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High hydrostatic pressure influences the *in vitro* response to xenobiotics in *Dicentrarchus labrax* liver

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ABSTRACT

Hydrostatic pressure (HP) increases by about 1 atmosphere (0.1 MPa) for each ten-meter depth increase in the water column. This thermodynamical parameter could well influence the response to and effects of xenobiotics in the deep-sea biota, but this possibility remains largely overlooked. To grasp the extent of HP adaptation in deep-sea fish, comparative studies with living cells of surface species exposed to chemicals at high HP are required. We initially conducted experiments with precision-cut liver slices of a deep-sea fish (*Coryphaenoides rupestris*), co-exposed for 15 h to the aryl hydrocarbon receptor (AhR) agonist 3-methylcholanthrene at HP levels representative of the surface (0.1 MPa) and deep-sea (5–15 MPa; *i.e.*, 500–1500 m depth) environments. The transcript levels of a suite of stress-responsive genes, such as the AhR battery *CYP1A*, were subsequently measured (Lemaire et al., 2012; *Environ. Sci. Technol.* 46, 10310–10316). Strikingly, the AhR agonist-mediated increase of *CYP1A* mRNA content was pressure-dependently reduced in *C. rupestris*. Here, the same co-exposure scenario was applied for 6 or 15 h to liver slices of a surface fish, *Dicentrarchus labrax*, a coastal species presumably not adapted to high HP. Precision-cut liver slices of *D. labrax* were also used in 1 h co-exposure studies with the pro-oxidant *tert*-butylhydroperoxide (tBHP) as to investigate the pressure-dependence of the oxidative stress response (*i.e.*, reactive oxygen production, glutathione and lipid peroxidation status). Liver cells remained viable in all experiments (adenosine triphosphate content). High HP precluded the AhR agonist-mediated increase of *CYP1A* mRNA expression in *D. labrax*, as well as that of *glutathione peroxidase*, and significantly reduced that of *heat shock protein 70*. High HP (1 h) also tended *per se* to increase the level of oxidative stress in liver cells of the surface fish. Trends to an increased resistance to tBHP were also noted. Whether the latter observation truly reflects a protective response to oxidative stress will be addressed in future co-exposure studies with both surface and deep-sea fish liver cells, using additional pro-oxidant chemicals. Altogether, data on *CYP1A* inducibility with *D. labrax* and *C. rupestris* support the view that high HP represses AhR signaling in marine fishes, and that only species adapted to thrive in the deep-sea have evolved the molecular adaptations necessary to counteract to some extent this inhibition.

1. Introduction

Laboratory and field studies support the notion that the vertical zonation of the marine biota into shelf, slope and abyssal species could be partly explained by the relative tolerance of their molecular arsenal to the linear increase of hydrostatic pressure (HP) with depth (Carney, 2005). Biological processes connected to positive (or negative) volume changes are inhibited (or favored) by HP (Pradillon and Gaill, 2007), so that achieving « life at low volume change » (Somero, 1990) is a prerequisite for thriving in high-pressure environments. Biochemical studies with cytosolic enzymes (dehydrogenases) and agonist-activated signaling systems (G protein coupled receptors) have notably shown that deep-sea proteins were less affected by HP than surface counterparts, even though species-specific inhibitory effects were noted in some instances (*e.g.*, Somero, 1990; Siebenaller et al., 1991; Siebenaller, 2003).

These observations suggest that HP might influence the response to and effects of xenobiotics in both surface and deep-sea fish, but this possibility remains largely overlooked. Three

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studies have initiated research in the field by focusing on the hyperbaric behavior of classical biomarkers of exposure to chemicals. In the first, liver of *Pagellus bogaraveo* juveniles exhibited reduced EROD activity and increased levels of carbonylated proteins following 14-day exposure of the animals to 0.5 MPa (Kopecka-Pilarczyk and Coimbra, 2010a). In the second, increased hepatic levels of carbonylated proteins were detected in silver eel (*Anguilla anguilla*) following 7-day exposure of the animals to 5 MPa (Kopecka-Pilarczyk and Coimbra, 2010b). In the third, precision-cut liver slices of the deep-sea fish *Coryphaenoides rupestris* (200–2600 m depth), incubated for 15 h with the AhR agonist 3-methylcholanthrene (3-MC; 25 μM), showed lower increases of *CYP1A* and *heat shock protein* (*HSP*) transcript levels with increasing HP (0.1–5–15 MPa). Non-monotonic changes of antioxidant enzyme (EAOX) expression were also noted (Lemaire et al., 2012). While only the latter study involved co-exposures to a chemical, results taken together suggest that detoxification systems targeting persistent chemicals (*e.g.*, CYP1A protein and the associated EROD activity) and reactive oxygen species (*e.g.*, EAOX) could well be impacted in both surface and deep-sea fish by HP levels typical of their environment.

We initiated a research project aimed at deciphering the extent of HP adaptation in detoxification systems of deep-sea fish. By making use of interspecies comparisons, we plan to see whether the protective response elicited by this fauna upon simultaneous exposure to chemicals and high HP is ameliorated compared to that elicited by surface species (*i.e.*, presumably not adapted to high HP). Confronting the two sets of data should provide important information regarding the possible adaptation of deep-sea fish detoxification capacities to HP.

As part of such interspecies comparison effort, we herein analyzed the response of precision-cut liver slices of a coastal fish (*Dicentrarchus labrax*; 10–100 m depth) to exactly the same scenario of co-exposure that was previously applied to the deep-sea species *C. rupestris* (Lemaire et al., 2012). Effects of high HP on the inducibility of components of the chemical defensome that are CYP1A, EAOX and HSP70 were studied. We also investigated the pressure-dependence of EROD activity in liver slices of *D. labrax*. Finally, the impact of high HP on the response of liver cells of the surface fish to pro-oxidant chemicals was also investigated in 1-h co-exposure studies with *tert*-butylhydroperoxide. Parameters analyzed subsequently were glutathione and lipid peroxidation status, and reactive oxygen production. Results are discussed in light of those obtained previously with liver cells of *C. rupestris*.

2. Material and methods

2.1. Chemicals

L-15 medium (Leibovitz's 15 without phenol red, adapted to 360 mOs l^{-1} with sodium chloride), PenStrep (Penicillin 10,000 U ml^{-1} and Streptomycin 10,000 μg ml^{-1}), FBS (fœtal bovine serum, lot 41G6281K) and HBSS (Hank's balanced salt solution, adapted to 360 mOs l^{-1}) were purchased from Gibco (Paisley, UK). CM-DCFHDA probe (5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; CAS 56-49-5), α-NF (alpha-naphtoflavone; CAS 60459-1), tBHP (*tert*-butylhydroperoxide; CAS 75-91-2), MS-222 (tricaine methanesulfonate; CAS 886-86-2) and other reagents were from Sigma–Aldrich (Milwaukee, USA). 3-MC and α-NF were first dissolved in dimethylsulfoxide (final concentration of 0.1%). tBHP was provided as an aqueous solution.

2.2. Animals

3-year-old (adult) male European seabass (*D. labrax*), weighing 250–350 g, were obtained from Aquanord (Gravelines, France) and reared in 600-l tanks with synthetic seawater (18 ± 1 °C; recirculation system) at a density of 25 fish per tank. They were fed twice a week Aquabio BDA48 pellets from Joosen-Luyckx (Turnhout, Belgium).

2.3. PCLS generation

Fish were euthanized by prolonged immersion in a bath of MS-222, and the musculature of a flank was dissected to allow access to the liver. Perfusion of the liver with ice-cold HBSS followed. This ensured the removal of blood constituents from the organ. Sectioning of the veins connecting the hepatic circulation to the heart was conducted shortly after liver whitening begun. This protocol complied with the *Code of Ethics of the World Medical Association* and was approved by the Institutional Animal Care and Use Committee. The process of liver excision, coring and slicing, with a Krumdieck MD-1100 from Alabama Research (Munford, USA), was conducted as previously (Lemaire et al., 2011). Single-lobe livers were devoid of alterations. Slices, obtained from cores generated at various liver locations, were 100-μm thick (8-mm diameter), and predominantly contained hepatocytes, with a smaller content of epithelial cells of the biliary system and endothelial cells (Hinton et al., 2004; Wolf and Wolfe, 2005). Noteworthy, all these cell types are involved in AhR signaling in fish liver (Sarasquete and Segner, 2000).

2.4. PCLS incubation systems

In the concentration-response analysis with 3-MC, liver slices from one fish were incubated for 21 h in 24-well plates at 15 °C and 0.1 MPa under moderate orbital agitation (175 rpm) with 500 μl of L15+ medium (*i.e.*, L-15 medium with 10% FBS and 1% PenStrep) and increasing concentrations of 3-MC (with or without α-NF), as previously (Lemaire et al., 2011).

In the concentration-response study with tBHP, liver slices from one fish were individually incubated for 1 h at 15 °C and 0.1 MPa in 4-ml Simport cryovials (Québec, Canada) filled with O_2-saturated HBSS and increasing concentrations of tBHP.

In co-exposure studies with 3-MC (6 or 15 h), liver slices from one fish ($n = 2$) were incubated in each custom-designed 500-ml hyperbaric chambers from Autoclave (Rantigny, France) filled with L15+ medium and 25 μM of 3-MC (a concentration leading to significant induction of *CYP1A* at atmospheric pressure—see Section 3). A Multitemp III circulating water bath from Pharmacia Biotech (Uppsala, Sweden), connected to the outer shell of the chambers (Lemaire et al., 2012), was set at 15 °C. The cylinders were mounted on a magnetic stirrer to allow a mild homogenization of media during incubation. A tube section with fiberglass grid on its top protected free-floating slices from the magnetic barrel (Lemaire et al., 2012). Pressurization and depressurization were set at 1 MPa s^{-1} with a M189 LVE air-driven liquid pump from Maximator (Zorge, Germany). Data obtained with liver slices of the individual fish were analyzed jointly (*i.e.*, inter-individual studies were not conducted). Inter-slice variation in the response, identified by the percentages of deviation from the means, were in most cases below 25% (see Table S1 for a representative example).

In studies on EROD activity under pressure, liver slices from one fish ($n = 2$) were individually incubated in 4-ml cryovials filled with O_2-saturated HBSS and 2 μM of 7-ethoxyresorufin, and placed in hyperbaric chambers filled with deionized water (15 °C). The rates of pressurization and depressurization of the hyperbaric chambers were as above. Data obtained with liver slices of the individual

fish were analyzed jointly (*i.e.*, inter-individual studies were not conducted). Inter-slice variation in the response, identified by the percentages of deviation from the means, were usually in the range of 20–30% (not shown).

In co-exposure studies with tBHP (1 h), liver slices from one fish ($n = 2$) were individually enclosed in 4-ml cryovials filled with O_2-saturated HBSS containing or not 5 mM of tBHP (a concentration leading to significant oxidative stress responses at atmospheric pressure—see Section 3), and 10 μM of CM-DCFHDA only for PCLS used to study ROS production. Cryovials were placed in hyperbaric chambers filled with deionized water (15 °C). The rates of pressurization and depressurization of the hyperbaric chambers were as above. Data obtained with liver slices of the individual fish were analyzed jointly (*i.e.*, inter-individual studies were not conducted). Inter-slice variation in the response, identified by the percentages of deviation from the means, were in most cases below 30% (not shown).

2.5. Adenosine triphosphate (ATP) content

The ATP content of individual slices was measured at 25 °C on a Berthold LB96P microplate luminometer (Bad Wilbad, Germany) against a standard of ATP with the ATP lite 1 Step kit from PerkinElmer, as previously described (Lemaire et al., 2011). The protein pellets obtained after centrifugation of the liver slice homogenates in perchloric acid 2% were used for protein content determination (see Section 2.12). Results were expressed as nmol mg^{-1} proteins.

2.6. Ethoxyresorufin-O-deethylase (EROD) activity

S9 fractions of individual slices from the concentration–response study with 3-MC were prepared according to previously published procedure (Lemaire et al., 2011), and kinetic fluorimetric measurements were done at 25 °C. 10-μl aliquots were kept for protein content determination (see Section 2.12). Results were expressed as pmol min^{-1} mg^{-1} proteins.

EROD activity of individual slices that had been incubated with 2 μM of 7-ethoxyresorufin at various HP levels was quantified as the sum of the activity (*i.e.*, the amount of resorufin) found in aliquots of media and slice homogenates (in phosphate buffer 100 mM, pH 7.4). End-point fluorimetric measurements were done with a Labsystems Fluoroskan microplate reader (Minneapolis, USA) set at 530–590 nm, against a standard of resofurin. 10-μl aliquots were kept for protein content determination (see Section 2.12). Results were expressed as pmol min^{-1} mg^{-1} proteins.

2.7. Antioxidant enzyme activities

Individual slices were homogenized in phosphate-buffered saline (pH 7.4) containing 1% of Triton X-100. Homogenates were first centrifuged for 10 min at 15,000 × *g* (4 °C) and subsequent assays were conducted at 25 °C on dilutions of supernatants as previously described (Lemaire et al., 2011). Briefly, catalase (CAT) activity was determined by luminometry on a Berthold LB96P microplate reader (Bad Wilbad, Germany) according to Janssens et al. (2000). Superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione *S*-transferase (GST) activities were assayed by spectrophotometry on a Molecular Devices Spectramax 190 microplate reader (Sunnyvale, USA). SOD assay was adapted from the method of Flohe and Otting (1984). GPX assay was adapted from the method of Paglia and Valentine (1967), using H_2O_2 (GPX_H) or tBHP (GPX_T) as substrates. GST assay was adapted from the method of Habig et al. (1974). 10 μl aliquots were kept for protein content determination prior to the centrifugation step (see Section 2.12). Results were expressed as U mg^{-1} proteins.

2.8. Quantitative PCR (qPCR) analyses

Total RNA from needle-disrupted slices was extracted with the Nucleospin RNA II kit from Macherey-Nagel (Düren, Germany) as per kit instructions (DNAse I treatment included), and the purity checked with $OD_{260/280}$ and $OD_{260/230}$ on a Nanodrop ND1000 from Thermo Scientific (San Jose, USA). Reverse transcription was performed on 400 ng of total RNA using oligo$(dT)_{20}$ and the Superscript III First Strand Synthesis kit from Invitrogen (Carlsbad, USA). Primer pairs, designed from sequences of *D. labrax* available in Genbank database, were selected with Primer Express 2.0 (Foster City, USA) and further analyzed *in silico* with Amplify 3.1.4 (Wisconsin, USA). Titration reactions were conducted to determine optimal primer pairs concentrations (Table 1). Quantitative PCR (qPCR) experiments were performed on a Step One Plus thermocycler according to Lemaire et al. (2011). A dissociation stage was always included in the thermal profile, and the specific amplification of the product of interest was verified by sequencing randomly-selected amplicons with the Big Dye Terminator 1.1 kit on a 3100 Genetic Analyzer, each from Applied Biosystems (Foster City, USA). The expression of *elongation factor 1 alpha* (*EF1α*), selected as reference gene, was not affected by treatments ($p = 0.31$).

2.9. Reduced glutathione (GSH) content

Quantification of GSH was conducted with a high-performance liquid chromatography (HPLC) method adapted from Cereser et al. (2001) and Gasparrini et al. (2006). Individual slices were homogenized in HBSS and 10 μl aliquots were removed for protein content determination (see Section 2.12). 100 μl aliquots of slice homogenates were first mixed with 400 μl metaphosphoric acid 6% and centrifuged for 7 min at 10,000 × *g* (4 °C). 100 μl aliquots of the deproteinized supernatant were then submitted to derivatization with 100 μl *ortho*-phthalaldehyde (OPA; 50 mg in 500 μl of methanol mixed with 10 ml of Borax buffer 0.1 M pH 9.9). Following 5 min incubation in the dark at 25 °C, 800 μl of sodium dihydrogen phosphate 50 mM (pH 7.0) was incorporated. A standard of GSH was prepared accordingly. To determine the sum of reduced (GSH) and oxidized (GSSG) forms, 100-μl slice homogenates in HBSS were first incubated for 30 min at 4 °C with 100-μl dithiotreitol 25 mM and 50-μl Tris buffer 0.1 M (pH 8.5). 1-ml metaphosphoric acid was then added, and the protocol of derivatization followed. The HPLC consisted of a Waters XTerra MS C-18 column (Milford, USA) coupled to a Spectra System P1000XR injector, an AS3000 autosampler and a SN4000 controller, each from Thermo Scientific (San Jose, USA). The fluorescence detector, set at 340 nm (excitation) and 420 nm (emission), was a FP2020 from Jasco (Tokyo, Japan). Samples were kept at 4 °C in the dark prior to analysis. Quantification of OPA-GSH adducts was conducted at a flow rate of 0.7 ml min^{-1} with a mobile phase consisting of sodium acetate 50 mM with 8% of acetonitrile (pH 6.2). Samples were eluted isocratically for 10 min at 35 °C and results expressed as nmol mg^{-1} proteins.

2.10. Reactive oxygen species (ROS) production

The cell-permeant fluorescein derivative CM-DCFHDA is a commonly used probe for the detection of reactive oxygen species (ROS) in vertebrate tissues (*e.g.*, King and Oh, 2004). Assays of ROS production were conducted with individual slices incubated with or without tBHP (5 mM). Endpoint measurements of the fluorescence of individual slices kept in 24-well plates with 500-μl HBSS plus 10 μM of the probe was performed before and after the incubation (25 °C). A Labsystems Fluoroskan (Minneapolis, USA)

Table 1
Characteristics of primer pairs and associated amplicons for reference (*Elongation factor 1α—EF1α*) and target (*Cytochrome P4501A—CYP1A*; *Catalase—CAT*; *CuZn Superoxide dismutase—CuZnSOD*; *Glutathione peroxidase—GPX*; *Glutathione S-transferase θ3—GSTθ3*; *Heat shock protein 70—HSP70*) genes in quantitative PCR experiments with liver slices of *Dicentrarchus labrax*. Accession numbers and optimal primer pair concentrations are also reported.

Gene	GenBank accession	Forward primer	Reverse primer	Product size (bp)	Concentration (nM)
EF1α	AJ866727	CACCGTTGCCTTTGTACCCAT	GCTCAACCTTCCATCCCTTGA	94	300
CYP1A	U78316	TCAGTGGCAGATCAACCATGAC	AAAACCATCACCTTCTCCCCC	122	300
CAT	FJ860003	GATCGAGGTTTGCCTGATGG	CATCGGCATTGACCAGTTTG	75	300
CuZnSOD	CX660893	TGCTGCAAAGATGGTGCTGAAA	TCCTGCTCAAAGTGAACGACCC	80	300
GPX	CX660442	TCCAGGAGCTGGAAGTTTGGT	TGCCCTGCAATCAGTTTGG	111	200
GSTθ3	CX660604	CCGATTGCAACTGGACTGGAT	TTTTCGCCGAGCTCCTTCTTC	85	200
HSP70	AY423555	GCACCTTCGTCGTGTCCAT	GACCATTCGGTTGTCAAAGTC	106	200

set at 485 nm (excitation) and 520 nm (emission) was used. 10-μl aliquots of slice homogenates were kept for protein content determination (see Section 2.12). Results were expressed as the increase of fluorescence detected post-incubation per protein weight of slices. Noteworthy, preliminary experiments with hydrogen peroxide confirmed that the sensitivity of the probe was not affected by HP ($p = 0.90$). Also, tBHP does not directly oxidize the probe (Martin et al., 2001).

2.11. Thiobarbituric acid reactive substances (TBARS) assay

Oxidative damage to lipids were measured as thiobarbituric acid reactive substances (TBARS) in culture media with a method adapted from Wey et al. (1993). At the end of the co-exposure period, 150 μl of media were mixed in glass tubes with 240 μl thiobarbituric acid 0.67% and 120 μl trichloroacetic acid 15%. A standard of malondialdehyde (MDA) was prepared accordingly. Glass tubes were sealed and incubated for 30 min at 95 °C. 100 μl aliquots were then mixed at 4 °C with 100 μl of butanol in black 96-well plates and the fluorescence was monitored by an endpoint measurement at 25 °C with a Labsystems Fluoroskan (Minneapolis, USA) set at 515 and 555 nm. Results were expressed as pmol of MDA equivalents mg^{-1} proteins.

2.12. Protein content

The protein content of liver slices was determined with the DC Protein Assay Kit (Bio-Rad, Richmond, USA). The vast majority of samples made available for this mode of data normalization (*i.e.*, 10 μl aliquots) were first diluted appropriately in 5% sodium dodecyl sulfate. In the case of ATP, protein pellets had first to be sonicated in 200 μl of 5% sodium dodecyl sulfate prior to appropriate dilution. Absorbance was measured at 750 nm with a Spectramax 190 spectrophotometer (Molecular Devices, Sunnyvale, USA), and samples were compared to a standard of bovine serum albumin.

2.13. Statistical analyses

Statistical analyses were conducted with Statistica 7.1 from Statsoft (Tulsa, USA). Experimental groups were compared by Kruskal–Wallis ANOVA, followed by Mann–Whitney one- or two-tailed U tests at the $\alpha = 0.05$ level (one-tailed tests were used for the concentration–response studies with 3-MC and tBHP at 0.1 MPa). The nomenclature for the different levels of statistical significance was as follow: * when $p \leq 0.05$; ** when $p \leq 0.01$; *** when $p \leq 0.0001$. Graphs were obtained with Sigma Plot 8.0 from Systat (San Jose, USA).

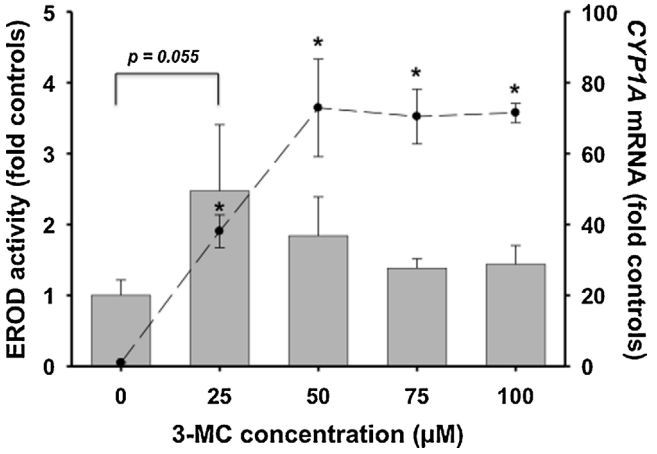

Fig. 1. Concentration–response analysis of *cytochrome P4501A* mRNA content (*CYP1A*; scatter plot) and 7-ethoxyresorufin-*O*-deethylase activity (EROD; vertical bars) in liver slices of *Dicentrarchus labrax* incubated for 21 h at 0.1 MPa in 24-well plates with L15+ medium containing increasing concentrations of 3-methylcholanthrene (3-MC). Data are expressed as fold solvent controls, and are presented as mean ± S.E.M. ($n = 4$ for *CYP1A*; $n = 6$ for EROD). Asterisks indicate significant differences ($p \leq 0.05$) from solvent controls.

3. Results

3.1. Validation of methods at 0.1 MPa

To identify possible modulating effects of HP on the response to and effects of selected chemicals in liver cells of *D. labrax*, we first identified concentrations effective to cause a moderate recruitment of cellular detoxification systems (3-MC) and the generation of a mild oxidative stress response (tBHP) at atmospheric pressure. We specifically analyzed effects of increasing concentrations of the AhR agonist on the transcript level and activity of *CYP1A* and EAOX, and of increasing concentrations of the pro-oxidant on the ATP and GSH contents.

3.1.1. Expression of CYP1A and antioxidant enzymes with 3-MC at 0.1 MPa

Slices individually incubated for 21 h (15 °C; 0.1 MPa) in 24-well plates with increasing concentrations of the AhR agonist exhibited a concentration-dependent increase of *CYP1A* transcript level. The transcript level was 38 fold in comparison to solvent controls in exposure to 25 μM of 3-MC and increased to 70 fold in exposures to higher concentrations. The induction was far less obvious for EROD activity, which was nearly significantly increased above controls only in exposure to 25 μM of 3-MC (Fig. 1). Noteworthy, a preliminary experiment aimed at validating EROD assay for use with liver cells of *D. labrax* showed a significant increase of activity from solvent controls with that concentration (Fig. S2). The magnitude of EROD induction was comparable betwen the two experiments. The ATP content of slices incubated with increasing AhR agonist

Table 2
Effects of increasing concentrations of 3-methylcholanthrene (3-MC; 21 h at 0.1 MPa in 24-well plates with L15+ medium) on activities (U mg^{-1} protein) of antioxidant enzymes (catalase—CAT; superoxide dismutase—SOD; glutathione peroxidase—GST; glutathione S-transferase—GST) and transcript levels of genes encoding them (fold solvent controls) in liver slices of *Dicentrarchus labrax*. Data are presented as mean ± S.E.M. ($n = 4$) and results with a p-value ≤ 0.05 were considered significant. Different letters indicate significant differences between concentrations.

Enzyme	Parameter	3-MC concentration (μM)					ANOVA
		0	25	50	75	100	
CAT	mRNA	1.00 ± 0.15	0.97 ± 0.09	1.15 ± 0.12	1.14 ± 0.08	1.35 ± 0.09	$p = 0.16$
CAT	activity	281 ± 33	225 ± 22	213 ± 16	249 ± 16	232 ± 22	$p = 0.33$
CuZnSOD	mRNA	1.00 ± 0.04	1.06 ± 0.03	1.14 ± 0.02	1.03 ± 0.05	1.11 ± 0.06	$p = 0.17$
SOD	activity	3.05 ± 0.68^{abc}	1.88 ± 0.54^{a}	3.41 ± 0.43^{ac}	5.54 ± 1.31^{bc}	5.15 ± 0.44^{b}	$p = 0.02$
GPX	mRNA	1.00 ± 0.27	1.17 ± 0.33	1.02 ± 0.10	1.23 ± 0.21	1.77 ± 0.31	$p = 0.42$
GPX	GPX_H activity	2.93 ± 0.21^{ab}	3.92 ± 0.31^{a}	2.00 ± 0.27^{b}	2.37 ± 0.32^{b}	3.04 ± 0.33^{ab}	$p = 0.01$
	GPX_T activity	4.90 ± 0.40	5.46 ± 0.53	4.85 ± 0.63	5.44 ± 0.93	6.42 ± 0.45	$p = 0.29$
GSTθ3	mRNA	1.00 ± 0.12	1.00 ± 0.11	1.09 ± 0.06	0.89 ± 0.02	1.12 ± 0.08	$p = 0.17$
GST	activity	2.31 ± 0.60	2.15 ± 0.25	2.67 ± 0.73	2.76 ± 0.28	4.66 ± 0.38	$p = 0.08$

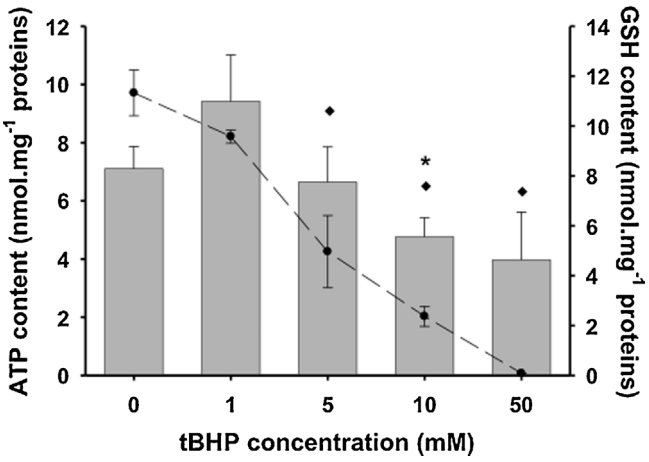

Fig. 2. Concentration–response analysis of adenosine triphosphate (ATP; vertical bars) and reduced glutathione (GSH; scatter plot) content (nmol mg^{-1} protein) in liver slices of *Dicentrarchus labrax* incubated for 1 h at 0.1 MPa in 4-ml cryotubes with oxygen-saturated saline solution and increasing concentrations of *tert*-butylhydroperoxide (tBHP). Data are presented as mean ± S.E.M. ($n = 3$). Results with a p-value ≤ 0.05 were considered significant. Asterisks indicate significant differences from controls without tBHP for ATP data and rhombuses for GSH data.

concentrations was never below that of solvent controls (data not shown).

There were no clear concentration-response relationships for antioxidant enzyme genes with 3-MC either at the mRNA or protein activity level (Table 2). SOD activity was lower in exposures to 25 μM than to 75 μM or 100 μM. In contrast, GPX_H activity tended to increase from solvent controls to 25 μM of 3-MC incubations ($p = 0.057$), and was significantly higher at that concentration than at 50 μM and 75 μM.

Altogether, these data led us to select 25 μM concentration for the 6- and 15-h co-exposure studies on the inducibility of stress-responsive genes with HP.

3.1.2. Resistance to tBHP at 0.1 MPa

Liver slices were individually incubated for 1 h (15 °C; 0.1 MPa) in 4-ml tubes with oxygen-saturated HBSS containing increasing concentrations of tBHP, up to 50 mM. With concentrations up to 5 mM, the ATP content of slices was not reduced from controls without pro-oxidant. A strong concentration-dependent reduction of the GSH content of liver cells was nevertheless achieved in slices incubated with tBHP. This was significant starting with 5 mM, a concentration responsible for roughly 50% reduction of the GSH content of liver slices (Fig. 2).

We thus selected 5 mM tBHP concentration for the 1-h co-exposure studies on the extent of oxidative stress experienced by liver cells at high HP.

3.2. Effects of high HP on the response to 3-MC

Slices were exposed to 3-MC (25 μM) at 5 or 15 MPa (15 °C; 6 or 15 h) to investigate effects of deep-sea pressure levels on the inducibility of stress-responsive genes in liver cells of *D. labrax*. As previously (Lemaire et al., 2012), controls for these experiments consisted of slices sampled just after slicing (*i.e.*, time 0 of culture; T0) and slices incubated for the duration of the exposure (*i.e.*, 6 or 15 h) in a hyperbaric cylinder at 0.1 MPa. In each experiment, we verified that the protocol of incubation did not impair cell viability, as judged from ATP content.

3.2.1. Effects of high HP on PCLS viability

Strikingly, a doubling of the ATP content from T0 controls was observed in slices incubated for 6 h with 3-MC at both 0.1 and 5 MPa. Liver cells exposed to 3-MC for that period at 15 MPa had ATP levels similar to T0 controls. A doubling from T0 controls was also found in slices incubated for 15 h with 3-MC at 0.1 MPa. This time, the corresponding ATP levels in PCLS exposed to 3-MC at high HP were similar to T0 controls (Fig. 3A and B).

3.2.2. Effects of high HP on gene expression

CYP1A expression increased to about 3 and 6 times that of T0 controls after 6 and 15 h exposure at 0.1 MPa, respectively. Still, *CYP1A* transcript abundance was always reduced at higher HP levels (*i.e.*, 5 and 15 MPa) from the level detected at atmospheric pressure. At the higher HP levels, *CYP1A* transcript level was similar to that of T0 controls (Fig. 3C and D). Very strong increases of *HSP70* mRNA content compared to T0 controls were also seen at 0.1 MPa (*i.e.*, about 170 and 620 fold controls after 6 and 15 h incubation, respectively). *HSP70* mRNA content increased to much lower levels after 6 and 15 h exposure at high HP (Fig. 3E and F). After 15 h of co-exposure, *HSP70* mRNA level at 15 MPa was more than twice that observed at 5 MPa.

EAOX transcript levels were only mildly affected by treatments (Table 3). Non-monotonic hyperbaric responses were suggested for both transcripts in the 15 h co-exposure studies. Thus, *SOD* transcript level increased from T0 controls at 0.1 MPa, and significantly declined from that level at 5 MPa but not ar 15 MPa. *CAT* transcript tended to exhibit an opposite pattern of expression. HP effects on *GPX* mRNA content closely resembled those seen with *CYP1A*. At atmospheric pressure, significant increases of *GPX* transcript level were found (*i.e.*, about 2 and 2.6 fold T0 controls after 6 and 15 h culture with 3-MC, respectively). This effect was abolished at high HP. A reduced level of *GSTθ3* transcript from T0 controls was seen

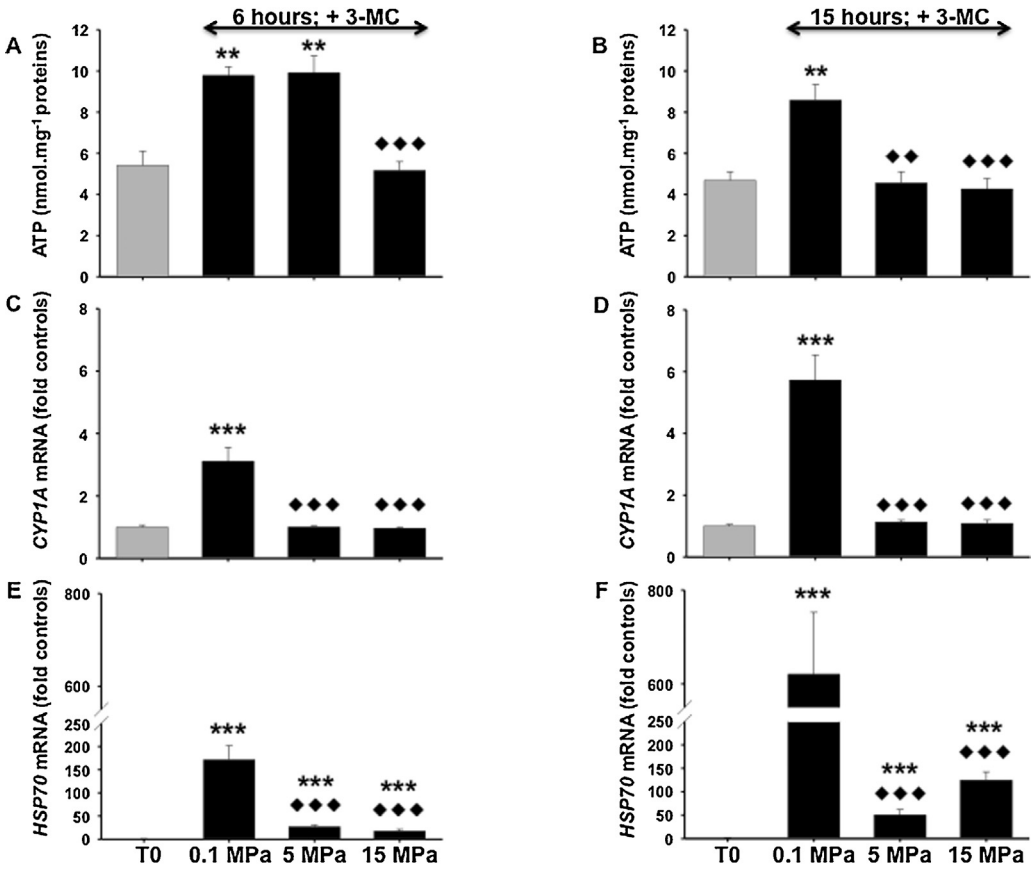

Fig. 3. Effects of 6 or 15 h exposure of liver slices of *Dicentrarchus labrax* to 3-methylcholanthrene (3-MC; 25 μM) at atmospheric (0.1 MPa) and deep-sea (5–15 MPa) pressure levels in hyperbaric chambers on: (A and B) adenosine triphosphate (ATP) content (nmol mg^{-1} protein; for the 6 h study, $n = 8$ for T0 and $9 \leq n \geq 10$ for other treatments, while for the 15 h study, $n = 12$ for each treatment); (C and D) *cytochrome P4501A* (*CYP1A*) transcript level (fold T0 controls; for the 6 h study, $n = 8$ for T0 and $n = 12$ for other treatments, while for the 15 h study, $n = 8$ for T0 and $10 \leq n \geq 11$ for other treatments); (E and F) *heat shock protein 70* (*HSP70*) transcript level (same mode of data expression and number of replicates as for *CYP1A*). Slices sampled prior to the incubation (T0) were used as controls for 3-MC effects at 0.1 MPa. Results are presented as mean ± S.E.M. Asterisks indicate significant ($p \leq 0.05$) differences from T0 controls and rhombuses from 0.1 MPa.

Table 3
Effects of 6 or 15 h exposure of liver slices of *Dicentrarchus labrax* to 3-methylcholanthrene (3-MC; 25 μM) at atmospheric (0.1 MPa) and deep-sea (5–15 MPa) pressure in hyperbaric chambers on transcript level of *catalase* (*CAT*), *CuZn superoxide dismutase* (*CuZnSOD*), *glutathione peroxidase* (*GPX*) and *glutathione S-transferase θ3* (*GSTθ3*). Slices sampled prior to the incubation (T0) were used as controls for 3-MC effects at 0.1 MPa. Results, expressed as fold changes from T0, are presented as mean ± S.E.M. ($n = 8$ for T0 and $10 \leq n \geq 12$ for other treatments). Different letters indicate significant differences (p-value ≤ 0.05) between treatments for a given transcript and co-exposure period.

Treatments	*CAT*		*CuZnSOD*		*GPX*		*GSTθ3*	
	6 h	15 h	6 h	15 h	6 h	15 h	6 h	15 h
T0	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.07	1.00 ± 0.05[a]	1.00 ± 0.08[a]	1.00 ± 0.07[a]	1.00 ± 0.03	1.00 ± 0.04[a]
0.1 MPa	1.04 ± 0.05	0.99 ± 0.03	1.09 ± 0.06	1.21 ± 0.07[b]	1.95 ± 0.16[b]	2.56 ± 0.30[b]	0.87 ± 0.04	0.80 ± 0.07[b]
5 MPa	1.07 ± 0.05	1.14 ± 0.04	1.28 ± 0.11	0.91 ± 0.02[a]	1.22 ± 0.18[a]	0.90 ± 0.06[a]	0.91 ± 0.03	0.66 ± 0.05[b]
15 MPa	1.16 ± 0.05	1.07 ± 0.07	1.25 ± 0.07	1.14 ± 0.09[ab]	1.25 ± 0.09[a]	1.06 ± 0.10[a]	0.96 ± 0.03	0.75 ± 0.07[b]
ANOVA	0.14	0.08	0.13	0.01	0.0007	<0.0001	0.16	0.006

after 15 h of incubation with the AhR agonist (regardless of the HP level).

3.3. Effects of high HP on EROD activity

To determine whether high HP would affect CYP1A-associated EROD activity, liver slices of *D. labrax* were first incubated for 21 h in 24-well plates at 0.1 MPa with 25 μM of 3-MC (as to increase the amount of CYP1A protein). They were then rinsed and individually placed in cryovials containing oxygen-saturated HBSS and the substrate of EROD (*i.e.*, 7-ethoxyresorufin). The cryovials were then incubated for 3 h at high HP (5–15 MPa) or atmospheric pressure (0.1 MPa controls). EROD activity was estimated from end-point measurements of EROD's product (*i.e.*, resorufin) in aliquots of media and slice homogenates. The activity tended to decline with increasing HP, but the differences were not statistically significant. Concomitantly, an increased content of resorufin in slices was observed at 15 MPa compared to the other two HP levels (Fig. 4).

3.4. Effects of high HP on the response to tBHP

We analyzed whether high HP would modulate the resistance of liver cells to a pro-oxidant. To this end, slices were individually placed in cryovials with oxygen-saturated HBSS containing 5 mM of tBHP. Cryovials were then incubated for 1 h at high HP (5–15 MPa) or atmospheric pressure (0.1 MPa controls). Controls for the generation of oxidative stress consisted of slices incubated without tBHP at each HP level. Biomarkers of oxidative stress (GSH content, ROS production and thiobarbituric acid reactive substances—TBARS)

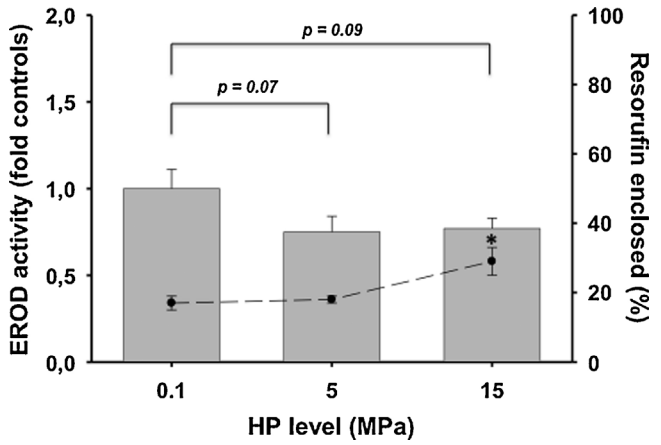

Fig. 4. Effects of a 3-h incubation of liver slices of *Dicentrarchus labrax* at high pressure (HP; 5–15 MPa) in hyperbaric chambers with 7-ethoxyresorufin on the level of 7-ethoxyresorufin-O-deethylase (EROD; vertical bars) activity and the associated retention of EROD's product in slices at the end of incubation (% of resorufin enclosed; scatter plot). Data were expressed relatively to the level of 0.1 MPa controls (mean ± S.E.M.; $n = 8$). Asterisks indicate significant ($p \leq 0.05$) differences from 0.1 MPa for the data on resorufin kept within slices only.

and cell viability (*i.e.*, ATP content) were used to assess the response of liver cells to tBHP at various HP levels.

As expected, the ATP content of slices incubated with tBHP was significantly reduced from controls without pro-oxidant at both 0.1 and 5 MPa. Surprisingly, this was not seen in liver slices incubated at 15 MPa. Trends to a reduced ATP content with HP in slices incubated without tBHP – which became significant at 15 MPa – and to a slight increase of the ATP content with HP in slices incubated with tBHP seem to explain this phenomenon (Fig. 5A).

Liver cells exposed to the pro-oxidant at both 0.1 and 5 MPa exhibited significantly higher levels of ROS production than controls without tBHP. Similarly to what was seen for ATP, there were no significant differences for this parameter between tBHP-treated and non-treated slices at 15 MPa. An important reduction of the level of ROS production was noted in tBHP-treated liver slices at 15 MPa. For slices incubated without tBHP, the production of ROS at 15 MPa tended to be slightly higher than at 0.1 MPa (Fig. 5B).

In accordance with the results of the dose-response study, the GSH content of slices incubated with 5 mM of tBHP at 0.1 MPa was reduced to about 50% of the corresponding controls without tBHP. Here, the same was noted at 5 MPa. The difference of GSH content between tBHP-treated and non-treated slices tended to decline at 15 MPa, though. This probably reflects the slight trend to a reduced GSH content with HP in slices incubated without tBHP (Fig. 5C).

Aliquots of media from co-exposed slices were sampled to quantify the amount of TBARS released by liver cells under the various experimental conditions. At all HP levels, the amount detected in media of slices incubated with tBHP was higher than in media of non-treated PCLS. In line with the other biomarkers, this effect tended to decrease with HP (Fig. 5D).

4. Discussion

Pioneering « eco-barotoxicological » (as quoted by Vevers et al., 2010) studies revealed that HP levels above atmospheric can modulate responses of classical biomarkers of chemical exposure in both surface and deep-sea fish (Kopecka-Pilarczyk and Coimbra, 2010a,b; Lemaire et al., 2012). This implies that there could be con-

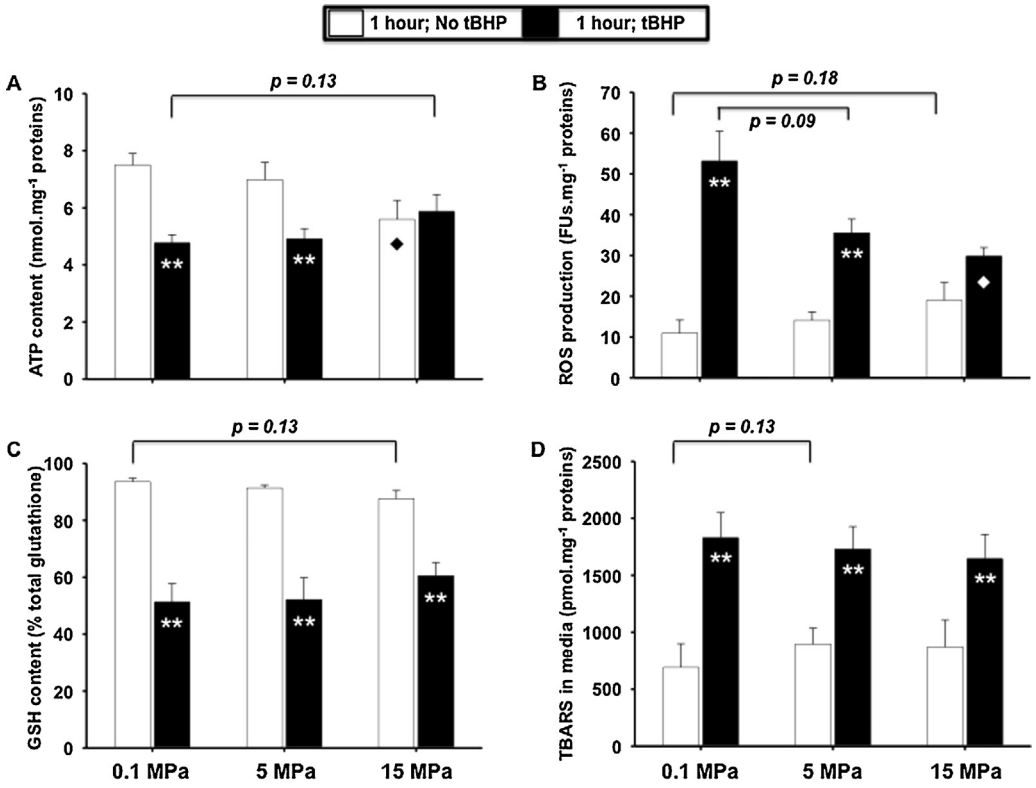

Fig. 5. Effects of a 1-h exposure of liver slices of *Dicentrachus labrax* to *tert*-butylhydroperoxide (tBHP; 5 mM) at atmospheric (0.1 MPa) and deep-sea (5–15 MPa) pressure levels in hyperbaric chambers on: (A) adenosine triphosphate (ATP) content (nmol mg^{-1} protein; $n = 8$); (B) reactive oxygen species (ROS) production (fluorescence units mg^{-1} protein; $n = 6$); (C) reduced glutathione (GSH) content (% of total glutathione in slices; $5 \leq n \geq 10$); (D) thiobarbituric acid reactive substances (TBARS) content in media (pmol mg^{-1} protein; $9 \leq n \geq 10$). Results are presented as mean ± S.E.M. Slices incubated without tBHP (white bars) were used to control for effects of tBHP at each pressure level (black bars). Asterisks indicate significant differences from controls without tBHP at a given pressure level and rhombuses from 0.1 MPa for a given tBHP treatment.

founding impacts of this thermodynamical parameter in field and laboratory studies, and that the crushing HP levels typical of the deep-sea might strongly affect the ability of the resident fauna to cope with pollutants.

Here, liver slices of a surface fish (*D. labrax*; presumably not adapted to high HP) were co-exposed for 1–15 h to chemicals (a model AhR agonist or a pro-oxidant) at various HP levels, and many of the detoxification and oxidative stress responses analyzed were affected by pressure. ATP levels were close to those of freshly-cut liver slices (*i.e.*, T0 controls) in all co-exposure studies, and values reported here were in the range of – or even higher than – those previously measured in functional liver slices of salmonids at 0.1 MPa (Singh et al., 1996; Lemaire et al., 2011). We conclude that liver cells were viable in all instances.

Slices incubated at atmospheric pressure in multi-well plates (21 h) or hyperbaric cylinders (6 or 15 h) were able to increase the transcript level of the AhR battery gene *CYP1A* in response to the agonist 3-MC, however, to different extents depending on the incubation system and exposure period. There was also an increased EROD activity at 21 h detected in multiwell plates in response to 25 μM of 3-MC. The lower EROD activity detected at higher 3-MC concentrations are suggestive of a competition between the AhR agonist and ethoxyresorufin during the assay (Lemaire et al., 2011). Altogether, these data support the view that functional liver cells were used in co-exposure experiments. The pressure-independence of EAOX transcript levels frequently seen in co-exposure studies with 3-MC, the stable expression of the reference gene and the increased content of *HSP70* mRNA at 15 MPa compared to 5 MPa (after 15 h with 3-MC) also support the view that patterns of gene expression do not reflect a global collapse of the transcriptional machinery at high HP.

In co-exposure studies with 3-MC, *CYP1A* transcript level was strikingly lowered from the level at 0.1 MPa at high HP. Similar observations were made with liver slices of *C. rupestris* (Lemaire et al., 2012). Still, significant increases from T0 controls were not observed at high HP in liver slices of *D. labrax*, as was the case with the deep-sea fish (*i.e.*, about 4.5 and 2.5 fold increase from T0 after 15 h at 5 or 15 MPa, respectively; Lemaire et al., 2012). Interestingly, co-exposing liver slices of *Salmo salar* to 3-MC at 15 MPa for 6 or 24 h also resulted in a limited increase of *CYP1A* mRNA content (*i.e.*, about 2 and 1.4 fold increase from T0, respectively; *unpublished*). Noteworthy, when at sea, *S. salar* can mostly be found over depths exceeding 1000 m (reviewed in Dadswell et al., 2010). Thus, only liver cells of the coastal fish failed to increase to some extent *CYP1A* mRNA level in response to the AhR agonist at high HP.

To explain the absence of *CYP1A* induction in *D. labrax*, a direct impact of high HP on the stability of CYP1A molecule can be ruled out. Indeed, covalent bonds are insensitive to pressure levels typical of the deep-sea (Silva et al., 2014). Furthermore, calculations of HP effects on the fugacity of dissolved oxygen (Ludwig and Macdonald, 2005) do not support the view that liver slices were subjected to hypoxia at high HP. This could have repressed *CYP1A* transcription (*e.g.*, Rahman and Thomas, 2012). One might ask whether a pressure-driven increased ordering of lipid bilayers limited to some extent the uptake of 3-MC in liver cells of *D. labrax*, thereby reducing the magnitude of *CYP1A* transcriptional response. There are several counterarguments to this proposition. The literature indeed suggests that 5 MPa only modestly increases the anisotropy of native membranes of terrestrial mammals (Williams et al., 2001), and that an increase of 10–13 MPa roughly corresponds to effects of a temperature decrease of 3–6 °C in model bilayers (Macdonald, 1984). Results we obtained for EROD activity at high HP (see below) give further support to the notion that membrane fluidity and permeability were not severely impacted at least at 5 MPa in liver slices of *D. labrax*. With this HP level, effects on *CYP1A* induction were already maximal.

We propose that the observed patterns of *CYP1A* mRNA mostly reflect an altered transcriptional capacity of the AhR pathway of marine fishes at high HP. Pressure might repress in some way(s) AhR signaling in both surface and deep-sea species, such as through effects on ligand binding and protein–protein interactions. Additionally, the possible implication of a pressure-induced change of nuclear cytoskeletal tension altering cell signaling cannot be ruled out (Ihalainen et al., 2015). As suggested previously (Lemaire et al., 2012), only the deep-sea fauna would have evolved the molecular adaptation(s) necessary to partly counteract this inhibition. In this regard, it is interesting to note that, within the same oceanic region, slope fish exhibit significantly lower levels of EROD activity than shelf species (*e.g.*, Sole et al., 2010).

EROD activity tended to decline with pressure in liver cells of *D. labrax*. In comparison, previous results with liver slices of *C. rupestris* showed that EROD activity was fairly pressure insensitive over the HP range applied herein (*unpublished*). Such a divergence between surface and deep-sea enzymes is commonly reported in the literature (*e.g.*, Brindley et al., 2008). Our data suggest that a limited permeability of liver slices to the substrate ethoxyresorufin cannot account for this apparent reduction of EROD activity with HP. First, measuring EROD activity with liver microsomes of *D. labrax* yielded results comparable to liver slices following incubations at 5 MPa, and there was even a greater decline of activity with microsomes than with liver slices for the 15 MPa treatment (not shown). In microsomal studies, the substrate does not need to cross cell membranes to reach the enzyme. Second, the proportion of resorufin that was retained in the slices at the end of hyperbaric incubations – a parameter that might give clues to cell permeability – was only modestly affected at 15 MPa (and not at 5 MPa). Still, HP effect on EROD activity was already maximal at 5 MPa.

Effects of high HP on *GPX* and *HSP70* expression resemble those seen for *CYP1A* (*i.e.*, time-dependent increases at 0.1 MPa and reductions from these levels at high HP). While this suggests there might be a partial repression of their transcription with increasing HP in *D. labrax*, strong time-dependent increases of *HSP70* transcript level from T0 controls were nevertheless detected at high HP. Also, a strong increase of *HSP70* expression from 5 MPa was seen at 15 MPa in the 15 h co-exposure study. The latter pattern could reflect the increasing needs for the chaperone, as time and HP increase, to cope with detrimental pressure effects on protein folding (for a review, see Mayer and Bukau, 2005). This is reminiscent of the increased *HSP70* transcript level detected in surface crustaceans upon exposure to deep-sea pressure levels (Cottin et al., 2012; Morris et al., 2015), and in sharp contrast with the pattern seen with *C. rupestris* (Lemaire et al., 2012). In the latter study, we hypothesized that the recompression of liver cells stabilized membrane fluidity and protein structure, thereby reducing the needs for HSP70 in liver cells.

It is suggested that high HP could increase the risk of free radical formation in biological systems, due to the increased chemical potential of oxygen (see Ludwig and Macdonald, 2005). Indeed, oxidative stress appears to be a significant component of the response of surface microorganisms to high HP (*e.g.*, Palhano et al., 2004; Aertsen et al., 2005). Whether this phenomenon could add to the pro-oxidant threat of many persistent chemicals found in the marine environment (Lushchak, 2011) is of prime importance. To investigate this question with fish liver cells, we used tBHP, a chemical whose mode of action involves thiol depletion and transition metal-driven ROS generation (Aherne and O'Brien, 2000; Martin et al., 2001). This pro-oxidant was selected to ensure a strong oxidative stress response over a short time scale in liver cells of *D. labrax*.

Increasing HP in the absence of tBHP led to an imbalance between pro- and anti-oxidant forces in liver slices as one would expect (*e.g.*, Aertsen et al., 2005). However, according to the parameters we measured, co-exposing cells to the pro-oxidant at high HP

tended to limit oxidative stress. This counter-intuitive result perhaps reflects the fact that P450 enzymes, whose activities might have been affected by the HP levels tested here (see above), participate in the conversion of tBHP to radical intermediates (Davies, 1989). Importantly, Palhano et al. (2004) found an increased baroresistance of *Saccharomyces cerevisiae* following pretreatment with hydrogen peroxide, indicating that the two stressors can induce overlapping responses in cells. The possibility therefore remains that high HP might antagonize the pro-oxidant potential of xenobiotics by eliciting a protective response in liver cells of marine fishes. Clearly, more work with agents that do not require enzymatic activation is required to solve this matter.

Present study, in combination with our previous work with *C. rupestris* (Lemaire et al., 2012) and *S. salar* (*unpublished*), notably reveals that *CYP1A* inducibility is detrimentally affected by HP levels typical of the deep-sea in both surface and deep-sea fishes. Although species adapted to thrive in the deep-sea appear to have evolved molecular adaptation(s) to partly overcome this problem, the limited transcriptional response to AhR agonists might detrimentally affect their ability to detoxify persistent pollutants. Our work also opens up the possibility that other stress-responsive gene pathways could be affected by high HP. In future studies with liver slices, focus will be put on comparing the resistance of surface and deep-sea fish to pro-oxidant chemicals at high HP. Overall, combining biochemistry, molecular biology and histology in future studies with liver slices should prove very useful in our understanding of fish tissue response to scenarios of co-exposure.

Conflict of interest

The authors declare that there are no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2016.01.004.

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