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EFFECT OF WATER TEMPERATURE INCREASE ON HO-1 EXPRESSION IN EUROPEAN SEA BASS (*Dicentrarchus labrax* L.) TISSUES

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Abstract

One of the most pertinent environmental factors influencing the marine organism life is temperature. It has been demonstrated that an increase of temperature is able to induce the synthesis of heat shock proteins (HSP). In this study we investigated the expression of HO-1 mRNA, also referred to as HSP32, in different tissues of European sea bass (*Dicentrarchus labrax,* L.) at several time points after increased temperature exposure (from 12°C to 30°C). Our results showed that HO-1 was not expressed in gills, heart, muscle and brain while it was expressed at a basal level in intestine. In liver, spleen and kidneys, HO-1 expression was influenced by temperature increases. In the spleen, we found a significant decrease of the HO-1 expression at the end of 4 weeks. In kidneys a very fast collapse of HO-1 expression level was recorded reaching null value as soon as one hour after exposure to 30°C. In liver, HO-1 expression increased from one hour of exposure to 30°C confirming HO-1 involvement to heat shock response in this organ. This increasing trend reached a 4.5-fold higher value than the initial level after 4 weeks.

Key words: *Dicentrarchus labrax*, gene expression, heme oxygenase-1, temperature increase, Heat Shock Protein 32.

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INTRODUCTION

One of the most pertinent environmental factors influencing the marine organism life is temperature (15). With the global warming and the worldwide decline of fishery stocks, increasing interest is accorded to study the effects of temperature increase on physiological and biochemical responses of fishes (8,11,19,20). Temperature exceeding the tolerance range can disrupt growth and reproduction of fish as well as larval development (27,30) and is considered as a real stress for organisms. Indeed an increase of normal growing temperature of 4 to 8°C is able to induce the synthesis of stress proteins (7). Classically named as heat shock proteins (HSP), their synthesis has been shown to be the first response to a large number of non-physiological stressors other than heat such as heavy metals, toxic chemicals, UV-light, etc... These proteins are synthesized to protect organisms from environmental stressors in order to maintain homeostasis and insure cell survival even if some display basal synthesis levels in normal unstressful conditions (17,34). This mechanism, commonly named stress adaptation or tolerance, is supposed to minimize the response of the organism to further exposures to the same stimulus (34). Genes coding for HSP represent an early biomarker of stress exposure in ecotoxicological studies (17). HSP are commonly classified by their molecular weight HSP25, HSP32, HSP60, HSP70 etc…(34).

Heme oxygenase-1 (HO-1), also referred to as heat shock protein (HSP32) is one of the three isoforms (in addition to HO-2 and HO-3) of the heme oxygenase enzyme (HO) responsible for the heme degradation into biliverdin (a cytoprotective antioxidant), carbon monoxide (a signaling gas) and iron. Biliverdin and its products prevent cells from oxidative damages (13,24,34). However, extensive research have demonstrated that HO-1 induction may have both antioxidant and pro-oxidant activities related to iron release (28). Immunomodulatory as well as anti-inflammatory and anti-apoptotic properties have also been revealed (4,5). Unlike HO-2 and HO-3 isoforms which are constitutively expressed in cells under normal conditions, the HO-1 isoform has been demonstrated to be inducible as an adaptative cellular defense against various stressors (1) such as heat shock, UVA radiation, hydrogen peroxide, sodium arsenite, heavy metals, endotoxin, etc… These stressors were reported to induce HO-1 expression (17 and references therein). Moreover, HO-1 plays a relevant role in cytoprotective process (34).

The European sea bass (*Dicentrarchus labrax,* Linne 1785) is one of the major species bred in aquaculture and is naturally present in the Mediterranean sea where temperature varies from 10°C to 28°C along the year (19). Growth and metabolism are dependent on water temperature (8 and references therein). Optimal growth temperature has been set between 22 and 25°C, whereas growth seems to stop at 11-15°C. Temperatures lower than 2°C and higher than 32°C are lethal to sea bass. Temperature interacts not only with biological factors but also with external ones like oxygen and salinity (18 and references therein). Other effects of non-optimal temperature, such as spleen contraction, increase in the erythrocyte number, hyperglycaemia, increase in leucocrit and swelling of erythrocytes reflecting an hypoxic state were observed (11). Since European sea bass represents a great commercial interest, it is relevant to study the impact of environmental variations that may

affect its welfare.

In the present study, we evaluate the effect of water temperature increase in *D. labrax* HO-1 gene expression in eight tissues (heart, liver, intestine, spleen, kidneys, gills, brain and muscle) at several time points in a warm temperature acclimation experiment.

MATERIALS AND METHODS

Animals and aquariums

All experiments were performed according to the guidelines of the Canadian Council on Animal Care under the appropriate approved animal care protocol described in «The care and use of fish in research, teaching and testing». Juvenile European sea bass were reared in a fish farm located at «La Seyne sur Mer» near the Mediterranean coast of France, within natural environmental conditions.

Eighteen one-year-old juveniles (80–100 g) were collected and transferred into 150 L filter aquariums in our zootechnical laboratory and held at 12°C for 5 weeks before the warm acclimation experiment. They were fed daily to satiation during the entire experiment.

Warm acclimation experiment

The warm acclimation experiment was conducted as previously described by Watabe et *al.* (33). The aquarium water temperature was increased by 1°C every hour from 12°C to 30°C, then maintained at 30°C for 4 weeks. Fishes were divided into groups of three for each of the six experimental conditions (12°C control group, and 30°C groups acclimated for 1h and from 1 to 4 weeks). The material used for this experiment included resistors (Shego, 300 W) connected in series with the thermostat (Thermostab, Aquael). The temperature was checked on the digital thermostat and another thermometer in the aquarium. The accuracy on the water temperature was +/−0.5°C. Other water parameters were also strictly controlled : salinity 30 g/L, pH 7.5, nitrite <0.3 mg/L and nitrate <12.5 mg/L.

Tissue collection and RNA extraction

Fish were sedated with 2-phenoxyethanol overdose and sacrificed by cervical dislocation.

Samples of liver, kidneys, spleen, brain, muscle, gills, heart and intestine were collected from freshly sacrificed fishes and immediately submerged in RNA*later* (Ambion, the RNA Company). Then they were stored at -80°C until further analysis.

Total RNA was extracted from tissues (50-100 mg) using 1 ml of EXTRACT-ALL (Eurobio, France) according to the manufacturer's instructions. After homogenization using FastPrep-24 and Lysing Matrix D (MP Biomedicals), 200 µl of chloroform were added and samples were vortexed for 15 sec and left at room temperature for 3 min. Then they were centrifuged at 12000 g for 15 min at 4°C, the aqueous phase was transferred to new tubes and 500 µl of isopropanol were added and the tubes were left for 10 min at room temperature.

After a second centrifugation (12000 g, 10 min, 4°C), supernatant was removed and total RNA pellet was washed in 75% ethanol.

Following the third centrifugation (7500 g, 5 min, 4°C), supernatant was removed and total RNA pellet was air dried for 10 min. Later the total RNA pellet was redissolved in 50 µl of distilled water.

A digestion with 2U/µl DNase I, RNase-free (Ambion) at 37°C for 30 min was performed in order to avoid any DNA contamination. An electrophoresis on an agarose gel showed a clear individual 28S and 18 rRNA bands and no smear confirming the integrity of extracted RNA (data not shown). Concentration and quality were assessed by spectrophotometry.

Reverse transcription and polymerase chain reaction

The cDNAs were generated following the standard protocol of Superscript II RNAse H Reverse Transcriptase kit (Invitrogen) using random Hexa-primers and 1µg of total RNA. Oligonucleotides used for the reverse transcription polymerase chain reaction (RT-PCR) are listed in Table 1. PCR conditions were as follow : 2 min at 94°C, 30 cycles of 45 sec at 94°C (DNA denaturation), 45 sec at 63°C (annealing), 2 min at 72°C (extension), followed by a 5 min final extension phase. The PCR was performed using specific primers (initial concentration : 10 pmol/µL) for HO-1 (forward : HO-1Dir201; reverse : HO-1Rev201), resulting in a 201 bp PCR product corresponding to the amplification between positions +494 and +694 of the HO-1 sequence HO-1 (GenBank accession no.EF139130). Normalization was carried with RPL17 sequence (GenBank accession no.AF139590) run under the same conditions and resulting in a 389 bp PCR product corresponding to the amplication between positions +18 and +407. Its expression is supposed to be invariable among individuals and experimental conditions (Table 1). The visualization of amplified cDNA was performed by a 1.5% TAE agarose gel electrophoresis (UV, coloration with ethidium bromide). The 100 pb Plus DNA Ladder (Invitrogen) was used to verify the size of amplified bands.

Gene expression and statistical analysis

Gene expression levels were quantified with ImageJ software (25) and normalized with RPL17 gene expression. The image analysis falls within the linear range of the densitometry. Analysis of the results was performed by ANOVA using the software XLSTAT. The null hypothesis supposing that no significant difference exists between groups was rejected for a probability under 0.05. The Tukey's multiple range test was used to determine significant differences between different groups.

RESULTS

Following one hour of heat stress, we noted a 2.5 fold increase in HO-1 expression in liver compared to the control group. From one hour to 3 weeks, HO-1 expression steadily increased to reach after 4 weeks of heat exposure, a 4.5-fold increase statistically different from the initial value (Fig. 1 and Fig. 2) ($P<0.05$).

In the spleen, we found a significant decrease of HO-1 expression at the end of the 4 week period. This decrease in HO-1 expression resulted in values lower than baseline values observed in fish group under control conditions (12°C) (data not shown).

A decrease in HO-1 expression was also observed in kidneys. This important decrease appeared earlier than in the spleen despite similar baseline levels. HO-1 gene expression reached null value as soon as one hour after exposure to 30°C and remained at this level for the rest of the experi-

Table 1. Primers used in polymerase chain reaction.

Primer	Sequence (5'→3')	cDNA position
HO-1Dir201	5'CCCTGAATTTCTAGTTGCCCATGC3'	494→517
HO-1Rev201	5'TCCGTCAGCTCCACGCTGTTCATC3'	671→694
RPL17Dir	5'GGTGGTTCATCTGGAGCCAAGTTCC3'	18→43
RPL17Rev	5'GCGTTAGAGGCTATCCGGGGCC3'	385→407

HO-1 (GenBank accession no.EF139130)
RPL17 (GenBank accession no.AF139590)

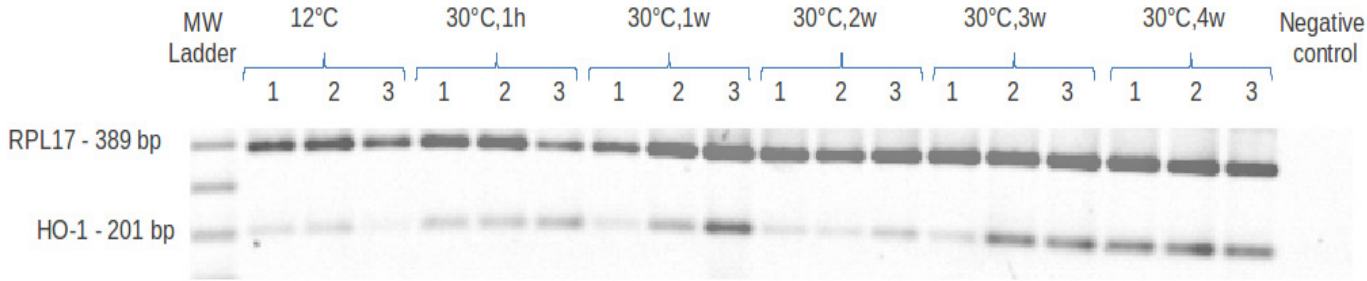

Figure 1. Electrophoresis result of HO-1 gene expression in sea bass liver tissue after increased water temperature exposure (MW- molecular weight, h- hour, w- week, bp- base pair, 1- first individual, 2- second individual, 3- third individual).

ment.

In intestine, HO-1 gene expression levels maintained the initial value throughout the experiment while no expression of HO-1 was observed in other tissues (heart, brain, muscle or gills) regardless of experimental conditions (data not shown).

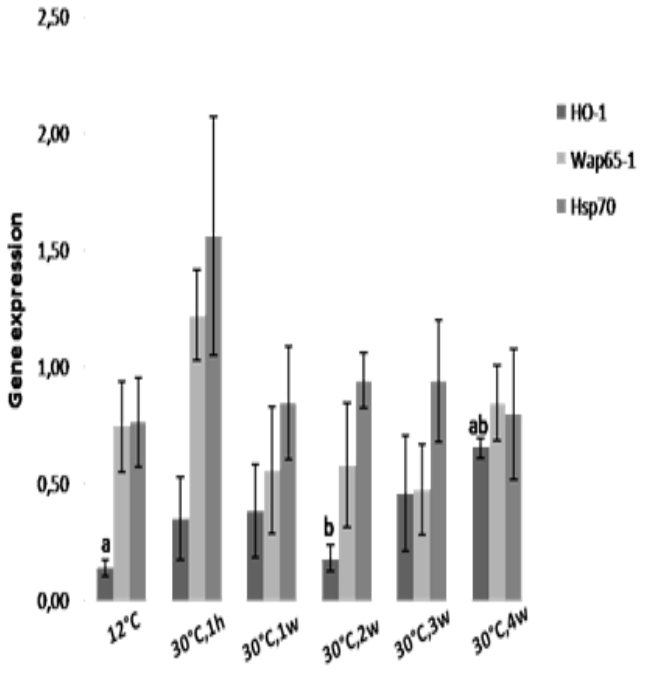

Figure 2. Average levels of HO-1, wap65 and hsp70 gene expressions in sea bass liver tissue after increased water temperature exposure. Wap65 and hsp70 levels were previously determined and published (20). Relative mRNA levels of HO-1 were detected by semi-quantitative PCR. Data shown are expressed as mean ± SEM. Different letters indicate significant statistical differences ($P<0.05$) after a Tukey's test.

DISCUSSION

In this study, we explored HO-1 gene expression in response to a heat stress in different sea bass tissues. Our results showed that HO-1 expression differs between tissues. HO-1 was not expressed in gills, heart, muscle and brain while it was expressed at a basal level in intestine in spite of the temperature increase. In liver, spleen and kidneys, where HO-1 appeared sensitive to the temperature increase, the pattern of HO-1 expression variation was specific to each tissue. In liver, HO-1 expression increased from one hour of exposure to 30°. It followed an increasing trend throughout the duration of the experiment and reached a value 4.5-fold higher than the initial level after 4 weeks showing a clear influence of temperature on HO-1 expression.

In a previous work (20), we investigated the effects of temperature increase on the expression of two other genes involved in temperature acclimation process in liver ; wap65-1 (an isoform of the "warm temperature acclimation-related 65kD protein) and hsp70 (a heat shock protein which acts as "chaperone" helping newly synthesized protein transport (17)). Our results on OH-1 expression varia tions after increased water temperature exposure are close to our previous findings. Indeed, all three genes of interest were expressed at 12°C even if HO-1 was expressed at a much lower level compared to wap65-1 and hsp70. Unlike HO-2 and HO-3 that are constitutively expressed, expression of HO-1 seems to be detectable only in stress conditions (17).

After one hour exposure to 30°C, a 2-2.5-fold increase for all three genes was recorded. Whereas HO-1 expression levels continued to increase even after one hour, both wap65-1 and hsp70 expressions decreased right after and

regained values close to those observed in control groups (Fig. 2).

In sea bass, wap65-1, hsp70 and HO-1 seem to be involved in a heat shock response. However wap65-1 and hsp70 responses are only involved at an early stage of experiment, while HO-1 expression increase is maintained and achieves values higher than baseline levels.

HO-1 expression level also increased in rat liver after hyperthermic treatment (42°C, 20 min) (24), but HO-1 expression pattern differs from the one found in the present study. Maximal HO-1 expression was observed as soon as one hour after heat stress to reach values 20 to 40-fold that of control groups. It then regains control group value by 6h after temperature increase treatment. In the same study, coordinated induction of HO-1 response was also demonstrated in rat kidneys and heart (24). In sea bass, we did not find similar results for these organs. Indeed, one hour after increased temperature exposure of fish, HO-1 expression in kidneys collapsed while it was not expressed at all in the heart throughout the experiment. The intensity and duration of exposure and mainly the species could explain such differences.

The contribution of HO-1 in renal cytoprotection has long been studied (21) and even if it is weakly expressed in kidneys in normal conditions, it is rapidly over expressed in renal injuries. It is clearly involved in protection again renal damages and even prevention and reversion of fibrosis (4,5,6). Only few works have focused on the pattern of HO-1 expression in response to stress in the kidneys of fish. In goldfish (*Carassius auratus*) HO-1 induction was sharply increased precisely in posterior kidney exposed to hypoxia conditions (31). Given that hyperthermia has been often suggested to induce hypoxia in central fish tissues and even to induce a collapse of the ventilatory and circulatory activities (12,22), it is interesting to compare our results to hypoxia-induced effects. It remains to be noted that in the study concerning goldfish, HO-1 was highly transcribed in posterior kidney, head kidneys, gills and in intestine and at lower level in heart, spleen and brain in non-stressed fishes. These findings are consistent with ours for the kidneys, intestine (where initial level of HO-1 was highly expressed), heart and brain (where initial level of HO-1 was lowly expressed) but interestingly differ from our data notably for the liver, spleen and gills (31). Results are also divergent concerning transcription of HO-1 after induction of stress where HO-1 was found up-regulated only in posterior kidney and gills in the goldfish, whereas in the sea bass it was down-regulated in kidneys and no expression was detected at any moment in the gills, muscle, heart and brain. Such a divergence in outcomes may be related to different molecular mechanisms involved in the reaction to each type of stress, as well as the fish species used. Therefore, further studies are needed to explore this mechanism and to understand why each organism reacts differently.

For the heart, it was proven that HO-1 was present and regulated in this organ and that it plays a key role in blood pressure regulation mediated by the carbon monoxide production.

In the heart, HO-1 was up-regulated after angiotensin administration as an adaptative response to angiotensin-induced cardiac damage. A cardioprotective role of HO-1 was also demonstrated in HO-1 knockout mice exposed to hypoxia (14 and references therein). In contrast, our data suggest that HO-1 is not expressed in the sea bass heart in normal conditions and that a heat shock does not induce HO-1 expression.

In the brain, the absence of HO-1 before and after temperature increase results from the fact that HO-1 does not represent the major isoform of heme oxygenase in this tissue. However, HO-1 expression has been detected in rat brain following an hyperthermia treatment (9) and heat shock was identified as the only stressor that can increase HO-1 transcripts in this tissue (10). Even if HO-1 was also found abundantly expressed in the sea bass brain (23), HO-2 is still by far the prominent isoform of heme oxygenase in the brain. HO-1 role in the nervous system remains unclear (2,16) despite the HO-1 neuroprotection and immunoreactivity that have been demonstrated in nervous tissues (14 and references therein).

In the spleen, in homeostatic state, HO-1 expression levels are similar to those found in other studies on the same species (23). Among all organs, the initial HO-1 expression is the highest in the spleen because hemoglobin degradation takes place mainly in this organ requiring continuous induction of HO-1 (3,21,29). However, following induction of a heat stress, we observed a notable unexplained decrease in HO-1 expression. Contrary to our findings, HO-1 expression was significantly up-regulated in rat spleen after exposure to other different types of stress such as hemolytic agents e.g aniline, phenylhydrazine and phenacetin (26,32).

Our data confirm HO-1 involvement in a heat shock response and suggest that HO-1 may be part of an array of genes that could be used as biomarkers to heat stress in the sea bass liver. Both its rapid response to stress and the long duration of its involvement make it interesting in such studies. Studies concerning HO-1 expression in response to heat stress in entire organism remain scarce. Fish species and temperature levels used in the experiment are highly variable as well as the duration of exposure to heat stress. Further studies are required to explore mechanisms of induction of HO-1 expression involved in fish acclimation.

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