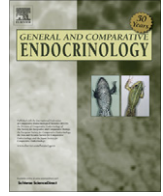




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## Elevated cortisol modulates Hsp70 and Hsp90 gene expression and protein in sea bass head kidney and isolated leukocytes

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### ABSTRACT

In fish, interactions between Hsps and cortisol are involved in stress modulated physiological processes including innate immune responses. Cortisol exerts a role in the regulation of Hsps synthesis. Fish head kidney is a lymphomieloid and endocrine organ releasing cortisol, and it is the central organ for immune-endocrine interactions. In sea bass, cortisol intraperitoneal injection and *in vitro* treatment of head kidney cells show that inducible Hsp70 and Hsp90 are modulated by this hormone. However, an inverse relationship between mRNA expression (real-time PCR) and Hsp70 and Hsp90 protein levels (densitometric band analysis) was found. Time-course assays indicate a cortisol-mediated regulation. Furthermore, Hsp70 gene modulation appears to be more susceptible to the cortisol action and the mRNA was transcribed within 3 h post-injection. The restoration of the homeostatic conditions was observed at a week p.i., when plasma cortisol baseline was reached. Although fish manipulation and injection exerted stressing effects as indicated by serological parameters, differences between cortisol treated specimens compared to untreated or sham fish are statistically significant. Similar results were found by examining *in vitro* total cells and isolated leukocytes from head kidney cultured for 3 h with increasing cortisol concentration. Finally, MTT test and DNA fragmentation experiments showed that the apoptotic effect expected in cortisol-treated cells could be counteracted by high Hsp70 intracellular levels.

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### 1. Introduction

Exposure of fish to stressing environmental factors gives rise to a series of biochemical and physiological changes mediated by the neuroendocrine system and characterized by increased circulating cortisol [47]. This stress hormone, synthesized by the head kidney interrenal cells, regulates osmolarity, metabolism and modulates immune responses [25,40,44]. Injection with exogenous cortisol mimics stress effects including cortisol-modulated responses such as enhanced serum levels of glucose, lactate and osmolarity [3,42], challenging a differential gene regulation [35]. Following cortisol binding, intracellular glucocorticoid receptors (GR) can exert their activity on the expression of the target genes by ligand-dependent transcription factor [33].

Heat shock proteins (Hsps) are key components in modulating stress responses [35]. They are highly conserved molecular chaperones, ubiquitously expressed, belonging to distinct multigenic families and, according to their molecular size, have been classified into several major categories e.g., Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small Hsp. In fish, like in mammals, the Hsp90 and Hsp70 have been related to cytoprotection, cell survival and im-

mune responses [16,5,2] exerting a protective role [19]. Two Hsp90 cytosolic isoforms have been reported: the Hsp90 $\beta$  is constitutive and mainly associated with early embryonic development and several cellular pathways [4], the Hsp90 $\alpha$  is inducible and associated with stress-induced cytoprotection. The Hsp70 constitutive form plays an important chaperoning role in unstressed cells, whereas an inducible form rapidly increases following cellular stress [31,49] and can be found in the cytoplasm as well as in extracellular compartments having a role in the innate immunity [2]. Although the induction of Hsps is a stress response [15,19], contrasting results have been reported on the cortisol-Hsps interplay. In salmonids, exogenous cortisol leads to changes in the expression of the Hsp70 gene [3,35]. Elevated levels of circulating cortisol significantly decrease the Hsp30 in the gill of cutthroat trout [1], as well as the hepatic and gill Hsp70 of rainbow trout [4]. Similarly, elevated cortisol suppresses hepatic and gill Hsp70 expression of the cichlid tilapia [4]. On the contrary, daily injections of cortisol into the silver sea bream do not alter hepatic Hsp70 levels after 24 h and 48 h p.i. [12], whereas a chronic stress, accompanied by high levels of plasma cortisol, stimulates rainbow trout hepatocytes to synthesize Hsp70 and 90 [43].

In previous papers we reported that high plasma cortisol levels due to seabass confinement stress [40], and *in vitro* cortisol treatment of peritoneal cavity leukocytes [44] affect innate immunity

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responses. In the present paper we report on head kidney Hsp70 and 90 modulations in sea bass inoculated with exogenous cortisol. The *in vivo* and *in vitro* experiments disclosed that the hormone causes an inverse relationship between the Hsp70 and Hsp90 mRNA expression and the level of the produced proteins.

## 2. Materials and methods

### 2.1. Animals and experimental design

Fishes (200–250 g) were obtained from ECO-ITTICA (Marsala, TP) fish farm. Three distinct fish groups were arranged in separated 100 L tanks with continuously flowing aerated sea water (18 °C). Fishes were acclimated at constant environmental conditions for at least 2 weeks, and fed with commercial pellets (Biomar for sea bass).

The fish groups (16 fish *per* group) were designated as untreated (Untr.), vehicle treated (group I) and cortisol treated (group II). For sampling, fishes were anesthetized with 0.05% of 3-amino-benzoic acid ethyl ester (MS-222) in seawater. Intraperitoneal injection of 20 mg hydrocortisone/kg body mass in 2.5 ml medium (vehicle: ethanol/1:4 diluted Hanks balanced salt solution, v/v) was given to each individual from group II. Fishes from group I were injected with the vehicle alone while untreated group were used as a baseline control. Four fishes from each group were sampled at 3 h, 24 h, 72 h and 1 week after injection. Fishes were sampled from the same tank, and data were recorded from fishes injected with cortisol compared to those from sham and untreated ones.

After anesthesia (5 min with 0.05% of MS-222 in sea water), blood was withdrawn from the heart into heparinized sterile plastic syringes, then fishes were killed with a lethal dose of 0.1% MS-222, and head kidney was excised. The plasma was obtained by centrifuging the blood samples at 800g for 10 min at 4 °C. The head kidney was divided in two parts, one half was placed in RNA later (Ambion) for RNA analysis, and the second part was stored at –80 °C for protein analysis.

To perform *in vitro* cortisol treatment, an additional fish group (16 individuals) was acclimated as reported above. Six fishes were sacrificed and head kidney surgically excised, the cell preparation from each organ was divided into two halves, one of the two was subjected to cell separation. Cells (unseparated or separated) from three specimens were used to evaluate the expression, and cells from the other three to evaluate the apoptotic effect (MTT and DNA fragmentation).

All experiments were performed in full compliance with the national rules and guidelines (D.Lgs 116/92 and subsequent amendments) and international European Commission Recommendation guidelines for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EC).

### 2.2. Hematological parameters

Plasma cortisol (ng/ml) level was estimated using an enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Neogen Corporation, Lansing, MI), and values recorded by a microplate reader ( $\lambda = 450$  nm, Labsystems Unishan I). The glucose and lactate levels were recorded by Accutrend GC kit (Boehringer, Mannheim) and Lactate Dry-Fast kit (Sentinel diagnostics, Italy) respectively. Osmolarity was estimated by an osmometer (Roebing, MESSTECHNIK, Berlin).

### 2.3. Tissue homogenate supernatant preparation (THS)

Tissue samples were crushed in liquid nitrogen, and kept in ice for 1 h with 1 ml of lysis buffer (RIPA: 0.5% sodium deoxycholate

minimum 97%; 1% NP40; 0.1% SDS with PBS-T (Na<sub>2</sub>HPO<sub>4</sub> 1 M, NaH<sub>2</sub>PO<sub>4</sub> 1 M, NaCl 1.5 M, 0.1% Tween 20) pH 7.5, supplemented with a cocktail of protease inhibitors: 2 µg/µl antipain, leupeptin and bestatin, 1 µg/µl aprotinin and pepstatin, 1 mM benzamidine, and 0.1 mM AEBSF.

Then the samples were centrifuged at 15,000g for 30 min at 4 °C, supernatants were collected, dialyzed against 50 mM Trizma base (Tris[hydroxymethyl] aminomethane) pH 7.5, and the total protein content was determined by using the method of Bradford [7].

### 2.4. Total RNA extraction and cDNA synthesis

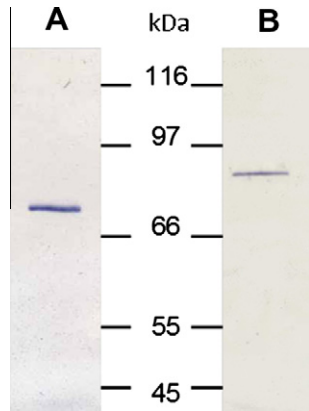
Total RNA was isolated from the tissue by using a RNAqueous™-Midi Kit purification system (Ambion), 1 µg total RNA, reverse-transcribed by the Kit Ready to Go™ T-primed first-strand using random primers (Amersham-Pharmacia Biotech, USA).

### 2.5. Real-time PCR analysis

Tissue expression of the Hsp genes (Hsp70 Accession No. AY423555; Hsp90 Accession No. AY395632) was detected by Real-time PCR using the Taqman method.

Primers and hybridization probes were designed using primer express software V.0 and synthesized commercially (Applied Biosystem, Foster City, USA). The Taqman probe sequence contained a 5'-FAM fluorophore and 3' MGB quencher for target genes (Hsp70, Hsp90) and 5'-VIC fluorophore and 3' MGB quencher for the housekeeping gene (*Dicentrarchus labrax* actin Accession No. AY148350). Real-time PCR analysis was performed using the Applied Biosystem 7500 real-time PCR system. Tissue or cell expression was performed in a 25 µl PCR containing 2 µl cDNA converted from 1 µg total RNA, 200 nM Hsp70 probe (ACCATGAAGTC CACTGTG), 800 nM Hsp70 of forward (5'-TGGCCTGGAGTCATA TGCTTT-3') and reverse primers, (5'-TCTTACCGCAAGCCTTTC A-3'), or 200 nM Hsp90 probe (AGCTGACCGCACCT), 600 nM forward (5'-GAAAATTGACATCATCCCCAACA-3') and reverse primers (5'-TGCCGATTCCAGTGTCCGAT-3'). Two hundred nM actin probe (ACCACAGCCGAGAGG), 600 nM actin forward (5'-CAGAGCGTGG CTACTCCTTCA-3') and reverse (5'-TCCTTGATGTCAGCAGGAT-3') primers, and 12.5 µl of Taqman PCR Master Mix (Applied Biosystem) were added. The 50 cycles of the two steps PCR program consisted of initial polymerase activation for 3 min at 95 °C followed by denaturing at 95 °C for 15 s, and annealing/extension at 60 °C for 45 s, in which fluorescent signal was detected. For every time point/treatment four individuals were sampled and each cDNA sample was run in triplicate together with negative controls. To obtain sample quantification, the RQ = 2<sup>-ΔΔCt</sup> method was used, and the relative changes in gene expression analyzed as described in the Applied Biosystem Use Bulletin N.2 (P/N 4303859). To validate Ct calculation, the approximately equal amplification efficiencies of the target and the reference gene were checked. To assess whether amplicons had the same efficiency, a sensitive method was used for checking how Ct varies with template dilution. Serial dilutions (1, 0.5, 0.2, 0.1, 0.005, 0.002, 0.001) of cDNA were amplified by real-time PCR using the target and the housekeeping gene specific primers and the Ct i.e., CT(Hsp70 or Hsp90)-CT(Actin) was calculated in 3 replicates for each cDNA dilution. Data were analyzed using the linear regression analysis. The amount of Hsp70 and Hsp90 transcript was normalized with actin. In a previous assay, samples from untreated fishes (at 3 h, 24 h, 72 h and 1 week) were analyzed. Since the values recorded did not show any time-dependent significant difference, a unique pooled control sample was chosen to perform the analysis. The relative Hsp70 and Hsp90 expression was calculated by dividing the normalized value of Hsp70 and Hsp90 by the normalized value obtained from the

untreated tissue. Consistent results were recorded and presented in Fig. 2 as averages.



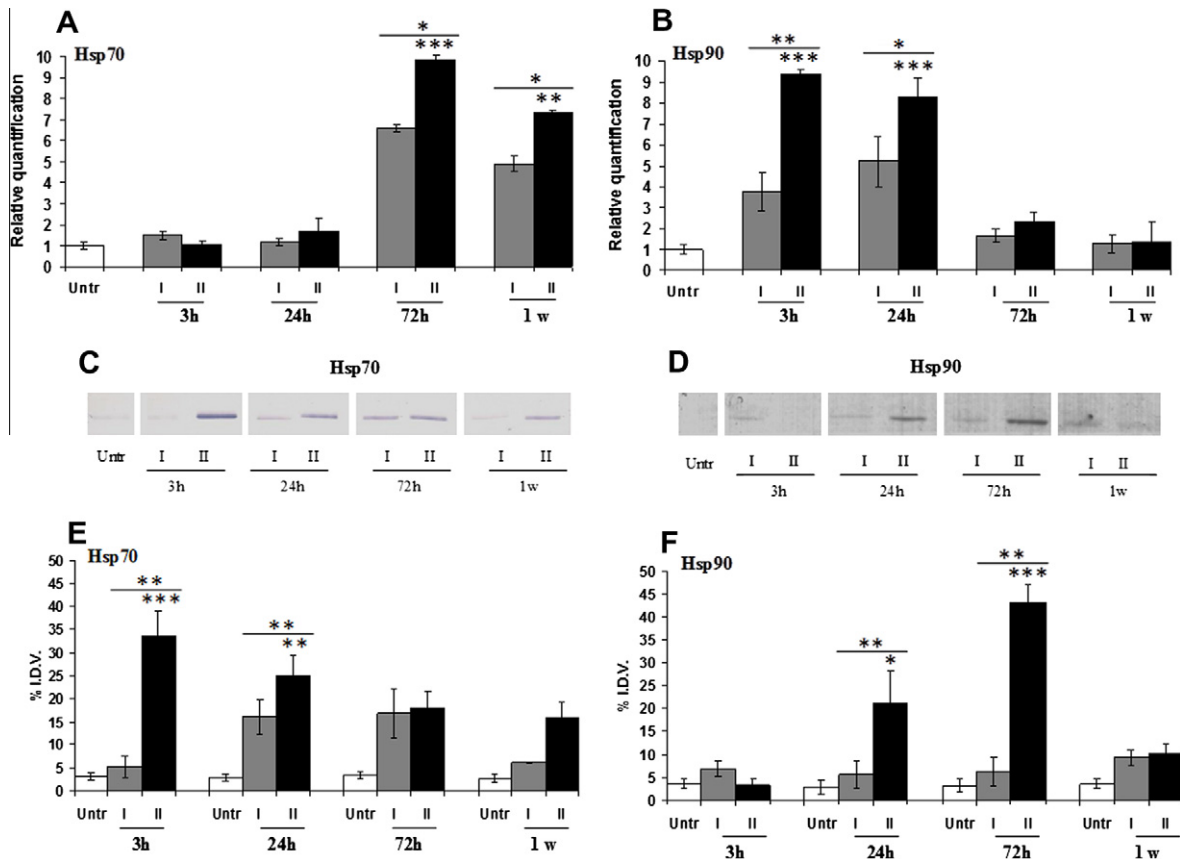
**Fig. 1.** Cortisol-treated fishes head kidney homogenate supernatant. The identity of the Hsps was analyzed by immunoblotting with a monoclonal anti-Hsp70 antibody (lane A) and a monoclonal anti-Hsp90 antibody (lane B). A high molecular weight marker (Sigma) was used: (from top to bottom:  $\beta$ -galactosidase from *E. coli* (116 kDa), phosphorylase b from rabbit muscle (97 kDa), albumin, bovine serum (66 kDa), glutamic dehydrogenase from bovine liver (55 kDa), ovalbumin from chicken egg (45 kDa).

## 2.6. SDS-PAGE and Western blot

The equivalent of 25  $\mu$ g of total lysates for each sample was separated on 7.5% SDS-PAGE under reducing conditions according to the method of Laemmli [21]. The SDS-polyacrilamide minigels were transferred to a nitrocellulose membrane by using a semidry transfer apparatus (BioRad) and blocked with 5% bovine serum albumin (BSA) in TBS-T (20 mM Trizma base pH 7.5, 300 mM NaCl, 0.1% (v/v) Tween 20 with 0.02% sodium azide) for 1 h at room temperature (r.t.). According to previous papers [24,8] commercial antibodies identified fish Hsps as described below. The membranes were incubated overnight with anti-mouse Hsp70 or Hsp90 mAbs (1:3000 dilution, 1 h at r.t., at 4 °C) from Sigma–Aldrich, washed with TBS-T (three times for 5 min), incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (1:10,000 for 1 h at r.t.). After washing with TBS-T (three times for 5 min), the membranes were incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (BCIP/NBT). Densitometric analysis of the immunoblotted bands was carried out using Alphamager software. Four specimens from untreated, I and II fish groups were examined at every time points. Each assay was repeated three times.

## 2.7. Separation of head kidney cells (HKCs)

The head kidney was cut under sterile conditions into small fragments, dissociated using a plastic pestle, and filtered through a cell strainer of 70  $\mu$ m nylon (Falcon). The resulting suspension (120  $\times$  10<sup>6</sup> cells/ml) was halved. An aliquot (60  $\times$  10<sup>6</sup> cells/ml)



**Fig. 2.** Effect of cortisol on Hsp70 (A) and Hsp90 (B) mRNA expression and protein level in sea bass head kidney. (C and D) Show a representative western blot of Hsp70 and Hsp90 respectively; integrated optical density histogram (IDV) of the Hsp70 and Hsp90 protein bands (E and F); Untr: untreated fishes; I: vehicle-treated fishes; II: cortisol-treated fishes. Statistical significant differences between cortisol-treated and vehicle-treated fishes are shown upon the bars (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). Significant differences between treated fishes (I, II) and untreated specimens are also indicated.

was layered on a gradient at 34–46% v/v Percoll/Hanks balanced salt solution (HBSS: 190 mM NaCl, KCl 5.36 mM, 5.54 mM glucose,  $\text{KH}_2\text{O}_4$  0.44 mM,  $\text{Na}_2\text{HPO}_4$  0.56 mM; pH 7.6, 370 mOsm). The gradient was centrifuged at 400g for 25 min at 4 °C and the band at the interface between 34% and 46% was collected and washed in L15-medium (Leibovitz medium, 100 units penicillin/ml, 100 units streptomycin/ml and 10 units heparin/ml, osmolarity adjusted to 370 mOsm). An aliquot of  $40 \times 10^6$ /ml separated cells was obtained from each organ. Dead cells less than 0.5% were revealed by the trypan blue (0.01%) exclusion test.

### 2.8. Treatment in vitro of the head kidney cells with cortisol

The cells ( $2.5 \times 10^6$ /ml) were incubated with increasing cortisol concentrations at 18 °C under mild shaking for 3 h. A cortisol stock solution was prepared by dissolving hydrocortisone (Sigma) in absolute ethanol diluted with Hanks balanced salt solution (HBSS). An aliquot was added to the cell suspension to reach 10 ng/ml, 100 ng/ml, 1000 ng/ml, 10,000 ng/ml cortisol final concentrations.

The control cells were treated in an equal manner and incubated in a medium containing 0.1% ethanol, without hydrocortisone. After treatment, cells were washed in HBSS (3 times) at 400 g for 5 min. The cells suspended in L15 medium were incubated in the dark for 60 min at 18 °C [10].

### 2.9. MTT assay for cell viability

The MTT assay was carried out according to the method of Mosmann [27]. Briefly, cells ( $20 \times 10^6$ /ml) were incubated with 5 mg/ml methylthiazolyl-diphenyl-tetrazolium bromide in L15 medium, for 3 h at 18 °C and sterilized (0.2  $\mu\text{m}$  filter). Then the microplates were centrifuged for 5 min at 50g at 4 °C, then 100  $\mu\text{l}$  of dimethyl sulfoxide (DMSO) was added and the mixture incubated for 10 min with gentle shakings. Aliquots (100  $\mu\text{l}$ ) of the resulting sample were transferred in 96-well plates and absorbance (550 nm) was recorded by a microplate reader (Labsystems Uniskan I).

### 2.10. DNA fragmentation assay

DNA was extracted by using the Sigma–Aldrich genelute mammalian genomic miniprep kit based on a silica-based membrane, specially selected for genomic DNA purification, in a convenient spin column format. To ensure macromolecule denaturation, a cell suspension ( $5 \times 10^6$ /ml) of total and separated HKCs cortisol-treated and untreated control, was lysed with a chaotropic salt-containing solution to insure the through denaturation of macromolecules. DNA was bound to the spin column membrane, the remaining lysate was removed by centrifugation (6500g) and cell debris by a filtration column. After washing, the DNA was

eluted with buffer, analyzed through standard 1% agarose gel electrophoresis and visualized with ethidium bromide (5  $\mu\text{g}/\text{ml}$ ) reaction.

The DNA fragmentation was analyzed using 1% agarose gel electrophoresis to check for a “ladder” pattern at  $\sim 200$ -BP intervals due to apoptosis or a random DNA fragmentation which forms a “smear” on agarose gels disclosing necrosis.

### 2.11. Statistical analysis

The experiments were performed using 4 specimens for each time point and the results were expressed as a mean value  $\pm$  SD. The data were compared using the analysis of variance (ANOVA). Homogeneity of variances was performed using Cochran's test prior to the ANOVA analysis. Whenever necessary the data were log-transformed. *Posthoc* comparisons were made using Student's *t* test and the significant level was set at  $P < 0.05$ ; the analysis was performed with Statistica 6.0 (StatSoft, Tulsa, OK, USA).

### 2.12. Chemicals

Unless otherwise reported all chemicals were from Sigma–Aldrich (Germany).

## 3. Results

### 3.1. Serological parameters

Sham fishes from group I examined at 3 h and 24 h *post* vehicle injection presented a significant increase in endogenous plasma cortisol ( $P < 0.01$ ;  $P < 0.05$  respectively) compared to untreated fishes (Table 1). Following intraperitoneal cortisol injection (fishes from group II), the hormone level was significantly ( $P < 0.001$ ) higher than sham fishes. Such an increase was evident at 3 h and 24 h *p.i.* (Table 1).

Glucose, lactate and osmolarity levels (Table 1) reached the highest levels at 24 h ( $P < 0.001$ ;  $P < 0.05$ ;  $P < 0.001$ , respectively).

### 3.2. Hsps identification

Fig. 1 shows the cross reaction of the head kidney Hsp70 (lane A) and 90 (lane B) contained in a homogenate supernatant and assayed with monoclonal antibodies.

### 3.3. Hsp70 and Hsp90 mRNA and protein expression levels after cortisol administration

The expression of Hsp70 mRNA was analyzed by real-time PCR. Fig. 2A shows that in the head kidney excised at 3 h and 24 h *p.i.* from sham and cortisol-treated fish (groups I and II), the mRNA

**Table 1**

Plasma Cortisol, glucose, lactate and osmolality levels of untreated (Untr), vehicle-treated (I) and cortisol-treated (II) sea bass at 3 h, 24 h, 72 h and 1 week.

Time post stress	3h			24h			72h			1w		
	Untr	I	II	Untr	I	II	Untr	I	II	Untr	I	II
Cortisol (ng/ml)	89 $\pm$ 44	260 $\pm$ 37 <sup>b</sup>	625 $\pm$ 25 <sup>c</sup>	95 $\pm$ 19	300 $\pm$ 70 <sup>a</sup>	590.6 $\pm$ 22 <sup>c</sup>	86 $\pm$ 21	145 $\pm$ 20	170 $\pm$ 31 <sup>a</sup>	70 $\pm$ 17	111 $\pm$ 46	116 $\pm$ 37
Glucose (mg/dl)	68 $\pm$ 15	126 $\pm$ 21 <sup>b</sup>	121 $\pm$ 2 <sup>b</sup>	70 $\pm$ 10	95 $\pm$ 11	260 $\pm$ 26 <sup>c</sup>	75 $\pm$ 19	91 $\pm$ 16	103 $\pm$ 26	71 $\pm$ 5.9	86 $\pm$ 26	90 $\pm$ 15 <sup>b</sup>
Lactate (mg/dl)	5.5 $\pm$ 2.1	24 $\pm$ 10	19 $\pm$ 4 <sup>b</sup>	8 $\pm$ 10	16 $\pm$ 5	18 $\pm$ 9 <sup>a</sup>	7 $\pm$ 16	17.8 $\pm$ 4.2	16.4 $\pm$ 3 <sup>a</sup>	4.5 $\pm$ 11	14 $\pm$ 6	27 $\pm$ 6
Osmolarity (mOsm/kg)	370 $\pm$ 12	374 $\pm$ 13	383 $\pm$ 19 <sup>b</sup>	360 $\pm$ 15	367 $\pm$ 7 <sup>a</sup>	428 $\pm$ 6 <sup>c</sup>	366 $\pm$ 20	382 $\pm$ 5	400 $\pm$ 16	351 $\pm$ 22	366 $\pm$ 12	366 $\pm$ 20

Values represent means  $\pm$  SD ( $n = 4$ ). The average value of the untreated samples was obtained by putting together results from four untreated specimens at every time point ( $n = 16$ ). Different letters denote significant differences between untreated and cortisol-treated fishes (<sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.01$ ; <sup>c</sup> $P < 0.001$ ) as well as between fishes from group I and II (<sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$ ) are shown.

levels did not change significantly. On the contrary, at 72 h and 1 week p.i., the specimens from group II presented a significant increase in Hsp70 mRNA compared to the untreated ( $P < 0.001$ ,  $P < 0.01$ , respectively) and sham fish ( $P < 0.05$ ). In untreated fish a constant level was found. In sham fish (group I) the values were higher than those from untreated ones, i.e., 7 times at 72 h and 5 times at 1 week. The exogenous cortisol increased the mRNA expression of 10 times at 72 h and 7 times at 1 week compared to the untreated specimens.

Real-time PCR analysis revealed that the Hsp90 mRNA level was increased both in samples from sham and cortisol-treated fish, as compared to the constant levels found in the untreated specimens (Fig. 2B). In sham fish, at 3 h and 24 h p.i., the Hsp90mRNA expression was respectively 3 and 5 times higher, then (72 h and 1 week p.i.) the expression lowered to control levels of the untreated specimens. In cortisol-treated fishes, at 3 h and 24 h p.i., the Hsp90 mRNA expression was 9 ( $P < 0.001$ ) and 8 ( $P < 0.001$ ) times higher than untreated ones. Then (72 h and 1 week p.i.) the mRNA lowered to control levels. Differences between sham and cortisol-treated fishes were significant at 3 h ( $P < 0.01$ ) and 24 h ( $P < 0.05$ ) p.i.

To assess the Hsp70 protein production, assays were performed by densitometry analysis of the immunoblotted bands. Fig. 2 shows that, in samples from groups I and II, the specific anti-mouse Hsp70 (Fig. 2C) and Hsp 90 mAbs (Fig. 2D) identified only 70 kDa and 90 kDa proteins, respectively. The densitometry profile (Fig. 2E) disclosed that the Hsp70 density was modulated in sham and cortisol-treated specimens, meanwhile no changes were observed in samples from the untreated ones. In sham fish, the band density values increased slightly at 3 h p.i. whereas they were higher (4 times, compared to the untreated fish) at 24 h and 72 h p.i. and at 1 week (2 times). In samples from cortisol-treated fish, the highest protein level (10 times higher) was found at 3 h p.i.; then it lowered at 24 h (8 times higher), 72 h and 1 week (5 times higher). Differences between sham and cortisol-treated specimens were significant ( $P < 0.01$ ) at 3 h and 24 h p.i., only.

In samples from the cortisol-treated fish, the densitometry highlights a significant change in Hsp90 density at 24 h and 72 h p.i. (Fig. 2F), whereas in samples from sham fish the protein density was almost constant and equal to the controls. In particular, the density was 7 and 13 times higher at 24 h and 72 h (respectively) after the cortisol injection ( $P < 0.05$  and  $P < 0.001$ ). Such a difference was also found by comparing samples from sham and cortisol-treated fishes ( $P < 0.01$ ).

#### 3.4. Hsp70 and Hsp90 mRNA expression and protein levels after cortisol *in vitro* treatment of head kidney cells

Cell populations from the head kidney include erythrocytes and leukocytes, while only leukocytes were contained in the Percoll separated populations.

The analysis of total head kidney cells treated for 3 h with cortisol at various concentrations (10, 100, 1000, 10,000 ng/ml) and compared to the untreated samples, showed no significant effect of 10–1000 ng/ml cortisol on the Hsp70 mRNA expression (Fig. 3A). Similarly, the Hsp70mRNA of isolated leukocytes remains at a constant level up to 1000 ng/ml cortisol, whereas only 10000 ng/ml cortisol lowered the mRNA in total cells and isolated leukocytes ( $P < 0.01$ ) (Fig. 3A).

Also the levels of Hsp90 mRNA from total and isolated cells did not change significantly (Fig. 3B). Only the treatment of isolated leukocytes with the highest cortisol concentration, compared to the medium-treated cells, significantly ( $P < 0.01$ ) decreased (about 30%) the Hsp90 mRNA expression. Fig. 3C1 and C2 shows that the anti-mouse Hsp70 mAbs cross-reacted with a 70 kDa band of the head kidney total cells, whereas it could be identified in the iso-

lated leukocytes samples only after the cells were treated with 10,000 ng/ml cortisol (Fig. 3C2). As shown in Fig. 3, the anti-mouse Hsp90 mAbs cross-reacted with a 90 kDa band present both in total cells (Fig. 3D1) and isolated leukocytes preparations (Fig. 3D2). Densitometry analysis of Hsp70 protein (Fig. 3E) shows a constant and significant increase in total cells samples. In particular, the density reached the highest level (6 times higher than the untreated samples) after a treatment with 10,000 ng/ml cortisol. The Hsp70 band density revealed after 10,000 ng/ml cortisol treatment of the isolated leukocytes was 10 times higher ( $P < 0.001$ ) than that observed in the untreated samples. The 90 kDa band identified in the total cell lysate did not indicate any significant density change following cortisol treatments (from 10 up to 10,000 ng/ml) (Fig. 3F) and a constant value (up to 1000 ng/ml cortisol) was found. On the contrary, when isolated leukocytes were treated with 10,000 ng/ml cortisol, the protein density underwent a significant ( $P < 0.05$ ) increase (Fig. 3F).

#### 3.5. Cortisol treated cells were not apoptotic

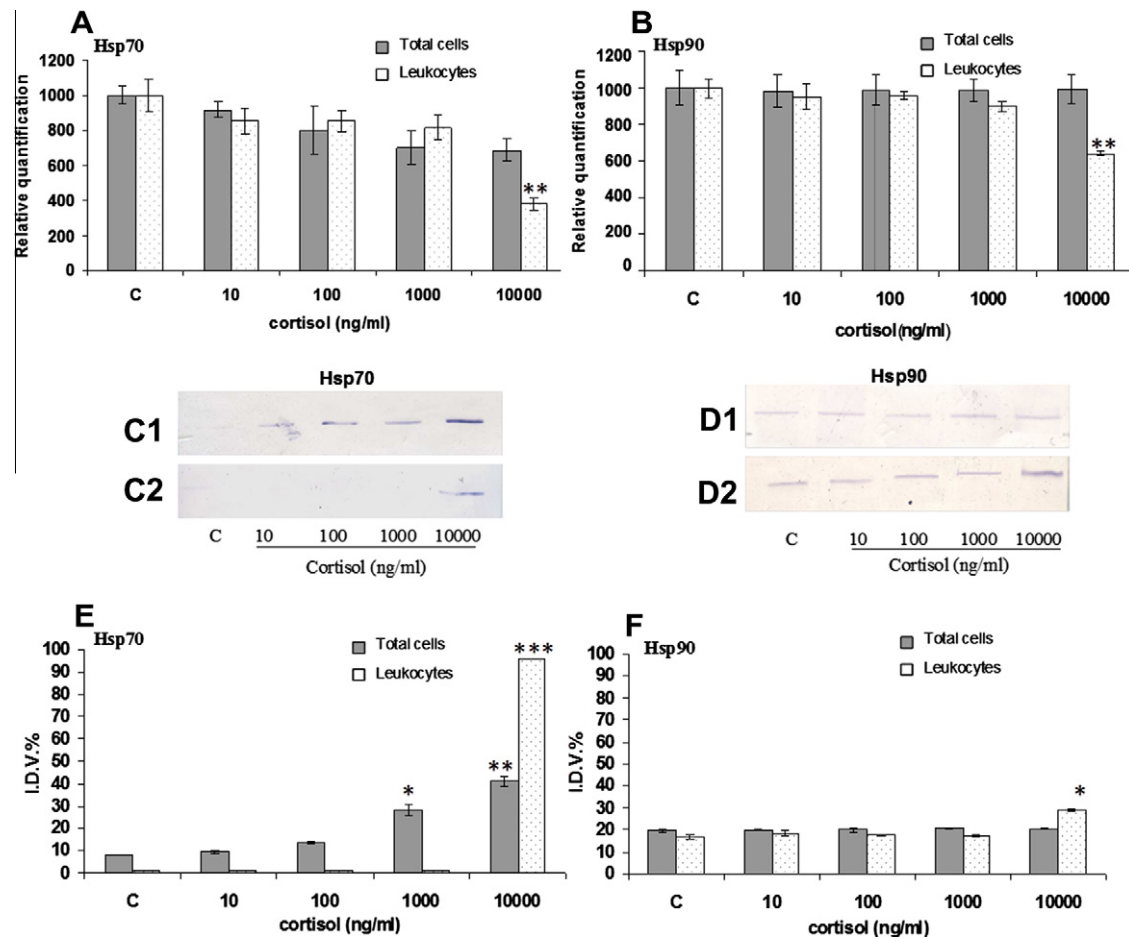
Head kidney cells (total and isolated) were viable after 3 h culture in cortisol-free medium. Although cell viability, revealed by MTT assay, slightly decreased in a dose-dependent fashion, Fig. 4A shows that increasing cortisol concentrations (up to 10,000 ng/ml) did not significantly change viability. A diminished viability (about 10–15% less) was only found at the highest cortisol concentration (10000 ng/ml), overcoming the expected effect of this hormone.

In agarose gel, DNA electrophoresis pattern of the unseparated cells did not show any typical feature of apoptotic DNA ladders (Fig. 4B).

## 4. Discussion

In fish, the Y shaped kidney placed along the body axis is composed with a lower part that works as a renal system, and two arms (aglomerular head kidney or pronephros) that perform hemopoietic and immune functions, i.e., phagocytosis, antigen processing, development of B lymphocytes, macrophages and granulocytes, production of IgM-like immunoglobulins, and immune memory [39,32,50]. The head kidney also is an important endocrine organ, homologous to mammalian adrenal glands, that releases cortisol produced by the interrenal tissue [18] and is considered the central organ for immune-endocrine interactions [37,39]. There is increasing evidence that cortisol may have a direct influence on the cellular stress response [6] including the possible interplay between cortisol and Hsps [36] that modulates several functions including both adaptive and innate immune responses [48]. Numerous studies have been focused on the effects of cortisol administration, both *in vitro* and *in vivo*, revealing the hormone immunomodulatory effects [39] that can be mediated *via* GRs already identified in fish leukocytes including seabass [39,44].

Our results show that in sea bass, following cortisol intraperitoneal injection or *in vitro* treatment of head kidney cells, a high cortisol level exerts modulating activity on the Hsps expression. To highlight the *in vivo* response, the cortisol-treated fishes were compared not only with untreated specimens but also with those injected with the vehicle (sham fish) i.e., the medium used to dissolve the hormone. The sea bass plasma cortisol baseline appeared to be higher than previously reported (at  $10 \pm 8$  ng/ml in reference [40]), however different environmental conditions and population density as well as fish drawing out from different farms could account for the difference. The exogenous cortisol circulated in the blood and mimicked stress conditions (mainly at 3 and 24 h p.i.)



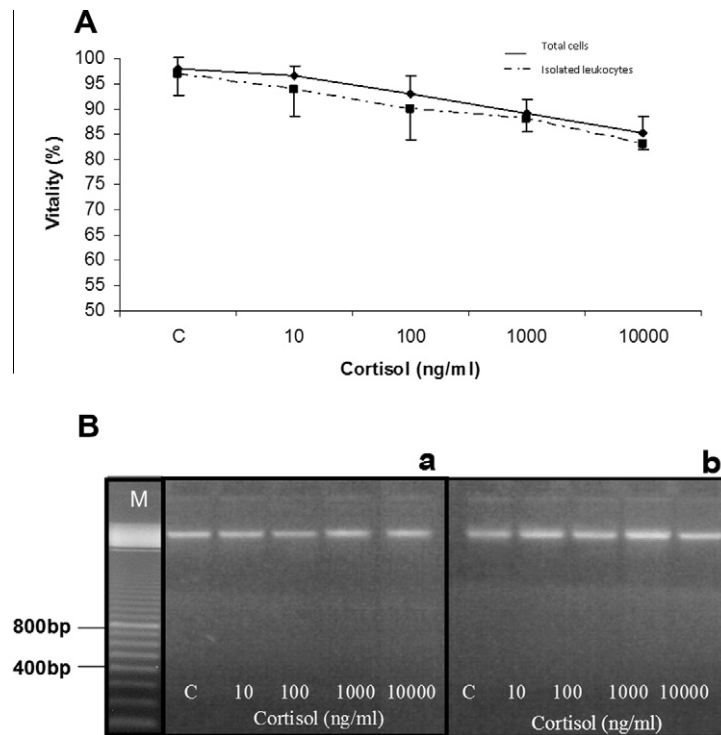
**Fig. 3.** Hsp70 and 90 expressions in head kidney total cells and isolated leukocytes treated *in vitro* with cortisol. (A): Hsp70 mRNA levels; (B): Hsp90 mRNA levels; (C1) and (D1): representative Western blot of Hsp70 and Hsp90 of total cells; C2 and D2: representative Western blot of Hsp70 and Hsp90 of isolated leukocytes; (E) and (F): integrated optical density values (IDV) respectively of the Hsp70 and Hsp90 protein bands abundance. Four specimens from untreated, I and II fish groups were examined at every time point. Each assay was repeated three times. Statistically significant differences between control and cortisol-treated kidney total cells and isolated leukocytes are shown upon the bars (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

as suggested by increased values of serological parameters (glucose, lactate, and osmolarity).

Real-time PCR analysis of the tissue from sham and cortisol-treated specimens clearly showed that, despite the enhanced plasma cortisol level, the Hsp70 mRNA expression was at a baseline value. A significant increase both in sham and cortisol-treated specimens was found at 72 h p.i., just when the cortisol level decreased, suggesting a delayed genomic modulation. Assays at intermediate time-points could establish the precise lowering-time. Although a decrease was observed at 1 week p.i., a high Hsp70 mRNA level was still observed indicating a prolonged gene activity after circulating cortisol started the response. It should be emphasized that, after cortisol administration, the Hsp70 and cortisol levels (3 h and 24 h sampling) were always significantly higher than those recorded in sham fish. An opposite trend characterized the Hsp70 protein identified by an immunoblotting method with anti-mouse Hsp70 mAbs. A single 70 kDa band was detected in the head kidney lysate supernatant, and densitometry analysis showed that the band density time-course approximately overlapped the dynamic of plasma cortisol. The protein reached the highest value at 3 h, then (24 h), although still high, decreased up to the non significant value found at 72 h just when the Hsp70 mRNA increased. A different behavior characterized the Hsp90 gene expression that was mainly upregulated at 3 h and 24 h reaching the base-line value at 1 week. The Hsp70 and Hsp90

genes upregulation may be related to the circulating cortisol suggesting a cortisol-dependent gene activation that could be arranged in order of occurrence. In this respect, the optical density of the immunoblotted Hsp90 protein detected by the anti-mouse inducible Hsp90 mAbs, suggests an inverse ratio (at 72 h p.i.). It is notable that, in the head kidney, the Hsp70 and Hsp90 appear to be inducible.

Since inducible Hsp expression in all organisms can be regulated by the heat shock transcription factors (HSFs), our results suggest that high Hsp protein level blocks the transcription. Several HSFs have been cloned and characterized in fish [34,46,28]. In all vertebrates, stress does not alter the quantity of HSF produced, but its activity is regulated on a post-transcriptional level through oligomerization, translocation and hyperphosphorylation [9], providing a mean of differential control of transcription. During stressful conditions, pre-formed Hsp70 and Hsp90 dissociated from HSF [26], move into the nucleus where activated gene transcription takes place [30]. In sea bass, the opposite trend between Hsp70 mRNA expression and amount of produced protein, suggests that after circulating cortisol was enhanced, the translation of pre-existing or just produced mRNA, blocks gene activation [23]. On the contrary, Hsp90 gene was upregulated by cortisol and then downregulated through the synthesized protein. In both cases a negative feedback mechanism could be invoked [45]. In other fish species, similar results have been reported [29,35] and



**Fig. 4.** (A) Cortisol effects on head kidney cells viability after 3 h treatment with increasing cortisol concentrations. The cell viability was analyzed by MTT assay. Viability of the control group after 3 h incubation was taken as 100% (mean  $\pm$  SD;  $n = 4$ ). Total cells (continuous line); isolated leukocytes (dotted line). Three specimens were examined and each assay was repeated three times. (B) Agarose gel electrophoresis analysis of apoptotic DNA fragments showing that the absence of patterns or smears is indicative of absence of apoptosis. Cells were treated with increasing cortisol concentrations. (a) DNA from total cells; (b) DNA from isolated leukocytes; (M) 100 bp DNA marker; (c) control. Three specimens were examined and each assay was repeated three times.

it has been shown a quick time (15–90 min) for Hsp70 mRNA transcription [17]. Differences in cortisol Hsp modulation reported in the literature [1,12,43] could be dependent on the organ and cell types examined in various fish species and/or by the experimental conditions. Since in seabass head kidney two distinct GRs (DIGR1 and DIGR2) [41,38] are differently modulated by exogenous cortisol [42], being DIGR1 upregulated, the possibility exists that the latter is involved in cortisol modulation of Hsps [42].

To check for the relationships between cortisol-modulated Hsps and head kidney leukocytes response, the cortisol effects were examined after 3 h incubation of total cells or isolated leukocytes with increasing hormone doses (10–100–1000–10,000 ng/ml). Both cell preparations presented Hsp70 mRNA expression that diminished inversely to the dose showing a significant ( $P < 0.01$ ) decrease at the pharmacological dose (10,000 ng/ml) [14,22] just when the protein level significantly increased (densitometry data). Although the protein bands were feeble, the profile of Hsp70 protein density revealed an evident dose-dependent increase inversely proportional to the mRNA levels, whereas the cell Hsp90-level remained almost unchanged up to 1000 ng/ml concentration. In this respect it is known that the Hsp70 is involved in numerous stress-dependent cell mechanisms whereas more specific and limited intervention have been imputed to the Hsp90 [8,35,31]. This is also supported by the highest Hsp70 production *in vitro* though at the cortisol pharmacological dose. The Hsp genes expression and protein density modulation were more evident in isolated leukocyte preparations which were deprived of erythrocytes which could affect the Hsps estimation. The treatments demonstrated that these cells were responsive to cortisol, they expressed mRNA and produced inducible Hsps. These findings are consistent with the *in vitro* cortisol effects on seabass peritoneal cavity leukocyte activity [44], namely modulation of oxygen reactive intermediates production usually associated to phagocyte activity. In spite of the

cortisol treatment that could cause apoptosis [13,11], no apoptotic effects were found in the cells that conversely presented high vitality levels (MTT) and did not show any DNA fragmentation. This finding may be related to the Hsp70 anti-apoptotic effect [13].

In conclusion, since fishes are constantly exposed to stressful conditions they have evolved neuroendocrine responses including cortisol production that modulates the expression of the head kidney inducible Hsps. In addition, according to Iwama et al. [20], the possibility exists that Hsp70 and 90 levels can vary in a same organ, in various tissues and species making their use, as stress indicators, a complex issue.

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