In: Teleosts
Editor: Skylar Carone
ISBN: 978-1-62948-754-0

Chapter 2

OCCURRENCE AND POSSIBLE ROLE OF HEAT SHOCK PROTEIN 70 IN THE DEVELOPING SEA BASS *DICENTRARCHUS LABRAX* (L.)

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ABSTRACT

Heat shock proteins (HSPs) are cellular proteins highly conserved to polypeptide folding, identified in cells subjected to high temperature (as the name indicate). Many members of three families (HSP90, HSP70 and HSPs with low molecular weight) are present in both invertebrates and vertebrates, including the fish. The production of HSPs is one of the most common ways cell responds to a stressor to maintain its integrity and function.

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From several years we have been undertaking immunohistochemical studies on the appearance and distribution of regulatory molecules during the larval stages of the sea bass, a fish widely studied for its commercial value. In this study the results of immunolocalization of HSP70 in sea bass larvae, both in control and in specimens subjected to a biological stressor (LPS), are reported.

The results showed a different pattern of immunoreactivity (IR) in LPS-treated vs. untreated sea bass larvae. LPS stress increases the amount of HSP70-IR in cells of skin, gills, terminal gut, liver and pituitary gland and induces the expression of HSP70 in the kidney collecting ducts; these effects are particularly evident in a short time (1 h). The present immunohistochemical data on the sea bass larvae suggest an involvement in stress-induced HSP-response of liver, gut, skin, gills, kidney collecting ducts and pituitary gland.

In previous our research it has been hypothesized that some molecules involved in stress responses, such as ACTH, nitric oxide and CRF, may play an active role, via autocrine/paracrine ways, in early immune responses of sea bass larvae, before the complete development of gut-associated lymphoid tissue (GALT). This hypothesis comes from observations of IR to these molecules in gut epithelium, liver, pronefric tubules, skin and from the different pattern of IR following LPS treatment carried out with the same experimental design as in the present work. In particular, the distribution of IR to HSP70 in 24 day-old larvae LPS treated is very similar to that described previously for ACTH-IR in the same larval stages LPS treated. The data of present work may indicate that HSP70 also is a member of the pool of molecules involved in the early immune responses of larval sea bass.

In the present research it has also been demonstrated, first in teleosts, HSP70-IR in untreated sea bass pituitary gland and its increase after LPS stress. HSP expression is already known for mammals. These data support the idea of a functional relationship between HSP expression and the hypothalamus-pituitary-adrenal axis that could be a common trait for vertebrates.

Keywords: stress, LPS, bacterial antigens, immune response, larval fish

INTRODUCTION

Heat shock proteins (HSPs) are cellular proteins highly conserved to polypeptide folding. They were identified in cells subjected to high temperature (as the name indicate), in which they stabilize the partially

denatured proteins making their folding easier. Their molecular function as a chaperone is also well-known in normal conditions of cellular growth [15].

Three families of HSPs were described: HSP90 (85-90 kDa), HSP70 (68-73 kDa) and HSPs with low molecular weight (16-47 kDa). Many members of these families are present in both invertebrates and vertebrates, including the fish [15, 10, 6]. The production of HSPs is one of the most common ways cell responds to a stressor to maintain its integrity and function. Both constitutive and inducible forms of HSP70 are expressed in fish; in particular the inducible HSP70 are detectable in fish subjected to several types of stressor [26]. The numerous potential stressors for fish have been grouped in three categories: environmental stressors, physical stressors and biological stressors [10].

In fish aquaculture many procedures, such as weighing, handling, transport conditions, or factors of management (feeding, temperature of water, infections) may cause stress. Thus, several research have been carried out on HSP70 expression in fish to evaluate the stress resulting from aquaculture practices [20].

Handling of rainbow trout, *Oncorhynchus mykiss* (Walbaum), did not influence the HSP70 expression in liver [23], gills, heart, muscles [24]. In the Mozambique tilapia, *Oreochromis mossambicus* (Peters), crowding modifies the expression of HSP70 in the liver, but not in gills or brain [5]. In developing sea bass, *Dicentrarchus labrax*, the expression of the constitutive and inducible forms of HSP70 has been considered as an indicator of stress caused by transport [20]. Immunoreactivity to constitutive HSP70 is distributed in many tissues and organs of this teleost in both stressed and control specimens, while inducible HSP70 is present only in skeletal muscles of stressed animals [20]. Moreover, the results of a study carried out on early developing *D. labrax* subjected to heat shock indicate a very early stress response with a marked increase of mRNA levels of inducible HSP70 [2].

From several years immunohistochemical studies have been undertaking on the appearance and distribution of regulatory molecules during the larval stages of the sea bass, a fish widely studied for its commercial value [12, 13, 14, 16, 17]. Active roles in the early immunological responses of some well-known molecules involved in the stress responses, such as adrenocorticotropic hormone (ACTH) [12, 13], nitric oxide [17] and corticotrophin-releasing factor (CRF) [14] have been proposed. These molecules are biologically active before complete differentiation of gut-associated lymphoid tissue (GALT), acting in a autocrine/paracrine way. In this study the results of immunolocalization of HSP70 in sea bass larvae, both in control and in specimens subjected to a biological stressor, are reported.

MATERIALS AND METHODS

Thirty sea bass larvae (*Dicentrarchus labrax*) at 24 days after hatching (10-12 mm in length) were obtained from the aquaculture fish-farm "Valle Figheri", Lova (Venice, Italy). In this fish-farm the rearing standard conditions are: 24 h light photoperiod at 16-18°C temperature and 40‰ salinity for the first 12 days. Subsequently the salinity is lowered to 30‰. The diet consists of rotifers supplied from day 5th to 16th and newly hatched *Artemia* starting from day 10th. Under these conditions 18 day-old larvae had just completed reabsorption of the yolk sac.

Twenty of these larvae 24 day-old were maintained in a tank containing 10 U/ml LPS (Lipopolysaccharide from *Escherichia coli* serotype 055:B5, Sigma Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Ten specimens were immediately fixed *in toto* in Bouin fluid, while the remaining ten animals were transferred to normal tap water for another 1 h at room temperature and then fixed. A third group of ten animals was fixed without LPS treatment. After fixation for 18 hs, all specimens were embedded in paraffin and cut in 7μm transverse sections.

Subsequently, they were stained with the biotin-avidin immunohistochemistry technique (BAS) utilizing the polyclonal antibodies from rabbit anti-HSP70/HSC70 (H-300) (Santa Cruz Biotecnology, Santa Cruz, CA, USA), titer 1:100. Slides were rinsed three times in 0.01M PBS, pH 7.4 and incubated for 30 min in 0.3% H_2O_2 to block endogenous peroxidase activity, then placed in PBS containing 0.3% Triton X-100 and blocked with 5% normal goat serum (Rockland, Gilbertsville, PA, USA) for 30 min. The slides were incubated with the primary antibody in a humid chamber at 4°C overnight. Subsequently, they were rinsed with PBS and incubated for 30 min at room temperature with the secondary biotinylated antibody goat anti-rabbit (titer 1:300) (Rockland). After rinsing in PBS and 0.1M Tris pH 7.6, the preparations were incubated with Avidin-Biotin Complex (ABC, Vector, Vectastain, Burlingame, CA, USA) in Tris for 45 min at room temperature. The reaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride (10mg/15ml Tris) (Sigma Aldrich). Reactions were allowed to develop for 8 min with 10-12 μl 30% H_2O_2. The specificity of the reactions was checked by negative controls incubating the sections with (1) PBS instead of the primary antibody, (2) normal serum instead of the primary antibody, (3) PBS instead of the secondary antibody, (4) preadsorbed primary antibody in liquid phase with the homologous antigen (50 μg/ml diluted antiserum) at 4°C for 24 h. All

sections were rinsed in Tris and *aqua fontis*, dehydrated and mounted in Eukitt, and observed at a Zeiss Axioscop microscope.

RESULTS

Untreated larvae showed the presence of immunopositive cells in the skin of the entire body and in the gill epithelium (Figures 1a, 1b). The apical border of medium and posterior intestinal epithelium was covered by an immunoreactive stripe (Figures 1a, 1c). In addition, immunoreactive hepatocytes (Figure 1a) and clusters of immunoreactive pituitary cells peripherally localized (Figure 1d) were observed.

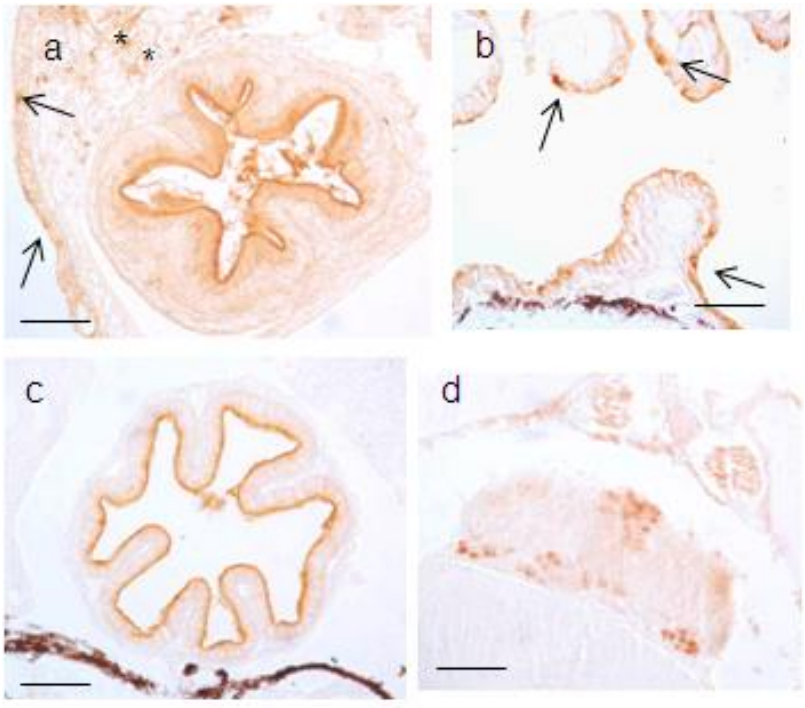

Figure 1. Transverse sections of *Dicentrarchus labrax* untreated larvae. – a. IR to anti-HSP70 in the cells of the skin (arrows), in the hepatocytes of liver (asterisks) and on the apical border of gut epithelium. – b. IR to anti-HSP70 in some cells (arrows) of gill epithelium. – c. Thin HSP70 immunopositive stripe on the apical border of midgut epithelium. – d. "Clusters"of positive cells in the periphery of the hypophysis. Bars, 50 μm.

Larvae treated for 1 h with LPS and immediately fixed revealed intensely immunopositive cells in the skin (Figures 2a, 2b) and gills. Some immunoreactive cells were also present in the epithelium of the posterior gut at rectal valve level (Figure 2c). In the medium and posterior intestinal regions the apical border of epithelium was covered with an immunoreactive stripe (Figures 2a, 2c). Moreover all the hepatocytes resulted more or less intensely immunopositive (Figure 2b). The IR appeared around the lumen of kidney collecting ducts (Figure 2a). An intense immunoreactivity was also seen in all pituitary cells (Figures 2d, 2e).

In the larvae fixed 1 h after LPS treatment the pattern of IR was similar to that found for larvae fixed immediately after LPS treatment for skin, gut and pituitary, but the collecting ducts (Figure 3a) and the liver (Figure 3b) showed the same pattern of untreated larvae.

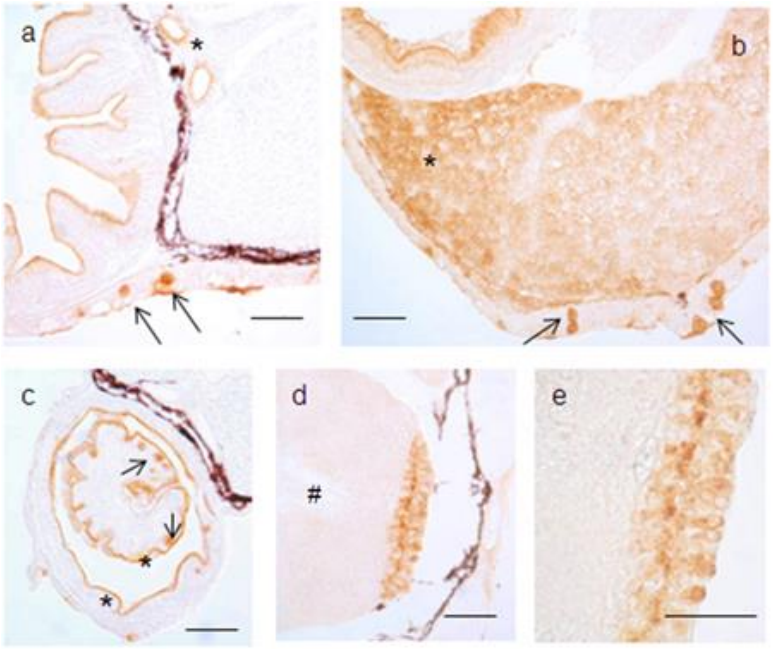

Figure 2. Transverse sections of *Dicentrarchus labrax* larvae treated with LPS and immediately fixed. – a. Immunopositive cells in the skin (arrows) and in kidney collecting ducts (asterisk). – b. IR in cells of skin (arrows) and the liver (asterisk). – c. Many epithelial positive cells (arrows) and a positive stripe (asterisks) at the rectal valve level of gut. – d. All pituitary cells are immunopositive. (#: third ventricle). – e. Enlargement of d. Bars, 50 μm.

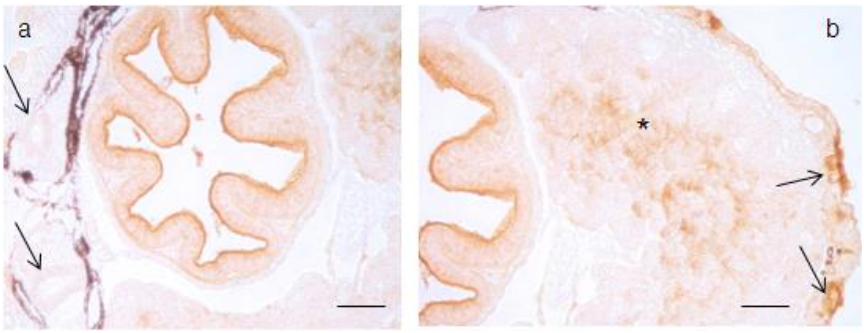

Figure 3. Transverse sections of D*icentrarchus labrax* larvae fixed 1 h after LPS treatment. – a. HSP70 immunopositive stripe on the apical border of midgut epithelium. The collecting ducts (arrows) are negative. – b. IR in cells of the skin (arrows) and in cells of the liver (asterisk). Bars, 50 μm.

All the control slides were negative.

CONCLUSION

The results showed a different pattern of IR in LPS-treated vs. untreated sea bass larvae. Indeed, LPS stress increases the amount of HSP70-IR in cells of skin, gills, terminal gut, liver and pituitary gland and induces the expression of HSP70 in the kidney collecting ducts; these effects are particularly evident in a short time (1 h).

Most of the evidences about how the generalized stress response and HSPs expression may be related comes from mammalian studies [see refs. 15, 6, 22]. Some data are available for fish, showing the increase in HSPs (60, 70, 90 kDa) in various tissues of different species subjected to stressors, such as heat shock, environmental contaminants and bacteria. In adult fish liver, kidney and gills seem to be the most responsive tissues to these stressors and several species also show the induction of a number of HSP proteins in response to stressors in primary cultures of hepatocytes [see ref. 10].

The present immunohistochemical data on the sea bass larvae indicate an involvement in stress-induced HSP-response of liver, gut, skin, gills, kidney collecting ducts and pituitary gland. This suggest that the HSP response involves a greater number of organs in larvae than in adults fish and that hepatocytes may be a preferential seat of responses to stressors by means of HSPs, both *in vitro* and *in vivo*, including the larval development. Indeed, the important role of HSPs in phenomena of intense cell division, as occurs during

larval development, has been indicated by their presence in adenomas of rats [11] and in human tumours [18].

A number of studies indicate that larvae and juveniles of fish respond to several stressors producing HSPs at higher level than adults [19, 4, 8]. Moreover an inducible or embryo-specific form of HSP70 is constitutively expressed during development in *Austrofundulus limnaeus* Schultz [19] and the Authors hypothesize that this expression of HSP70 during development may protect embryos from stress more quickly than relying of an inducible form.

In *D. labrax* larvae 25 and 40 days-old, fry 80 days-old and adults, has been demonstrated that the expression of HSP70 is an indicator of physical stress caused by fish transport [20]. Increased expression of the inducible HSP70 form is evident only in 40 days-old stressed larvae and higher in 80 days-stress fry than in controls. In adult sea bass inducible HSP70 is present only in skeletal muscles of stressed animals [20].

In the present research a biological stressor (LPS) has been used and the findings indicate an HSP increased response already in 24 days-old larvae stressed than in controls. It is possible that a biological stressor may cause an earlier HSP response.

There is some literature on the possible role of HSP70 in fish immune response [see ref.21]. Bacterial infections cause an increase of HSP70 in liver and kidney of teleosts [7, 1] and a HSP70 mRNA expression increase in head kidney, spleen, thymus and gill [3, 27]. The HSP70 gene is inducible and involved in the fish immune response [3]. Considering these findings, the increase in HSP70-IR following LPS treatment in sea bass larvae may be included in the early mechanisms of immune responses.

In our previous research [12, 13, 14, 17] it has been hypothesized that some molecules involved in stress responses, such as ACTH, nitric oxide and CRF, may play an active role, via autocrine/paracrine ways, in early immune responses of sea bass larvae, before the complete development of GALT. This hypothesis comes from observations of IR to these molecules in gut epithelium, liver, pronefric tubules, skin [12, 17] and from the different pattern of IR following LPS treatment [13, 14] carried out with the same experimental design as in the present work. In particular, the distribution of IR to HSP70 in 24 day-old larvae LPS treated is very similar to that described previously for ACTH-IR in the same larval stages LPS treated [13]. The data of present work may indicate that HSP70 also is a member of the pool of molecules involved in the early immune responses of larval sea bass, a fish very studied for its commercial value. In spite of the immune system of *D. labrax* is best known

among marine teleosts, few data are present on early larval immunity. It is possible that new finding on immune responses in this crucial phase of life could provide useful informations for rearing *D. labrax*.

In the present research it has been demonstrated, first in teleosts, HSP70-IR in untreated sea bass pituitary gland and its increase after LPS stress. HSP expression is already known for mammals. Indeed, in unstressed rats, a number of cells in the anterior pituitary gland show strong immunostaining for HSP25 [25]. Moreover, in rat thermic shock leads to a marked increase in HSP27 in many organs, including pituitary gland [9]. Lastly, hypophysectomized rats did not show HSP gene expression in response to stress and addition of ACTH induced HSP expression in the adrenals glands [10]. All these data support the idea of a functional relationship between HSP expression and the hypothalamus-pituitary-adrenal axis that could be a common trait for vertebrates.

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