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Abstract

It has been previously demonstrated that "Warm temperature Acclimation-related 65kD Protein" (WAP65) is involved in temperature acclimation, response to intoxication and infection, as well as in development. The expression of wap65-1 was investigated in the liver of European sea bass (*Dicentrarchus labrax*) during exposure to the increased temperature (from 12 °C to 30 °C) and during intoxication with four heavy metals: lead, cadmium, copper and zinc. Post temperature increase wap65 expression was highest after one hour at 30 °C. After 1 to 4 weeks at 30 °C wap65 transcript levels did not differ from the 12°C control group, similar to observations regarding the heat shock protein, hsp70. Upregulation of wap65 was detected after treatment (intoxication) with cadmium (0.5 µg/l). In contrast, a slight, but significant down regulation of wap65 was seen after copper (5 µg/l) intoxication. These data indicate that functional analyses of WAP65 are needed to understand the differential regulation of this gene by metals. The role of WAP65 may be similar to that of HSP70, which has generalized functions in responding to certain stressors and maintaining normal cell physiology.

Key words: *Dicentrarchus labrax*, gene expression, hsp70, RNA, wap65, Warm Temperature Acclimation-related.

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Abbreviations: **ATP**: adenosine triphosphate; **Cd**: cadmium; **Co**: cobalt; **Cu**: copper; **DPASV**: Differential Pulse Anodic Stripping Voltammetry; **GST**: glutathione S-transferase; **HDPE**: high-density polyethylene; **Hg**: mercury**; HSP**: heat shock protein; **MT**: metallothionein; **Ni**: nickel; **PAH**: polycyclic aromatic hydrocarbon; **Pb**: lead; **PCB**: polychlorobiphenyl; **PCR**: Polymerase Chain Reaction; **TBT**: tributyltin; **UV**: ultraviolet; **WAP65**: Warm temperature Acclimation-related 65kD Protein; **Zn**: zinc.

INTRODUCTION

Changes in marine environmental conditions include both natural events, e.g. temperature fluctuations (long-term such as global warming, and short-term seasonal variations), and anthropogenic actions such as chemical contamination. Among pollutants, metals are of a great importance. Their sources in aquatic environment are various, but significant contributions are man-made. Metals concentrations in marine environments are often relatively high, because their elimination in wastewater treatment plants is often limited (4).

Due to environmental changes, multiple dynamic equilibrium regulation mechanisms in marine organisms are aimed at maintaining efficient and optimal behavior in new environmental conditions (reestablishing homeostasis). Therefore, a variety of responses on cellular and molecular levels occurs. Natural

changes in environmental conditions such as seasonal changes in water temperature provoke acclimatory responses. In poikilothermic fish, such as the European sea bass, this response involves, among others, regulation of a set of genes that includes myosin, lactate dehydrogenase, Δ^9-desaturase, warm temperature acclimation-related 65kDa protein gene (wap65) and ATP synthase (in cold temperature) (23 and references therein, 39). Responses and adaptations differ in case of sudden temperature perturbations, which provoke short-term adjustments, exemplified by the production of heat shock proteins (HSPs) (18). Water composition also influences the quality of commercially valuable fish, because of the accumulation of the pollutants inside the body (3,7,9). Contamination with metals has a profound effect on gene expression patterns in fish. One of the responses to metal intoxication is the upregulation of metallothioneins (MT), which can mitigate the harmful effects of various metals (19). Moreover, cytochrome P4501A, vitellogenin and heat shock proteins have been examined in several metal toxicity studies on fish (43).

On the basis of responses to environmental perturbations, different biomarkers have been proposed in order to assess exposure of aquatic organisms to pollutants and to identify certain substances with low concentrations levels (34, for fish reviewed in 44). There is a strong interest in finding novel genetic markers to monitor environmental perturbations that may affect fish. It is especially important for species with important commercial value like the European sea bass (*Dicentrarchus labrax*), in order to assure high fish quality. In 2009, the global sea bass capture fisheries production was ~11 933 tons with France accounting for 57% of the production (10). In the past 25 years aquaculture of this species has developed rapidly, reaching a global production amount of ~113 653 tons in 2009.

It has been shown that in fish the expression of the gene coding for WAP65, the "Warm temperature Acclimation-related 65kDa Protein" changes as a result of environmental perturbations. However, its function has yet to be fully characterized. It resembles mammalian hemopexin (in amino acid sequence and functional sites), a gene encoding a plasma glycoprotein that is synthesized in the liver and functions as a scavenger of free heme. In contrast to the single isoform of hemopexin found in mammals, there are two paralogous wap65 genes in fish (wap65-1, wap65-2) (6 and references therein), presumably as a result of the teleost whole genome duplication event (2). These forms probably differ in function. The evolution rate of wap65-2 is much lower than the one of wap65-1 and its tissue distribution restricted (mainly liver). Therefore, it is more similar to mammalian hemopexin. WAP65 in fish is known to play a role in the warm temperature acclimatory response. The protein (or mRNA coding for it) has been found in goldfish (*Carassius auratus*) and carp (*Cyprinus carpio*) acclimated to 30°C for at least 1 month (21,23,45). It also appeared in the muscle tissues of goldfish within 5 days of high temperature exposure and was maintained at high concentrations for at least 9 days (20). Increased wap65-1 mRNA levels were also found in liver within four hours after exposure to 30°C, with maximum expression observed after 3 days. After 3 weeks wap65 transcript levels diminished, but remained 10 times higher than before the warm water exposure (22). The role of WAP65 in acclimatory processes appears to vary considerably among fish, as significant upregulation of this gene was not recorded in pufferfish (*Takifugu rubripes*) and medaka (*Oryzias latipes*) exposed to high water temperature for one month (14,15).

Moreover, it was demonstrated that wap65 expression levels change in reaction to pathogen infections (e.g. 38,39,40), during fish development (15,29,38) and in the reaction to intoxication with certain heavy metals and other pollutants (1,25,26). However, in comparison to the number of studies concerning wap65 expression after water temperature increase, knowledge concerning regulation of this gene after intoxication with pollutants remains scarce.

In the present study we investigated: (i) the delay between the temperature increase and the change in wap65 expression, and (ii) wap65 expression in reaction to heavy metal intoxication.

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». Fish used for our experiments were collected from a fish farm located on the Mediterranean coast at La Seyne sur Mer (south of France), where they were kept in floating cages in natural environmental conditions and fed once a day *ad libitum* with commercial fish diet (Le Gouessant, France). One year old sea bass fish (average weight of 80–

100 g) were used for the experiments. Experiments were conducted in 150 L aquariums filled with sea water. The sea water was filtered with a mechanical silica filter, sterilized with ultraviolet (UV), filtered with biological filter with nitrifying bacteria and with the microfiltration system with different mesh sizes. Before the experiments, aquariums were decontaminated with hydrochloric acid (0.1%) and rinsed three times with sea water.

Experiment: increased water temperature

Fish were held in aquariums at 12°C for 4 weeks and fed *ad libitum*. In the first experiment, the aquarium water temperature was increased by 1°C every hour from 12°C to 30°C, and maintained at 30°C for 4 weeks. There were three fish for each experimental condition (12°C control group and 30°C groups acclimated for 1 h, and from 1 to 4 weeks). Temperature was regulated using a series of resistors (Shego, 300 W) connected with the thermostat (Thermostab, Aquael) which resulted in an accuracy of the water temperature between +/−0.5 °C.

Experiment: intoxication with heavy metals

Fish intoxication experiments were performed in water containing four heavy metals: lead (Pb), cadmium (Cd), copper (Cu) and zinc (Zn). Experimental conditions were defined accordingly to the metals levels measured in water pumped from the Bay of Toulon (south of France) used to fill the aquariums (32) and directly in the aquariums (to check the cleaning) and compared to concentrations recorded along transects in the Bay of Toulon (Jean, Université du Sud Toulon-Var, France- personal communication). Sample treatment and analysis was adapted from Louis *et al*. (27). Water samples were filtered through 0.45 µm pre-cleaned cellulose nitrate filters (Sartorius), and stored at 4 °C in the dark until analysis in pre-cleaned 60 mL HDPE (Nalgene) for trace - metal measurements (preservation by acidification at pH < 2 using suprapur HNO_3). Total dissolved metal concentrations were determined in acidified samples by Differential Pulse Anodic Stripping Voltammetry (DPASV). Measurements were carried out with a voltammetric analyser µAutolabI (EcoChemie) controlled by GPES 4.9 software (EcoChemie) coupled with a three - electrode cell of 663 VA Stand (Metrohm). The analytical procedure, fully automated, was based on 3 successive steps : (a) simultaneous measurement of Cd, Cu and Pb by DPASV with standard additions (using Cavro XL 3000 Syringe Pump) of standard solution containing the 3 metals, (b) addition of a 4 M sodium acetate solution (from sodium acetate suprapur, Merck) to increase the pH to 4, (c) measurement of Zn by DPASV with standard additions of Zn standard solution. Deposition time of the DPASV procedures was adapted accordingly to metal levels in samples. Metal levels measured in water sampled in pre-cleaned aquarium were on the level of those obtained on water pumped from the Bay of Toulon used to fill the aquariums, proving the efficiency of aquarium cleaning procedure. In comparison to metal concentrations recorded in various zones of the Bay of Toulon (Jean, Université du Sud Toulon-Var, France- personal communication), these values correspond to the levels obtained in the north of the small Bay of Toulon, largely higher than open-sea values reported for the western Mediterranean Sea (28), reflecting the significant contamination of the Bay due to numerous anthropogenic activities and low sea water renewal.

Accordingly to the measured levels, two concentrations of each metal were used: the first (M1), slightly higher than the one found in the natural environment and the second (M2), 100 higher than M1 (Table 1). Metals concentrations measurement in the control systems (*i.e.* in the absence of fish) showed that concentrations remained stable (*i.e.* they do not adsorb significantly neither on aquariums' walls nor on filters) either on non-spiked aquariums or with M1 and M2 concentrations. Before the experiment, fish were kept in aquariums at 20 °C for 4 weeks and fed *ad libitum*. There were 12 fish for each experimental condition (6 fish per aquarium). Four metals were tested, each of them at two concentrations (Table 1). Control without contaminants contained 14 fish. The intoxication experiment lasted for 96 h (acute intoxication).

Table 1. Concentrations of metals in the aquariums during metals exposure experiment.

Metal	Pb	Cd	Cu	Zn
M1, µg/l	1	0.5	5	10
M2, µg/l	100	50	500	500

Tissue collection, RNA extraction, reverse transcription and polymerase chain reaction

Samples of 100 mg tissue were immediately harvested from the liver of freshly scarified fish. They were stored in 1 mL of RNAlater (Ambion, the RNA Company) at 4 °C overnight for complete infiltration in the tissue and then kept at -80 °C until the analysis. Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. A DNAse treatment (DNAse I Amp Grade, Invitrogen) was performed after the extraction. RNA quality was assessed by electrophoresis on an agarose gel, showing clear individual 28S and 18S rRNA bands and no smears, confirming integrities of all prepared RNA. The cDNAs were generated following the standard protocol of the SuperScript II RNAse H Reverse Transcriptase kit (Invitrogen) using random Hexa-primers and 1 µg of RNA. The polymerase chain reactions (PCR) were performed using a Mastercycler ep gradient thermocycler (Eppendorf). For the temperature increase experiment PCR started with initial denaturation step of 94 °C for 2 min, followed by 24 cycles of denaturation step at 94 °C for 1 min, primer hybridization step at 54 °C for 1 min and elongation step at 72 °C for 2 min. The final elongation step was performed at 72 °C for 5 min. For the metal intoxication experiment it started with 2 min at 94 °C followed by 26 cycles at 94 °C for 1 min, 50 °C for 1 min and 65 °C for 1 min. A 15 min step at 65 °C ended the reaction.

In temperature increase experiments PCR reactions were performed to simultaneously amplify fragments of three genes : β-actin, wap65-1 and hsp70. β-actin was used to standardize the results of DNA amplification, as its expression is not supposed to differ among individuals and experimental conditions. Expression of heat shock protein (hsp70) was investigated for comparative purposes.

The primers used to amplify β-actin and wap65 genes were identical to those previously used by Pierre *et al*. (33). The primers for hsp70 amplification were designed with Primer3 software (37) using the published sequence of this gene of *D. labrax* (Genebank accession no. : AY423555). The following primers were chosen: HSPdir (5'-GAGAACAAGATCACAATCAC-3') and HSPrev (5'-

CTTCTGTTGATGTTCATACTC-3'). Each PCR mixture contained 1 µL of first strand cDNA, 2 µL of each primer (3 primer pairs) (10 pmol/ µL), 0.3 µL of 5U/µL HotMaster Taq DNA Polymerase (5 PRIME), 5 µL of 10× HotMaster Taq buffer (with 25 mM of Mg^{2+}) and 2 µL dNTPs at 5 mM. The volume was brought to 50 µL with RNAse DNAse free H_2O. For samples derived from the metal intoxication experiments the PCR mixture contained the same proportions of reagents, however only the β-actin and wap65 genes were examined.

Gene expression quantification and statistical analysis

The visualization of amplified cDNA was performed by a 2% TAE agarose gel electrophoresis (UV, coloration with ethidium bromide). The 1 Kb Plus DNA Ladder (Invitrogen) was used to check the size of amplified bands. Gene expression levels were quantified with ImageJ software (36) and standardized with β-actin. The ratios of wap65/β-actin and hsp70/β-actin (in case of temperature increase experiment) were calculated and compared among individuals.

In order to test the significance of differences in gene expression levels among groups of fish subject to different metal concentrations the Mann–Whitney test was performed in R environment (35).

RESULTS

Wap65 expression after water temperature increase

The wap65 and hsp70 genes expression levels differed among individuals and among experimental conditions. Both genes were expressed in the controls at 12°C. The highest expression levels for these two genes were recorded after 1 h of exposure to 30°C (Fig. 1). These data indicated a 2-2.5-fold increase in either wap65 or hsp70 transcript levels following the temperature increase. The patterns of expression of both genes from 1 to 4 weeks of exposure to 30°C were similar and these levels were close to the ones recorded at 12°C (control group) (Fig. 2).

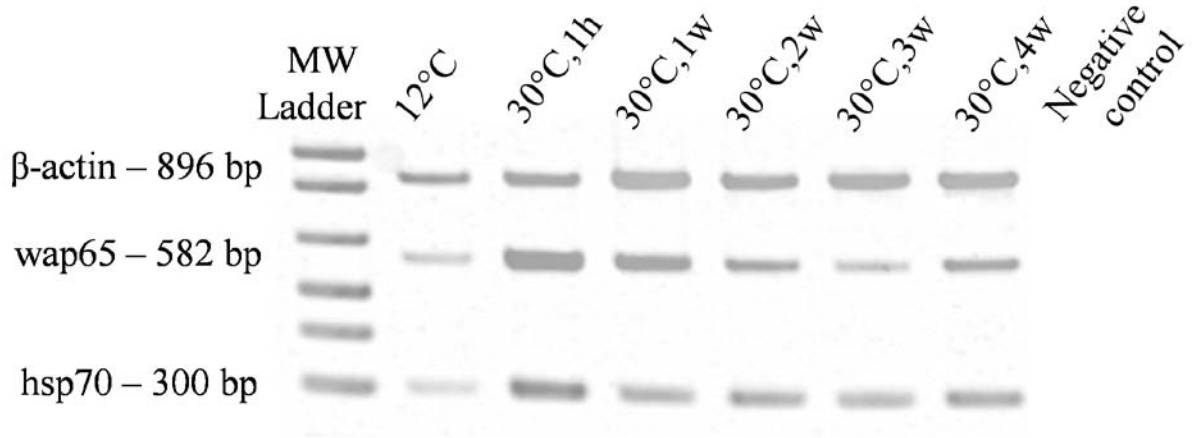

Figure 1. Examples of wap65 and hsp70 genes expression patterns in sea bass liver tissue after the exposition to increased water temperature (MW- molecular weight, h- hour, w- week).

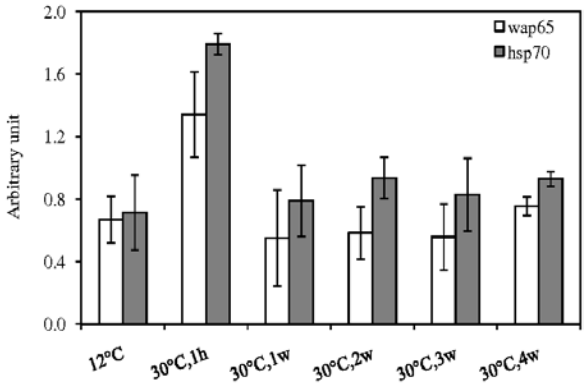

Figure 2. Average levels of wap65 and hsp70 genes expression in sea bass liver tissue after the exposition to increased water temperature. Wap65 and hsp70 expression levels were standardized with ß-actin expression level (h- hour, w- week).

Wap65 expression after intoxication with heavy metals

After treatment with Pb at the M1 concentration (Table 1), which was slightly higher than the concentrations found in the natural environment, the level of wap65 expression did not significantly change. However, after intoxication with Pb at the concentration M2, which was 100 times higher than M1, wap65 expression fluctuated greatly (either down or up). Wap65 was upregulated after intoxication with Cd ($p < 0.05$) at M1, but not at M2. After the intoxication with Cd at M1, 70% of individuals demonstrated wap65 expression levels twice as high as the control. Treatment with Cu at M1 provoked a slight downregulation of wap65 ($p < 0.05$), which was not observed at M2.

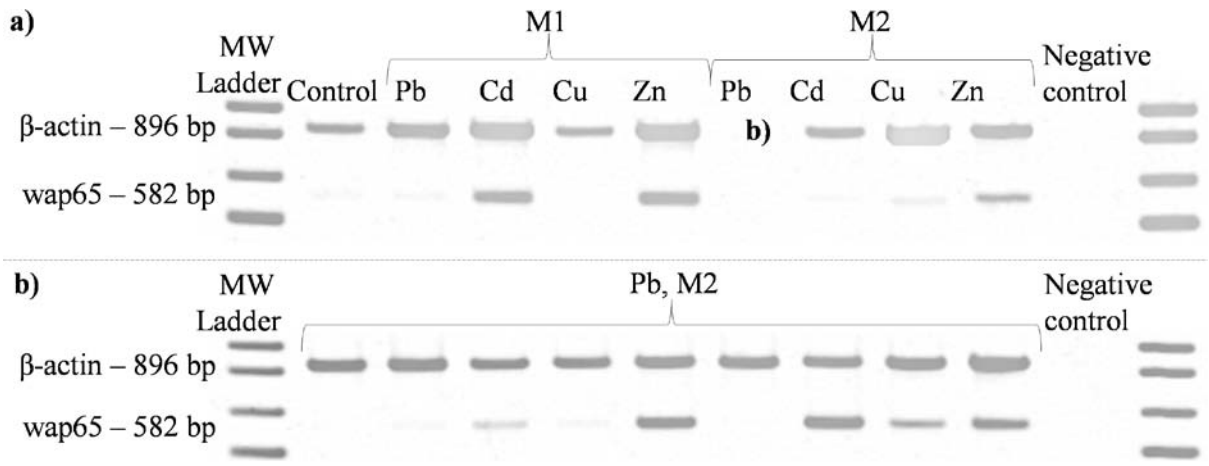

Figure 3. a) Examples of wap65 gene expression patterns after intoxication with heavy metals at the concentrations M1 and M2 (see Table 1), **b)** Wap65 expression after intoxication with lead (Pb) at M2 concentration (perturbing effect; either upregulation or downregulation) (MW- molecular weight).

The exposure to zinc resulted in an upregulation of wap65 at both concentrations, but there was a high level of variability among individuals. For only two metals, Cu (downregulation) and Zn (upregulation), the tendency in expression pattern was similar between M1 and M2 concentrations (Fig. 3, Fig. 4).

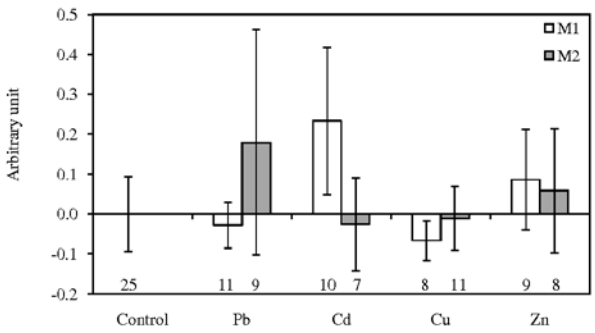

Figure 4. Average level of wap65 expression in sea bass liver tissue after intoxication with heavy metals at the concentrations M1 and M2 (see Table 1) in respect to the control sample. Wap65 expression level was standardized with ß-actin expression level. The numbers correspond to the numbers of individuals from each experimental condition included in the data analysis.

DISCUSSION

Expression of wap65 after water temperature increase

In the European sea bass wap65-1 upregulation was recorded as soon as one hour after exposure to 30 °C and its expression returning to baseline after ~1 week. The wap65 expression pattern was similar to that of the heat shock protein, hsp70. Based upon these results, we speculate that in *D. labrax*, WAP65 is involved in a "shock" response to rapid perturbation, rather than in a more long term acclimatory process. However, there are only a few studies on the expression of wap65 after a heat shock (6,22). As for the European sea bass, detailed study of the expression patterns between one hour and one week of warm temperature exposition would help to estimate more exactly the time after warm water exposition, when wap65 is not upregulated anymore.

Our study suggests that WAP65-1 in European sea bass probably has little function in the acclimatory response. However, such function was found for WAP65-1 in goldfish and carp (21,23,45). It has been suggested that WAP65-1 underwent neofunctionalization, and the form which maintained function resembles the ancestral one, functioning in acclimation to warm temperature or in mediating an immune response. For sea bass, this would correspond to WAP65-2 (14,15,38). In support for this idea it has been shown that in the channel catfish (*Ictalurus punctatus*) the transcriptional response of wap65-2 was quite slow, occurring days after warm temperature treatment (acclimatory response) or bacterial infection (39). However this may not be generalizable since in the Antarctic plunderfish (*Harpagifer antarcticus*) after the increase of water temperature (from 0 °C to 6 °C), a strong downregulation of wap65-2 was recorded within 2-8 hours. Recovery to near control levels took place within 48 hours (6).

A clear phylogenetic distinction between ancestral (e.g. Cypriniformes, like goldfish and carp) and modern (e.g. Perciformes, like sea bass) teleosts was found for both wap65-1 and wap65-2 sequences (38). It is therefore probable that different taxa acclimate to temperature increase in different ways. However, the number of studied species remains too low for detailed conclusions and, for example, in goldfish, both acclimatory reaction and rapid response to increased temperature (after 4 hours) was demonstrated (21,22).

Expression of WAP65 after intoxication with heavy metals

There are only a few studies on the expression of wap65 after intoxication with heavy metals. Exposure to Hg contaminated sediment provoked its upregulation in cod (*Gadus morhua*) (30). Wap65 upregulation was also seen in swordtail fish (*Xiphophorus helleri*) after contamination with Cu for 24 h (upregulation was detected at 50 µg/l and was highest at 1000 µg/l after 24 h) (1). We did not observe upregulation of the sea bass wap65 after the intoxication with copper for 96 h (slight downregulation was found at M1), although the concentrations tested (5 µg/l and 500 µg/l) were within the range previously tested (1). If metals intoxication provokes a rapid response, in our experiments, the expression levels could have returned to baseline before our measurements were taken. Therefore, our and the previous results are not necessarily contradictory. The M1 concentrations used were slightly higher than the ones found in the natural environment. The M2 concentrations were 100 times higher than M1 and may mimic spills or other concentrated events. The latter concentrations were used to test the reaction to acute pollution. This acute intoxication did not provoke any mortality, proving some resistance of fish at least to the short-term exposure to these metals at the concentrations examined. As for the concentrations that could be encountered in the natural environment (M1), wap65 is regulated after the contamination with cadmium (upregulation) and probably with copper (slight downregulation) and zinc (high level of variability among individuals with a global tendency of upregulation). In case of copper and zinc the study should be repeated using a higher number of individuals and a range of different concentrations to confirm the recorder patterns.

Although, the response of wap65 to metals intoxication has not been much studied before, there are some available results concerning other pollutants. Wap65 upregulation was demonstrated in cod experimentally exposed to alkylphenols (26). Its downregulation was also found in this species from Store Lungegårdsvann, in Bergen, Norway, which is polluted with polycyclic aromatic hydrocarbons (PAHs), tributyltin (TBT), polychlorobiphenyls **(**PCBs), Cd, and Hg (25).

Intoxication with metals influences numerous metabolic pathways and physiological processes in fish. In response to a toxic insult, fish may increase their metabolic activity in order to mobilize protective measures against the pollutant involved. Therefore, enzymes involved in energy production, such as 6-phosphofructo-2-kinase (key regulatory enzyme in glycolysis) might be upregulated (16). The reaction to heavy metals intoxication may also provoke a general inflammatory reaction, potentially resulting in the expression of glutathione S-transferases (GSTs) (17). WAP65 has been demonstrated to play a role in the inflammatory processes (e.g. 38), so the inflammation provoked by intoxication with some metals could lead to its upregulation. Moreover, some metals, like cadmium (upregulated wap65 in sea bass) bind to fish gills provoking their damage (17) and leading to tissue hypoxia. Upregulation of wap65 was detected during hypoxia conditions in goby *Gillichthys mirabilis* (13). Therefore, the increased wap65 expression in sea bass that we observed may be due to hypoxic stress induced by binding of cadmium to the gills. On the other hand, our data did not reveal an increase in wap65 expression levels after intoxication with copper. This metal can also induce gill damage and it has been proposed as one of the possible explanations for wap65 upregulation after the copper intoxication in the previous studies (1).

Various metals like Cd, Cu, nickel (Ni) and cobalt (Co) can affect heme homeostasis (e.g. 41 and references therein). Free iron can promote free radical formation resulting in oxidative stress (30). It has been demonstrated that the intoxication of fish with cadmium induces the expression of genes responsive to oxidative stress (46,47). Oxidative stress in fish might be also provoked by copper (12), lead (41 and references therein) or zinc (11). The oxidative stress induced by exposure to mercury (Hg) increased transcription of wap65, but also of transferrin and heme oxygenase, genes involved in iron metabolism, suggesting that WAP65 might play a role in these mechanisms (30).

The mammalian homologue of WAP65, hemopexin, participates in transporting heme from the plasma to the liver and preventing oxidative stress (8,31,42). Induction in hemopexin expression has been shown to increase the rate of the protein binding to heme. The binding of heme-hemopexin-like complexes with the hemopexin receptor located on the cell surface of the liver provokes among others, the induction of metallothionein gene expression (1 and references therein). Increased synthesis of metallothioneins and their binding to heavy metals reduces the amount of freely available metals (in the host) thus decreasing their potential toxicity (7,24). Therefore, a crucial question in uncovering the function of WAP65 after exposure to heavy metals is whether WAP65 has an activity similar to hemopexin. There are many hypotheses based on the structure of the putative protein concerning the ability of WAP65 (either of the two isoforms) to have a hemopexin-like activity (5,15,33,38). Although functional analyses of proteins are still scarce, it has been demonstrated that shark hemopexin binds heme (8).

It is possible that WAP65 is involved in maintaining homeostasis, likely by preventing high concentration of free heme and free metals within the body, thereby reducing toxicity (15). However, functional studies of WAP65 proteins are needed to confirm these hypotheses. Moreover, cadmium, which is the only metal inducing wap65 upregulation, is not the only one influencing heme homeostasis and causing oxidative stress in fish. The lack of significant wap65 upregulation after intoxication with other metals should be confirmed by additional experiments.

WAP65- not only "warm temperature acclimation-related protein"

Numerous studies suggest that WAP65 is not only involved in temperature acclimation, but also acts as a multifunctional agent in several biological protection processes. Taking into account the multitude of functions and the rapidity of reaction to stress, there is some similarity between WAP65 and heat-shock proteins (HSP), which has a general role in cell physiology. Initially heat shock proteins were thought to be linked only with environmental temperature increases, but it is now widely known that these proteins are upregulated in cells that are exposed to a broad range of stressors (18). Similarly, the term ''warm-temperature protein" was assigned to a 65-kDa protein due to its high abundance in various tissues after warm temperature acclimation, however, WAP65 is involved in many other host processes including in development, immune response, as well as mediating certain chemical stress responses (e.g. 15,38,39).

In the Mediterranean Sea, from where the European sea bass was sampled, the water temperature changes from 12°C in the winter to over 30°C in the summer. Therefore, the experimental temperature range reflects the one encountered by this poikilothermic species in its natural environment. The fact that there is no increased wap65 expression after more than a week of exposure to 30°C (for example during acclimatory reaction to the seasonal change of water temperature), makes wap65 a potential marker for a rapid detection of heat shock. This is particularly important in aquaculture, where fish in cages do not have a possibility to move to deeper, colder water when the surface water temperatures increase.

Due to the reaction to different physical and chemical stimuli wap65 could also be a potential marker to detect stress in fish. However, variability among individuals in expression patterns after exposure to heavy metals was considerable. Therefore, further studies with additional chemical substances and wider concentrations range are needed in order to verify the utility of wap65 in ecotoxicological studies as a possible biomarker of exposure. Some attempts to include wap65 in DNA microarrays, where its expression is analyzed in parallel with many other genes, has already been undertaken (25,26,30). Understanding the physiological pathways involved in wap65 regulation will lead to a better understand of the function of this protein and the reasons behind the differences in expression patterns seen in response to different pollutants.

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