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Heat shock protein (HSP) expression and mitogen-activated protein kinase (MAPK) phosphorylation during early embryonic developmental stages of the Gilthead sea bream (*Sparus aurata*)

CHRYSOULA ROUFIDOU[1,4], KONSTANTINOS FEIDANTSIS[1], ELENI MENTE[2], ELENA SARROPOULOU[3] and EFTHIMIA ANTONOPOULOU[1]

[1] Department of Zoology, School of Biology, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
[2] Department of Ichthyology and Aquatic Environment, School of Agricultural Sciences, University of Thessaly, 38446 Volos, Greece
[3] Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, 71003 Heraklion, Crete, Greece
[4] Department of Zoology, Stockholm University, S-106 91 Stockholm, Sweden

Corresponding author: eantono@bio.auth.gr
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Abstract

Both heat shock proteins (HSPs), which have key roles in vital cell functions, as well as members of the mitogen-activated protein kinases (MAPKs), which adjust gene expression by transducing cellular signals to the nucleus, are necessary for normal embryonic development in vertebrates. Therefore, protein expression levels of HSP70 and HSP90 and the activation of members of the MAPK protein family, such as p38 MAPK, ERKs, and JNKs were studied in the early developmental stages of the Gilthead sea bream, *Sparus aurata* Linnaeus, 1758. The protein expression of HSP70 and the phosphorylation ratio of JNKs remained at equal levels at all examined developmental stages, while the other examined proteins exhibited a differential profile. HSP90 levels were mostly increased at the 16-cell stage and towards the morula stages, and the lowest values were observed at the two- to four-cell and one-half epiboly stages. While p38 MAPK phosphorylation ratio exhibited increased values mostly in the early developmental stages, the opposite was observed concerning ERK phosphorylation ratio, where increased values were observed in the later embryonic stages (high blastula to one-half epiboly stages). These differential profiles of the examined protein expression levels highlight the importance of these proteins during embryogenesis and pave the way for further research to unveil their distinct role in early development.

Keywords: Embryonic development; cellular functions; HSP70; HSP90; p38MAPK; ERKs; JNKs.

Introduction

The developing oocytes and fertilized eggs of fish are particularly influenced by the endocrine status of the female during oogenesis, by husbandry practices and broodfish diet, as well as by environmental conditions, pollutants, and xenobiotic substances to which the eggs are exposed. These conditions can lead to incomplete development, lower hatching success and decreased viability (Lahnsteiner *et al.,* 2004; Ostrach *et al.,* 2008; Witeska *et al.,* 2014). Fish egg quality is also determined by the egg's endogenous factors, such as the genes, maternal mRNAs, proteins, nutrients, and macromolecules that are provided to the egg throughout oocyte development during oogenesis (Tata, 1986). While contribution of both paternal and maternal genes determines embryo quality, after fertilization the egg quality is initially regulated by maternally provided molecules (Brooks *et al.*, 1997). Therefore, in the aquaculture sector the larval stage, which is set up during embryogenesis, has been determined to be the most decisive period in the production of marine fish (Pavlidis & Mylonas, 2011).

The development of fish larvae is determined as in other animals during embryogenesis. At the transcript level, several studies have been performed showing differential expression during early development (Sarropoulou *et al*., 2005; Yufera *et al*., 2012; Kaitetzidou *et al*., 2015). In a study of Fernández *et al.* (2012), the mRNA expression of genes with putative involvement in the regulation of early development and egg quality (Ibabe *et al*., 2005; Traverso *et al*., 2012) was investigated in the Gilthead sea bream, *Sparus aurata*, a species of commercial interest in the Mediterranean basin. The present work supplements the previous results of Fernández *et al.* (2012) by investigating the expression levels of two members of the heat shock proteins (HSPs), the HSP70 and HSP90, as

well as the activation (via phosphorylation) of the members of the mitogen-activated protein kinase (MAPK) family [p38 MAPK, ERKs (extracellular signal regulated kinase), and JNKs (c-Jun N-terminal kinase)] members. In addition to the role of these proteins in mediating the response to stressful conditions (Schlesinger, 1990; Widmann *et al*., 1999), certain proteins are also involved in vertebrate reproduction and embryonic development (Krens *et al*., 2006; Fevurly *et al*., 2012). Specifically, the heat shock response (HSR) is induced by a variety of environmental stimuli (Iwama *et al.,* 1999). Additionally, the expression of heat shock genes is necessary for normal cell growth during embryonic development of both invertebrates and vertebrates, and is both constitutive and inducible (Walsh *et al.,* 1997; Morimoto, 1998). Distinct members of the MAPK superfamily, including ERKs, JNKs and p38 MAPK are involved in responses to growth factors and hormones (Nishida & Gotoh, 1993) and in transducing responses to several stresses and pro-inflammatory cytokines, such as UV irradiation, hyperosmolarity, heat shock, endotoxins, tumour necrosis factor and interleukin-1 (Cano & Mahadevan, 1995) resulting in the regulation of multiple genes (Snaar-Jagalska *et al*., 2003). Moreover, MAPKs' role in cell growth, proliferation, survival, and development is indisputable (Bogoyevitch & Court, 2004; Roux & Blenis, 2004; Johnson *et al.,* 2005; Xu *et al.,* 2012). In this context, and due to the fact that HSPs and MAPKs hold key roles in cell homeostasis and signal transduction during early embryonic development, the aim of the present study was to determine their expression during seven embryonic stages (from the two- to four-cell stage to the one-half epiboly stage) of the Gilthead sea bream.

Material and Methods

All procedures involving the handling and treatment of fish used during this study were approved by the HCMR Institutional Animal Care and Use Committee following the Three Rs (3Rs: Replacement, Reduction, Refinement) guiding principles for the more ethical use of animals in testing, firstly described by Russell and Burch in 1959. Fish were anaesthetized using 100-200 mg/L MS222 (tricaine methanesulphfonate, Sigma-Aldrich, USA) depending on fish size.

Chemicals

All biochemicals were purchased from Sigma (Darmstadt, Germany), Cell Signaling (Beverly, MA, USA) and Biorad (Hercules, CA, USA). All other chemicals were obtained from Sigma (Darmstadt, Germany), Merck (Darmstadt, Germany) and Applichem (Gatersleben, Germany) and were of analytical grade.

Tissue Sample Collection

Early embryonic samples from fertilized eggs of the Gilthead sea bream were obtained from the Institute of Marine Biology, Biotechnology, and Aquaculture of the Hellenic Centre for Marine Research in Crete, Greece as described in Fernández *et al.* (2012). In total, six different batches (biological replicates) of seven early developmental stages were collected: (S1) two- to four-cell stage, (S2) 16-cell stage, (S3) towards morula, (S4) morula, (S5) high blastula, (S6) start of gastrula, and (S7) 50% epiboly, according to Divanach (1985). All the developmental stages were collected from the same stripping event and were carefully examined and identified under stereoscope. All collected samples were frozen immediately in liquid nitrogen and stored at −80°C prior to protein extraction.

SDS-PAGE and immunoblot analysis

Frozen embryonic samples of different developmental stages were homogenized in 3 mL g^{-1} of cold lysis buffer [20 mM β-glycerophosphate (G9422, Sigma, Darmstadt, Germany), 50 mM NaF (A3904, Applichem, Gatersleben, Germany), 2 mM EDTA (A2937, Applichem, Gatersleben, Germany), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, HEPES (A3724, Applichem, Gatersleben, Germany), 0.2 mM Na_3VO_4 (S6508, Sigma, Darmstadt, Germany), 10 mM benzamidine (B6506, Sigma, Darmstadt, Germany), pH 7, containing 200 μM leupeptin (A2183, Applichem, Gatersleben, Germany), 120 mM pepstatin (A2205, Applichem, Gatersleben, Germany), 10 μM trans-epoxy succinyl-L-leucylamido-(4-guanidino) butane (A2157, Applichem, Gatersleben, Germany), 5 mM dithiothreitol, DTT (A2948, Applichem, Gatersleben, Germany), 300 μM phenyl methyl sulphonyl fluoride, PMSF (A0999, Applichem, Gatersleben, Germany) and 1% v/v Triton X-100 (A1388, Applichem, Gatersleben, Germany)], and were extracted on ice for 30 min. The samples were centrifuged (10,000*g*, 10 min, 4°C) and the supernatants were collected and then boiled with 0.33 volumes of SDS/PAGE sample buffer [330 mM Tris-HCl (A1086, Applichem, Gatersleben, Germany), 13% v/v glycerol (A2364, Applichem, Gatersleben, Germany), 133 mM DTT (A2948, Applichem, Gatersleben, Germany), 10% w/v sodium dodecyl sulphate, SDS (A1502, Applichem, Gatersleben, Germany), 0.2% w/vbromophenol blue (114391, Sigma, Darmstadt, Germany)]. The protein concentration was determined using the BioRad protein assay (Ref. No. 5000001, BioRad, Hercules, CA, USA).

Equivalent amounts of protein (50 μg) were separated on 10% (w/v) acrylamide (A1154, Applichem, Gatersleben, Germany) and 0.275% (w/v) bisacrylamide (A1096, Applichem, Gatersleben, Germany) lab gels and then transferred electrophoretically on to nitrocellulose membranes (0.45 μm, Schleicher and Schuell, Keene N.H. 03431, USA). All nitrocellulose membranes were dyed with Ponceau stain in order to assure a good transfer quality of transfer and equal protein loading. Non-specific binding sites on the membranes were blocked by

incubation for 30 min at room temperature with 5% (w/v) non-fat milk in TBST (Tris buffered saline-Twin 20) [20 mM Tris-HCl (A1086, Applichem, Gatersleben, Germany), pH 7.5, 137 mM NaCl (A1149, Applichem, Gatersleben, Germany), 0.1% (v/v) Tween 20 (A1389, Applichem, Gatersleben, Germany)]. Subsequently, the membranes were incubated overnight with the appropriate primary antibodies, which included the following: 70 kDa monoclonal mouse anti-heat shock protein, (H5147, Sigma, Darmstadt, Germany); polyclonal rabbit anti-heat shock protein, 90 kDa (4874, Cell Signaling, Beverly, MA, USA); monoclonal rabbit anti-phospho p44/42 MAPK (Thr202/Tyr204) (4370, Cell Signaling, Beverly, MA, USA), polyclonal rabbit anti-phospho-p38 MAP kinase (Thr180-Tyr182) (9211, Cell Signaling, Beverly, MA, USA), monoclonal mouse anti-phospho-SAPK-JNK (Thr183-Tyr185) (4668, Cell Signaling, Beverly, MA, USA), monoclonal rabbit anti-p44/42 MAPK (4695, Cell Signaling, Beverly, MA, USA), polyclonal rabbit anti-p38 MAP kinase (9211, Cell Signaling, Beverly, MA, USA) and polyclonal rabbit anti-SAPK-JNK (9252, Cell Signaling, Beverly, MA, USA). After washing in TBST (3 x 5 min), the blots were incubated with horseradish peroxidase-linked secondary antibodies, polyclonal goat anti-mouse immunoglobulins (7076, Cell Signaling, Beverly, MA, USA) and polyclonal goat anti-rabbit immunoglobulins (7074, Cell Signaling, Beverly, MA, USA) and washed again in TBST (3 x 5 min). The bands were detected by enhanced chemi-luminescence (7003S, Cell Signaling, Beverly, MA, USA) and were exposed to Fuji Medical X-ray films. The films were quantified by a laser-scanning densitometry (GelPro Analyzer Software, Media Cybernetics).

Statistics

Changes in the induction of HSPs and phosphorylation ratios of MAPKs, in the early developmental changes were tested for significance at the 5% level by using one way (GraphPad Instat 3.0) analysis of variance (ANOVA). Values are presented as means ± S.D. of $n = 6$ biological replicates. Post-hoc comparisons were performed using Bonferroni test.

Results

The induction levels of HSP70 and HSP90 during Gilthead sea bream embryogenesis are depicted in Figure 1. HSP70 levels had no differences between the different developmental stages, while HSP90 levels were significantly higher in the S2 and S3 developmental stages. Stages S4, S5 and S6 showed increased (but not statistically significant) HSP90 levels compared to S1 stage, while in the last stage (S7 - 50% epiboly), HSP90 levels were statistically lower compared to stages S2 to S6.

The MAPKs phosphorylation ratios (phosphorylation levels to constantly expressed levels) of p38 MAPK, ERKs and JNKs as well as representative blots are depicted in Figure 2. While p38 MAPK levels and p38 MAPK phosphorylation levels were found to be increased in the S5, S6 and S7 stages compared to the first four developmental stages, the phosphorylation ratio of p38 MAPK remained high in the first 4 developmental stages (S1, S2, S3, and S4), while at the S5, S6, and S7 developmental stages this ratio was statistically significant lower. In contrary to the p38 MAPK levels, the ERKs levels were found to be equal at all developmental stages while the ERKs activation levels were most profound in the last

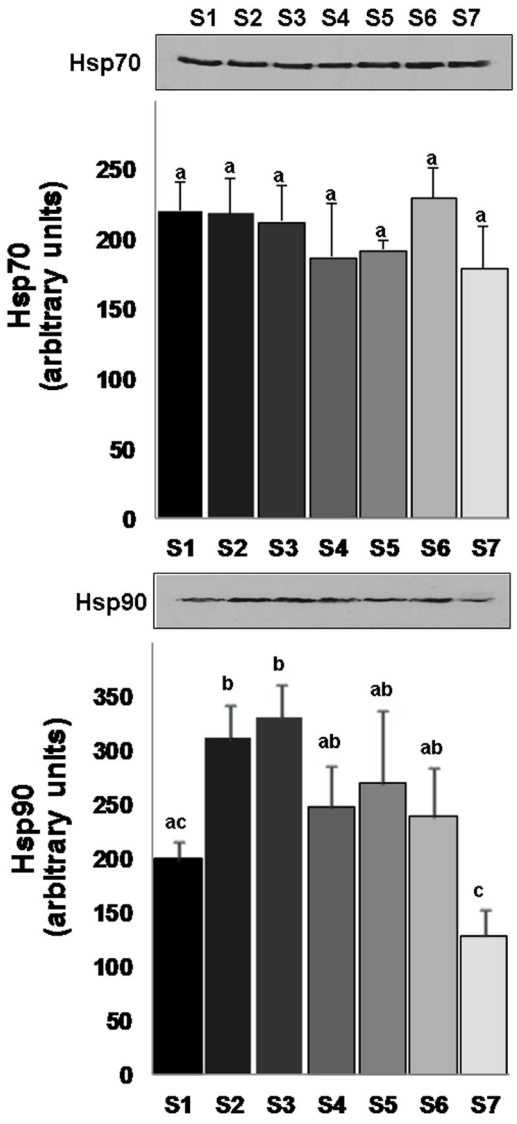

Fig. 1: HSP70 and HSP90 protein levels in the early developmental stages of the Gilthead sea bream. (S1) 2,4 cell stage, (S2) 16 cell stage, (S3) towards morula, (S4) morula, (S5) high blastula, (S6) start of gastrula and (S7) ½ epiboly. Representative immunoblots are shown. Values represent means ± SD; $n = 6$ biological replicates. The statistically significant difference ($P < 0.05$) between the developmental stages is indicated by different letters. Means sharing the same superscript are not statistically significant.

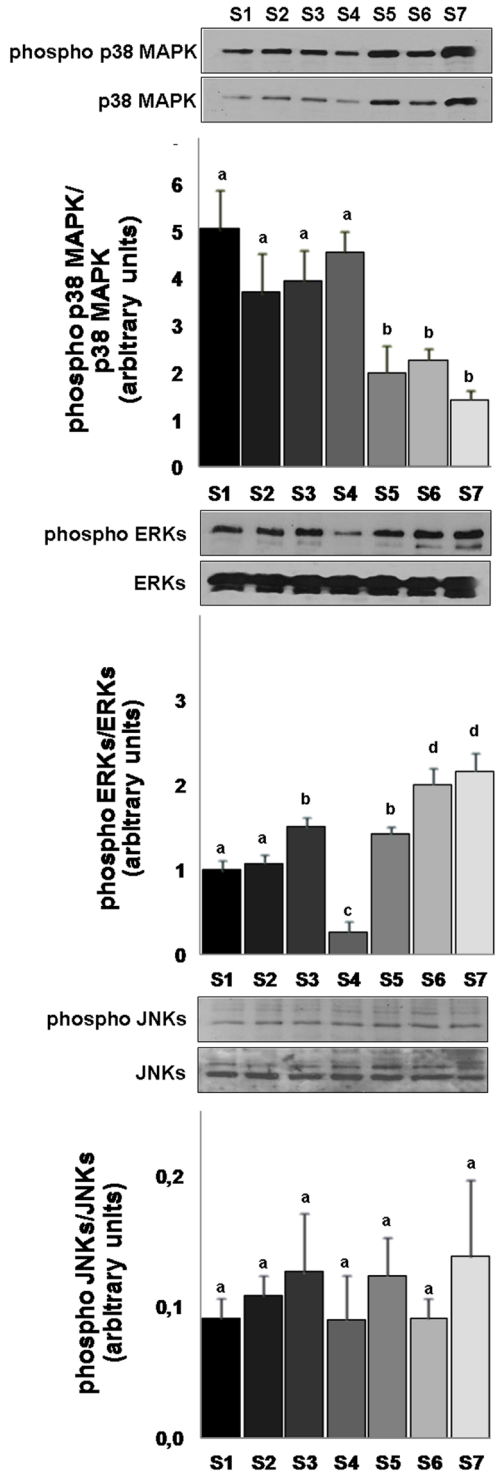

Fig. 2: Phosphorylation ratio of p38 MAPK (phospho p38 MAPK / p38 MAPK), ERKs (phospho ERKs / ERKs and JNKs (phospho JNKs / JNKs) in the early developmental stages of the Gilthead sea bream. (S1) 2,4 cell stage, (S2) 16 cell stage, (S3) towards morula, (S4) morula, (S5) high blastula, (S6) start of gastrula, and (S7) one half-epiboly. Representative immunoblots are shown. Values represent means ± SD; $n = 6$ biological replicates. The statistically significant difference ($P < 0.05$) between the developmental stages is indicated by different letters. Means sharing the same superscript are not statistically significant.

examined developmental stages. Thus, the phosphorylation ratio of ERKs was more intense in the later S6 and S7 developmental stages compared to the other stages and especially compared to the first stages of S1 and S2. A milder increase was depicted in the S3 and S5 stages compared to S6 and S7 stages, while in ERK phosphorylation levels were the lowest in the S4 stage. The phosphorylation ratio of JNKs remained at equal levels among the examined developmental stages, similar to the results obtained with the expression of HSP70.

Discussion

Due to the fact that the larval stage is the most decisive period in the production of marine fish, information regarding the expression of different genes and proteins and their role in fish embryogenesis is of great importance (Pavlidis & Mylonas, 2011). The present study is focusing on the differential protein expression of HSP70 and HSP90, as well as the protein levels and phosphorylation ratio of the MAPK members in seven stages (S1, S2, S3, S4, S5, S6, and S7) of early development in the Gilthead seabream.

Concerning HSP induction, only the HSP90 showed a different profile between the different developmental stages examined. The highest levels of expression were observed at the (S2) 16-cell stage and the (S3) towards morula stage while the lowest expression levels were observed at the (S7) one half-epiboly stage. Information in HSP90 protein levels in fish during embryogenesis is scarce and its role remains to be thoroughly investigated. In developmental stages of Zebrafish (*Danio rerio* Hamilton, 1822), however, two *hsp90* genes (*α* and *β*) are present. Although *hsp90β* gene expression is high during all normal embryo developmental stages under control conditions, *hsp90α* gene expression is low and it is activated mostly under thermal stress (Krone & Sass, 1994). The two different HSP90 isoforms probably play different roles during early embryogenesis and the subsequent larval stages. Moreover, Krone and Sass (1994) observed that levels of the *hsp90β* isoform levels were mostly decreased through the transition to the mid-blastula phase. On the contrary, HSP70 levels remained equal at all developmental stages. Although much research on HSP expression has been conducted, its role in embryonic development is not fully understood (Rupik *et al*., 2011). Similar to our results, Lele *et al*. (1997) and Evans *et al*. (2005) found that HSP70 levels remained low and unchanged between different stages of embryogenesis in Zebrafish and that expression is induced only when the embryos are exposed to different stressors. Despite the fact that HSP70 levels are low in fish embryonic development, they are necessary for proper tissue development (Evans *et al*., 2005). Similarly, Krone *et al*. (1997) observed that in response to thermal stress, *hsp70* gene expression in Zebrafish embryos is mostly elevated at the gastrulation and somitogenesis of 2 to 3 day-old larvae. Other genes that are closely related to the *hsp70* gene,

are highly expressed during normal embryogenesis (especially during somitogenesis and neurogenesis) while their expression is slightly increased under thermal stress (Santacruz *et al*., 1997). While, it had been previously shown that the transition from maternal mRNA to zygotic mRNA in Zebrafish occurs at the mid-blastula stage and is known as the mid-blastula transition (Kane & Kimmel, 1993), Santacruz *et al*. (1997) observed that HSP70 is maternally provided to the Zebrafish embryo as maternal mRNA. Thus, it is not clear if other HSP family members are maternally provided to the embryos and if this is also the case in other fish species.

In the Silver sea bream (*Sparus sarba* Forsskal, 1775), a species closely related to the Gilthead sea bream, Deane & Woo (2003) found that although Hsp70 mRNA and protein remained at equal levels during the early developmental larvae stages (1-14 days post hatch, dph), HSP90 exhibited a differential expression profile during larval development. This is similar to the pattern we found in the Gilthead sea bream. It appears that the roles of HSP70 and HSP90 during embryogenesis and development in fish are different and their patterns remain to be investigated.

Concerning MAPKs, the present results showed a differential MAPK activation in the early developmental stages of the Gilthead sea bream, probably due to the different roles of each MAPK member during embryogenesis. Specifically, although total p38 MAPK and p38 MAPK phosphorylation levels were mostly increased at S5-S7 stages, the respective phosphorylation ratio was found to be mostly elevated in S1 to S4 developmental stages. The increased phosphorylation ratio of p38 MAPK at the S1 to S4 stages is expected, given its vital role in the cell cycle regulation, transcription factor regulation (see review by Zarubin & Han, 2005), and early embryonic development (Krens *et al*., 2006). In Zebrafish p38α activation is required for symmetric and synchronous cleavage (Fujii *et al.,* 2000), while Keren *et al.* (2005) have shown that p38MAPK activation is involved in myogenesis during *Xenopus* early development. p38β, p38γ and p38δ knockout mice however, exhibited normal survival and fertility (Kuida & Boucher, 2004; Beardmore *et al.,* 2005; Sabio *et al.,* 2005). Similar to our results, p38 MAPK phosphorylation in the sea urchin embryos is more obvious in the 60-cell embryo stage and remains active throughout development but at low levels (Bradham & McClay, 2006). The elevated total levels as well as the elevated phosphorylation levels of p38 MAPK at S5, S6 and S7 stages in the present study contradict the elevated *p38α* and *p38δ* mRNA levels in the first four stages found by Fernández *et al*. (2012). This could be attributed to the transition at the mid-blastula stage, as well as the transcription and translation of the embryonic genome (Kane & Kimmel, 1993).

Contrary to p38 MAPK, the ERKs phosphorylation levels and phosphorylation ratio seemed to be increased in the Gilthead sea bream embryos, a finding parallel to the embryo development stages, with protein levels being increased at the later stages. This result may be attributed to the fact that ERKs play an important role in cell differentiation, proliferation and segmentation in several chordates such as mice, chicken, *Xenopus,* and Zebrafish (Krens *et al*., 2006; Sawada *et al*., 2001; Schohl & Fagotto, 2002). In mice, immunochemistry studies proved that ERKs were spatially and chronically activated during embryogenesis (Corson *et al*., 2003). Moreover, MEK as an activator of ERK has been shown to be an essential regulator for cell survival and normal embryonic development in mice (Xu *et al.,* 2012). Similar to the *p38* genes, *erk1* mRNA levels were found to be increased at the first five (S1-S5) embryonic developmental stages in the Gilthead sea bream (Fernández *et al*., 2012). The opposite pattern of ERKs phosphorylation probably reveals post-translational control of the embryo with no maternal origination. Because ERKs levels remain equal among the examined developmental stages in the present study, it is assumed that their role should be of great importance in embryogenesis and that the basal levels are always available to be activated when needed. This assumption is also supported by the ERK's essential role in the developing Central Nervous System (CNS) at early segmentation stages in Zebrafish (Shinya *et al.,* 2001; Krens *et al.,* 2006) and in *Xenopus* (Nishimoto *et al.,* 2005). Additionally, ERKs are essential for mesoderm differentiation and normal vascular development in mice during embryonic development (Yao *et al.,* 2003) probably because of ERK's involvement in endothelial cell integrity (Hayashi *et al.,* 2004).

Based on the present and previous results (Fernández *et al*., 2012) the same scenario could be assumed for the JNKs expression at the protein and mRNA level, respectively. The JNKs phosphorylation ratio, as well as the JNK total and phosphorylation levels, remained fairly equal at all developmental stages of the Gilthead sea bream, demonstrating the crucial role of JNKs in early embryonic development (Kuan *et al*., 1999), as JNKs are involved, inter alia, in the development as well as in the developmental apoptosis regulation, actin reorganization and cell transformation (Kyriakis & Avruch, 2001). In Zebrafish embryos, increased JNK activation levels were mostly observed during the late stages of gastrulation (Seo *et al*., 2010) and down-regulated JNKs directly inhibited ovary differentiation during early ontogenetic stages (Xiao *et al*., 2013). Moreover, Xiao *et al*. (2013) found that the *jnk1* gene was required for Zebrafish development, as the knockdown of this gene resulted in serious retardation and malformations in Zebrafish embryos since JNKs are essential for the expression of anterior neural genes (Kim *et al.,* 2005). Moreover, Kuan *et al.* (1999) have shown JNKs' crucial role in neuronal development in chicken embryos.

Comparing the present protein levels results with the ones obtained at the transcriptome level (Fernández *et al*., 2012), no close relationship between the MAPK mRNA levels and the MAPK protein levels in the early developmental stages of Gilthead sea bream is observed. Such a discrepancy between the transcription and translation

levels is not unusual, as it is known that gene expression levels have limited correlation to the relative protein quantities in the same cells (Griffin *et al*., 2002). Particularly in the oocytes, the mRNA and protein levels may not be of the same nature and origin. Maternal origin molecules are stored for further use during embryo development. Furthermore, in mature oocytes, the translation is suspended in order for the existing transcription products to be controlled by maternal factors (Tadros & Lipshitz, 2009). Additionally, not all the mRNAs molecules are translated into proteins, because the transcriptional and translational control includes RNA processing and degradation. In human oocytes it has been shown that during follicle maturation and mostly during oocyte growth, high transcription and translation rates are followed by differential transcriptional silencing and mRNA degradation (Eichenlaub-Ritter & Peschke, 2002; Schultz, 2002).

In conclusion, the early developmental stages of the Gilthead sea bream exhibit a differential HSP induction and MAPK activation suggesting a vital role during embryonic development which remains to be investigated in subsequent studies. Moreover, the discrepancies between the MAPK mRNA levels (Fernández *et al*., 2012) and the MAPK protein levels probably suggest a differential transcriptional and translational control concerning the regulation of the MAPK family in the early embryonic stages. Although, studies of embryonic development have shown that maternal factors regulate several developmental processes (Tata, 1986), future studies need to be conducted in order to illuminate the vital role and the origin of several proteins and RNAs during embryonic development. Our understanding of the role and significance of the HSPs and MAPKs expression in relation to farmed fish development is still limited, but their potential value in aquaculture sector is very important.

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