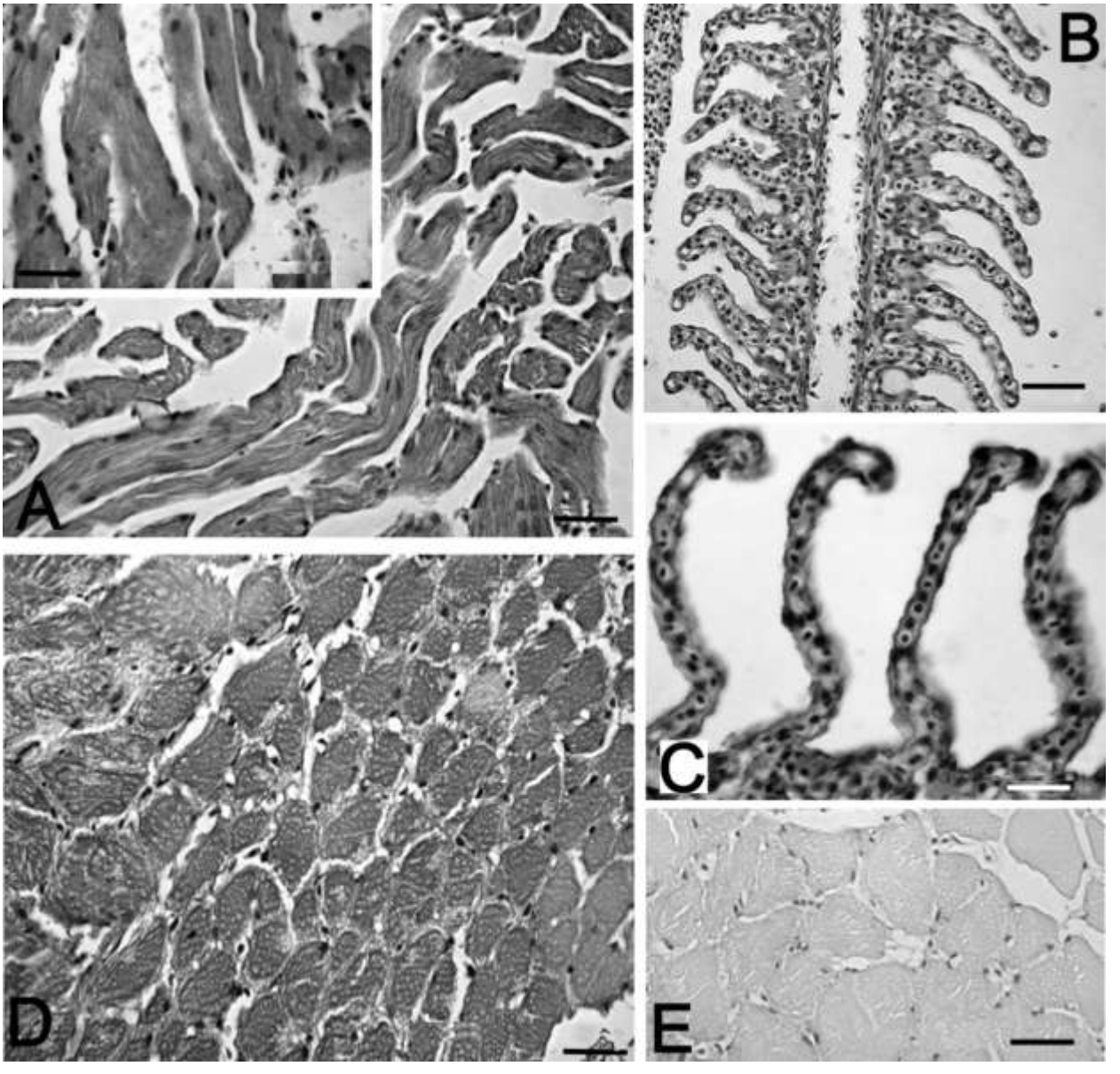

Figure 6. A-B-C. Immunohistochemical localization of HSC70 in adults of sea bass. A-B: stressed animals. *Inset* and C: Control animals. All panels are counterstained with haematoxylin. A) Heart of stressed animal showing immunostaining to HSC70 antibody. The *inset* shows the same pattern in a control animal. B-C) In gills of both control and stressed animals respectively, the immunostaining is present at the level of the epitelium of filaments. D-E. Immunohistochemical localization of HSP70 in adults of sea bass. Stressed animals. All panels are counterstained with haematoxylin. D) Transverse section of red muscle immunopositive to HSP70 antibody. E) Transverse section of white muscle immunonegative to HSP70 antibody. *Bars* 20 μm (A, *inset* in A, B, D-E), 10 μm (C).

tions (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, Zarate and Bradley 2003, Gornati *et al.* 2004), information regarding the cellular localization of both HSC70 and inducible HSP70 is lacking. In the literature there are few descriptions of the cellular localization of HSP70 by immunohistochemistry (Burkhardt-Holm *et al.*, 1998, Kilemade and Mothersill 2001, Rendell and Currie, 2005).

The two commercial antibodies used for immunohistochemistry in this study distinguish between the constitutive and inducible forms of HSP70. Technical specifications from the company indicate that these antibodies detect inducible HSP70 and HSC70 proteins by SDS-PAGE immunoblots in samples from different vertebrates, including fish. Moreover, the positive control performed on sections from fish exposed to heat shock demonstrated that the antibody recognizes the inducible HSP70

protein in several tissues.

The expression of HSC70 has been evaluated by immunohistochemistry because there is no information regarding cellular localization of the protein. In our study, HSC70 immunoreactivity was found in several tissues throughout development and growth of both stressed and control animals confirming that there is a constitutive production of this protein which is required in various aspects of protein metabolism to maintain cellular homeostasis (Fink and Goto 1998; Iwama *et al.* 2004). In future we hope to use Real-Time PCR to examine if transport stress can affect HSC70 mRNA expression even though no differences have been observed in terms of protein production between control and stressed animals.

Our immunohistochemical study of inducible HSP70 in sea bass provides novel information on the cellular localization of this protein during development and growth of this species. No immunoreactivity for inducible HSP70 was found in larvae and fry of both stressed and control animals, although a faint immunolabelling was evident in skeletal musculature of stressed fry. Lack of immunoreactivity in the other tissues from stressed larvae and fry is in contrast to the expression of inducible HSP70 mRNA in the same animals suggesting that at this point the mRNA has not been translated into a protein. Interestingly, although an expression of inducible HSP70 mRNA was observed in all tissues from stressed adult animals, inducible HSP70 immunoreactivity was found only in red muscle but not in any other tissues from the same animals confirming that mRNA has not been translated into a protein as in larvae. It is important to note that the immunopositivity observed in muscle is in agreement with the Real-Time PCR results (although we did not distinguish between red and white muscle) since muscle also represents the tissue which exhibited the highest expression of inducible HSP70 mRNA. The presence of inducible HSP70 protein in red muscle of stressed animals is in agreement with an increased muscular activity (Barton and Iwama 1991) and with the fact that inducible form (HSP70) is important for allowing cells to cope with acute stressor insults, especially those affecting the protein machinery (Boone and Vijayan 2002).

In conclusion, the conservation in structure of HSPs as well as the consistency with which they are induced by a high spectrum of stressor insults has made HSPs potential candidates to describe fish welfare. This work indicates that the expression of inducible HSP70 increases in sea bass in muscle and skin after a transport stress..

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