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XENOBIOTIC EXPOSURE UNDER HYPOXIC CONDITIONS IN AQUATIC ORGANISMS: INTERACTIONS BETWEEN THE SIGNALING ROUTES MEDIATED BY THE HIF-α AND AHR TRANSCRIPTION FACTORS

International Ph.D. Thesis submitted by

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For the degree of

Philosophiae Doctor

2015

THIS WORK WAS FUNDED BY:

The **Basque Government** through the pre-doctoral grant to Joshua Martos Bernal, through the grant to Consolidated Research Groups GIC07/26-IT-393-07, though a mobility grant (Chapter 4) and through the projects ETORTEK-K-EGOKITZEN (IE06-216) (Chapters 1 and 2) and SAIOTEK SA-2010/38 (Chapters 1 and 2).

The **Research Council of Norway** through its Center of Excellence funding scheme (223268/F50).

The **University of the Basque Country** through the grant to Unit of Formation and Research (UFI 11/37).

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I. INTRODUCTION

Abbreviations used in the introduction:

AHR	Aryl hydrocarbon receptor
ARNT, HIF-1β	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine triphosphate
bHLH-PAS	basic helix-loop-helix Per/Arnt/Sim family
cTAD	C-terminal activation domain
CYP1A	Cytochrome p450
DO	Dissolved oxygen
EROD	Ethoxyresorufin-O-deethylase
ETC	Electron transport chain
Fe2+	Ferrous iron
Fe3+	Ferric iron
FIH-1	Factor inhibiting HIF-1
Gya	Gigayears ago
HIFs	Hypoxia-inducible factors
HLH	Helix-loop-helix
HREs	Hypoxia responsive elements
MOC	Meridional overturning circulation
nTAD	N-terminal activation domain
ODD	Oxygen-dependent degradation domain
OMZs	Oxygen minimum zones
PAHs	Polycyclic aromatic hydrocarbons
PAS	PER/ARNT/SIM homology domain
PCBs	Polychlorinated biphenyls
PHD	Prolyl hydroxylase domain family of enzymes
PKM2	Pyruvate kinase
pVHL	Von Hippel-Lindau tumor suppressor protein
ROS	Reactive oxygen species
Sirt	Sirtuins
TCA	Tricarboxylic acid cycle
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
XREs	xenobiotic responsive elements

1. Oxygen availability in the oceans.

1.1. Atmosphere, hydrosphere, oxygen and living organisms.

The atmosphere of Earth is a dynamic layer of gasses surrounding the planet composed mainly by nitrogen (78.08 %) and oxygen (20.95 %). Composition and characteristics of the atmosphere are in close relationship with the hydrosphere of Earth, which is the combined mass of water found on, under, and over the surface of the planet. The deeply reductive early atmosphere allowed the presence of this hydrosphere. Evidences of oceans and tectonic plate subduction similar to those existing nowadays have been recorded to have occurred at least as soon as 4.4 gigayears ago (Gya), while the planet was formed 4.54±0.05 Gya (Wilde et al. 2001; Hopkins et al. 2008). Apparently, complex processes such as the "cool early Earth" and the "faint Sun paradox" enabled the presence of liquid water by buffering the extreme environmental conditions of Earth (Kasting & Catling 2003). This water allowed the apparition of life (anaerobic organisms), whose oldest traces have been discovered in a rock sedimented more than 3.7 Gya (Ohtomo et al. 2013). The moment life begun in Earth is being continuously delayed in time as research in the matter advances; recently, Bell and colleagues (2015) found that the oldest known biogenic material to date was formed 4.1 Gya. It is now hypothesized that life survived the impact of a massive number of asteroids (previously regarded as sterilizing events) during the so called "Late Heavy Bombardment" (4.1-3.8 Gya) and afterwards re-colonized the planet from the depths of the oceans (Abramov & Mojzsis 2008).

Both, the atmosphere and the hydrosphere have been influencing each other, altering their composition and features. The most outstanding milestone in this regard took place 2.3 Gya in the oceans, where cyanobacteria and newly appeared eukaryotes managed to produce enough oxygen in the water (byproduct of their photosynthetic processes) to switch the reducing atmosphere to an oxidizing one in what it is termed "Great Oxygenation Event" (Holland 2006). As a consequence, most of anaerobic organisms were extinct and the apparition and evolutionary radiation of aerobic forms of life was boosted by the presence of oxygen (Kopp et al. 2005; Cannio et al. 2000). This shift of the characteristics of the atmosphere and the hydrosphere did not bring

stability, for instance several mass extinctions have been influenced by oscillations in the oxygen levels of both Earth compartments (Lewis & Dorne 2006). Furthermore, the hypothetical causes of the most severe extinction event known to date, the Permian-Triassic extinction event that occurred 252 million years ago, include the drastic reduction in oxygen availability in the seas (Figure 1) (Isozaki 1997; Wignall & Twitchett 2002). In any case, the concentration of oxygen in the atmosphere and the hydrosphere has oscillated through time to finally reach the present levels (Figure 2).

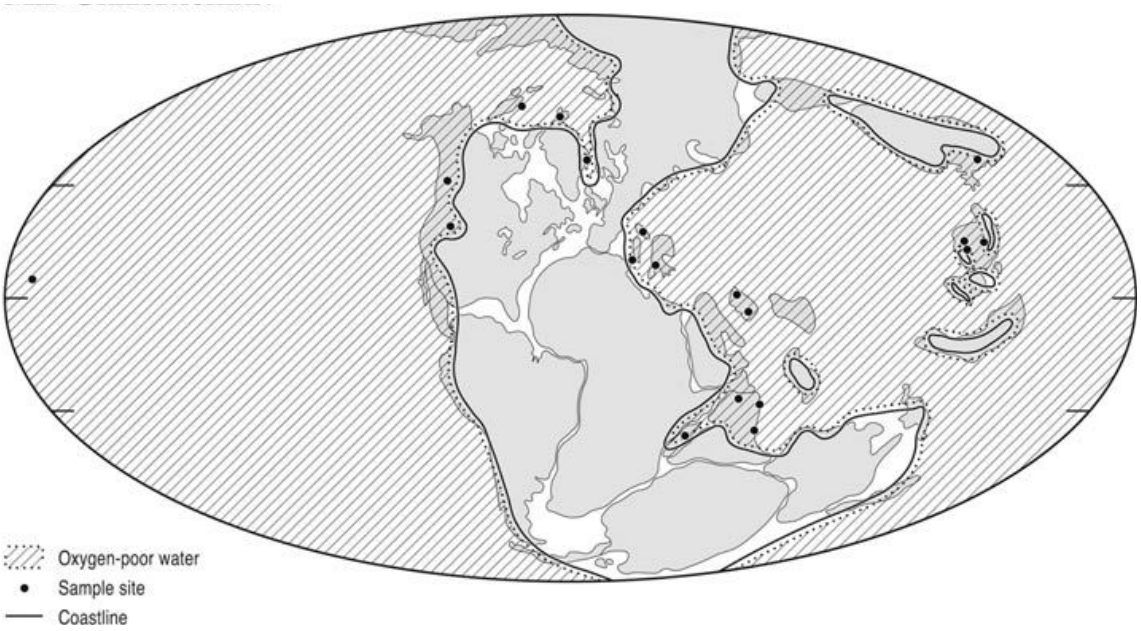

Figure 1: Extent of shallow-marine water under poor-oxygen conditions during the peak of Permian-Triassic superanoxic event 251 million years ago (Wignall & Twitchett 2002).

1.2 Oxygen cycle and the meridional overturning circulation.

The biogeochemical cycle of oxygen reflects how this element flows among the different Earth compartments. The most important reservoir of this gas is the lithosphere, followed by the atmosphere, the biosphere and finally by the hydrosphere. While the lithosphere retains a major part of oxygen, the atmosphere houses the part that is free to circulate and susceptible to be used by most of the living organisms. This circulating oxygen is synthesized during photosynthesis, while the catabolic processes of respiration and decomposition balance the equilibrium by consuming it. Besides being essential for a major part of the living organisms, the

atmospheric oxygen led to the formation of the ozone layer which is essential for life since it absorbs harmful ultraviolet radiation (Parson 2003).

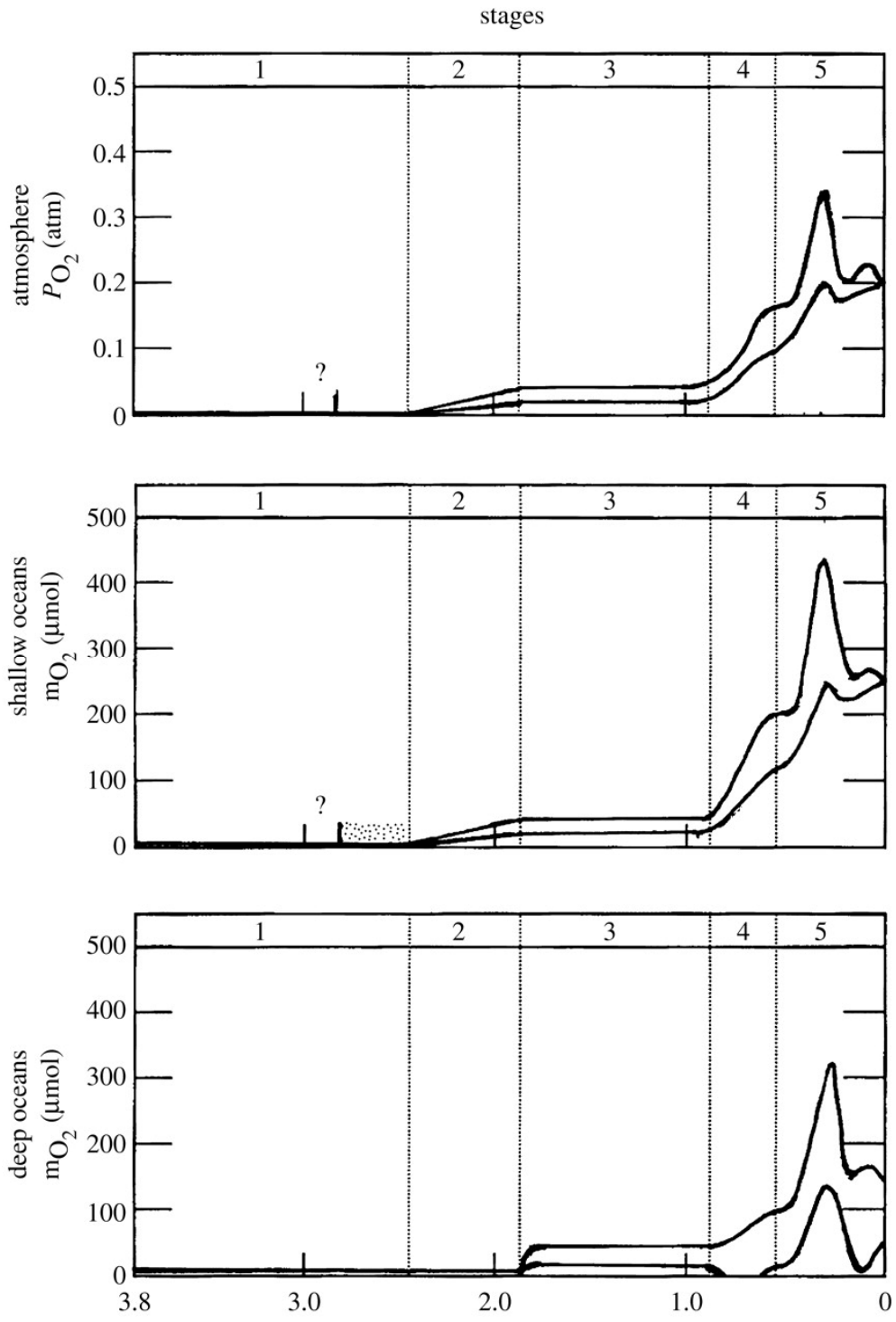

Figure 2: Estimated evolution of atmospheric partial pressure (P_{O_2}) and concentration of oxygen in shallow and deep oceans 3.8 Gya to present. Initially, there was no oxygen (stage 1). Apparition of photosynthetic forms of life caused synthesis of oxygen; however, this oxygen was absorbed by the lithosphere and oceans (stage 2). The saturation of oceans pushed oxygen to the atmosphere and the ozone layer was then formed (stage 3). During stages 4 and 5 oxygen continued to accumulate, reaching the levels existing nowadays (Holland 2006).

Even if the oxygen levels of the hydrosphere, which are dependent of the equilibrium with the atmosphere, are relatively small, they are extremely important for the organisms living there. The vast majority of the hydrosphere is represented by the oceans, and the thin upper layer of water in contact with the atmosphere is the main oxygen exchange area. The vertical mixing of the water column allows the distribution of oxygen from the enriched surface; such process being ruled by the physicochemical conditions of water, which also set the global circulation of the oceans. Regarding the previously mentioned flows, the meridional overturning circulation (MOC) is of special interest (Rahmstorf 2003). The temperature and salt content of each water layer determine its density and therefore the depth it will occupy in the column. In the MOC the cold and salty water sinks, travels as deep water for as long as 1000 years and finally upwells as surface water again (Figure 3). In the process, energy (in form of heat) and matter (nutrients and dissolved gasses) are transported in a global exchange process that defines the climatology and features of the nearby ecosystems (Curry & Mauritzen 2005; Quadfasel 2005). For example, the upwelling areas are among the most productive ecosystems, since the nutrient-enriched water is lifted to the photic zone. The MOC also prevents the mid and bottom zones of the ocean from being oxygen-depleted due to their lack of gas exchange with the atmosphere (Toggweiler & Key 2001).

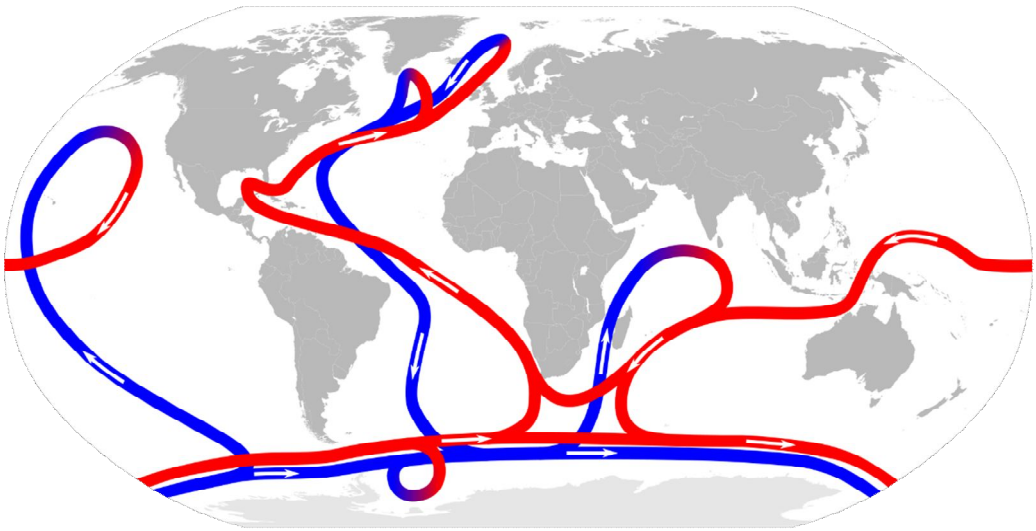

Figure 3: Schematic representation of the meridional overturning circulation, showing the dense deep water flows in blue and the lighter surface streams in red (Wikimedia commons, accessed 19-7-2015: https://commons.wikimedia.org/wiki/File:Thermohaline_Circulation.svg).

Alterations in the oxygen biogeochemical cycle or the MOC eventually lead to situations of oxygen scarcity situations in the different water bodies of the hydrosphere. The partial reduction in the dissolved oxygen (DO) levels in water results in hypoxia, whereas its absolute absence is termed anoxia. Such events have been occurring, as paleoindicators prove, and occur nowadays, affecting the local populations of organisms in varying degrees (Isozaki 1997; Wignall & Twitchett 2002; Diaz & Rosenberg 2008; Song et al. 2014).

1.3. Hypoxia in the oceans.

Hypoxia in water is defined as a situation in which DO is partially drained, reaching levels below those necessary to sustain the majority of the biota in the system. Healthy waters are usually close to saturating oxygen levels (around 90±10 % of oxygen capacity), while saturations under 25±5 % are considered detrimental for biota. Diaz and Rosenberg (1995) initially judged hypoxia to occur when oxygen levels are below 2.8 mg O_2/L, but recently most of definitions for hypoxic conditions in marine waters set the threshold at 2.1 mg O_2/L. In general, growth of most of marine animals is blocked below 5 mg O_2/L and non-sessile animals leave the area if DO drops to levels under 2 mg O_2/L (Farrell & Richards 2009).

Hypoxia has been occurring in the history of the Earth under diverse circumstances, having consequences of different magnitude. Natural hypoxic events occur nowadays, affecting the biotic diversity of the affected ecosystems. Short-lived low DO situations may be caused for example by temporary local water column stratification phenomena, which prevent the oxygen saturated surface water from mixing with the deeper layers (Keeling & Garcia 2002). However, hypoxic events of great duration and extent can also naturally arise even with the buffering influence of the MOC; the oxygen minimum zones (OMZs) are a representative example. These areas, which are subjected to extreme hypoxic conditions (less than 0.5 mg O_2/L, about 7.5 % of saturation) for thousands of years, can be found for example in the eastern Pacific Ocean, in the southeast Atlantic off West Africa, and in the northern Indian Ocean (Helly & Levin 2004). Sluggish water circulations and elevated decay of sinking organic matter, primarily due to high primary production promoted by upwelling events in the

surface, cause partial depletion of the oxygen in deeper waters (usually around 200-1000 m depth) (Wyrtki 1962; Glessmer et al. 2009). Such particular conditions of oxygen scarcity promote the development of specialized biotic communities (Levin 2002). Although specific communities thrive in the more steadily affected areas, an enlargement of the OMZs would drastically reduce the diversity of the neighboring ecosystems and affect the biogeochemical cycles (Ulloa & Pantoja 2009).

1.4. Aquatic hypoxia derived from anthropic impact.

Despite the primal state of the oceans and coastal areas includes hypoxic events in different areas or situations, certain anthropogenic influences can push some aquatic ecosystems towards an artificial critical point in terms of oxygen availability. Currently, it is common for large hypoxic events to be caused by excessive anthropogenic input of nutrients and organic matter into water bodies with poor circulation, creating artificial OMZs (Rosenberg et al. 1991). In the late 1940s the industrially produced nitrogen fertilizers began to be massively employed and, consequently, DO explosively declined in the 1960s. The number of dead zones has doubled each decade since then. Nitrogen, which usually constitutes a limiting factor for the marine ecosystems, is swept by the rivers from the farmlands causing eutrophication in the coastal zones (Diaz & Rosenberg 2008). As previously explained, the sinking of excessive organic matter created by the primary producers prompts excessive decay in deeper waters, causing abnormally low DO conditions.

Among the marine systems, data showing a decline in the oxygen concentration was first gathered for the northern Adriatic Sea in the 1950s, as showed by Justic and co-workers (1987). In successive years, other hypoxia-affected places were discovered in different marine systems (Figure 4), including the Black Sea, the Kattegat, the Baltic Sea, Chesapeake Bay, the Gulf of Mexico and the East China Sea among others (Rabalais & Turner 2001; Karlson et al. 2002; Mee et al. 2005; Kemp et al. 2005; Chen et al. 2007; Carstensen et al. 2014). The paleoindicators studied in relation to these areas do not show a natural origin for such hypoxic events (Johannessen & Dahl 1996; Kemp et al. 2005; Rabalais et al. 2007). The overall forecast predicts an increased deterioration in a global scale, with increased occurrence, frequency, intensity, and

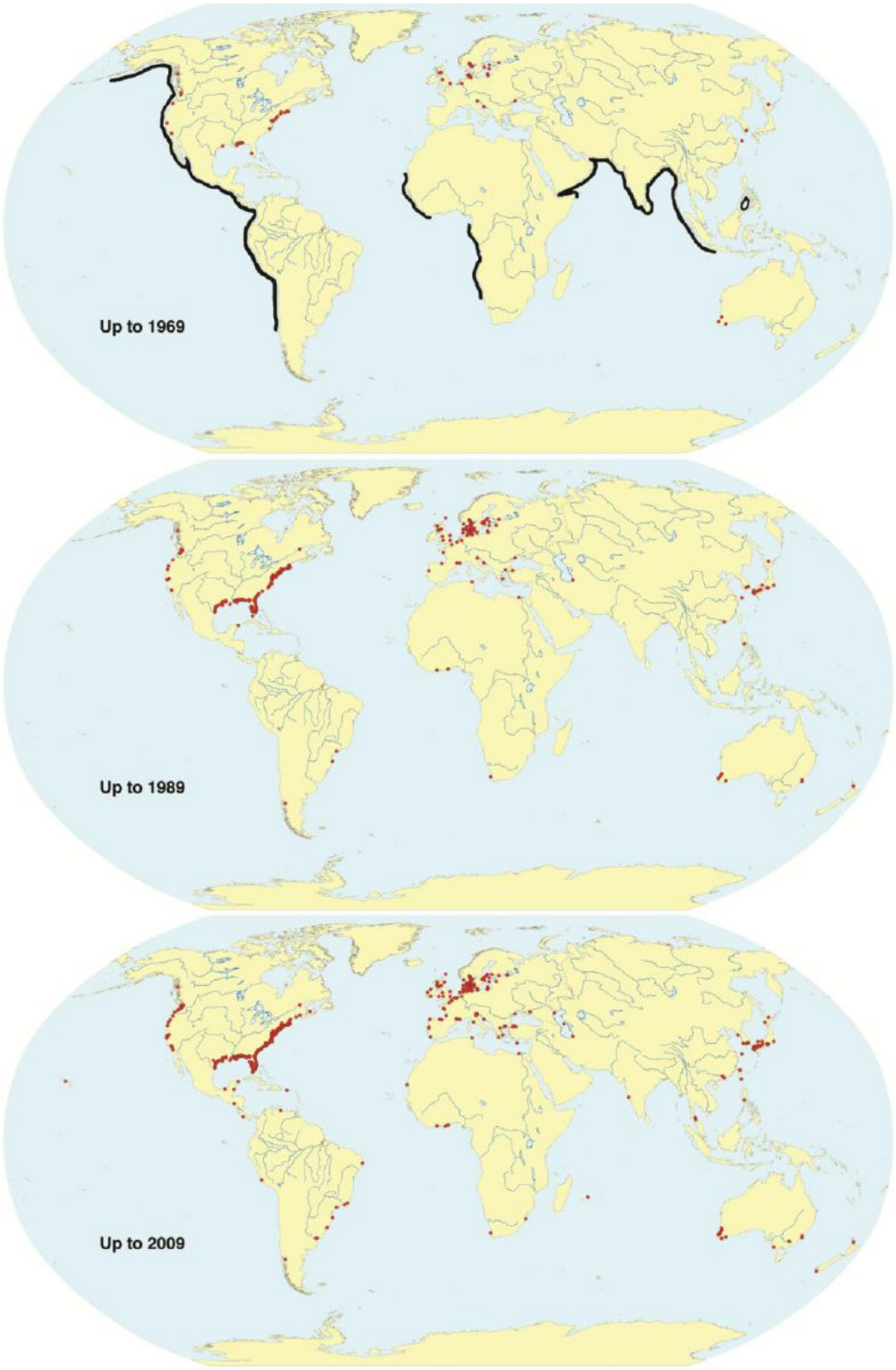

Figure 4: Global scenario and historic development of coastal areas affected by hypoxia. Each dot represents an hypoxic area generated as a consequence of human activities (accumulative through time) while the black lines represent areas threatened by the expansion of oxygen minimum zones (Rabalais et al. 2010).

duration of the hypoxic events (Rabalais et al. 2010). Water masses affected by hypoxic events are also found in the Basque Coast, including the Nervion, Oka, Oiartzun, Artibai, Urola and Bidasoa estuaries. In general, these areas suffer transient oxygen deprivation events that are specially marked in summer (Valencia & Franco 2004; Iriarte et al. 2010).

Among the variables ecologically relevant to marine ecosystems, DO is the one showing the deepest variations in such a short period of time, and still can be more drastically modified (Diaz & Rosenberg 2008). Climate change is predicted to bring further ocean deoxygenation: warmer surface water can house less DO and, in addition, may increase the stratification of the water column, and thus deoxygenation of deep waters (Sarmiento et al. 1998; Keeling & Garcia 2002; Keeling et al. 2010). In a broader scenario, the climate change is expected to modify oceanic currents. For instance, the slowing of the MOC has already been suggested (Quadfasel 2005). Changes in this global system of water circulation may lead to oceans with sluggish movements of water that hamper the distribution of oxygen. Such situation contributed in the past to the greatest of all biotic disasters registered, the Permian-Triassic extinction event (Song et al. 2014).

2. Detection and response to hypoxia.

2.1. Oxygen scarcity: a challenge for the aerobic organisms.

In some exceptional ecosystems oxygen does not play a main role in the biological processes, but as the thermodynamically most favorable electron acceptor for the oxidation of organic matter its presence is essential for a big proportion of the biota in Earth. More than the 95% of the oxygen consumed by a fish in normoxia is derived to the synthesis of adenosine triphosphate (ATP) via the mitochondrial electron transport chain (ETC) that performs the oxidative phosphorylation. Thus, oxygen scarcity leads to the utilization of alternative anaerobic mechanisms that are less productive sources of ATP. In this way, aerobic catabolism of 1 mole of glucose renders around 30 moles of ATP, while the anaerobic catabolism of the same amount of glucose through glycolysis yields only 2 moles of ATP. This reduction of the efficiency in the production of ATP

difficults the maintenance of the cellular energy balance. During undesirable situations, the survival of cells can depend on their ability to reduce their metabolic demand and on the availability of substrates suitable for oxygen-independent ATP synthesis (Richards 2011).

Aside from the DO levels in water, the length and the speed of development of the hypoxic events have to be taken into account. A steep collapse of the DO levels may directly kill the affected organisms, while a gradual decrease could allow for correct adaptation and survival. Moreover, the same organism might trigger different adaptive strategies depending on the duration of the hypoxic event.

2.2. Basic physiological and behavioral animal strategies to endure hypoxia.

The naturally occurring OMZs favor the presence of specialized biotic communities as abundance of nutrients, diminished competition for food and reduced presence of predators partially compensate the disadvantages of low DO levels. While some specialized bacteria from these areas are able to live without employing oxygen, the majority of organisms (including all metazoans such as molluscs, crustaceans or fish) show extremely efficient adaptations to optimize oxygen uptake from the water (Levin 2002).

However, most of the biota in marine ecosystems lives in relatively well oxygenated environments and thus they only need to deploy mechanisms of response to deficient oxygenation conditions when they occur. Such mechanisms differ among species depending on their particular strategies and tolerance to hypoxic conditions. Focusing in fish responses, the most basic and widely used resort during hypoxia is hyperventilation. In these circumstances, the water volume ventilated by the gills is increased to optimize the extraction of DO from the water (Shingles et al. 2005; Vulesevic et al. 2006; Leite et al. 2007). A slightly more specialized strategy deployed by fish is aquatic surface respiration. Some fish species emerge to the water surface and breathe directly this oxygen-rich water, improving their chances to survive during hypoxic events (Shingles et al. 2005; Mcneil & Closs 2007; Lefrançois et al. 2009). More extreme physiological strategies are also employed during hypoxia. Some fish species have evolved bimodal respiration (retaining their gills but being able to breathe air)

and they can increase their reliance on the oxygen extracted directly from the atmosphere, as reviewed by Chapman & McKenzie (2009). There are species that increase their swimming activity when subjected to hypoxic conditions in what it has been considered an avoidance strategy (Herbert & Steffensen 2005; Herbert & Steffensen 2006). Opposed to this, other species relay on an energy-saving response based on the reduction of the swimming activity, which is regarded as a major component of the energy budget of active fish (Dalla Via et al. 1998; Claireaux & Lefrançois 2007; Behrens & Steffensen 2006). Besides, fish can also change their effort in parental care or reproduction in general (Hale et al. 2003). These strategies can be simultaneously performed in different combinations in response to hypoxia, comporting behavioral changes that modify the ecological interactions of affected fish. Predator-prey relationships deserve special attention since the balance between the species that interact in such terms under low DO conditions may be shifted in one sense or the other (Domenici et al. 2007).

Sedentary or sessile organisms have deeper problems than motile ones when facing oxygen scarcity, since the extent of the hypoxic event could easily encompass an area too large for them to escape. That is the case for marine bivalves; several species inhabit areas that present drastic temporal oscillations of DO content (intertidal zones for instance, where they are temporally exposed to the air). Wang & Widdows (1991) studied the behavior of pediveliger larvae of *Mytilus edulis* mussels during their settlement and found no alterations when hypoxic conditions were applied. As seen for fish, bivalves base their reactions to low DO conditions on an enhancement of their water pumping/ventilation system that increases the oxygen intake in gills (Tran et al. 2000). If the detrimental conditions remain or get worse, some species have been identified to also reduce their feeding activity in order to save energy (Sobral & Widdows 1997).

2.3. Hypoxic conditions at the cellular level: hypoxia-inducible factors.

Oxygen became biologically relevant early in the story of Earth, being bound to living organisms initially as a harmful compound and eventually as an essential participant of their energy synthesis mechanisms. Such linkage suggests an early apparition of

general mechanisms developed by biota to detect and adapt to changes in the levels of this gas. The organisms have diverse conspicuous strategies to face hypoxia, but the essential components for the detection and adaptation are found at the cellular level. When oxygen deprivation in the environment translates in tissue and cellular hypoxia, its detection and subsequent adaptation relies in particular molecular mechanisms taking place inside each cell. The detailed mechanics of such important pathways are yet matter of discussion; however, the essential players of the route have been identified (at least in vertebrates) and their roles described.

Of special interest is the helix-loop-helix (HLH) family of transcriptional regulatory proteins, which are fundamental for the control of a wide array of biological processes such as early cell determination and differentiation, cell cycle maintenance, and homeostasis or stress response pathways (Massari & Murre 2000). The focus of this work is centered around a specific branch of the basic HLH group of proteins that contains the PER/aryl hydrocarbon receptor nuclear translocator (ARNT)/single minded (SIM) homology domain (PAS): the so called bHLH/PAS sub-family of proteins (Kewley et al. 2004). The PAS homology domain is present in proteins within the archaea, bacteria and eukaryote domains, and allows the detection of different stimuli such as the level of oxygen, presence of specific ligands or light and redox potential among others (Taylor & Zhulin 1999). In the molecular response to hypoxia, the hypoxia-inducible factors (HIFs) (members of this bHLH/PAS sub-family) have been linked to sensing the levels of cellular oxygen since the apparition of metazoans (Rytkönen et al. 2011). The generic name for these proteins was coined by Semenza & Wang (1992), who found a nuclear factor induced under hypoxic conditions that was able to bind to the promoter region of the human erythropoietin gene and which they termed hypoxia-inducible factor-1 (HIF-1). Afterwards, they purified and characterized this nuclear factor discovering that it was indeed a heterodimer consistent of the union of 2 bHLH/PAS family proteins, namely HIF-1α and HIF-1β (Wang & Semenza 1995). Finally, HIF-1β was identified as ARNT and HIF-1α was recognized to be the key molecule for the metazoan oxygen sensing mechanism (Wang et al. 1995). HIF-1 has been reported to similarly accumulate in cells of diverse animal groups (mammals, fish or molluscs) to perform its functions during exposure to hypoxic conditions (Guo et al 2011, Soitamo et al 2001, Fei & Feng 2008).

Despite the PAS domain has been detected in proteins of non-metazoan organisms, HIF-α molecules seem to have appeared in evolution with the emergence of the early metazoans that bound the PAS domain with a bHLH DNA binding domain (Rytkönen & Storz 2011). As reviewed by Rytkonen and colleagues (2011), the ancestral *hif-α* appeared in first metazoans (as *Trichoplax adhaerens*) and was conserved in the lineage until the whole genome duplications that occurred during early vertebrate evolution resulted in the apparition of 3 different functional copies of the gene (*hif-1α*, *hif-2α* and *hif-3α* paralogs) that were conserved thereafter. The first duplication gave rise to a clade ancestral to *hif-3α* and to another one ancestral to *hif-1α* and *hif-2α* (separated in the second duplication). Both duplication events occurred prior to the divergence between teleosts and tetrapods (Powell & Hahn 2002). The rest of metazoans retained the original *hif-α* gene (Figure 5). Most of the functional studies have been performed in vertebrate species, and, among the three paralogs, HIF-1α is the best studied paralog. ARNT protein is also found in metazoans (arthropods, molluscs, fish, birds, mammals), with its main domains (and thus their specific functions) highly conserved among those groups of animals (Cai et al. 2014).

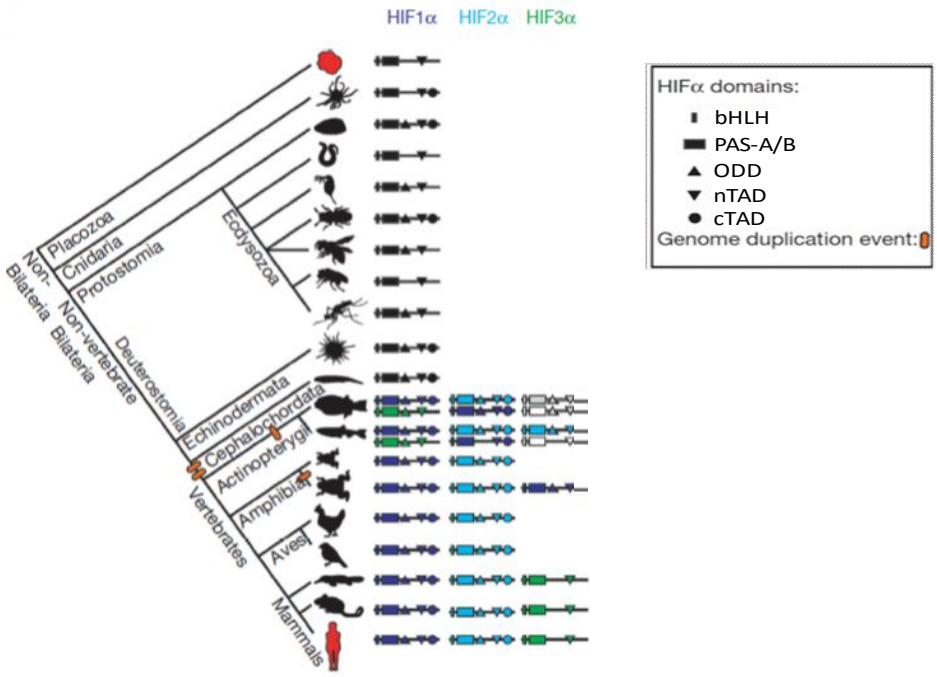

Figure 5: Structural evolutionary analysis of the 3 hypoxia-inducible transcription factor paralogs in metazoans. Ancestral *hif-α* appeared early and it was conserved until the whole genome duplication events occurred during the vertebrate evolution resulted in the actual 3 functional paralog genes *hif1α*, *hif2α* and *hif3α* that found in mammals and teleosts. Structural domains in the proteins coded by each gene are depicted by different geometrical figures (modified from Loenarz 2011).

2.4. Characterization of HIF-1α protein.

The basal HIF-α of amphioxus, previous to the vertebrate diversification, shows similarities with the HIF-1α protein in vertebrates but requires deeper characterization (Gao et al. 2014). The best studied example of the HIF-1α transcription factor (the one belonging to *Homo sapiens*) contains 826 amino acids (Wang & Semenza 1995). Its fish ortholog contains 777 amino acids, in the case of *Danio rerio* (Rojas et al. 2007). Phylogenetic analyses show a high degree of similarity between fish, tetrapod and avian HIF-1α proteins; the amino acid sequence of the protein being also highly conserved among different fish species (Richards 2009).

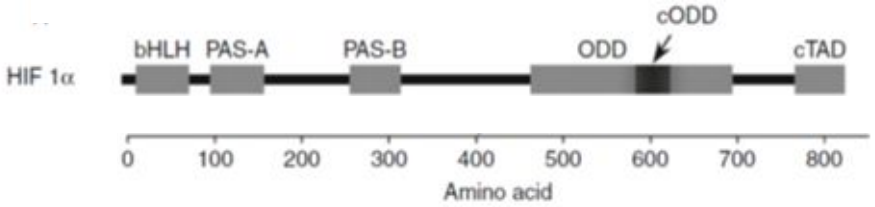

Figure 6: Structural analysis of the human HIF-1α amino acid sequence. Position and size of the basic helix-loop-helix (bHLH), the 2 Per/ARNT/Sim (PAS-A and PAS-B), the oxygen-dependent degradation (ODD) and the C-terminal transactivation (cTAD) domains are depicted (Richards 2009).

As a member of the bHLH/PAS family, HIF-1α features all the domains expected within this family of transcription factors (Figure 6). The bHLH region of the protein consists on several basic amino acids (responsible for direct DNA binding) adjacent to 2 amphipathic α helices separated by a loop of variable length, which forms the primary dimerization interface (Pugh et al. 1997). The PAS domain encompasses 200–300 amino acids containing 2 hydrophobic regions of approximately 50 amino acids termed PAS A and PAS B and forming a secondary dimerization interface with other family members in addition to other roles (Reviewed in Bracken et al. 2003). The molecule contains 2 transactivation domains located in its C-terminal halve: the nTAD or oxygen-dependent degradation domain (ODD, which contains 2 conserved proline residues in its core) and the cTAD (containing a conserved asparagine residue) (Jiang et al. 1997, Reviewed in Kaelin 2005). A high degree of sequence similarity exists between HIF-1α and HIF-2α vertebrate proteins, being the ODD domain non-core region the only zone lacking certain amino acid identity (Powell & Hahn 2002; Rahman & Thomas 2007).

HIF-3α also presents extense sequence similarity with the other two paralogs, with the exception of the cTAD domain which is absent (Gu et al. 1998).

As an example of HIF-α in invertebrates, HIF-α in the bivalve mollusc *Crassostrea virginica* is shorter than in vertebrates with only 701 amino acids. This HIF-α shows significant sequence similarity with its putative vertebrate orthologs sharing key functional domains, which has been interpreted as a clear hint of the highly conserved function of these proteins throughout the evolutionary history of animals (Piontkivska et al. 2011).

2.5. Oxygen sensing system of metazoans.

The classic notion of the molecular mechanism regulated by HIF-αs in response to low oxygen availability describes its regulation to occur post-translationally, both at the protein stability and at transactivation activity levels (Semenza 2011). As proved at least in cells of mammals, birds and fish, HIF-1α resides in the cytoplasm; and under normal oxygenation conditions it is hydroxylated (Figure 7) in one or both of its two proline residues located in the nTAD domain by members of the prolyl hydroxylase domain family (PHD) (Ivan et al. 2001). The enzymes of the PHD family are part of the 2-oxoglutarate and iron (II)-dependent oxygenase superfamily, therefore their activity depends on the levels of available oxygen (Jaakkola et al. 2001). This reaction generates a binding point with the von Hippel-Lindau tumor suppressor protein (pVHL), a member of the ubiquitin-ligase complex, for the polyubiquitination of HIF-1α and its subsequent degradation via proteasome (Kaelin & Ratcliffe 2008). Furthermore, the factor inhibiting HIF-1 (FIH-1), member of the extended 2-oxoglutarate and iron (II)-dependent oxygenase superfamily, is able to hydroxylate the asparagine residue located on the cTAD domain (Elkins et al. 2003). Such reaction blocks the union of the p300/CBP co-activator and thus prevents the activation of HIF-1α that could have escaped from proteolytic degradation (Lando et al. 2002; Kaelin 2005). Both PHDs and FIH-1 are dioxygenases; they employ one oxygen atom to transform 2-oxoglutarate in succinate while the other atom oxidizes a specific amino acid of HIF-1α. The rate of these reactions depends on the concentration of molecular oxygen in the cytoplasm and constitutes the mechanism that allows HIF-1α working as an oxygen sensor (Hirsilä

et al. 2003). As reviewed by Kaelin and Ratcliffe (2008), the K_M for PHDs is close to 100 µM of oxygen (slightly lower for FIH-1), higher than oxygen levels observed in tissues (10 - 30 µM), allowing to modulate their activity depending on the availability of molecular oxygen in all its physiological range.

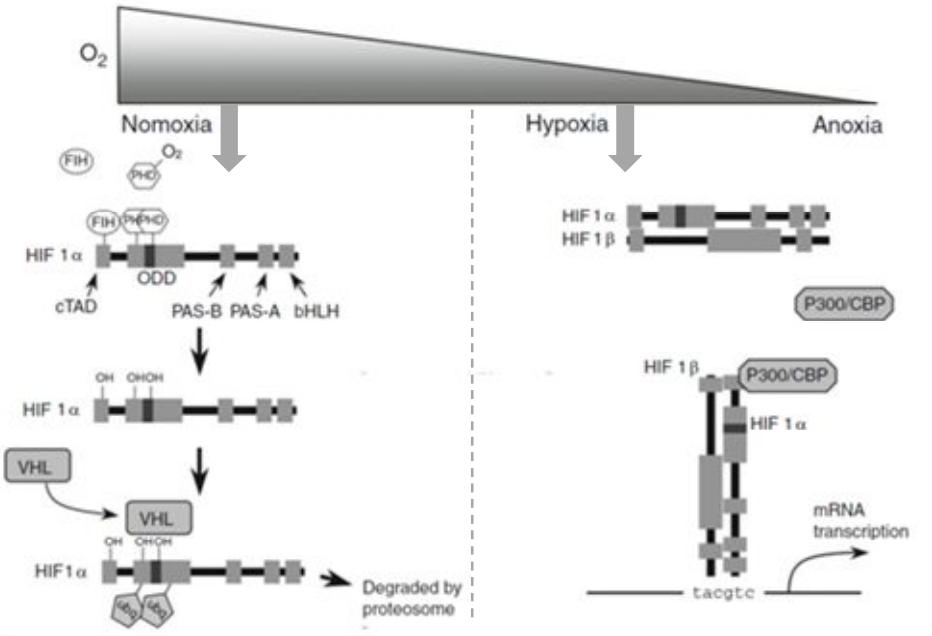

Figure 7: Oxygen-dependent regulation of HIF-1α in chordates. During normoxia, prolyl hydroxylases (PHD) and factor inhibiting HIF (FIH) hydroxylate HIF-1α, labelling it for binding to the von Hippel-Lindau (VHL) protein, ubiquitination and degradation via proteasome. Under oxygen scarcity, PDH and FIH cease their activity, allowing the accumulation of HIF-1α in the cytosol that heterodimerizes with HIF-1β (or ARNT), enters the nucleus, recruits its coactivators (p300/CBP) and binds to HREs on the promoter region of genes involved in the adaptive response to hypoxia (modified from Richards 2009).

The hydroxylation capacity of PHDs and FIH-1 is obstructed by the decrease of the oxygen levels in the cytoplasm. Inhibition of their activity allows HIF-1α to escape degradation and thus, concentration of the protein increases in the cytosol (Greer et al. 2012). Active HIF-1α is able to enter the cell nucleus, where it heterodimerizes with ARNT by means of their bHLH and PAS domains (Kaelin & Ratcliffe 2008). The heterodimer is then able to recruit the p300/CBP co-activator and bind to the hypoxia responsive elements (HREs, containing the core sequence RCGTG) in promoter regions of specific genes to trigger the hypoxia adaptive transcriptional response (Semenza 2007).

Even if the expression of HIF-1α is mainly regulated by post-translational means (Soitamo et al. 2001; Leveelahti et al. 2011), previous studies performed in different

fish species under hypoxic conditions have shown transcriptional up-regulation of this gene (Rahman & Thomas 2007; Terova et al. 2008; Borley et al. 2010; Kopp et al. 2011). As an example, Rimoldi and colleagues (2012) found *hif1α* to be transcriptionally up-regulated in *Perca fluviatilis* under chronic hypoxia while no alterations in transcription levels were observed after acute hypoxia.

The great sequence similarity observed between HIF-1α and HIF-2α suggests a similar activity, regulation pathway and mode of action for both paralogs at the molecular level, including degradation (involving pVHL), protein stabilization (by inactivation of PHDs and FIH-1), heterodimerization with ARNT and DNA binding to HREs (Rahman & Thomas 2007). However, the differences of HIF-2α in comparison to HIF-1α seem to confer it different sensibility to hypoxia (due to differences in the ODD domain) and mRNA expression patterns, as well as the possibility to regulate unique target genes (Powell & Hahn 2002; Raval et al. 2005; Pouysségur & Mechta-Grigoriou 2006; Rahman & Thomas 2007; Keith & Simon 2007). The previously described differences between HIF-1α and HIF-2α sequences allow regulation of a series of genes which mediate not completely overlapping biological responses (Majmundar et al. 2010). Besides, their transcriptional regulation differs depending on the characteristic of the hypoxic conditions and the tissue involved, as observed in diverse fish species (Law et al. 2006; Mohindra et al. 2013). For instance, long-term hypoxia caused a similar up-regulation of both paralogs in ovaries of *Micropogonias undulatus*, while short-term hypoxia (3 days) only increased *hif-1α* mRNA levels without noticeable effect in *hif-2α* transcription (Rahman & Thomas 2007).

On the other hand, HIF-3α conserves the general mode of action described for HIF-1α and HIF-2α but it lacks the cTAD domain, which prevents hydroxylation by FIH-1 (Gu et al. 1998; Greer et al. 2012). In addition, sequence motifs have been found in splice variants of HIF-3α that are absent in HIF-1α and HIF-2α proteins (Maynard et al. 2003). This paralog was initially thought to participate in a negative feedback loop activated by the HIF-1 heterodimer, repressing the hypoxia induced gene expression pathways in vertebrates (Hara et al. 2001; Makino et al. 2007). A recent study in *Danio rerio* embryos described HIF-3α as a transcription factor with its own battery of hypoxia responsive genes, only partially overlapping with that of HIF-1α and HIF-2α proteins (Zhang et al. 2014). Furthermore, HIF-3α was described as the most sensitive of the 3

paralogs due to its quick and deep transcriptional up-regulation in different tissues of *Ictalurus punctatus* held under acute hypoxia (Geng et al. 2014). However, at least six HIF-3α splicing variants have been found in mammals, displaying variable oxygen-sensitivity, ability to dimerize with ARNT, and transcription regulatory functions (reviewed in Dengler et al. 2014).

Loenarz and collaborators (2011) proved the presence of a basal oxygen sensing mechanism present in all metazoans, constituted by the HIF-α-PHD-pVHL triad. However, the prolyl hydroxylases are previous to the apparition of metazoans, as they function as molecular sensors for instance in plants, algae and protozoa controlling their growth and development among others (Gorres & Raines 2010). Metazoans then recruited them to form their oxygen sensing mechanism (Rytkönen et al. 2011). As an example regarding an invertebrate group of animals, accumulation of HIF-α protein was observed for the first time in molluscs by Fei & Feng (2008) in the neural tissue of *Lymnaea stagnalis* after 6 under mild hypoxic conditions. Kawabe & Yokoyama (2012) also reported an increase in *hif-α* mRNA and HIF-α protein levels in gills of oysters *Crassostrea gigas* during an acute hypoxia. Piontkivska and colleagues (2011) characterized the HIF-α and PHD of *C. virginica*, displaying significant sequence similarity and sharing key functional domains with HIF-α and PHD orthologs from vertebrates and other invertebrates. However, the functions and properties of the vertebrate and invertebrate HIF-α and PHD orthologs and the hypoxia adaptive pathways can be defined as similar but not identical. For example, hypoxia tolerance is widespread amongst invertebrates, while the ability to survive and recover from hours to days of oxygen scarcity is rarely found in vertebrates (Gorr et al. 2006).

2.6. Adaptive response to hypoxia.

A cross-kingdom comparison of the transcriptional responses of bacteria, fungi, plants and animals to hypoxia shows shared patterns: increased sugar transport and ROS-related countermeasures, carbon metabolism reconfiguration (increased glycolysis and fermentation) and reduced aerobic respiration and ribosome biogenesis. Nevertheless, the transcription factors and signaling components of the oxygen sensing mechanism have been found to be different in the living organisms compared (Mustroph et al.

2010). Numerous genes have been identified to be regulated by HIF-1 heterodimer in mammals (Table 1). The proteins that these genes encode play key roles in critical developmental and physiological processes such as angiogenesis and vascular remodeling, erythropoiesis, glucose transport, glycolysis, iron transport and cell proliferation and survival (Semenza 2002). Nonetheless, the importance of HIF-1 as a regulatory molecule is not limited to stress situations caused by oxygen deprivation. For instance, this heterodimer results critical in the coordination of development in vertebrates, a process in which low oxygen cellular tensions naturally occur (Semenza 2012a). The regulation of the adaptive responses to hypoxia has gained huge relevance also in the field of cancer research in recent years, especially in the study of the alterations that allow tumor cells to survive in the hypoxic microenvironment usually found in their neighboring areas (Semenza 2013; Kumar & Gabrilovich 2014; Jensen 2015; Chang et al. 2015).

Table 1: General overview of relevant genes regulated by HIF-1 and the diverse cellular processes in which they participate in mammals. A more detailed description of the transcriptional regulation performed by the hypoxia-inducible factors can be found in previous reviews (Wenger et al. 2005; Semenza 2012b; Tsai & Wu 2012; Dengler et al. 2014).

General process	Complete gene name	Abbrev.
Proliferation and Apoptosis	5'-nucleotidase	*cd73*
	Connective tissue growth factor	*ctgf*
	Endoglin	*eng*
	Insulin-like growth factor-binding protein 3	*igfbp3*
	Trefoil Factor 3	*itf*
	MET Proto-Oncogene, Receptor Tyrosine Kinase	*met*
	Nuclear Receptor Subfamily 4, Group A, Member 1	*nr4a1*
	DNA-Damage-Inducible Transcript 4	*redd1*
	Retinoic acid receptor-related orphan receptor-α 4	*rorα4*
	Serine/Threonine Kinase-15	*stk15*
	Telomerase reverse transcriptase	*tert*
	Transforming growth factor β 3	*tgfβ3*
	Wilms tumor 1	*wt1*
Stemness and self-renewal	Adrenomedullin	*adm*
	Endothelin 1	*edn1*
	Erythropoietin	*epo*
	Glucose phosphate isomerase	*gpi*
	Inhibitor of DNA binding 2	*id2*
	Insulin-like growth factor 2	*igf2*
	Phosphoglucomutase	*pgm*
	Octamer-binding transcription factor 4	*oct-04*
	Telomerase reverse transcriptase	*tert*
	Transforming growth factor α	*tgfα*
	Vascular endothelial growth factor	*vegf*

Table 1 (Continued)

General process	Complete gene name	Abbrev.
Metabolic reprogramming	Fructose-bisphosphate aldolase A	*aldoa*
	Fructose-bisphosphate aldolase B	*aldob*
	BCL2/adenovirus E1B interacting protein 3	*bnip3*
	BCL2/adenovirus E1B interacting protein 3-like	*bnip3l*
	Carbonic anhydrase 9	*ca9*
	Cbp/P300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	*cited2*
	Cytochrome c oxidase subunit IV isoform 2	*cox4i2*
	Deleted in esophageal cancer 1	*dec1*
	Deleted in esophageal cancer 2	*dec2*
	Enolase 1	*eno1*
	V-Ets avian erythroblastosis virus E26 oncogene homolog 1	*ets1*
	Glyceraldehyde-3-phosphate dehydrogenase	*gapdh*
	Glucose transporter type 1	*glut1*
	Glucose transporter t 2	*glut3*
	Glucose phosphate isomerase	*gpi*
	Hexokinase 1	*hk1*
	Hexokinase 2	*hk2*
	Inhibitor of DNA binding 2	*id2*
	Lactate dehydrogenase A	*ldha*
	Lon Peptidase 1, mitochondrial	*lonp1*
	Monocarboxylate transporter 4	*mct4*
	Monocarboxylate transporter 4	*mxi1*
	Na^+/H^+ exchanger isoform 1	*nhe1*
	Pyruvate dehydrogenase kinase, isozyme 1	*pdk1*
	Phosphoenolpyruvate carboxykinase	*pepck*
	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1	*pfkfb1-4*
	Phosphofructokinase, liver	*pfkl*
	Phosphoglycerate kinase 1	*pgk1*
	Phosphoglucomutase 1	*pgm*
	Pyruvate kinase, muscle	*pkm2*
	Transketolase	*tkt*
	Transketolase-like 2	*tktl2*
	Triosephosphate isomerase 1	*tpi1*
Redox homeostasis	Glutathione peroxidase 3	*gpx3*
	Heme Oxygenase 1	*hmox1*
	Superoxide Dismutase 2	*sod2*
Metastasis and invasion	Autocrine motility factor	*amf*
	Angiopoietin-like 4	*angptl4*
	Cathepsin C	*ctsc*
	Chemokine (C-X-C motif) ligand 12	*cxcl12*
	Chemokine (C-X-C motif) receptor 4	*cxcr4*
	L1 cell adhesion molecule	*l1cam*
	Lectin, galactoside-binding, soluble, 1	*lgals1*
	Lysyl oxidase	*lox*
	Lysyl oxidase-like 2	*loxl2*
	Lysyl oxidase-like 4	*loxl4*
	Matrix metallopeptidase 1/2/9/14	*mmp1/2/9/14*
	Plasminogen activator, urokinase receptor	*plaur*
	Stanniocalcin 2	*stc2*
	Twist Family BHLH Transcription Factor 1	*twist1*

Table 1 (Continued)

General process	Complete gene name	Abbrev.
Oxygen supply and angiogenesis	Adrenomedullin	*adm*
	Angiopoietin 1	*angpt1*
	Angiopoietin 2	*angpt2*
	Atrial natriuretic peptide	*anp*
	ATP-binding cassette sub-family G member 2	*brcp*
	Ceruloplasmin	*cp*
	Chemokine (C-X-C motif) ligand 12	*cxcl12*
	Endothelin 1	*edn1*
	Erythropoietin	*epo*
	Ferrochelatase	*fech*
	Vascular endothelial growth factor receptor 2	*flk1*
	Vascular endothelial growth factor receptor 1	*flt1*
	Glucose phosphate isomerase	*gpi*
	Heme Oxygenase 1	*hmox1*
	Leptin	*lep*
	Nitric oxide synthase (inducible)	*nos2*
	Nitric oxide synthase (endothelial)	*nos3*
	NADPH oxidase 2	*nox2*
	platelet-derived growth factor β	*pdgfβ*
	Placental growth factor	*pgf*
	Serpin Peptidase Inhibitor, Clade E , Member 1	*serpine1*
	Transferrin	*tf*
	Vascular endothelial growth factor	*vegf*
Epithelial-mesenchymal transition	Inhibitor of DNA binding 2	*id2*
	Snail family zinc finger 1	*snai1*
	Snail family zinc finger 2	*snai2*
	Transcription factor 3	*tcf3*
	Transforming growth factor α	*tgfα*
	Vimentin	*vim*
	Zinc finger E-box binding homeobox 1	*zeb1*
	Zinc finger E-box binding homeobox 2	*zeb2*

In essence, the overall metabolism of the cell is reprogrammed during oxygen scarcity by HIF-1 activation. Pivotal energetic pathways such as oxidative phosphorylation and the tricarboxylic acid cycle (TCA - main source of reducing power to fuel the production of ATP) are regulated by HIF-1 (Semenza 2013). Briefly, HIF-1 optimizes the utilization of O_2 and glucose under hypoxic conditions to generate ATP in a more efficient way (Figure 8), mostly by enhancing anaerobic glycolysis (involving lactic acid fermentation) in detriment of oxidative phosphorylation (Semenza 2007). This switch causes a decrease of ATP production; therefore, a general decrease of the metabolic ATP demand is coordinated by HIF-1 (mostly by limiting the maintenance of electrochemical gradients and the protein turnover) to reach a bioenergetic balance (Wheaton & Chandel 2011). The optimized regulation of this metabolic switch by HIF-1

seems to be quite basal in the evolutionary history of metazoans, and probably a key to their adaptive success (Webster 2003).

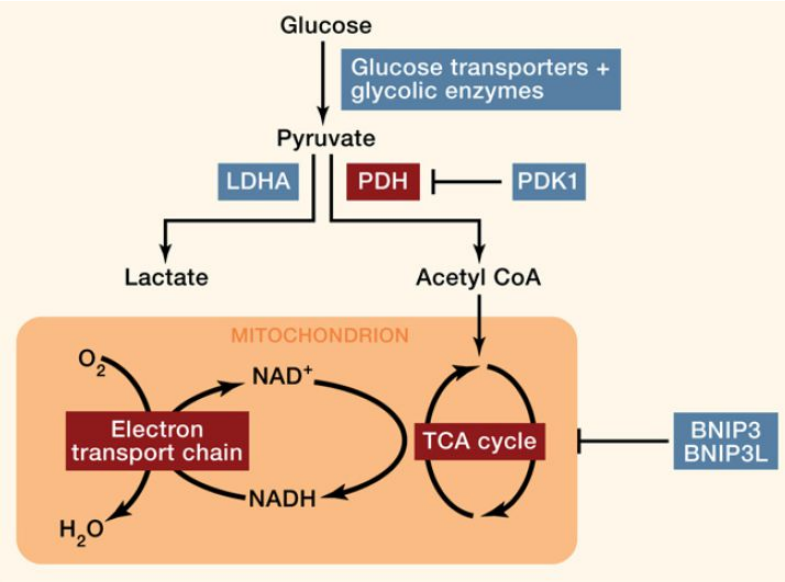

Figure 8: Under hypoxic conditions, hypoxia-inducible factor 1 (HIF-1) up-regulates glucose transporters, genes coding for glycolytic enzymes (increasing the flux of glucose to pyruvate) and lactate dehydrogenase (LDHA). It also enhances pyruvate dehydrogenase kinase (PDK1) which inactivates pyruvate dehydrogenase (PDH), blocking the synthesis of acetyl CoA and thus the tricarboxylic acid cycle. In addition, HIF-1 triggers the expression of proteins causing mitochondrial selective autophagy (BNIP3 and BNIP3L). In brief, glucose tends to be converted into lactate, instead of fueling the electron transport chain, reducing ATP production while preventing excessive ROS production.(Semenza 2012a)

Accordingly, in aquatic animals, the general sequence of adaptation to hypoxia begins with an increase in the capacity to deliver oxygen; and continues with the boosting of the efficiency of the remaining metabolic processes that has just been described (deriving energy from anaerobic sources) in combination with a reduction of the energy expenditure (Wu 2002). Such metabolic constraints cause a reduction in growth and fecundity, which in certain populations may subsequently lead to population decline.

Studies performed in different fish species analyzed their response to hypoxic events using expression microarrays; the results showed slight variations among tissues, but the gene expression profiles corroborated the sequence of adaptive pathways mentioned (Gracey et al. 2001; Ton et al. 2003; van der Meer et al. 2005; Ju et al. 2007; Zhang et al. 2012). In summary, under hypoxic conditions, fish increase their gill surface area, enhance erythropoiesis, synthesis of hemoglobin and angiogenesis, up-regulate glycolysis (in detriment of aerobic energy sources), elevate glucose synthesis

and transport and suppress their growth and energy expenditure (Nikinmaa & Rees 2005). In this regard, less information is available for invertebrate species; however, the expected metabolism switch from aerobic to anaerobic energy production was observed in *C. gigas* under hypoxic and anoxic conditions (Moullac et al. 2007; Kurochkin et al. 2009). Accordingly, the same species was found to coordinately decrease its ATP demand during hypoxia (David et al. 2005). Besides, a linkage between hypoxia and thermal stress has been proposed, since hypoxic events increase the transcription of heat shock-related genes in mollusc species (Fei & Feng 2008; Kawabe & Yokoyama 2009; Kawabe & Yokoyama 2011).

2.7. Regulation of the classical hypoxic response independent of oxygen levels.

The amount of functions affected by the response to low oxygen concentrations apparently require a wide battery of messengers towards a coordinated set of actions. The biology of this response has been extensively studied, unraveling signals different from oxygen availability that participate in the regulation of the described adaptive pathways. The proteins regarded as oxygen sensors in animals, PDHs and FIH-1, are also regulated by reactive oxygen species (ROS), ascorbate and TCA intermediaries due to the nature of their catalytic iron centre (Figure 9) (Ratcliffe 2013). ROS are able to change the state of the required ferrous (Fe^{2+}) cofactor to ferric iron (Fe^{3+}), hampering the activity of the PDHs and FIH-1 and therefore increasing the half-life and the activity of HIF-αs. Ascorbate, in return, regenerates Fe^{3+} to Fe^{2+} iron in the catalytic center; thus, scarcity of this antioxidant could also block the activity of these hydroxylases. Hydroxylation of HIF-αs in turn produces succinate as by-product, and elevated concentrations of this TCA intermediary also causes the inhibition of these hydroxylases (Pouysségur & Mechta-Grigoriou 2006).

ROS can be generated by NADPH oxygenases, but the main source is the mitochondrial ETC, producing even more ROS during hypoxic events (Cash et al. 2007). Seemingly, nitric oxide (also a reactive species) is related to the response to hypoxia, but the attempts to describe the specific mechanisms has yielded controversial results so far (Webb et al. 2009).

A group of novel deacetylases able to sense the changes in ROS levels and in NAD^+/NADH ratio, called sirtuins (Sirt), have been recently discovered to play a role in the adaptive response to hypoxia as well (Majmundar et al. 2010). They feature Sirt1, which reduces the activity of HIF-1α, while identical deacetylation of HIF-2α causes the increase of its activity. Besides, Sirt3 reduces the presence of ROS (and probably succinate), increasing the activity of the HIF-α hydroxylases and therefore destabilizing HIF-1α in an indirect way (Greer et al. 2012). Sirt6 can additionally regulate glucose homeostasis by inhibiting HIF-1α, but also by directly modifying glycolytic gene expression (Majmundar et al. 2010).

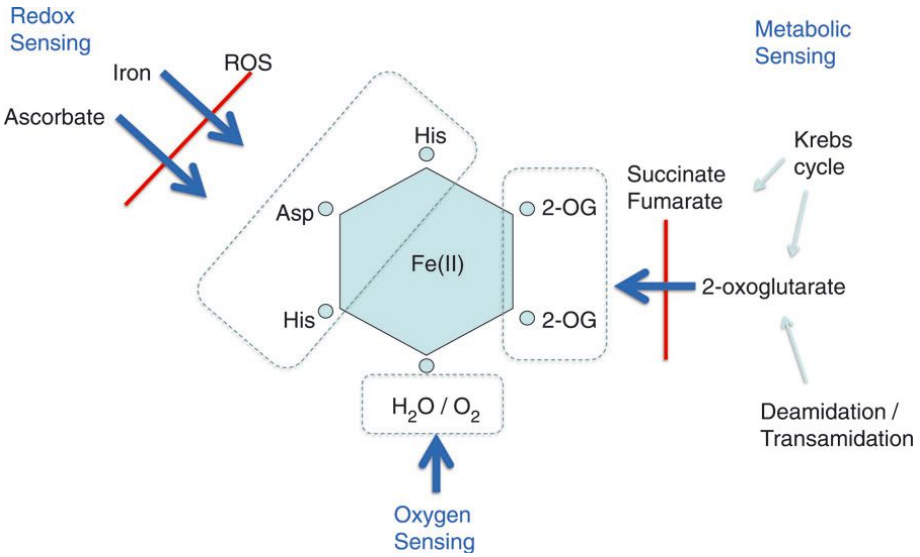

Figure 9: Representation of the catalytic iron centre of a 2-oxoglutarate-dependent dioxygenase. 3 of the 6 co-ordinate positions of the prosthetic group have the role of redox sensing, another 2 are in charge of the metabolic sensing and the last one receives oxygen during the reaction, acting as an oxygen sensor (Ratcliffe 2013).

Moreover, different cell growth stimuli (as the IGF/PI3K/Akt pathway for example) increase the concentration and activity of HIF-1α, as a mechanism to balance the increased oxygen consumption occurring during cell growth (Wenger et al. 2005). On the other hand, AMP activated kinase increases its activity if the concentration of AMP and/or ROS increase, reducing the synthesis of proteins and increasing glucose uptake and glycolytic activity as an oxygen-independent reaction to hypoxia (Zhu et al. 2013). In addition HIF-αs can also be acetylated or even SUMOylated (by small ubiquitin-like modifiers) and subsequently degraded via proteasome (Webb et al. 2009).

Positive feedback loops have also been described for this kind of responses: the M2 isoform of pyruvate kinase (PKM2), that catalyzes the last reaction of the glycolysis, is up-regulated by HIF-1α; and in turn, PKM2 increases the activity of HIF-1α (Greer et al. 2012). However, the HIF-1 cascade under normoxia and sustained hypoxia is only transiently active due to different negative feedback loops, including the synthesis of anti-sense RNA for HIF-α, the enhanced transcription of HIF-α hydroxylases and the production of CITED2 that sequesters the co-activators of the HIF-αs (Webb et al. 2009).

3. Interaction of the hypoxia adaptive and the xenobiotic detoxification pathways.

3.1. Ligand-activation of the aryl hydrocarbon receptor.

As it has been said before, the bHLH-PAS protein sub-family in metazoans includes transcription factors involved in many different signaling mechanisms during development and in response to a wide array of changes in the cellular environment besides oxygen availability (Hahn et al. 2003; Goldstone et al. 2006). The large amount of environmental fluctuations to be monitored by the cell, their possible combinations and the relatively small amount of sensors available in the cells allow thinking about molecular cross-talk among the molecules regulating adaptive pathways as a quite frequent phenomenon.

ARNT (also termed HIF-1β) was described for the first time as the heterodimeric partner required by the aryl hydrocarbon receptor (AHR), another member of the bHLH-PAS sub-family, to trigger the transcriptional responses allowing detoxification of different environmental pollutants and toxic bioactive chemicals (Hoffman et al. 1991). In the cytosol, AHR detects and binds xenobiotic compounds such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Poland et al. 1976). Endogenous ligands for this transcription factor have remained largely unknown (Denison & Nagy 2003). Several candidates, such as the tryptophan metabolites FICZ and ITE or indoxyl sulfate, have been proposed recently (Tian et al. 2015). Binding such ligands promotes the translocation of AHR to the nucleus (Figure 10). There, it forms a heterodimer with

ARNT, the activated heterodimer then binds to the xenobiotic responsive elements (XREs) found in the promoter region of target genes. Many of such genes, like those coding for several cytochrome p450s (CYPs), are related to the drug/xenobiotic detoxification metabolism and are up-regulated by the active AHR/ARNT heterodimer (Zhou et al. 2010).

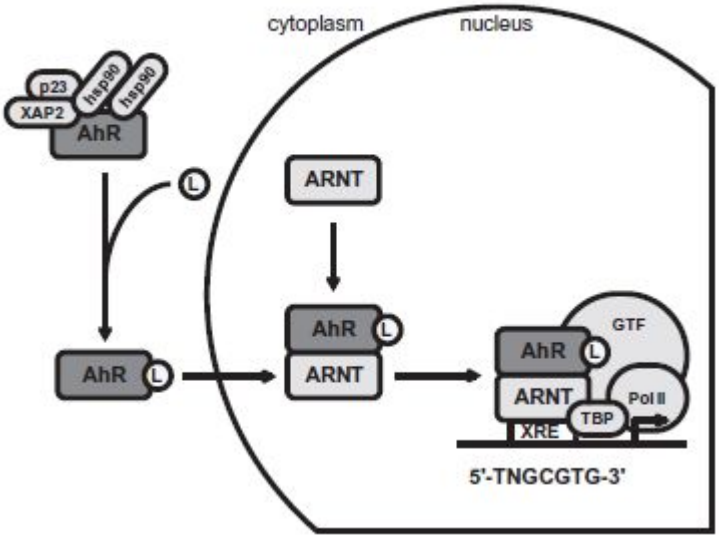

Figure 10: The aryl hydrocarbon receptor (AHR) signaling pathway. In absence of ligand (L), AHR is bound to various repressors in the cytoplasm. After ligand binding AHR is activated, allowing its translocation to the nucleus and the subsequent dimerization with ARNT. This heterodimer (assisted by diverse co-activators) binds to specific xenobiotic responsive elements (XREs) in the promoter region of genes related to the xenobiotic metabolism (Vorrink & Domann 2014).

In general, chordates up-regulate the expression of AHR upon exposure to its agonists (Denison et al. 2002). In the case of fish, specific events of gene duplication and diversification have resulted in the appearance of at least two AHR paralogs, increasing the diversity of the AHR gene family (Hahn 2006). In mammals the xenobiotic detoxification response is unchained by their only AHR gene (*ahr1*), while in fish both genes participate in the process being *ahr2* the paralog that is most regulated at the transcriptional level (Hahn 2005).

Butler and colleagues (2001) described the first mollusc AHR sequence (DNA and protein) in the soft-shell clam *Mya arenaria*; it clustered with invertebrate sequences which resembled AHR of vertebrates due to the presence of similar transactivation domains. However, these proteins seem to present different ligand-binding domain characteristics, suggesting that the dioxin responsiveness of the protein appeared in the chordate lineage while the invertebrate “AHR-like protein” agonists (if any) are yet

to be discovered. Similar sequences have been found thereafter in other mollusks, and in *Dreissena polymorpha* mussels increased transcription levels of *ahr* have been described after exposure to metoprolol (a β-adrenergic blocking agent). On the other hand, exposure to levonorgestrel (a synthetic progestin) caused the *ahr*-like mRNA levels to decrease (Contardo-Jara 2010, 2011). Both compounds are processed by phase I metabolism enzymes in vertebrates, so this questions the direct involvement of the AHR-CYP axis in their detoxification in molluscs. However, Tian and associates (2013) observed a transcriptional up-regulation of *ahr, arnt* and *cyp1a1* after moderate exposure of *Chlamys farreri* scallops to benzo[a]pyrene (B[a]P), suggesting an involvement of AHR in the regulation of CYP1A1 under B[a]P exposure.

3.2. Competitive cross-talk for ARNT.

The fact that two transcriptional regulatory cascades (hypoxia adaptive and xenobiotic detoxification pathways) converge on a single pivotal protein, ARNT, allows to hypothesize that competition could exist between HIF-αs and AHR to bind their heterodimeric partner. The potential exposure of organisms to environmental pollutants under hypoxic conditions could thus severely affect their ability to adapt to both stressors at once due to competition for ARNT (Vorrink & Domann 2014).

Some studies have been performed with the aim of studying such competition. Most of the results obtained indicate interplay between both pathways, in some cases centered at the ARNT level (Figure 11), but the precise mechanism by which the interference occurs remains elusive yet (Vorrink & Domann 2014). Initial studies on mammalian cell lines for example suggested that the wide range of toxic effects of TCDD were caused by the sequestration of ARNT by active AHR from other heterodimeric partners such as HIF-1α, changing the capacity of those transcription factors to regulate the expression of specific genes (Nie et al. 2001). Later, Allen and colleagues (2005) studying mouse hepatocytes exposed to 3-methylcholanthrene under hypoxic conditions, deduced that hypoxia reduced the activity of AHR but not through depletion of existing ARNT. This one-way interaction was also studied in human lung endothelial cells, in which hypoxia also reduced the constitutive transcription of *ahr* and *cyp1a* in a HIF-2α-dependent manner (Zhang & Walker 2007).

On the other hand, in human cell lines a two-way interaction was described (hypoxia reducing AHR activity and xenobiotics doing the same with HIF-1α activity); while PHD2 expression was found also to be regulated in an AHR-dependent way. In any case, the interaction between both pathways is not caused by competition for ARNT nor by PHD2 modulation (Seifert et al. 2008). Schults and co-workers (2010) also reported such a two-way interaction in human lung carcinoma cells exposed to B[a]P under simulated hypoxic conditions (HIF-1α stabilization by $CoCl_2$). They concluded that this was caused by competition for ARNT. Human skin- and liver-derived cell lines exposed to PBC-126 reduced their AHR activation and CYP1A1 expression levels under hypoxic conditions, and such effect was discussed as, at least partially, caused by competition for ARNT (Vorrink et al. 2014).

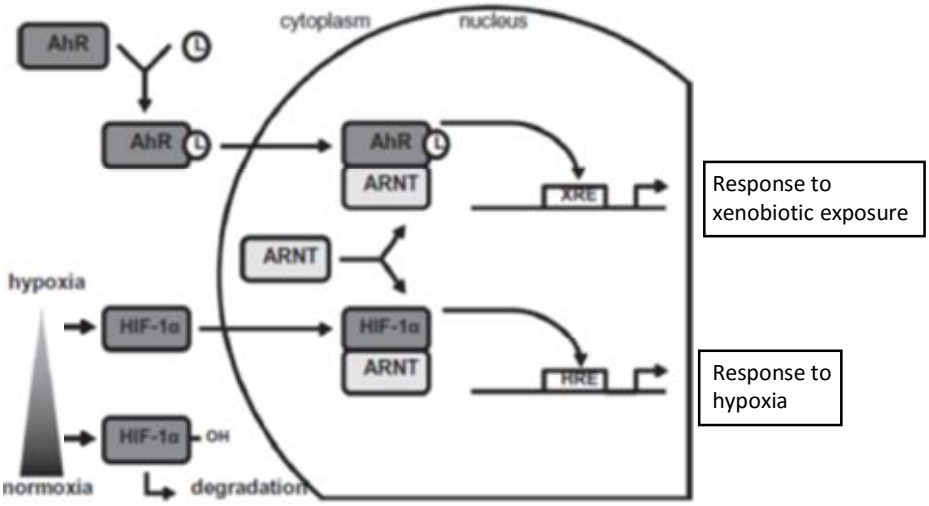

Figure 11: Crosstalk at the AHR-ARNT-HIF-1α signaling node. AHR ligand represented by (L). Both pathways require ARNT to be active (modified from Vorrink & Domann 2014).

Aquatic animals such as fish constitute an optimal matrix for the study of the effects of hypoxic conditions and, therefore, for the study of the cross-talk at the ARNT level (Nikinmaa & Rees 2005). Hypoxic conditions modified the response of zebrafish embryos to TCDD exposure (decreased *cyp1a* transcription levels); while no evidence of any effect in the hypoxia responsive genes was found (Prasch et al. 2004). Anyway, the level of ARNT could not be defined as the bottleneck in this process. On the contrary, in the study performed by Kraemer & Schulte (2004) in which *Fundulus heteroclitus* were injected with PCB-77 prior to a reduction in oxygen supply for 3 days, the induction of the glycolytic enzymes caused by hypoxia alone was blocked. In

opposition to both previous studies, exposure of *Cyprinodon variegatus* embryos (whose tissues present oxygen tensions that prevent degradation of HIF-αs) to pyrene caused the up-regulation of *cyp1a1*, whereas *vegf* was unaltered (Hendon et al. 2008). This suggested that intracellular levels of ARNT were sufficient to support both adaptive pathways. However, and contributing to increase the complexity of the matter, Yu and colleagues (2008) observed enhanced hepatic transcription levels of several hypoxia-related genes while xenobiotic-related ones were unaltered, after exposing *Epinephelus coioides* to B[a]P under hypoxic conditions. Such response was apparently caused by the increased production of ROS. In a posterior study employing a *Poeciliopsis lucida* cell line separately exposed to B[a]P, PCB-126 and benzo[k]fluoranthene under hypoxic conditions, Fleming and co-workers (2009) observed a reduction of the activity of AHR elicited by exposure alone in all cases, which was abolished by over-expression of ARNT (transfection of ARNT expression vectors). In the same study, B[a]P was the only compound able to reduce the activity HIF, an effect maintained even after over-expression of transfected ARNT. This suggests a one-sided crosstalk in which HIF-1α can sequester ARNT from AHR but not *vice versa*. Divergent results were also found in simple treatment experiments: acute hypoxia (3-48 h) increased hepatic *cyp1a2* mRNA levels and ethoxyresorufin-O-deethylase (EROD) activity in *Gasterosteus aculeatus* (Leveelahti et al. 2011), while liver *cyp1a* mRNA levels and EROD activity were down-regulated under chronic hypoxia (1-4 weeks) in *Micropogonias undulatus* (Rahman & Thomas 2012).

Although several mechanisms have been proposed to explain the interferences observed between the hypoxia adaptive and the xenobiotic detoxification pathways, none has proved to be fully explanatory so far. The role of different key players such as ARNT and ROS in that interplay needs to be revealed yet.

3.3. Gulf of Mexico: an example of the presence of AHR agonists in hypoxic waters.

On April 20, 2010, the workers of the mobile offshore drilling unit *Deepwater Horizon* were temporarily closing the exploratory Macondo oil well, 67 km Southeast of Venice, Luisiana (U.S.). At 10 p.m., U.S. Coast Guards were reported of an explosion and fire aboard the platform, in which 11 workers finally died. On April 29, the incident was

declared Spill of National Significance, becoming the first time such designation was used by the U.S. Federal Government. On May 17, the flow rate from the source was estimated to be between 1.91×10^6 and 3.02×10^6 L of oil per day by a technical team. On September 19, it was announced that the Macondo well was effectively sealed (National Response Team 2011). Finally, the *Deepwater Horizon* oil spill in the Gulf of Mexico released about 6.52×10^8 L of crude oil (Figure 12), constituting the largest accidental marine oil spill to date (McNutt et al. 2012). Close to 1.7×10^{11} g of C_1-C_5 hydrocarbons (the larger ones were not estimated) were released; besides, since it was a deep water oil spill, high residence times far from the atmosphere caused petroleum compounds to be dissolved in the water column to an abnormally high extent (Reddy et al. 2011). The surface oil slick reached a maximum of 75000 km^2 on May 24, 2010 (analyzed by Skytruth 2010).

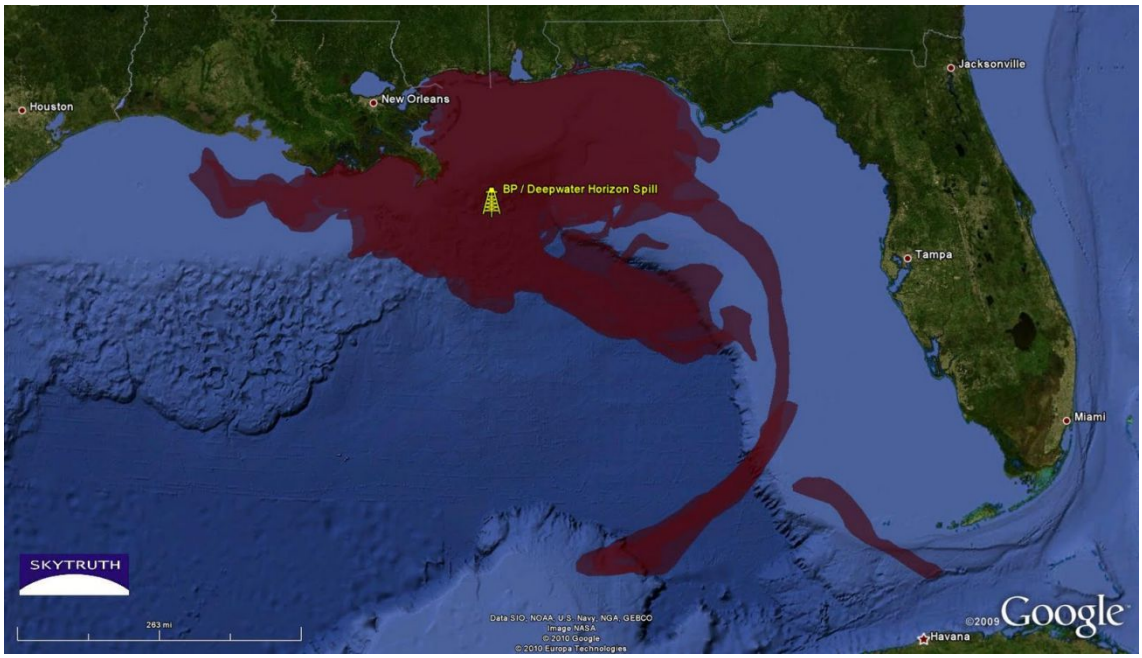

Figure 12: Extension of the surface oil slick caused by the Deepwater Horizon spill in the northern Gulf of Mexico. Satellite images indicated that the surface slick reached a maximum of 75000 km^2. It was estimated that the total oil-slick footprint reached 176119 km^2. Satellite images analyzed by Skytruth (2010).

On the other hand, the Gulf of Mexico houses the second largest anthropic OMZ of the planet. The Mississippi River forms a watershed of 3.27×10^6 km^2 which discharges on average 580 km^3 of freshwater per year in the Gulf of Mexico through its 2 main distributaries, the birdfoot delta (Close to New Orleans, Louisiana, U.S.) and the Atchafalaya River delta (200 km to the west). This way, 210×10^6 Mg of sediment and

$1.6x10^6$ Mg of nitrogen per year are discharged in the area, especially affecting the continental shelf ecosystem to the west of the birdfoot delta (Reviewed by Rabalais et al. 2007). The local OMZ is caused by the combination of water column stratification and high phytoplankton growth fueled by the elevated influx of nutrients (Figure 13). Increased productivity raises the amount of sinking organic material that decomposes, depleting the oxygen in the water column below the pycnocline, which in turn prevents it from mixing with the oxygenated upper water layers (Bianchi et al. 2010). The discharge of nutrients has doubled or tripled since 1950s, mostly due to extensive corn and soybean row crops in tile-drained fields. Paleoindicators and models relate the presence of the hypoxic zone and the nitrogen load of the Mississippi, stating it as a large-scale phenomenon from the early 1970s (reviewed by Rabalais et al. 2010). The area of bottom water affected by the described hypoxic conditions (dissolved oxygen below 2 mg O_2/L) reached a maximum of 22000 km^2 in 2002, and it was above 15000 km^2 in 2006 (Rabalais et al. 2006).

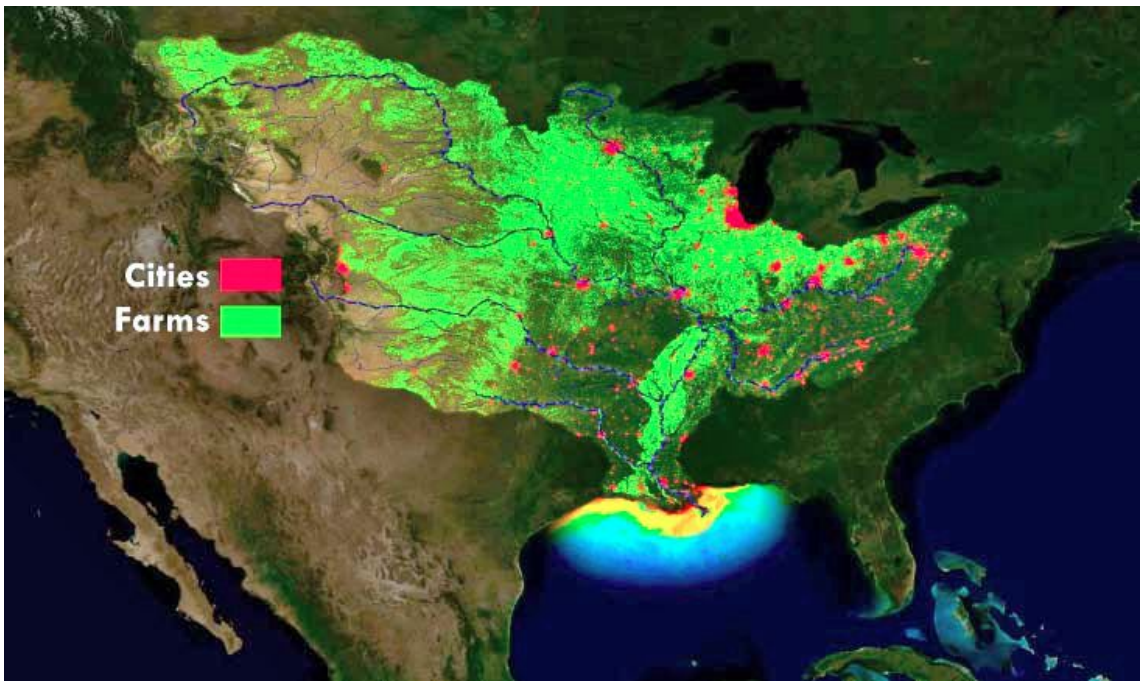

Figure 12: Hypoxic zone in the Northern Gulf of Mexico. Cities and farms located in the Mississippi River basin are shown in pink and green respectively. The extent of the hypoxic affection is shown in light blue, yellow and red, this last one indicates the most deeply affected areas (Image by NOAA). The area of water affected by hypoxia peaked at 22000 km^2 in 2002 and averaged 13500 km^2 between 1985 and 2009 (Rabalais et al. 2010).

In the case of the oil spill, a clear shift towards more carnivorous and scavenger benthic communities was reported right after the environmental catastrophe (Bik et al.

2012). The released xenobiotics were also detrimental for the fish affected (Incardona et al. 2014). The naphthalene metabolite levels found in demersal fish *Lopholatilus chamaeleonticeps* (240 μg of naphthalene equivalents per g of wet bile) captured in 2012 in the Gulf of Mexico were amongst the highest concentrations measured in fish to date (Snyder et al. 2015). Apparently, it will take more than decades for the northern Gulf of Mexico to recover from the disturbances caused by the Deep Water Horizon oil spill (Montagna et al. 2013).

Regarding the hypoxic conditions of the described OMZ, the major biotic community shifts began in the mid 1950s, as prevalence of eutrophication/hypoxia foraminiferan indicators show (Osterman et al. 2009). The local macrofauna has also been affected by oxygen scarcity, as seen for the disrupted distribution of fish *Micropogonias undulatus* and shrimp *Farfantepenaeus aztecus* (Craig & Crowder 2005). In addition, reproductive impairment has been reported in *M. undulatus* in the zone, which could lead to long-term impacts on population abundance (Thomas & Rahman 2012).

The previously described interactions between the hypoxia adaptive and the xenobiotic detoxification pathways gain relevance in situations such as the one of the northern Gulf of Mexico, where hypoxic conditions and presence of xenobiotics (more specifically, AHR agonists) co-occur. The cross-talk between the pathways regulated by AHR and HIF-αs would narrow the window of molecular tolerance to simultaneous exposure to hypoxic/polluted waters, predisposing animals to suffer increased toxicity and, therefore, raising the importance of studying the areas affected by both stressors.

4. Selected experimental organisms and biomarkers.

4.1. Fish.

The most studied fish species in developmental biology are zebrafish *Danio rerio* and medaka *Oryzias latipes*, considered model organisms for the study of human diseases. Nowadays, the suitability of fish to perform basic and applied research has been proven, as evidenced by the constant growth in the number of species included in scientific studies (Schartl 2013). In this context, fish are considered adequate model organisms to analyze biological effects of environmental variables, including pollution

and oxygen scarcity (Van der Oost et al. 2003; Nikinmaa & Rees 2005; Kane et al. 2005; Zhou et al. 2008).

In relation to environmental health assessment, it is conventionally said that a good pollution sentinel species has to fulfill several conditions: having a wide geographical distribution, a high trophic status and the ability to bio-accumulate pollutants while being easy to capture, study and maintain in captivity (Suter 1993). Having a well-known biology and being sensitive but also resilient to exposure to pollutants is also advisable. As reviewed by Ortiz-Zarragoitia and colleagues (2014), mugilids constitute an appropriate group of fish for this purpose, given their widespread distribution and their ability to endure and respond to highly stressing situations. The most representative mugilid species in the Basque Coast is the thicklip grey mullet (*Chelon labrosus*), whose distribution ranges from the African coast in front of the Canary Islands to the coast of Norway, including the Mediterranean Sea and the British Islands. Thicklip grey mullets are relatively easy to collect and handle, have adequate size and lifespan (even in laboratory conditions) and are relatively tolerant to chemical exposure. Habitat and behavior of thicklip grey mullet (usually found in highly polluted and eutrophic areas) allow thinking that it should be a quite hypoxia-tolerant fish, and thus a good candidate to analyze adaptive changes to cope with low oxygen availability in polluted waters. This species has been employed as a pollution sentinel in different coasts and estuaries (Antovic & Antovic 2011; Baptista et al. 2013), including the Bay of Biscay (Bizarro et al. 2014; Ortiz-Zarragoitia et al. 2014; Sardi et al. 2015).

Furthermore, *C. labrosus* mullet has also proved to be a valuable species to run diverse *in vivo* laboratory experiments, including studies in which fish were exposed to AHR agonist compounds (de Cerio et al. 2012; Bilbao et al. 2010a; Bilbao et al. 2010b; Pujante et al. 2015)

In 1959, Russel & Burch defined the “Three Rs: Replacement, Reduction and Refinement” as a guideline to be followed in order to substitute the use of animals in experimentation with *in vitro* methods. In the last decades, an increasingly high number of *in vivo* experiments has been substituted (or at least complemented) by alternative *in vitro* tests in an effort to implement the “3R´s” philosophy (Balls 2010). *In vitro* methods constitute cost- and time-effective procedures to test the effects of stressors; however, they usually lack the complexity derived from the real topology of

the real organism yielding simplified mechanistic results (Eisenbrand et al. 2002). Even so, they have the advantage of reducing the individual and experimental variability of the tests and take place under defined, controlled and stable conditions (Iguchi et al. 2006). Nowadays, a wide number of *in vitro* techniques have been implemented to screen and rank the toxicity of diverse chemical compounds, in addition to risk assessment purposes including potential hazards for humans. Great effort has been done to establish adequate *in vitro* methodologies with mammalian cell cultures that provide a coherent regulatory framework for testing environmental chemicals and drugs (Worth & Balls 2002; Andersen & Krewski 2009). Still, *in vitro* methodologies have to be considered screening tests, and performed in parallel to *in vivo* assays in order to obtain results biologically meaningful. In this regard, two different types of cell cultures exist: primary cultures, in which cells are taken from the organism and then maintained *in vitro* for a relatively short period of time, and cell line cultures, which arise from subculturing primary cultures of normally transformed cells (Schaeffer 1990).

The first piscine cell cultures were performed in the 1950s and 1960s. Diversity, complexity and general uses of such cultures have been increasing since then, especially for the methods applied in aquatic toxicology (Ganassin et al. 2000). The number of fish species (and tissues among them) whose cells is possible to culture has also increased regarding both, cell lines and primary cell cultures (Segner 1998; Segner & Cravedi 2000; Lakra et al. 2011). With regard of *in vitro* testing techniques in aquatic toxicology, one of the most extended methodologies is the culture of freshly extracted primary fish hepatocytes, being *Oncorhynchus mykiss* the most studied species (Schirmer 2006; Rissanen et al. 2006; Tollefsen et al. 2008; Petersen & Tollefsen 2011; Tollefsen et al. 2012). Cells of primary cultures (unlike those of cell lines) do not suffer dedifferentiation, retaining for instance the molecular machinery in charge of triggering the detoxification metabolism (AHR pathway) so typical of the differentiated hepatocytes and making possible to measure the activity of CYP1A enzyme in response to xenobiotic exposure (Segner & Cravedi 2000). Such primary cultures are also suitable to study the mechanisms of response to hypoxic conditions, since the molecular machinery involved in the response to hypoxia (HIF-α pathway) is also retained (Soitamo et al. 2001).

4.2. Bivalves.

Bivalves are an important clade of invertebrates in terms of number of species, biomass and position in the ecosystems (Gonzalez-Rey et al. 2014). They play a key role in human nutrition, being commercially relevant. Indeed 13.6 million metric tons of these molluscs were globally produced in 2005 (Pawiro 2010). Most of bivalve species are filter feeders with little capacity to move, including several completely sessile species (Meglitsch & Schram 1991). Among them, *Mytilus* constitutes a noteworthy genus given its commercial importance and wide geographic distribution (Gosling 1992). A large number of mussel species live in the marine intertidal zone, where quick changes in environmental conditions caused by tidal oscillations and human impact induce critical adaptive pressures. Mussels possess sensible and precisely regulated mechanisms for the detection and adaptation to physicochemical changes in their unstable environment (Cajaraville et al. 2000). Such features of mussels make them suitable to study the effects of bioavailable pollutants (Orbea & Cajaraville 2006). In addition, mussels and other bivalves are exposed to hypoxic conditions twice a day in intertidal zones. When exposed to air, these mussels increase their reliance on anaerobic metabolism (glycolysis essentially) to generate ATP after closing their shells (Widdows et al. 1979; Michaelidis et al. 2005).

Mussels have gained significance as pollution sentinel organisms from the 1970s, when the environmental monitoring program "Mussel Watch" was established (Goldberg 1986). Since then, they have been implemented as sentinel organisms in multiple monitoring programs around the globe (Phillips & Rainbow 1993; O'Connor 1998; Cantillo 1998; Monirith et al. 2003; Marigómez, Garmendia, et al. 2013)

The mussel species in the southern Bay of Biscay and the Cantabrian Sea is the Mediterranean mussel *Mytilus galloprovincialis*, whose geographical distribution includes the Atlantic coast of Europe (from the Mediterranean Sea to the English Channel and the British Islands), with additional populations in California, Japan, South Africa and Australasia (Gosling 2003). Habitat distribution and biological characteristics of *M. galloprovincialis* allow selecting it as an adequate sentinel species for the Basque coast. In this way, this species has been employed in multiple previous laboratory

based studies and in pollution monitoring campaigns (Marigómez et al. 2013; Gonzalez-Rey et al. 2014; Izagirre et al. 2014; Ruiz et al. 2014).

4.3. Brief summary of biomarkers in ecotoxicology.

Each kind of stress elicits a defined sequential order of responses in a biological system; this has encouraged the effort to establish useful early-warning measurable signals of those biological answers: biomarkers. Biomarkers are usually defined as changes in a biological response (ranging from molecular through cellular and physiological responses to behavioral changes) which can be related to the exposure to or toxic effects of environmental chemicals (Peakall 1994). In environmental toxicology, this allows certifying uptake of toxicants by the organism, their distribution among tissues and their toxicity before damage has gone beyond the point of any possible recovery (McCarthy & Shugart 1990). However, in some exceptional cases it still has to be demonstrated that biomarkers respond in a regular and predictable fashion to exposure (Suter 1990). Overall, biomarkers have proved to be a useful and reliable tool in the assessment of the effects caused to the biota living under stressing environmental conditions. Thus, this approach to assess exposure to chemical compounds was quickly applied in diverse aquatic organisms including bivalves, crustaceans, and fish (Solé et al. 1995; Pedersen et al. 1997; Teh 1997). Nowadays, their use in aquatic toxicological studies is widespread (Valavanidis et al. 2006; Fonseca et al. 2011; Cravo et al. 2013; Liang et al. 2013; Koenig et al. 2013). Since one of the goals is to use them as early-warning tools, the establishment of mRNA level measurements of key genes as biomarkers of exposure to specific stressors is of special interest, given that transcriptional changes represent the initial step of response and adaptation/maladaptation to environmental changes.

Regarding pollution by toxic chemical compounds, induction of CYP1A is the most studied biomarker in environmental biomonitoring of aquatic ecosystems (Reviewed in Van der Oost et al. 2003). The protein levels and the activity of this enzyme in fish is directly proportional to the levels of aromatic and polychlorinated aromatic hydrocarbons in the organisms (Tairova et al. 2012; Valdehita et al. 2012; Allen 2013). The increase in the CYP1A protein levels is preceded by an increase in the transcription

of *cyp1a*; therefore, the increase of *cyp1a* mRNA levels is a suitable biomarker of exposure to certain environmental organic pollutants (Bucheli & Fent 1995; Bilbao et al. 2010; Won et al. 2013).

As elsewhere explained, most of organisms share a common strategy to adapt and survive to hypoxia; and a huge number of genes have been regarded as regulated by HIF-1 (Table 1). Previous studies measured changes in the expression (at mRNA, protein and/or activity level) of some of such key genes in aquatic animals in an attempt to unveil reliable biomarkers of hypoxia (Ju et al. 2007; Woo et al. 2011; Zhang et al. 2012; Mohindra et al. 2013). Discrepancies exist in this regard and therefore no clear biomarkers of hypoxia have been established yet for fish, especially at the transcriptional level (reviewed in Zhang et al. 2009). However, HIF-α proteins and transcript levels have been regarded as good biomarkers to identify situations of oxygen scarcity at the cellular level in aquatic organisms (Soitamo et al. 2001; Terova et al. 2008; Rahman & Thomas 2012).

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II. STATE OF THE ART, HYPOTHESIS AND OBJECTIVES

STATE OF THE ART

Superficial water in healthy environments is usually close to oxygen saturation levels; however, there are situations in which the gas equilibrium between atmosphere and water is disrupted. When a certain threshold is surpassed (water saturation below 25±5% of oxygen) hypoxic situations arise, a disadvantageous event for the organisms living in such places. In the past, changes in the amount of available oxygen in oceans and the occurrence of hypoxia have contributed to cause massive extinctions. Nowadays, the consequences of the global climate change are altering the biogeochemical cycle of oxygen and are contributing to general ocean outgassing. In local scenarios, activities linked to human settlements result in the disposal of diverse byproducts and residues that end up in the oceans (urban, agricultural, industrial and sewage runoff discharges), causing eutrophication and subsequent problems of hypoxia. In this way, the number of hypoxic areas has doubled each decade since the 1960s and future projections foresee that this problem will become worse.

In the majority of organisms the process of energy synthesis (ATP essentially) is based on the utilization of oxygen; thus, hypoxic events force to deploy alternative anaerobic energy-yielding pathways that are less productive than aerobic ones. Depending on the situation, the initial response of aquatic organisms to hypoxia involves fleeing from the areas affected, boosting the ventilation rate of their respiratory system and/or decreasing their activity to reduce the energy expenditure. In the occasions where hypoxic conditions affect the cellular and molecular processes in metazoans, molecular adaptive mechanisms have to be triggered; for which the HIF-α proteins (members of the bHLH-PAS family of transcription factors) are the key oxygen level sensors. HIF-αs suffer continuous degradation in the cytosol during normoxia, but hypoxic conditions block the activity of PHDs and FIH-1 (responsible of triggering proteolysis of HIF-αs) allowing their stabilization. Stabilized HIF-αs then enter the nucleus and heterodimerize with ARNT (also member of the bHLH-PAS family), binding the promoter region of specific genes whose expression unchains the response to hypoxia. In general, cells optimize the utilization of oxygen and glucose to generate ATP in an efficient way by enhancing anaerobic glycolysis in detriment of oxidative

phosphorylation. This switch reduces the energy supply; therefore, a general decrease of the metabolic ATP demand is also a consequence.

Another member of the bHLH-PAS family of transcription factors, AHR, is in charge of detecting the presence of xenobiotics (such as PAHs and PCBs) in cells. After binding its ligand, AHR enters the nucleus to heterodimerize also with ARNT and activate the promoter region of specific genes to trigger the xenobiotic detoxification metabolism in metazoan cells. Both hypoxia adaptive and xenobiotic detoxification pathways share ARNT as heterodimeric partner, allowing to think that cross-talk in form of competition could exist between HIF-αs and AHR to bind ARNT in situations of xenobiotic exposure in hypoxic sites. Recent studies have pointed out that exposure of chordates to xenobiotics under hypoxic conditions generates a maladaptive interplay between both, the hypoxia adaptive and the xenobiotic detoxification pathways. However, the role of ARNT in that interaction remains elusive yet. In nowadays-global scenario, when the prevalence of xenobiotic compounds in the environment is common as hypoxic events keep increasing, improving the knowledge about the adaptation constraints caused by the alleged interplay results of capital importance.

The strong industrial history in settlements along the Basque Coast, the level of human uses along the main rivers and estuaries and the agricultural activities performed on the Basque basins suggest that exposure to xenobiotics during seasonal hypoxic events must be common in different locations. Thus, it is important to describe the effects of this phenomenon in the organisms inhabiting Basque estuaries. In this regard, relevant pollution sentinel species of the Basque Coast (mullets *Chelon labrosus* and mussels *Mytilus galloprovincialis*) were selected to perform different laboratory and field *in vivo* studies in this PhD work. In addition, and to develop high throughput tools to analyze the hypoxia adaptive and the xenobiotic detoxification pathways, primary cultures of rainbow trout *Oncorhynchus mykiss* hepatocytes were analyzed in *in vitro* studies under hypoxic conditions and exposure to benzo[a]pyrene. The main effort was focused on measuring the transcriptional responses of a selected battery of key genes that have been described as early-warning biomarkers of exposure to xenobiotics and/or hypoxic conditions in vertebrates.

HYPOTHESIS

Exposure of fish and mussels to xenobiotics (aryl hydrocarbon receptor-AHR- agonists) under hypoxic conditions results in a loss of transcriptional control of the xenobiotic and hypoxic adaptive pathways as a consequence of the competition of hypoxia inducible factors αs (HIF-αs) and AHR for transcriptional activation.

OBJECTIVES

In order to prove this hypothesis true, the present work attempts to address the following objectives:

1. To improve the information of gene sequences related to the thicklip grey mullet *Chelon labrosus* and the Mediterranean mussel *Mytilus galloprovincialis*, used as pollution sentinel species in the Basque Coast, by sequencing fragments of genes putatively involved in their hypoxia adaptive pathways.
2. To characterize *hif-α* transcription levels in different tissues of *Mytilus galloprovincialis* and to assess its transcriptional regulation, together with that of other genes theoretically related to the hypoxia adaptive pathway, in the digestive gland of mussels exposed to benzo[a]pyrene under hypoxic conditions in a laboratory *in vivo* experiment.
3. To quantify transcription levels of genes related to the xenobiotic detoxification and the hypoxia adaptive pathways in juvenile *Chelon labrosus* exposed to benzo[a]pyrene under hypoxic conditions in an *in vivo* laboratory experiment, in order to study the interaction between both molecular pathways.
4. To apply the transcriptional tools developed under laboratory conditions in an *in vivo* field study, deploying juvenile *Chelon labrosus* in a chronically polluted Basque estuary affected by periodical hypoxic events and assessing the interactions between the hypoxia/xenobiotic responses at the level of ARNT in environmentally relevant conditions.
5. To study the interactions between HIF-αs and AHR adaptive pathways in *in vitro Oncorhynchus mykiss* primary hepatocyte cultures by measuring the transcriptional expression of key selected genes under different oxygen concentration incubation conditions and under benzo[a]pyrene exposure.

All fish handling and experimental procedures were performed following the specifications agreed with the Ethics Committee for Animal Experimentation of the University of the Basque Country under approved experimental protocol (CEEA/151/2010/CANCIO).

III. RESULTS AND DISCUSSION

Chapter 1

Transcription profiles of HIF-α and glucose metabolism genes in mussels (*Mytilus galloprovincialis*) exposed to benzo[a]pyrene under hypoxic conditions

Parts of this chapter have been presented at:

J. Martos-Bernal, E. Bilbao, I. Cancio. *Transcriptional regulation of genes involved in adaptation to hypoxia in Mediterranean mussels (*Mytilus galloprovincialis*) exposed to Benzo[a]Pyrene under hypoxic conditions.* 17th INTERNATIONAL SYMPOSIUM ON POLLUTANT RESPONSES IN MARINE ORGANISMS. Faro, Portugal. 5th-8th May 2013. Poster.

J. Martos-Bernal, E. Bilbao, I. Cancio. *Regulation of hypoxia and xenobiotic exposure related genes in fish (*Chelon labrosus*) and mussels (*Mytilus galloprovincialis*) held under acute hypoxic conditions*. 28th EUROPEAN SOCIETY OF COMPARATIVE PHYSIOLOGY AND BIOCHEMISTRY. Bilbao, Spain. 2nd-5th September 2012. Poster.

This chapter is in preparation to be submitted as:

J. Martos-Bernal, E. Bilbao, I. Cancio. *Transcription profiles of HIF-α and glucose metabolism genes in mussels (*Mytilus galloprovincialis*) exposed to benzo[a]pyrene under hypoxic conditions.* Marine genomics.

Abbreviations:

AHR	Aryl hydrocarbon receptor
ALDOLASE	Fructose-bisphosphate aldolase
ARNT, HIF-1β	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine triphosphate
B[a]P	Benzo[a]pyrene
COX-4	Cytochrome c oxidase subunit IV
CYP1A	Cytochrome p450
DMSO	Dimethyl sulfoxide
ETC	Electron transport chain
HIFs	Hypoxia-inducible factors
HK	Hexokinase
NCBI	National Center for Biotechnology Information
PAS	PER/ARNT/SIM homology domain
PEPCK	Phosphoenol pyruvate carboxykynase

Abstract

Habitat distribution and biological characteristics set bivalves as candidates to suffer from xenobiotic pollution under low oxygen availability conditions in anthropized zones. Similarities between molluscan and vertebrate hypoxia inducible factor-α and the possible involvement of the aryl hydrocarbon receptor-cytochrome p450 axis in bivalves exposed to benzo[a]pyrene (B[a]P) have been described. Therefore, a better understanding of hypoxia and xenobiotic adaptive pathways is important by itself as well as to assess the possibility of interactions between both pathways as it has been described in vertebrates. An *in vivo* lab experiment was carried out to assess the hypoxia adaptive pathway, keeping Mediterranean mussels *Mytilus galloprovincialis* under hypoxia (2.5 mg O_2/L), under exposure to benzo[a]pyrene (0.4 mg/L) and to hypoxia/B[a]P for 1, 7 and 14 days. Fragments of genes coding for *hypoxia inducible factor-α* (*hif-α*), *cytochrome c oxidase subunit IV* (*cox-4*), hexokinase (*hk*), *fructose-bisphosphate aldolase* (*aldolase*) and *phosphoenolpyruvate carboxykinase* (*pepck*) were first sequenced. Transcriptional profile analyses of *hif-α* revealed that transcription occurs in all studied tissues of mussels, being gills the organs where *hif-α* is most highly transcribed. Transcription levels of *hif-α, cox-4*, *hk* and *pepck* genes were measured in digestive gland (target organ for xenobiotic metabolization) of mussels under hypoxia and B[a]P exposure. A decay of *hif-α* mRNA levels during hypoxia and a non-homogeneous distribution of *hk* transcript levels among the different treatments were observed, while transcription levels of *cox-4* and *pepck* remained unaltered. Mussels apparently rely in metabolic rate depression during hypoxic events rather than in an enhancement of the glycolytic activity. However, they could also be deploying a negative feedback strategy in the hypoxia adaptive pathway as protection against re-oxygenation. Both processes seemed to be negatively affected at the transcriptional level by co-exposure to B[a]P, suggesting interferences between the referred pathways in this bivalve species.

Key words: hypoxia, Benzo[a]pyrene, *Mytilus galloprovincialis*, transcription, HIF-α.

Resumen

Las características biológicas de los bivalvos y su distribución geográfica les predisponen a sufrir exposición a xenobióticos bajo condiciones de escasez de oxígeno en zonas antropizadas. Estudios recientes reflejan una notable similitud entre los factores inducidos por hipoxia-α (HIF-αs), así como en la activación del eje "receptor de hidrocarburos arilo-citocromo p450" bajo exposición a benzo[a]pireno (B[a]P) en vertebrados y moluscos. Por lo tanto, mejorar el conocimiento sobre las rutas moleculares de adaptación a hipoxia y exposición a xenobióticos podría permitir evaluar la potencial existencia de interacciones entre ambas rutas, como sucede en vertebrados. Para estudiar la ruta molecular de adaptación a hipoxia se realizó un experimento de laboratorio en el que se mantuvieron mejillones *Mytilus galloprovincialis* bajo hipoxia (2.5 mg O_2/L), expuestos a B[a]P (0.4 mg/L) y bajo hipoxia/B[a]P durante 1, 7 y 14 días. En primer lugar se secuenciaron fragmentos de los genes *factor inducido por hipoxia-α* (*hif-α*), *citocromo c oxidasa subunidad VI* (*cox-4*), *hexoquinasa* (*hk*), *fructosa-bisfosfato aldolasa* (*aldolasa*) y *fosfoenolpiruvato carboxiquinasa* (*pepck*). Los análisis transcripcionales revelaron que *hif-α* se transcribe en todos los órganos estudiados, pero especialmente en branquia. Se midieron los niveles de transcripción de *hif-α, cox-4*, *hk* y *pepck* en glándula digestiva (principal órgano en el metabolismo de xenobióticos) de mejillones sometidos a hipoxia y expuestos a B[a]P. Los niveles de mRNA de *hif-α* descendieron bajo hipoxia, mientras que *hk* mostró niveles de transcripción no homogéneos entre los diferentes tratamientos. Los niveles transcripción de *cox-4* y *pepck* permanecieron estables. Aparentemente, los mejillones tienden a reducir su metabolismo basal durante situaciones de hipoxia, en vez de incrementar su actividad glicolítica. Sin embargo, podrían estar desplegando mecanismos de retroalimentación negativa en la ruta de adaptación a hipoxia para protegerse de una eventual reoxigenación. Al parecer, la co-exposición a B[a]P afectó de manera negativa a los dos procesos adaptativos analizados a nivel transcripcional, sugiriendo la presencia de interferencias entre ambas rutas moleculares en esta especie de bivalvos.

Palabras clave: hipoxia, benzo[a]pireno, *Mytilus galloprovincialis*, transcripción, HIF-α, genes metabolismo energético.

Laburpena

Bibalboak, beraien banaketa geografikoa eta ezaugarri biologikoak direla eta, gune antropogenizatuetan kutsadura eta oxigeno eskuragarritasun baxudun baldintzak pairatzera behartuta daude. Muskuilu eta ornodunetan hipoxiak induzitutako α-faktoreak dituen antzekotasunez gain, bibalboetan benzo[a]pirenoaren (B[a]P) eraginpean ari den arilo hidrokarburoen hartzaileak eta p450 zitokromoak osatzen duten ardatzaren aktibazioa ere deskribatu izan da azkenaldian. Horrela, hipoxia eta xenobiotikoen pean adaptaziorako molekula mailako bidezidorrak ulertzeak, bidezidor bien artean eman daitezkeen ustezko elkarrekintzen berri emango luke, ornodunetan deskribatu bezala. Hipoxia baldintzapeko molekula mailako adaptazioa ebaluatzeko *Mytilus galloprovincialis* mediterraneoko muskuiluak hipoxia (2.5 mg O2/L), B[a]P (0.4 mg/L) eta bien konbinazio pean mantendu ziren 1, 7 eta 14 egunez. Lehendabizi *hipoxiak induzitutako α-faktorea* (*hif-α*), *c zitokromo oxidasaren VI subunitatea* (*cox-4*), *hexokinasa* (*hk*), *fruktosa-bisfosfato aldolasa* (*aldolasa*) eta *fosfoenolpirubato karboxikinasa* (*pepck*) kodifikatzen dituzten geneen zatiak sekuentziatu ziren. *hif-α* genea, aztertutako muskuiluen ehun guztietan transkribatu zen, zakatzetan maila nabarmenagoa erakutsiz. Hipoxia eta B[a]P-aren eraginpean mantendutako muskuiluetan *hif-α, cox-4, hk* eta *pepck* geneen liseri-guruineko transkripzio mailak (xenobiotikoen eraldaketa metabolikorako itu-organoa) neurtu ziren. Hipoxia pean *hif-α* genearen mRNA mailaren gutxitzea eta *hk*-ren transkripto mailen banaketa ez-homogeneoa behatu ziren; *cox-4* eta *pepck* geneen transkripzio mailak ordea, egonkor mantendu ziren. Ematen duenez, hipoxia pean muskuiluen jarduera glikolitikoa handitu beharrean, oinarrizko metabolismoak murrizteko joera erakutsi du. Hala ere, hipoxia peko adaptazio-bidezidorren feedback negatiboa ematen egon liteke berroxigenaziotik babesteko mekanismo gisa. Hipoxia eta B[a]P-ak, bidezidor bietako transkripzio mailetan eragin negatiboa izan zuen, muskuiluen molekula mailako bidezidorren arteko elkarrekintzak egon daitezkeela iradokiz.

Gako hitzak: hipoxia, Benzo[a]pireno, *Mytilus galloprovincialis*, transkripzioa, HIF-α.

1. Introduction

A large number of bivalve species live in the intertidal zone where adaptive pressure, caused by quick changes in environmental conditions (mostly due to tidal oscillations) and human impact, is critical. The vast majority of those molluscs are filter-feeders with limited mobility, including several completely sessile species (Meglitsch & Schram 1991), so they have no possibilities to escape from areas affected by deleterious conditions. Therefore, bivalves could be expected to possess sensible and effective mechanisms for detection and adaption to physicochemical changes in their surrounding environment (Cajaraville et al. 2000). Events of oxygen deprivation and presence of xenobiotic compounds are bound to co-occur in coastal waters affected by human activities. Hypoxia inducible factor-α (HIF-α) is the key molecule required by metazoans to adapt to hypoxic conditions (Wang & Semenza 1995; Loenarz et al. 2011), while the aryl hydrocarbon receptor (AHR) triggers the adequate response to metabolize xenobiotics in vertebrates (Hoffman et al. 1990). In molluscs, the molecular pathways related to HIF-α and AHR transcription factors are far from being understood, with very limited information being available to date.

Initial studies with bivalves exposed to air detected a decrease in dissolved oxygen levels within the shell-enclosed water, with an increase in the prevalence of anaerobic-end products identified within the animal soft tissues (Widdows et al. 1979; Michaelidis et al. 2005). This late observation would be in consonance with known strategies to face hypoxia in other more studies animal groups. David et al. (2005) screened the multi-tissue transcriptional response of oysters *Crassostrea gigas* chronically subjected to hypoxia: responses such as enhancement of the anaerobic metabolism together with a decrease in the ATP demand could be envisaged as relevant in adaptation to low oxygen availability. Moreover, a switching from aerobic to anaerobic energy production was observed in *C. gigas* during chronic hypoxic and anoxic conditions (Moullac et al. 2007; Kurochkin et al. 2009).

Loenarz and colleagues (2011) suggested the oxygen-sensing mechanism to be highly conserved among metazoans, since the central core proteins in the hypoxia adaptive pathway (HIF-α) can be found in all the animals analyzed (including the mollusc *Lottia*

gigantea). In vertebrates under normoxic conditions, HIF-1α is hydroxylated, ubiquitinated and subsequently degraded via proteasome; but when oxygen is scarce the hydroxylation does not occur (Greer et al. 2012). HIF-1α is then accumulated and enters the nucleus to heterodimerize with the aryl hydrocarbon receptor nuclear translocator (ARNT) and unchain the adaptive response to hypoxia (Kaelin & Ratcliffe 2008). Accumulation of HIF-α protein was observed in molluscs for the first time by Fei & Feng (2008) in the neural tissue of *Lymnaea stagnalis* after 6 days under mild hypoxic conditions. Piontkivska and colleagues (2011) also found an increasing trend of *hif-α* mRNA levels in *Crassostrea virginica* gills after 14 days of hypoxia. Kawabe & Yokoyama (2012) also reported an increase in *hif-α* mRNA and HIF-α protein levels in gills of oysters *C. gigas* during acute hypoxia. Furthermore, transcriptional up-regulation of *hif-α* in gills and haemocytes of *Haliotis diversicolor* abalones under acute hypoxia was reported by Cai and collaborators (2014). The complexity of the transcriptional regulation during hypoxic events was reflected in the study of Woo and coworkers (2011) in *M. galloprovincialis* subjected to hypoxic conditions for 72 h. At that time, changes in the transcription levels of phosphoglycerate mutase among others lead the authors to suggest a shifting to a cell signaling pathway different from the hypoxia adaptive one identified at earlier time points. As seen for vertebrates, exposure of molluscs to hypoxia has been linked to production of reactive oxygen species (De Zoysa et al. 2009; Choi et al. 2013).

On the other hand, first mollusc AHR (gene and protein) was described in *Mya arenaria*, the sequence clustering together with invertebrate AHR orthologs and resembling vertebrate AHR due to the presence of similar putative transactivation domains (Butler et al. 2001). However, characteristics of the ligand-binding domain in the molluscan protein are different from those in vertebrate AHRs, suggesting that the "dioxin-like compound binding"-mediated transactivation of the receptor protein appeared later in evolution and it is exclusive to the chordate lineage. Similar observations have been reported for the *Ruditapes philippinarum* AHR ortholog (Liu et al. 2010). In any case, exposure to benzo[a]pyrene (B[a]P) has been shown to increase the transcription levels of *ahr* in different bivalve species, suggesting its involvement in

the xenobiotic detoxification metabolism in this group of molluscs (Tian et al. 2013; D. Liu et al. 2014).

Habitat distribution and biological characteristics set bivalves as candidates to suffer from xenobiotic exposure under conditions of low oxygen availability in zones with anthropic influence. It is still unknown whether *hif-α* plays a role during hypoxia in molluscs or whether this is an adaptive pathway exclusive for vertebrates (Kawabe & Yokoyama 2011). However, features of the molluscan adaptive pathway previously described and the structural similarities between molluscan and vertebrate HIF-α proteins could suggest similar functions for both molecules (Cai et al. 2014). The regulation of the hypoxia adaptive pathway in presence of B[a]P is of interest due to the cross-talk described in vertebrates involving HIF-1α, AHR and ARNT (Vorrink & Domann 2014). *Mytilus galloprovincialis* mussel is considered a well suited pollution sentinel species and it has been widely used in pollution monitoring campaigns and in lab based studies aiming the analysis of chemical toxicity mechanisms (Marigómez et al. 2013; Gonzalez-Rey et al. 2014; Izagirre et al. 2014; Ruiz et al. 2014). In this study, sequences of genes related to the hypoxia adaptive pathway were sequenced for the first time in the mussel *M. galloprovincialis*, and the transcriptional profile of *hif-α* was characterized in different tissues of mussels. Further, transcription levels of selected genes were measured in digestive gland to assess their changes after exposure to B[a]P under normoxic and hypoxic conditions.

2. Materials and Methods

2.1. Experimental animals

One batch of mussels *Mytilus galloprovincialis* (3.5-4.5 cm in shell length) was collected in the intertidal zone of Txatxarramendi (Sukarrieta, 43°39´N; 2°69´W) in the estuary of the Biosphere Reserve of Urdaibai. 10 mussels were immediately dissected to obtain muscle, gill, mantle and digestive gland. After immersion in RNA later® (Ambion-Invitrogen Life Technologies, Carlsbad, USA), samples were frozen in liquid nitrogen and maintained at -80°C.

Another batch of mussels taken from the intertidal zone of Mundaka (43º40´N; 2º69´W), also in the estuary of the Biosphere Reserve of Urdaibai, was acclimatized for 2 months in a 300 L tank with clean seawater (33.3‰ salinity, 47 mS conductivity, pH 7.8) supplied from an aquaculture farm pipe (Getaria, 43º30´N; 2º19´W). Water was decanted and sequentially filtered through 100, 50 and 5 µm pore size meshes while receiving ultraviolet treatment, then stabilized at 18ºC prior to the final filtering step through a 0.45 µm pore size mesh. Normal photoperiod provided 12 hours light supply to the tank. Residual metabolites (ammonium, nitrite and nitrates) were maintained at 0-0.5 mg/L, 0-0.5 mg/L and 5-10 mg/L respectively; assessment performed using commercial analysis kits (Sera GmbH, Heinsenberg, Germany) every 3 days. Animals were daily fed during the acclimatization period with Sera Marin Coraliquid (Sera GmbH, Heinsenberg, Germany). A week prior to the experiment mussels were transferred to 60 L tanks (oxygen concentration = 9±0.2 mg O_2/L), where the experiment would take place, and fed every 3 days.

2.2. Exposure to B[a]P and hypoxic conditions during experiment.

Tanks, containing an electrically fed air source and a spigot on the lid to avoid overpressure, were sealed. Tanks that contained mussels under hypoxic conditions were provided with a nitrogen gas source, and an Oxi::lyser probe (s::can, Messtechnik GmbH, Vienna, Austria) was used to measure temperature and oxygen concentrations. Such parameters were monitored by a Con::lyte terminal (s::can, Messtechnik GmbH, Vienna, Austria). Whenever water oxygen concentration exceeded 2.5 mg O_2/L, a relay-connected valve automatically pumped nitrogen into the tanks to maintain the desired oxygen concentration. Initially, dissolved oxygen was between 9.2 and 8.9 mg O_2/L, concentration which was gradually decreased in the hypoxic tanks to achieve 2.5 mg O_2/L in 4 h.

Animals in all experimental groups were exposed to 0.01% (v/v) dimethyl sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MI, US), as it was used as vehicle to dose benzo[a]pyrene (B[a]P, Sigma–Aldrich) in the two B[a]P exposure groups. These two groups received 0.4 mg B[a]P/L dissolved in DMSO. Both hypoxia tanks, one in combination with B[a]P exposure, were maintained at 2.5 mg O_2/L (close to the

average definition of hypoxia at 2.1 mg O_2/L, Farrell & Richards 2009). Food was supplied every 3 days, prior to partial water renewal that was accompanied by new B[a]P and DMSO dosage. Digestive gland samples were taken from 7 mussels per treatment after 1, 7 and 14 days in the experimental tanks. After immersion in RNA later® (Ambion-Invitrogen), samples were frozen in liquid nitrogen and maintained at -80°C .

2.3. RNA extraction and cDNA synthesis

A piece of 50-100 mg of each tissue was separately homogenized with zirconia/silica beads (Biospec, Bartlesville, USA) in a Precellys® 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 6 m/s for 20 s twice. Total RNA was extracted using the TRIzol® (Ambion-Invitrogen) method and purified with the MinElute PCR Purification kit (Qiagen, Hilden, Germany). Final concentration and purity were spectrophotometrically measured, accepting A260/A280 ratios between 1.8 and 2. Retrotranscription was performed using the Superscript™ First-Strand cDNA Synthesis kit (Ambion-Invitrogen) with random hexamers in an iCycler™ thermocycler (Bio-Rad, San Diego, USA). Synthesized cDNA was stored at -40°C.

2.4. Sequencing of target genes

Since no sequence information was available at the international data bases, fragments of Mediterranean mussel *hypoxia-inducible factor-α* (*hif-α*), *cytochrome c oxidase subunit IV* (*cox-4*), hexokinase (*hk*), *fructose bisphosphate aldolase* (*aldolase*) and *phosphoenolpyruvate carboxykinase* (*pepck*) had to be first sequenced. Sequences from mollusc species phylogenetically close to *M. galloprovinciallis* and available in the GenBank (National Center for Biotechnology Information, NCBI) were aligned with ClustalW2 (Larkin et al. 2007). Conserved regions allowed designing primers (Table 1) to amplify the named fragments, which were visualized in ethidium bromide stained 1,5% agarose gel electrophoresis. Specific amplicons were sequenced in the Sequencing and Genotyping Service of the University of the Basque Country. Blastx analysis (NCBI) was used to search for homology and deduced amino acid identity of amplified sequences. Only identity values above 50% and e-values below $1e^{-30}$ were

considered. Structural analyses of sequenced fragments were contrasted with the NCBI conserved domain database (Marchler-Bauer et al. 2014).

Table 1. Primer sequences and temperatures used to amplify *hif-α, cox-4*, *hk, aldolase* and *pepck* fragments of *Mytilus galloprovincialis*. The expected length of each fragment is also included.

Gene	Primer sequence (5´-3´)		T. (ºC)	Lenght (bp)
	Fw	Rv		
hif-α	TGTAATGCCCTTCCTGTTCC	GCTCTCCAACGGCAATGTAG	54	514
cox-4	GAAGTTGTAACCCTTTCACAGACA	TTCTGGGGTCATACTTTCTGG	54	413
hk	CTGGGATGTACATGGGAGAGA	TGGACAGAATCAGCAATCCA	54	488
aldolase	TTGGAAAGAGATTTGCATCAAT	AACATGATGATCTGACAATGCTT	51	570
pepck	ACTGACCCTGCTGATGTCG	TCTGGATTACATGGCCAATGGTT	59	409

Phylogenetic trees based on the deduced amino acid sequences of the newly sequenced *M. galloprovincialis hifα* and *cox-4* genes were designed to assess their identity within their respective gene families. To perform the HIF-α tree, protein sequences belonging to *Homo sapiens* (NP_001521, NP_001421, NP_690007), *Megalobrama amblycephala* (ADF50043, ADF50044, ADF50045), *Haliotis diversicolor* (AGE97172), *Crassostrea virginica* (AED87588) and *Crassostrea gigas* (EKC33928) were aligned. In the case of the COX-4 tree, protein sequences belonging to *Homo sapiens* (AAH62437, NP_115998), *Heterocephalus glaber* (EHB09422), *Panthera tigris* (AGU01722), *Myotis davidii* (ELK31409), *Pteropus alecto* (ELK04070), *Bos taurus* (NP_001180115), *Fukomys damarensis* (KFO31496), *Urechis caupo* (AAA74396), *Ixodes scapularis* (AAY66918), *Zootermopsis nevadensis* (KDR18465), *Litopenaeus vannamei* (AFY10818) and *Tribolium castaneum* (NP_001164085) were employed. Sequences were aligned using the MUSCLE software in the MEGA 6 program (Tamura et al. 2013), and the phylogenetic analysis was based on the maximum likelihood method.

2.5. Quantitative PCR (qPCR) analyses.

Basal transcription levels of *hif-α* were quantified in muscle, gill, mantle and digestive gland of 10 mussels using a Viia 7 thermocycler (Applied Biosystems, California, USA). cDNA was amplified in triplicates with a total reaction volume of 20 µl, containing 10 µl of SYBR Green (FastStart Universal SYBR Green Master, Roche, Indianapolis, USA). Primers concentration was 2.5 pmol/µL (Table 2) and PCR conditions were set as

follows: 50°C for 2 min, 95°C for 10 min; 40 cycles at 95°C for 15 s followed by 59ºC (melting temperature) for 30 s. Each primer set specificity was certified by analyzing the dissociation curve of amplicons to verify the presence of a single melting peak. cDNA concentration of each sample was measured with the Quant iT OliGreen ssDNA Assay Kit (Life Technologies), following manufacturer instructions. Plates were read in a FLX800 fluorometer (Biotek) using the 480-520 nm excitation-emission wavelength pair. Relative transcription levels (RQ) were determined using muscle as calibrator and correcting efficiency (E) of each PCR:

$$RQ = {(1 + E)^{-\Delta CT}}/{\mu g\ cDNA}$$

In digestive gland of treated mussels, transcription levels of *hif-α*, *cox-4*, *hk* and *pepck* were normalized using *18S rRNA* (accession number L33451) as reference gene (its coefficient of variation within the experiment was below 5%). qPCRs were performed following the specifications depicted above, in a 7300 Real Time PCR System (Life Technologies). cDNA concentration, primer sequences and concentrations and melting temperatures are detailed in Table 2. Relative transcription values were calculated based on the Delta-Delta Ct method corrected for PCR efficiency (modified from Pfaffl 2001).

Table 2. Primer sequences and melting temperatures used for the amplification of target genes by qPCR. A final concentration of 0.8 ng cDNA/µL was used for each qPCR, except for *18S rRNA* (measured as reference gene) that contained 2.5 pg cDNA/µL. Final primer concentration used for the amplification of all cDNA fragments was 1.25 pmol/µL.

Gene	Primer sequence (5´-3´)		T. (ºC)
	Fw	Rw	
hif-α	AGTCTACTAGAGTTTCTTCATCCATGTGA	CTTCATACGAAGAAACAACGACTTCT	55
cox-4	ATTATCCCTTCCCAGCACTCAA	TCCTCCATGGTTAGATTCTTCCA	55
hk	AAATCTGTAAAGCGAGCCATCAA	AAGGGCTGCCTTCCTGTCA	58
aldolase	CAATGTTTTAGCCAGATATGCTAGCA	CAGTATAAAGATCGTGATCACCATCA	55
pepck	TCAGTAAAATTGGGATACAGCTGACT	TCTCCCAAAGTTTCCCAAACG	55
18S rRNA	ACGGCTACCACATCCAAGGA	GCCTCGAAAGAGTCCCGTATT	56

2.6 Data analysis

Since data proved to be non-parametric, the Kruskal-Wallis one-way analysis of variance test was employed to assess gene transcription level differences among groups and tissues. Dunn´s post-hoc test was then applied to perform the final

multiple comparison. Significant differences were established at $p<0.05$. Statistical analysis was performed using the SPSS 22.0 software.

3. Results

3.1. Sequencing of target genes

Used primers allowed the amplification of specific cDNA fragments. Upon sequencing and analysis based on Blastx, obtained amplicons were identified as *hif-α*, *cox-4*, *hk*, *aldolase* and *pepck* (Table 3).

Table 3. Name, GenBank accession number and length in base pairs of the obtained *Mytilus galloprovincialis hypoxia-inducible factor-α* (*hif-α*), *cytochrome c oxidase subunit IV* (*cox-4*), *hexokinase* (*hk*), *fructose bisphosphate aldolase* (*aldolase*) and *phosphoenolpyruvate carboxykinase* (*pepck*) gene fragments, followed by the E-value, maximal amino acid identity and percentage of the total open reading frame obtained for each gene in comparison to its closest available ortholog sequence (organism) as revealed by Blastx analysis.

Gene (Acc. number)	Length (bp)	E-value	Identity	ORF	Organism (Acc. number)
hif-α (JN595864)	453	3,E-40	51%	20,4%	*Crassostrea virginica* (AED87588)
cox-4 (JN595863)	321	4,E-34	56%	60%	*Tribolium castaneum* (NP_001164085)
hk (JN595865)	488	5,E-54	66%	34,86%	*Crassostrea gigas* (EKC38890)
aldolase (JN595862)	483	2,E-93	84%	43,95%	*Meretrix meretrix* (ADP37376)
pepck (JN595866)	369	2,E-46	65%	18,96%	*Crassostrea gigas* (CAJ28913)

Structural analysis of the deduced amino acid sequence of *hif1-α* confirmed the presence of a complete PAS domain with its putative active site and heme pocket. The phylogenetic analysis of the deduced *M. galloprovincialis* HIF-α revealed that it grouped together with mollusc orthologs, in a clade that is not specifically linked to any of the three HIFα paralogs described for vertebrates (Figure 1).

Regarding *M galloprovincialis cox-4*, its deduced amino acid sequence contains the main part of the cytochrome c oxidase subunit IV region; including several subunit IV interfaces (I, Va, Vb, VIIb and VIIb) and the putative ATP/ADP binding site. In the phylogenetic analysis, the deduced sequence of *M. galloprovincialis* COX-4 grouped with COX-4 sequences belonging to invertebrate species in a clade not specifically linked to any of the two isoforms described for mammals (Figure 2).

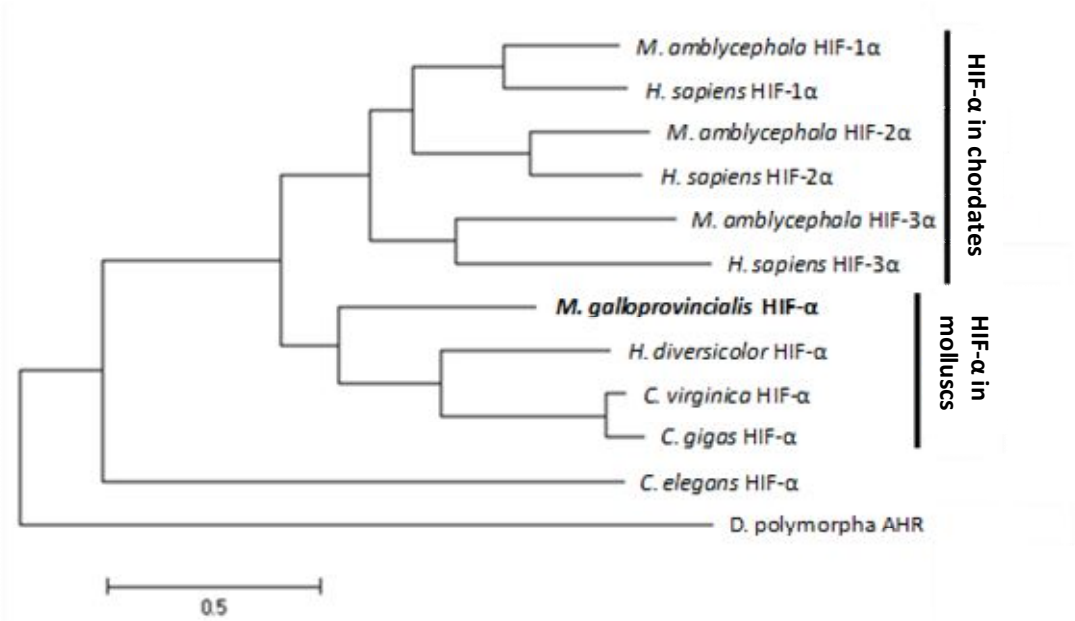

Figure 1. Phylogenetic tree for the correct unambiguous annotation of the *M. galloprovincialis hif-α* fragment sequenced. Deduced amino acid sequences belonging to *Homo sapiens* (NP_001521, NP_001421, NP_690007), *Megalobrama amblycephala* (ADF50043, ADF50044, ADF50045), *Haliotis diversicolor* (AGE97172), *Crassostrea virginica* (AED87588), *Crassostrea gigas* (EKC33928) and *M. galloprovincialis* (JN595864) *hif-αs* were aligned using MUSCLE software in the MEGA 6 program. The phylogenetic analysis was carried out based on the maximum likelihood method. Amino acid sequences belonging to *Caenorhabditis elegans* HIF*α* and *Dreissena polymorpha* AHR were included as outgroups (NP_508008 and AAZ83700, repectively).

In the case of the *M. galloprovincialis hk* fragment obtained, its deduced amino acid sequence features a part of a region that frames it within the hexokinase 2 superfamily of enzymes. The portion of the *aldolase* gene sequence obtained encodes about half of a glycolytic domain that belongs to fructose-bisphosphate aldolase class-I proteins, although the particular isoform cannot be discerned. Finally, the deduced amino acid sequence of the obtained *M. galloprovincialis pepck* fragment contains part of the ATP-binding cassette transporter nucleotide-binding domain. Blastx analysis (NCBI) relates this fragment with the GTP-dependent PEPCK enzymes.

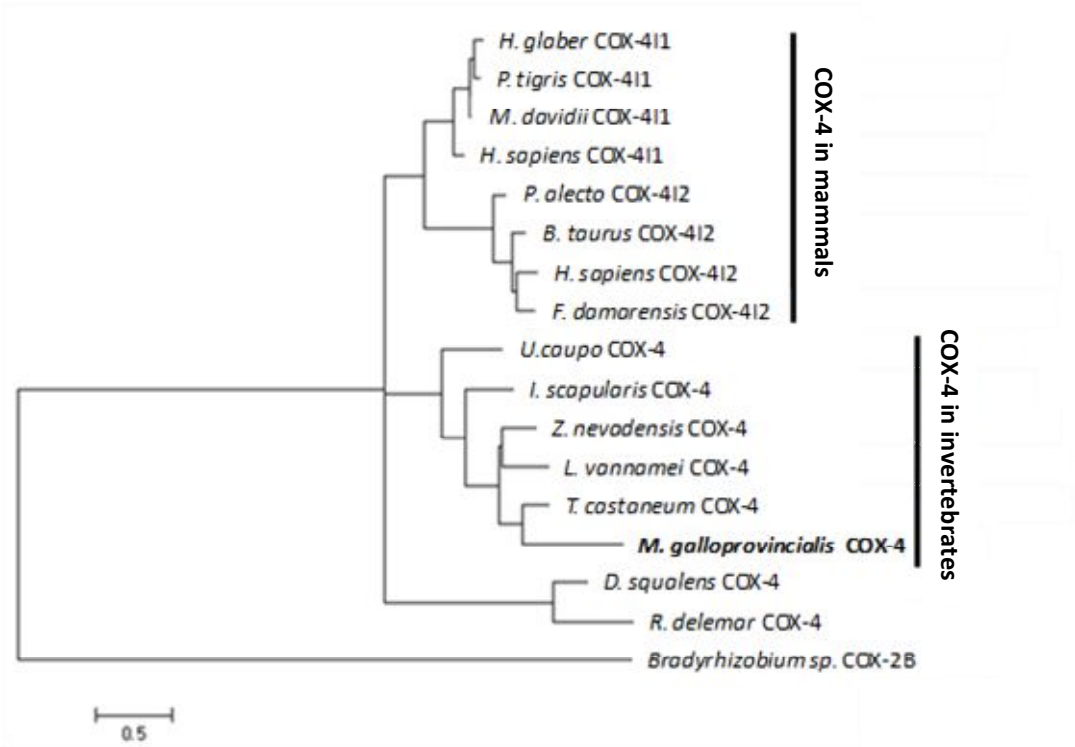

Figure 2. Phylogenetic tree for the correct unambiguous annotation of the *M. galloprovincialis cox-4* fragment sequenced. Deduced amino acid sequences belonging to *Homo sapiens* (AAH62437, NP_115998), *Heterocephalus glaber* (EHB09422), *Panthera tigris* (AGU01722), *Myotis davidii* (ELK31409), *Pteropus alecto* (ELK04070), *Bos taurus* (NP_001180115), *Fukomys damarensis* (KFO31496), *Urechis caupo* (AAA74396), *Ixodes scapularis* (AAY66918), *Zootermopsis nevadensis* (KDR18465), *Litopenaeus vannamei* (AFY10818), *Tribolium castaneum* (NP_001164085) and *M. galloprovincialis* (JN595863) *cox-4* genes were aligned using MUSCLE software in the MEGA 6 program. The phylogenetic analysis was carried out based on the maximum likelihood method. Amino acid sequences belonging to *Dichomitus squalens* and *Rhizopus delemar* COX-4 and *Bradyrhizobium sp.* COX-2 were included as outgroups (XP_007361501, EIE80435 and ERF84418, respectively).

3.2. hif-α tissue specific transcription levels

hif-α was found to be transcribed in all organs tested, including gills, mantle, digestive gland and muscle, of mussels coming directly from the field. Mussel gills presented the highest *hif-α* transcription levels, around 40% higher than those of muscle (Figure 3). Both, gills and muscle, presented transcription levels significantly higher than those found for digestive gland, which showed the lowest *hif-α* values of all tested tissues. The levels of transcription in the digestive gland and mantle were quite homogeneous in the individuals analyzed, while gills and muscle showed individuals with very different transcription levels for this gene.

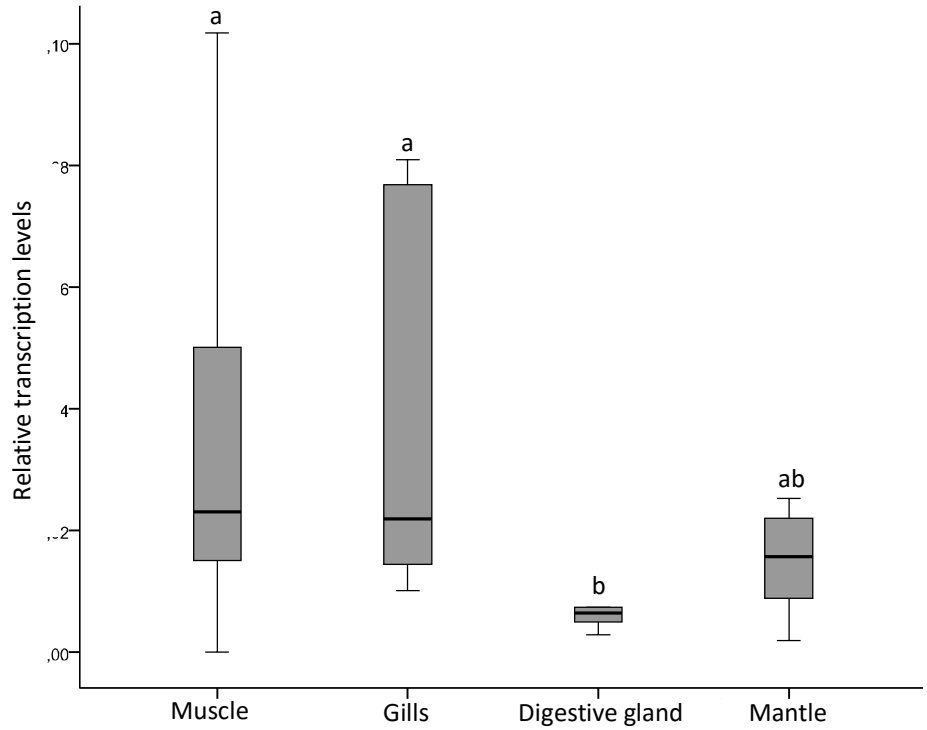

Figure 3. Relative transcription levels of *hif-α* in different organs of *M. galloprovincialis* mussels. Box plots represent the 25th, 50th and 75th percentiles, while whiskers represent the standard deviation. Significant differences ($p<0.05$) between groups are identified by different letters. Statistical differences were based on the Kruskal-Wallis one-way analysis of variance followed by the Dunn´s post-hoc test.

3.3. Experimental set up and oxygen levels

Oxygen levels measured in the two experimental groups maintained under hypoxia were constant in the range of 2.46 and 2.53 mg O_2/L. Slight and punctual oscillations were recorded that in no case were above 2.8 or below 2.3 mg O_2/L, during the 14 days that the experiment lasted. No mortality was observed in any of the treatments.

3.4. Transcription levels in digestive gland under hypoxia and B[a]P exposure

hif-α transcription levels measured in all treatment groups remained stable and similar to those measured in the control group, being mussels exposed to hypoxia the exception (Figures 4 and 5). *hif-α* mRNA levels were at their lowest after the hypoxic treatment, but significant differences were only found at day 7 in comparison to B[a]P treated mussels. Even when *hk* presented a general non-homogeneous transcriptional profile among treatments, no significant differences between treatments were found

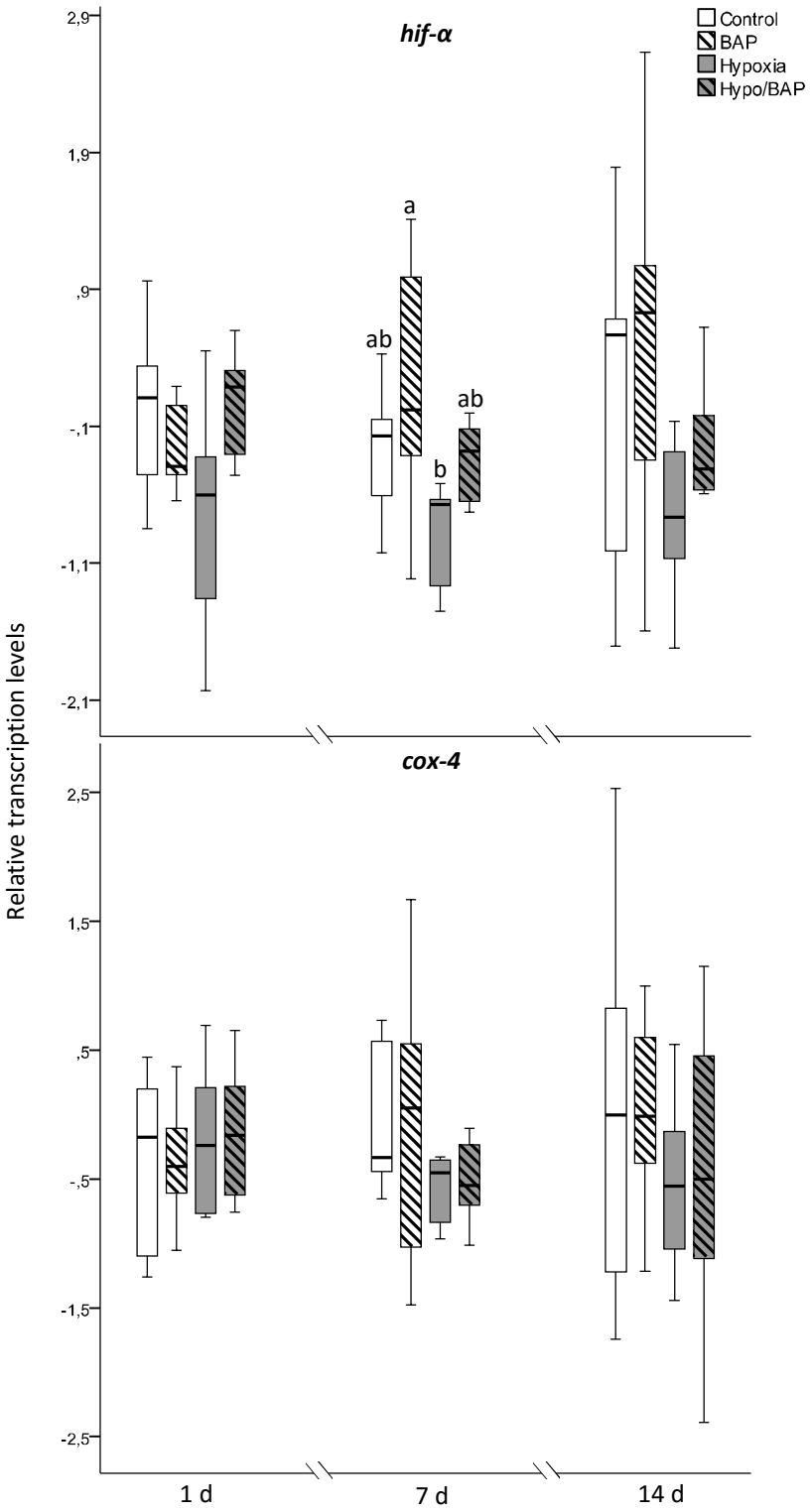

Figure 4. Relative transcription levels of *hif-α* and *cox-4* in the digestive gland of *M. galloprovincialis* under control and hypoxic conditions in absence and presence of B[a]P for 1, 7 and 14 days. Box plots represent the 25th, 50th and 75th percentiles, while whiskers represent the standard deviation. Significant differences ($p<0.05$) between treatments (control, hypoxia, B[a]P, and hypoxia/B[a]P) for the same time point are identified by different letters. Statistical differences were based on the Kruskal-Wallis one-way analysis of variance followed by the Dunn´s post-hoc test.

(Figure 4). Transcription levels of *cox-4* and *pepck* did not vary in a significant way (Figures 4 and 5). Optimized qPCR conditions for the specific and unique amplification of *aldolase* revealed the presence of unspecific amplicons in the dissociation curve; thus, quantification of *aldolase* transcription levels was discarded.

4. Discussion

Nucleotide sequence information of genes that could be related to the hypoxia adaptive pathway of mussels *M. galloprovincialis* was obtained and applied in a laboratory experiment in order to assess changes in their transcription levels. Fragments of *hif-α*, *cox-4*, *hk*, *aldolase* and *pepck* genes were successfully sequenced. The organ specific transcription profile of *hif-α* was characterized in mussels taken directly from the field; mRNA levels being at their highest in gills, followed by muscle, then mantle and finally digestive gland. After 1, 7 and 14 days of exposure to hypoxic conditions, B[a]P or combined exposure to B[a]P under hypoxic conditions, mRNA levels were analyzed in digestive gland in an attempt to assess whether such genes are transcriptionally regulated. Selection of this organ was based on its role in xenobiotic detoxification and due to the low individual variability recorded for *hif-α* transcription levels. qPCR analysis showed a decay in *hif-α* transcription levels during hypoxia and a large variability in *hk* mRNA levels among the different treatments. Transcription levels of *cox-4* and *pepck* remained unaltered.

The key genes of the hypoxia adaptive pathway in vertebrates are the 3 HIF-α paralog genes, which are specifically involved in the response to low oxygen availability in cells (Ratcliffe 2013). Piontkivska and colleagues (2011) characterized a *hif-α* ortholog of *C. virginica* which had significant sequence similarity with HIF-α orthologs from vertebrates and invertebrates, sharing key functional domains with them. This implies a highly conserved function of the molecule throughout the metazoan evolutionary history. Same authors constructed a minimum evolution tree with different HIF-α amino acid sequences in which those belonging to invertebrates clustered together; clearly differentiated from the vertebrate clade that was divided in the three extant paralogs (HIF-1α, 2α and 3α), so no specific number could be assigned to invertebrate HIF-α. Similar trees have been constructed in other studies with different molluscan

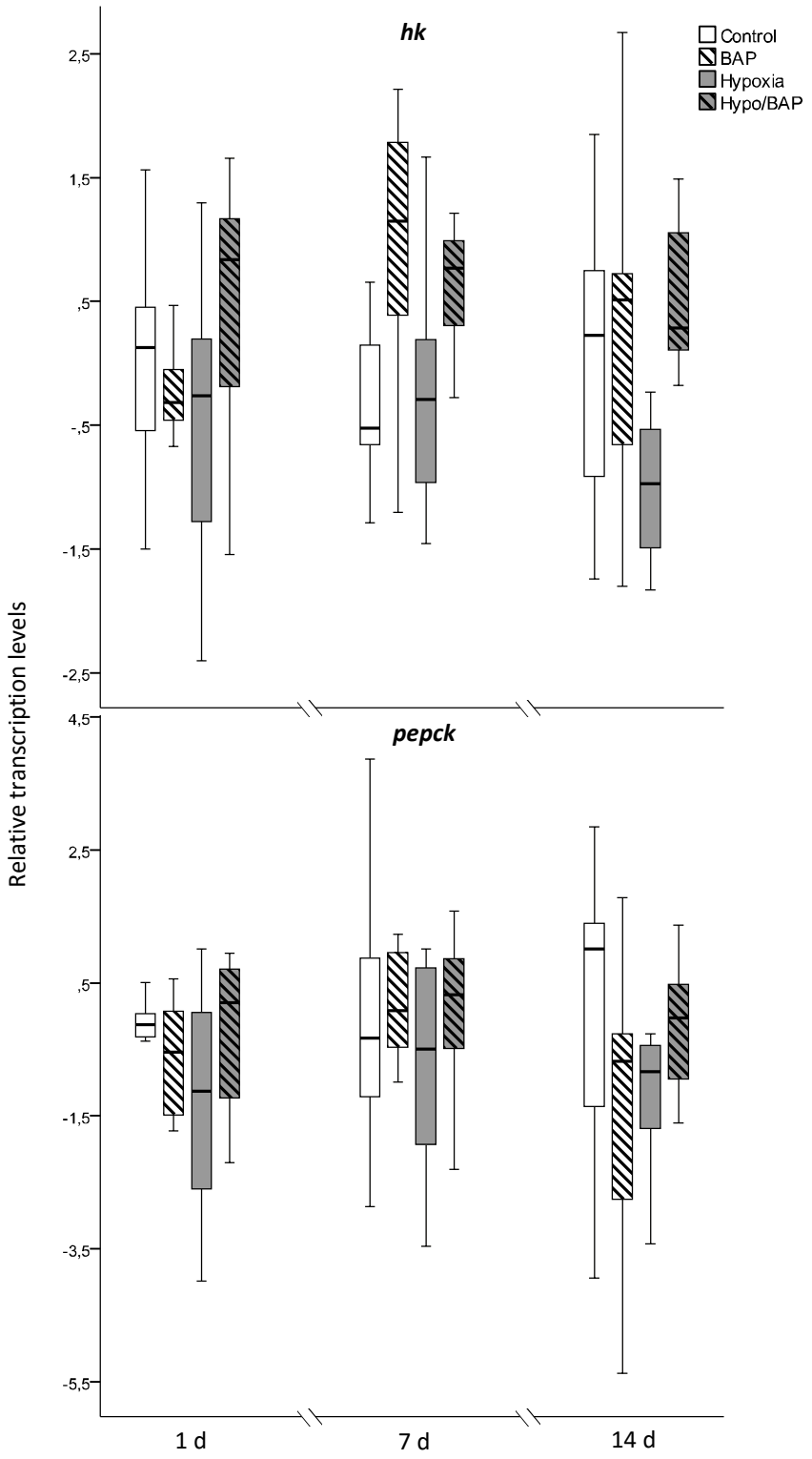

Figure 5. Relative transcription levels of *pepck* and *hk* in the digestive gland of *M. galloprovincialis* under control and hypoxic conditions in absence and presence of B[a]P for 1, 7 and 14 days. Box plots represent the 25th, 50th and 75th percentiles, while whiskers represent the standard deviation. No significant differences ($p<0.05$) were found, based on the Kruskal-Wallis one-way analysis of variance followed by the Dunn´s post-hoc test.

HIF-αs (Liu et al. 2014; Cai et al. 2014; Kawabe & Yokoyama 2012; Piontkivska et al. 2011). In concordance, the maximum likelihood tree constructed in this experiment clustered *M. galloprovincialis* HIF-α within the invertebrate clade, apart from the 3 vertebrate paralogs.

The organ specific transcriptional profile of *hif-α* has been previously studied in different aquatic invertebrate species. The magnitude of the differences found in the transcription level among tissues varies depending on the species analyzed. However, all studies coincide in pointing at gills as the tissue in which the basal transcription level of *hif-α* during normoxia is the highest (Soñanez-Organis et al. 2009; Piontkivska et al. 2011; Kawabe & Yokoyama 2012; Cai et al. 2014). Normoxic *M. galloprovincialis* mussels transcribed *hif-α* in the four analyzed organs. Based on similar results, Cai and colleagues (2014) discussed that the HIF pathway appears to be ubiquitous in all animal tissues, but the important role of gills in respiration would boost the presence of *hif-α* transcripts in such tissue. However, the high degree of individual variability quantified for the *hif-α* transcription levels in gills encouraged us to perform further analysis centered in the digestive gland, which displayed small inter-individual variability in that regard. In addition, digestive gland is a suitable target organ for the measurement of the potential interaction between, the hypoxia adaptive and the xenobiotic detoxification pathways in molluscs, since it is the main organ involved in xenobiotic accumulation and detoxification and one of the most metabolically active organs in bivalves (Liu et al. 2014; Liu et al. 2015).

Although post-translational regulation of the 3 HIF-α paralogs is accepted as the main mechanism to regulate their activity in vertebrates, examples of transcriptional changes that underpin the importance of gene transcription regulation at the mRNA level have been also described (Wiener et al. 1996; Rossignol et al. 2002; Rahman & Thomas 2007; Kopp et al. 2011; Rimoldi et al. 2012). It has been suggested that hypoxia-tolerant species could be able to increase *hif-α* mRNA levels under hypoxic conditions (Rissanen et al. 2006). This could be the case of most mollusc species that naturally endure periodical hypoxic events. Accordingly, several studies with this group of invertebrates have reported up-regulation of *hif-α* after hypoxia (Piontkivska et al. 2011; Kawabe & Yokoyama 2012; Cai et al. 2014). Opposed to this, we observed a

down-regulation trend for *hif-α* in the digestive gland under hypoxia. Comparable results were also observed in gills, muscle and digestive gland of *Litopeanus vannamei* shrimps, in which *hif-α* transcription levels were down-regulated under hypoxia in a time-dependent manner (Soñanez-Organis et al. 2009). Perhaps enhanced HIF-α protein stability leads to a negative feedback regulation as it has been described in other animals (Makino et al. 2007; Webb et al. 2009; Ratcliffe 2013). Similar processes have been suggested for bivalves, arguably to avoid the potential damage of re-oxygenation after hypoxic or anoxic stress (David et al. 2005; Ivanina et al. 2010).

During hypoxic events, invertebrates are known to switch from aerobic energy production pathways to anaerobic ones (Larade & Storey 2002; Wheaton & Chandel 2011) while the mitochondrial electron transport chain (ETC) remains at least partly active (Tielens & Van Hellemond 1998). Therefore, regulation of the ETC-related genes could be envisaged to occur in such situations. In vertebrates, *cox-4* (the gene coding for the 4th subunit of the ETC) has been shown to respond to changes in cellular oxygen concentration (Little et al. 2010). However, discrepancies have been reported regarding regulation of the two extant isoforms of this group of chordates (Hüttemann et al. 2001; Hüttemann et al. 2007; Kocha et al. 2015). COX-4 isoform 1 is the general form of the subunit, while isoform 2 prevails in well-oxygenated tissues and its concentration increases under hypoxic events. Strategies of ETC subunit regulation have been described in organisms basal to the metazoan lineage. For instance, *Saccaromyces cerevisiae* and *Dictiostellum discoideum* also switch between two protein isoforms related to the vertebrate COX-4 in response to low oxygen availability (Hüttemann et al. 2007). A linkage between vertebrate *cox-4* isoform regulation and enhanced ETC efficiency has been suggested, apparently to manage excessive reactive oxygen species generated during hypoxic events (Pierron et al. 2012). As expected, mussel COX-4 sequence clustered with those from invertebrates, in a clade unrelated to any of the two vertebrate isoforms (Figure 2). Despite regulation of COX subunits being related to both oxygen and reactive oxygen species levels in different metazoans, no transcriptional regulation was observed in our experimental set-up. Ivanina and co-workers (2010) reported up-regulation of *cox-4* in *C. virginica* oysters under anoxia. Besides, oysters *C. gigas* under hypoxic conditions for 7-10 or 24 days to

hypoxic conditions showed down-regulation of *cox-2* in both time points. On the other hand, *cox-3* was initially up-regulated while *cox-1* was up-regulated only after 24 days (David et al. 2005). Similarly, *M. galloprovincialis* mussels under hypoxia showed different transcriptional responses of ETC subunits depending on the time of exposure and the tissue analyzed (Woo et al. 2011).

Genes related to the glucose metabolism were also studied, a process regarded to be regulated in vertebrates under low oxygen availability (Semenza 2012). In the first place, *hk,* coding for the enzyme that catalyzes the first step of the glycolysis, has been described to be up-regulated under hypoxia, at least in the case of its II isoform (Mathupala et al. 2001; Greijer et al. 2005). The obtained mussel *hk* fragment was part of the hexokinase II superfamily. Its transcriptional profile in the animals of the experiment was non-homogeneous, but no significant differences were found between specific treatments. Hypoxia tended to down-regulate *hk* at day 14, and B[a]P exposure always resulted in higher transcription levels than its respective non-exposed counterpart (B[a]P vs control, B[a]P-hypoxia-B[a]P vs hypoxia). Absence of up-regulation of glycolytic genes was also recorded in *C. gigas* under hypoxia (David et al. 2005). Ivanina and colleages (2010) found *hk* transcription to be unaffected in *C. virginica* under anoxia. Unfortunately, mussel *aldolase* transcription levels could not be measured, probably because the obtained sequence fragment and the generated primer nonspecifically amplify different isoforms of the aldolase class I family of enzymes. In order to assess gluconeogenesis, transcription levels of *pepck* (which encodes the enzyme that converts oxaloacetate into phosphoenolpyruvate and carbon dioxide) were measured. It has been described that hypoxia-mediated HIF-1 transactivation of *pepck* plays a critical role in glucose homeostasis through the increased expression of gluconeogenic enzymes in rat hepatocytes (Choi et al. 2005). A mitochondrial and a cytosolic isoform have been described, the second one (PEPCK-C, present in metazoans and fungi) being regarded as hypoxia responsive (Hanson & Reshef 1997). Sequenced *M. galloprovincialis pepck* fragment was similar to that found in *C. gigas* (Table 2) by Moullac and colleagues (2007), which was described as cytosolic and being up-regulated in muscle after 10 days of hypoxia. That study pointed to the transcriptional regulation as the main mechanism to increase PEPCK

activity. Present results in mussels showed no mRNA level alterations for *pepck* under hypoxia, B[a]P exposure or both together. Similarly, *C. virginica* oysters under anoxia showed no transcriptional changes in *pepck* (Ivanina et al. 2010). This general unresponsiveness could be due to the fact that molluscs could to rely in a metabolic rate depression rather than in an enhancement of the glycolysis in situations of oxygen scarcity (Brooks & Storey 1997).

The sequence similarities between HIF-α molecules in vertebrates and invertebrates (Cai et al. 2014) and the possibility of the AHR-CYP axis being also involved in the detoxification of B[a]P (low concentrations, not at higher ones) in bivalves, as proposed in *Chlamys farreri* (Tian et al. 2013), encouraged conducting this preliminary study to see whether any interactions could occur between the hypoxic and xenobiotic detoxification pathways in mussels. The slight transcriptional down-regulation of *hif-α* and *hk* genes under hypoxia was not observed when mussels were co-exposed to B[a]P. Previous studies in vertebrate organisms reported an attenuation on the hypoxia adaptive responses caused by co-exposure with AHR agonists (Kraemer & Schulte 2004; Seifert et al. 2008; Schults et al. 2010). The results obtained do not allow to conclude anything on the presence of the suggested interaction between both adaptive pathways in molluscs, at least under the analyzed B[a]P exposure and oxygen concentrations tested.

In conclusion, our results suggest Mediterranean mussels rely in a metabolic rate depression during hypoxic events rather than in an enhancement of the glycolytic activity. In addition, mussels could be deploying a negative feedback strategy to protect themselves from re-oxygenation, a scenario they experience twice during the daily tidal cycle. The fact that both processes seemed to be (although not in a statistically significant way) negatively affected at the transcriptional level by co-exposure to B[a]P allowed thinking about interferences between both adaptive pathways in this bivalve species. Both, the xenobiotic detoxification and the hypoxia adaptive pathways, have to be studied in a deeper extent at the transcriptional and at the protein level to define their performance and to assess the presence of possible cross-talks in molluscs.

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Chapter 2

Exposure to benzo[a]pyrene under hypoxic conditions in the thicklip grey mullet *Chelon labrosus*: interactions of hypoxia and xenobiotic detoxification adaptive pathways at the transcriptional level?

Parts of this chapter have been presented at:

J. Martos-Bernal, E. Bilbao, I. Cancio. *Regulation of hypoxia and xenobiotic exposure related genes in fish (*Chelon labrosus*) exposed to Benzo[a]Pyrene combined with hypoxia*. 17th INTERNATIONAL SYMPOSIUM ON POLLUTANT RESPONSES IN MARINE ORGANISMS. Faro, Portugal. 5th-8th May 2013. Oral presentation.

J. Martos-Bernal, E. Bilbao, I. Cancio. *Regulation of hypoxia and xenobiotic exposure related genes in fish (*Chelon labrosus*) and mussels (*Mytilus galloprovincialis*) held under acute hypoxic conditions*. 28th EUROPEAN SOCIETY OF COMPARATIVE PHYSIOLOGY AND BIOCHEMISTRY. Bilbao, Spain. 2nd-5th September 2012. Poster.

This chapter has been submitted as:

J. Martos-Bernal, E. Bilbao, I. Cancio. *Exposure to benzo[a]pyrene under hypoxic conditions in the thicklip grey mullet* Chelon labrosus*: interactions of hypoxia and xenobiotic detoxification adaptive pathways at the transcriptional level?* Aquatic toxicology.

Abbreviations:

AHR	Aryl hydrocarbon receptor
ALDOLASE	Fructose-bisphosphate aldolase
ARNT, HIF-1β	Aryl hydrocarbon receptor nuclear translocator
B[a]P	Benzo[a]pyrene
bHLH-PAS	Basic helix-loop-helix Per/Arnt/Sim family
CYP1A	Cytochrome p450
DMSO	Dimethyl sulfoxide
EROD	Ethoxyresorufin-O-deethylase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HIFs	Hypoxia-inducible factors
NCBI	National Center for Biotechnology Information
PAS	PER/ARNT/SIM homology domain
PEPCK	Phosphoenol pyruvate carboxykynase
PHDs	Prolyl hydroxylase domain family of enzymes
qPCR	Quantitative PCR
ROS	Reactive oxygen species
VEGF	Vascular endothelial growth factor

Abstract

Fish populations on areas affected by human activities are likely to suffer impact of multiple stressors simultaneously, as it is the case for xenobiotic exposure under hypoxic conditions. Molecular pathways responsible for detection and adaptation to hypoxia and to xenobiotic exposure require the same transcription factor, aryl hydrocarbon receptor nuclear translocator (ARNT): it heterodimerizes with the two master transcription factors controlling adaptive pathways to both stressing situations, hypoxia inducible factor-α (HIF-α) and aryl hydrocarbon receptor (AHR). HIF-αs regulate the transcription of hypoxia response genes and AHR that of xenobiotic responsive genes. Thus, competition for ARNT could block the adequate response to each of both stimuli. In the present study, an *in vivo* lab experiment was carried out to assess this phenomenon exposing juvenile thicklip grey mullets *Chelon labrosus* to hypoxic conditions (2.5 mg O_2/L), to benzo[a]pyrene (B[a]P, 0.4 mg/L) and to hypoxia/B[a]P (same concentrations) for 1, 7 and 14 days. Gene fragments coding for hypoxia inducible factor-1α (*hif-1α*), 2α (*hif-2α*), 3α (*hif-3α*), *vascular endothelial growth factor* (*vegf*), *fructose-bisphosphate aldolase* (*aldolase*) and *glyceraldehyde-3-phosphate dehydrogenase* (gapdh) were first sequenced. Then, transcriptional levels of *hif-1α*, *hif-2α*, *hif-3α*, *arnt*, *vegf*, *aldolase*, *gapdh*, *phosphoenol pyruvate carboxykynase* (*pepck*) and *cytochrome p450 1a1* (*cyp1a1*) genes were measured in liver and gills. General histology of gills was assessed at the end of the experiment (14 days), showing increased prevalence of aneurysm affection in mullets under hypoxia, which was attenuated under co-exposure to B[a]P. Hypoxia/B[a]P treatment reduced the transcriptional levels of *hif-α*s and *arnt* (especially in liver) and tended to increase the *cyp1a1* up-regulation elicited by exposure only to B[a]P. B[a]P also increased the mRNA levels of *hif-2α* in gills. Alterations in glucose metabolism related genes were also detected in the combined treatment. Thus, the response to xenobiotic exposure seems to be favored over adaption to hypoxia, which may have implications under the envisaged future trends that point out to an increase in hypoxia events in our coastal areas.

Keywords: hypoxia, benzo[a]pyrene, *Chelon labrosus*, transcription, HIF-α, ARNT

Resumen

Las poblaciones de peces localizadas en áreas afectadas por actividades humanas pueden sufrir el efecto simultáneo de múltiples factores estresantes, éste es el caso de la exposición a xenobióticos en condiciones de hipoxia. Las rutas moleculares encargadas de detectar y adaptarse a las condiciones hipóxicas y a la exposición a xenobióticos requieren el mismo factor de transcripción, el translocador nuclear del receptor de hidrocarburos arilo (ARNT). Éste heterodimeriza con los factores de transcripción clave para ambas rutas de adaptación, el factor inducido por hipoxia-α (HIF-α) y el receptor de hidrocarburos arilo (AHR). Los HIF-αs regulan la transcripción de los genes de respuesta a hipoxia, mientras que AHR hace lo propio con los genes de respuesta a xenobióticos. Por lo tanto, la competición por ARNT podría bloquear la respuesta adecuada a cada uno de los estímulos. En este estudio se analizó dicho fenómeno mediante un experimento de laboratorio en el que mubles *Chelon labrosus* juveniles fueron sometidos a condiciones de hipoxia (2.5 mg O_2/L), exposición a benzo[a]pireno (B[a]P, 0.4 mg/L) y a hipoxia/B[a]P (mismas concentraciones) durante 1, 7 y 14 días. Primero se secuenciaron fragmentos de los siguientes genes: *factor inducido por hipoxia-1α* (*hif-1α*), *2α* (*hif-2α*), *3α* (*hif-3α*), *factor de crecimiento endotelial vascular* (*vegf*), *fructosa-bisfosfato aldolasa* (*aldolasa*) y *gliceraldehído-3-fosfato deshidrogenasa* (*gapdh*). Después se midieron lo niveles de transcripción de *hif-1α*, *hif-2α*, *hif-3α*, *arnt*, *vegf*, *aldolasa*, *gapdh*, *fosfoenolpiruvato carboxiquinasa* (*pepck*) y *citocromo p450 1a1* (*cyp1a1*) en hígado y branquia. Se realizó también una evaluación histológica de las branquias que mostró un aumento de la prevalencia de aneurismas en los mubles sometidos a hipoxia. Dicho aumento fue atenuado por la co-exposición de los peces a B[a]P. El tratamiento hipoxia/B[a]P redujo los niveles de transcripción de los *hif-α*s y *arnt* (especialmente en hígado), a la vez que se observó una tendencia a aumentar la sobrerregulación causada por la exposición sólo a B[a]P. Los niveles de mRNA de *hif-2α* también aumentaron en branquia tras la exposición a B[a]P. El tratamiento combinado causó también alteraciones en los genes relacionados con el metabolismo de glucosa. En resumen, la respuesta a xenobióticos parece prevalecer sobre la de adaptación a hipoxia, lo que puede resultar relevante ante las previsiones futuras de un aumento de los eventos de hipoxia en nuestras áreas costeras

Palabras clave: hipoxia, benzo[a]pireno, *Chelon labrosus*, transcripción, HIF-α, ARNT

Laburpena

Giza jardueren eragina jasaten duten lekuetan bizi diren arrain populazioak faktore estresagarri anitzen pean bizi dira, esate baterako, hipoxia egoeretan ematen den xenobiotikoen esposizioa. Hipoxiaren detekzio eta hipoxiaren aurreko adaptazioaz, zein, xenobiotikoen esposizioaz arduratzen diren molekula mailako bidezidorrek transkripzio faktore beraren beharra daukate, aril hidrokarburoen hartzailearen translokatzaile nuklearra (ARNT). Honek bi adaptazio bidezidorretan gakoak diren transkripzio faktoreekin heterodimerizatzen du, hipoxiak induzitutako □-faktorearekin (*hif-*□) eta hidrokarburo ariloen hartzailearekin (AHR). HIF-α faktoreek hipoxia peko erantzuna ematen duten geneen transkripzioa erregulatzen dute, AHR-ek aldiz, xenobiotikoen peko geneena. Beraz, ARNT-rekiko duten lehiak kinada bakoitzaren aurrean duten erantzun egokia blokea lezake. Ikerketa honetan, fenomeno hau aztertzeko asmoz, laborategiko *in vivo* esperimentu bat burutu zen *Chelon labrosus* korrokoi heldugabeak hipoxia pean (2.5 mg O_2/L), benzo[a]pireno pean (B[a]P, 0.4 mg/L) eta hipoxia/B[a]P pean (kontzentrazio berdinak) 1, 7 eta 14 egunez mantenduz. Lehenengo, *hipoxiak induzitutako 1α-faktorea* (*hif-1α*), *2α* (*hif-2α*), *3α* (*hif-3α*)*, hazkuntza faktore endotelial baskularra* (*veaf*), *fruktosa-bifosfato aldolasa* (*aldolasa*) eta *glizeraldehido-3-fosfato deshidrogenasa* (*gadph*) geneen zatiak sekuentziatu ziren. Ondoren *hif-1α*, *hif-2α*, *hif-3α*, *arnt*, *vegf*, *aldolasa*, *gapdh*, *fosfoenolpirubato karboxikinasa* (*pepck*) eta *p450 1a1 zitokromoaren* (*cyp1a1*) transkripzio mailak neurtu ziren gibel eta zakatzetan. Gainera, zakatzen azterketa histologiko orokorra burutu zen, hipoxia pean mantendutako korrokoietan eta aneurismen prebalentziaren areagotzea behatu zen. Areagotze hori ahuldu egin zen B[a]P-a gehitzean. Hipoxia/ B[a]P tratamenduak *hif-α* eta *arnt* geneen transkripzio mailak murriztu zituen (gibelean batez ere), era berean, B[a]P-ak bakarrik erregulazioa areagotu zuen. *hif-2α*-ren mRNA mailak ere zakatzetan areagotu egin ziren B[a]P-pean. Tratamenduen konbinazioak ere, glukosaren metabolismoarekin erlazionaturiko geneetan aldaketak eragin zituen. Laburbilduz, xenobiotikoen peko erantzuna gailendu egiten da hipoxiarekiko adaptazioaren gainetik, eta hau kezkagarria izan daiteke gure kostaldeko hainbat gunetan aurreikusita dagoen hipoxia egoeren handipenari lotuta.

Hitz gakoak: hipoxia, benzo[a]pyrenoa, *Chelon labrosus*, transkribapena, HIF-α, ARNT

1. Introduction

Coastal and estuarine areas worldwide are the places where most of the human population is concentrated. Therefore, water bodies in those areas are likely to suffer from human impact, as it is the case for xenobiotic contamination derived from farming, urban and industrial activities (Ravindra et al. 2008; Tobiszewski & Namieśnik 2012). Furthermore, aquatic ecosystem functions might be altered by other factors, sometimes causing situations of low dissolved oxygen availability (Levin 2002; Helly & Levin 2004). Human influence is known to aggravate such hypoxic events (Keeling & Garcia 2002; Bopp et al. 2002; Diaz & Rosenberg 2008; Keeling et al. 2010; Rabalais et al. 2010; Bijma et al. 2013). Thus, animal populations in these affected ecosystems will need to cope with multiple stressors simultaneously and trigger the adequate adaptive responses to achieve homeostasis.

Metazoan genomes feature a family of genes coding for transcription factors known as basic helix-loop-helix Per/Arnt/Sim (bHLH-PAS) proteins, some of them involved in sensing environmental variables; for instance the presence of small molecules such as hormones or xenobiotics, or the reduction in available oxygen levels (Goldstone et al. 2006). The aryl hydrocarbon receptor nuclear translocator (ARNT) was described for the first time as a transcription factor required by the aryl hydrocarbon receptor (AHR), both members of the bHLH-PAS family, to trigger transcriptional responses leading to the detoxification of different environmental pollutants such as polycyclic aromatic hydrocarbons or polychlorinated aromatic compounds (Hoffman et al. 1991). Later, Wang & Semenza (1995) found ARNT, which they termed hypoxia inducible factor-1β, to be needed by hypoxia inducible factor-1α (HIF-1α), another member of the bHLH-PAS family, as heterodimeric partner to trigger specific transcriptional responses under low oxygen availability conditions. Such responses under hypoxia would modify glucose metabolism, enhance angiogenesis, increase gill surface area or reduce growth rate; among other functions (Wu 2002; Nikinmaa & Rees 2005; Semenza 2012). Therefore, both transcription factors, AHR and HIF-1α (along with HIF-2α and HIF-3α, its paralogs in fish species), require ARNT to regulate the transcription of xenobiotic related genes in one case and hypoxia related genes in the other. Co-occurrence of

pollutants under hypoxic conditions could thus severely affect the ability of organisms to adapt through competition for ARNT (Vorrink & Domann 2014).

Besides, interferences at levels that do not include competition for ARNT have also been proposed. For example, the generation of reactive oxygen species (ROS) as byproduct of the processes leading to xenobiotic detoxification may shift the state of the iron contained in prolyl hydroxylases (PHDs), blocking their activity in the constitutive degradation of HIF-1α that occurs under normoxic conditions. Thus, exposure to xenobiotics itself could be causing a hypoxia mimicking state (Pouysségur & Mechta-Grigoriou 2006; Cash et al. 2007).

According to this scenario, events such as the Deepwater Horizon oil spill in the Gulf of Mexico (the second largest seasonal hypoxic zone in the world, Thomas & Rahman 2012), highlight the importance of studying the effects derived from the potential molecular crosstalk for adaptation to exposure to AHR agonists in conditions of low oxygen availability. In that sense, alterations in xenobiotic detoxification pathways under hypoxic conditions have been reported in fish even in the absence of AHR agonists (Leveelahti et al. 2011; Rahman & Thomas 2012). In fish exposed to both stressing situations hints of this crosstalk have been described (Prasch et al. 2004; Kraemer & Schulte 2004), where activation of one of the pathways affected the other one. For instance, Yu and colleagues (2008) found benzo[a]pyrene (B[a]P) to alter the transcription of hypoxia responsive genes in *Epinephelus coioides* during hypoxia while xenobiotic responsive genes were unaffected by hypoxia.

In this study juvenile *Chelon labrosus*, considered a useful pollution sentinel species in the monitoring of the coastal areas of the Bay of Biscay (Bilbao et al. 2010; Ortiz-Zarragoitia et al. 2014; Pujante et al. 2015), were studied as experimental organisms. Habitat and behavior of thicklip grey mullets (Ortiz-Zarragoitia et al. 2014) allows to think that this species is quite tolerant to hypoxia, and thus a good candidate to analyze adaptive changes under low oxygen availability in polluted waters. During experimentation, fish were maintained under 3 different conditions (hypoxia, exposure to B[a]P and the combination of hypoxia and B[a]P exposure) to assess interactions between hypoxia and xenobiotic detoxification molecular pathways. In this context,

the transcription levels of genes related to both adaptive molecular pathways were analyzed through qPCR. *hif-1α*, *hif-2α*, *hif-3α* and *arnt* genes were selected as key transcription factors, vascular endothelial growth factor (*vegf*) in relation to angiogenesis, fructose-bisphosphate aldolase (*aldolase*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and phosphoenol pyruvate carboxykynase (*pepck*) to assess glucose metabolism and cytochrome p450 1a1 (*cyp1a1*) in relation to xenobiotic detoxification. In addition, general morphology and presence of histopathological alterations in gills of mullets were assessed.

2. Materials and Methods

2.1. Experimental animals

Immature thicklip grey mullets *Chelon labrosus* were captured with a fishing rod to a final number of 126 (12-17.5 cm in length) in the estuary of the Butroi river in Plentzia (43º24´N; 2º57´N), a relatively non-polluted place (Orbea & Cajaraville 2006). Mullets were acclimatized to laboratory conditions in a 300 L tank with clean seawater (33.3‰ salinity, 47 mS conductivity, pH 7.8) supplied from an aquaculture farm pipe (Getaria, 43º18´13.26´´N; 2º14´21.96´´W) for 2 months. Water was decanted and sequentially filtered through 100, 50 and 5 µm pore size filters while receiving ultraviolet treatment, then stabilized to 18ºC prior to a final filtering step through a 0.45 µm pore size filter. Normal photoperiod was set providing 12 h of light supply. Animals were daily fed during the acclimatization with Vipagran (Sera GmbH, Heinsenberg, Germany). A week prior to the experiment fish were transferred to 60 L tanks (oxygen concentration = 9±0.2 mg O_2/L) where the experiment would take place, and fed every 3 days. The experimental procedures were performed following the specifications agreed with the Ethics Committee for Animal Experimentation of the University of the Basque Country under approved experimental protocol (CEEA/151/2010/CANCIO).

2.2. Maintenance of fish under hypoxic conditions and exposure to benzo[a]pyrene

Tanks, containing an electrically fed air source and a spigot on the lid to avoid overpressure, were sealed. Tanks that contained fish under hypoxic conditions were provided with a nitrogen gas source, and an Oxi::lyser probe (s::can, Messtechnik

GmbH, Vienna, Austria) was used to measure temperature and oxygen concentrations. Such parameters were monitored by a Con::lyte terminal (s::can, Messtechnik GmbH, Vienna, Austria). Whenever water oxygen concentration exceeded 2.5 mg O_2/L, a relay-connected valve automatically pumped nitrogen into the tanks to maintain the desired oxygen concentration. Initially, dissolved oxygen was between 9.2 and 8.9 mg O_2/L, concentration which was gradually decreased in the hypoxic tanks to achieve 2.5 mg O_2/L in 4 h.

Animals in all experimental groups were exposed to 0.01% (v/v) dimethyl sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MI, US), as it was used as vehicle to dose benzo[a]pyrene (B[a]P, Sigma–Aldrich) in the two B[a]P exposure groups. These two groups received 0.4 mg B[a]P/L dissolved in DMSO. Both hypoxia tanks, one in combination with B[a]P exposure, were maintained at 2.5 mg O_2/L (not reaching hypoxia levels, but close to the average definition of 2.1 mg O_2/L, Farrell & Richards 2009). Food was supplied every 3 days, prior to partial water renewal that was accompanied by new B[a]P and DMSO dosage. Fish behavior was monitored during the whole experiment. Samples (liver and gills) were taken from 7 fish per treatment after 1, 7 and 14 days in the experimental tanks. After immersion in RNA later® (Ambion-Invitrogen, Life Technologies, Carlsbad, USA) both mullet tissues were frozen in liquid nitrogen and maintained at -80^{o}C until further needed. In addition, a sample of gills was taken from 7 individuals per treatment after 14 days, placed in histological cassettes and immersed for fixation in 10% neutral buffered formalin at 4°C for 24 h. Afterwards, tissues were transferred to 70% ethanol and stored at 4°C until further processing.

2.3. RNA extraction and cDNA synthesis

Mullet liver and gills were homogenized using a Hybaid Ribolyser™ cell disrupter (Hybaid, Ashford, UK) with zirconia/silica beds (Biospec, Bartlesville, USA) for 40 seconds at 4 m/s. In order to extract total RNA, TRIzol® (Ambion-Invitrogen) method was followed. Extracted total RNA was treated with the MinElute PCR Purification kit (Qiagen, Hilden, Germany) to eliminate contamination. Final concentration and purity were measured in a Biophotometer (Eppendorf, Hamburg, Germany), accepting

A260/A280 ratios between 1.8 and 2. Retrotranscription was performed using the Superscript^{TM} First-Strand cDNA Synthesis kit (Ambion-Invitrogen) with random hexamers in a 2720 Thermal Cycler (Life Technologies). Synthesized cDNA was stored at -40^{o}C.

2.4. Sequencing of target genes

Since no sequence information was available at the international data bases regarding hypoxia inducible factor-1α (*hif-1α*), hypoxia inducible factor-2α (*hif-2α*), hypoxia inducible factor-3α (*hif-3α*), vascular endothelial growth factor (*vegf*), fructose-bisphosphate aldolase (*aldolase*) and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) genes of *C. labrosus*, they had to be sequenced. Sequences from fish species phylogenetically close to *C. labrosus*, and available in the GenBank (National Center for Biotechnology Information, NCBI), were aligned with ClustalW2 (Larkin et al. 2007). Conserved regions allowed designing primers to amplify fragments belonging to such target genes. Electrophoresis in 1.5 % agarose gel stained with ethidium bromide were used to visualize the PCR products. Positive fragments were sequenced in the Sequencing and Genotyping Service of the University of the Basque Country. Blastx analysis (NCBI) was used to check for homology of amplified sequences. Only identity values above 70% and e-values below 1e^{-50} were considered. Structural analyses of amplified fragments were contrasted with the NCBI conserved domain database (Marchler-Bauer et al. 2014).

A phylogenetic tree based on the deduced amino acid sequences of the amplified *C. labrosus hif-α* gene family was designed to assess the relations among *hif-1α*, *hif-2α* and *hif-3α*. Protein sequences belonging to *Homo sapiens* (NP_001521, NP_001421, NP_690007), *Megalobrama amblycephala* (ADF50043, ADF50044, ADF50045), *Schizothorax prenanti* (AFU07561, AFU07569, AFU07576), *Clarias batrachus* (AGT15726, AGT15727, AGT15728) *hif-α*s and the amino acid sequences deduced from cloned and sequenced *Chelon labrosus* fragments were aligned using the MEGA 6 program (Tamura et al. 2013). The phylogenetic analysis was based on the maximum likelihood method.

2.5. Quantitative PCR (qPCR) analyses.

Transcriptional levels of *hif-1α*, *hif-2α*, *hif-3α*, *arnt*, *vegf*, *aldolase*, *gapdh*, *pepck* and *cyp1a1* were normalized using *β-actin* as reference gene (its coefficient of variation within the experiment was below 5%). qPCR analyses were performed in a 7300 Real Time PCR System (Applied Biosystems). The cDNA of at least 5 individuals per experimental group was amplified in triplicates in a total reaction volume of 20 µl, containing 10 µl of SYBR Green (FastStart Universal SYBR Green Master, Roche, Indianapolis, USA). cDNA and primer concentrations are detailed in Table 1. PCR conditions were set as follows: 50°C for 2 min, 95°C for 10 min; 40 cycles at 95°C for 15 s followed by the melting temperature (see Table 1) for 30 s. Each primer set specificity was certified by analyzing the dissociation curve of amplicons to verify the presence of a single melting peak. The Delta-Delta Ct method, corrected for PCR efficiency, was used to assess the relative transcription levels of selected genes (modified from Pfaffl 2001).

2.6. Gill histology

Gill histological samples were processed under vacuum conditions in an ASP300 Tissue Processor (Leica Microsystems, Nussloch, Germany). Plastic molds were used to produce the paraffin blocks. 5 µm-thick sections were cut in a RM2125RT microtome (Leica Microsystems), slides were stained with hematoxylin/eosin in an Auto Stainer XL (Leica Microsystems) and mounted in DPX (Sigma-Aldrich) by means of a CV5030 Robotic Coverslipper (Leica Microsystems). Histopathological alterations were observed in 7 samples per group using a BX51 light microscope (Olympus, Tokyo, Japan). The extent of aneurysm affection in each individual was quantified according to the following formula:

$$\text{Aneurysm extent (\%)} = \frac{x \cdot \left({}^{y}/_{2}\right)}{z} \cdot 100$$

Where “x” represents the number of secondary lamellae affected by severe aneurysm (causing a complete structural disruption of the secondary lamella), “y” by mild aneurysm (morphology of the secondary lamella visible yet) and “z” the total of primary lamellae found in each sample.

Table 1. Primer sequences and melting temperatures used for the amplification of target genes by qPCR, followed by cDNA concentration used from each of the studied organs. Final primer concentration used for the amplification of all cDNA fragments was 1.25 pmol/µL.

			[cDNA]	
Gene	**Primer sequence (5´-3´)**	**T. (°C)**	**Liver (pg/µL)**	**Gills (pg/µL)**
hif-1α Fw	CACAGTTGGACAAAGACGTCTCA	56	200	200
hif-1α Rv	GCTCCCTGTCCCATGATCTC			
hif-2α Fw	CTGGAAGCGGATTCGGTAAA	56	200	200
hif-2α Rv	TGACGGYGCGTCCTCTGTT			
hif-3α Fw	CCACGTCCAGGTCCGAGTAA	59	200	200
hif-3α Rv	CCTGCTGGTCAGTGTGCTCTT			
arnt Fw	GACTTGAAGACAGGAACAGTGAAGAAG	55,5	200	200
arnt Rv	TGCCACATCTCATTCTGCAGAT			
vegf Fw	ATGCAGGAGTATCCGGAGGAA	57	200	1000
vegf Rv	TGCACTGCAGCATCTCATCA			
aldolase Fw	CGTGGGTGGCGTCATCTT	55,5	25	200
aldolase Rv	GCCAACAACAATGCCCTTGT			
gapdh Fw	CTGGAGAAAGCCGCCAAGTA	55,5	25	200
gapdh Rv	CGACCTGATCCTCTGTGTATGC			
pepck Fw	CCCAGTGCCTGCGGTAAA	54	200	1000
pepck Rv	CGAACTTCATCCACGCAATG			
cyp1a1 Fw	CTGCACCACAAAAGACACATCTC	55,5	200	200
cyp1a1 Rv	CCACAGCTCAGGATCATGGTT			
ß-actin Fw	ACCCAGATCATGTTCGAGACCTT	57	200	200
ß-actin Rv	TGACGATACCGGTGGTACGA			

2.7. *Data analysis*

Statistical analysis of gene transcription levels and the extent of aneurism affection in gills were based in the Kruskal-Wallis one-way analysis of variance test in order to establish statistical differences between groups. Dunn´s post-hoc test was then applied to perform the final multiple comparison. Significant differences were established at a $p<0.05$. Statistical analysis was performed using the SPSS 22.0 software.

3. Results

3.1. Sequencing of target genes

Used primers allowed the amplification of specific fragments. Upon sequencing and analysis based on Blastx, obtained transcript amplicons were identified as *hif-1α*, *hif-2α*, *hif-3α*, *vegf*, *aldolase* and *gapdh* (Table 2). Structural analysis of the deduced

amino acid sequence of *hif-1α* confirmed the presence of the complete hypoxia-inducible factor-1 region. Sequenced *hif-2α* fragment showed a partial helix-loop-helix domain and a complete PAS domain, while *hif-3α* featured 3 partial PAS domains. On the other hand, the *vegf* fragment showed the complete platelet-derived and vascular endothelial growth factors family domain and the major part of the VEGF heparin-binding domain. The sequenced *aldolase* fragment contained a large portion of the active site of the enzyme and a small fragment of its intersubunit interface. Finally, the *gapdh* fragment included most of the C-terminal domain of the coding domain sequence.

Table 2. GenBank accession number and length in base pairs of obtained *Chelon labrosus hypoxia inducible factor-1α* (*hif-1α*), *hypoxia inducible factor-2α* (*hif-2α*), *hypoxia inducible factor-3α* (*hif-3α*), *vascular endothelial growth factor* (*vegf*), *fructose-bisphosphate aldolase (aldolase*) and *glyceraldehyde-3-phosphate dehydrogenase (gapdh*) gene fragments, followed by the e-value, maximal amino acid identity and percentage of the total open reading frame (ORF) obtained for each gene in comparison to its closest available ortholog sequence (organism) as revealed by Blastx analysis.

Gene (Acc. number)	Length (bp)	E-value	Identity	ORF	Organism (Acc. number)
hif-1α (JF732801)	871	1.E-124	79%	38.54%	*Sander lucioperca* (ABO26718)
hif-2α (KM402135)	593	2.E-114	87%	23.1%	*Micropogonias undulatus* (ABD32159)
hif-3α (KM402136)	395	2.E-58	73%	20.37%	*Megalobrama amblycephala* (ADF50045)
vegf (JX983145)	471	6.E-99	90%	62.3%	*Notothenia coriiceps* (XP_010768739)
aldolase (KP981393)	418	6.E-83	91%	36.54%	*Epinephelus coioides* (AER42679)
gapdh (KP981394)	388	7.E-84	95%	38.74%	*Kryptolebias marmoratus* (ACJ64842)

Phylogenetic analysis of the three *C. labrosus hif-α* sequenced fragments clearly identified them as each of the three different fish *hif-α* paralogs (Figure 1).

3.2. Experimental setup and fish behavior

Oxygen levels measured in the two experimental groups maintained under hypoxia were constant in the range of 2.46 and 2.53 mg O_2/L. Slight and punctual oscillations were recorded that in no case were above 2.8 or below 2.3 mg O_2/L, during the 14 days that the experiment lasted. Fish under low oxygen availability conditions were less active than those maintained under oxygen saturating conditions. In addition, no aquatic surface respiration was observed for the fish in any of the treatments. No mortality was observed either.

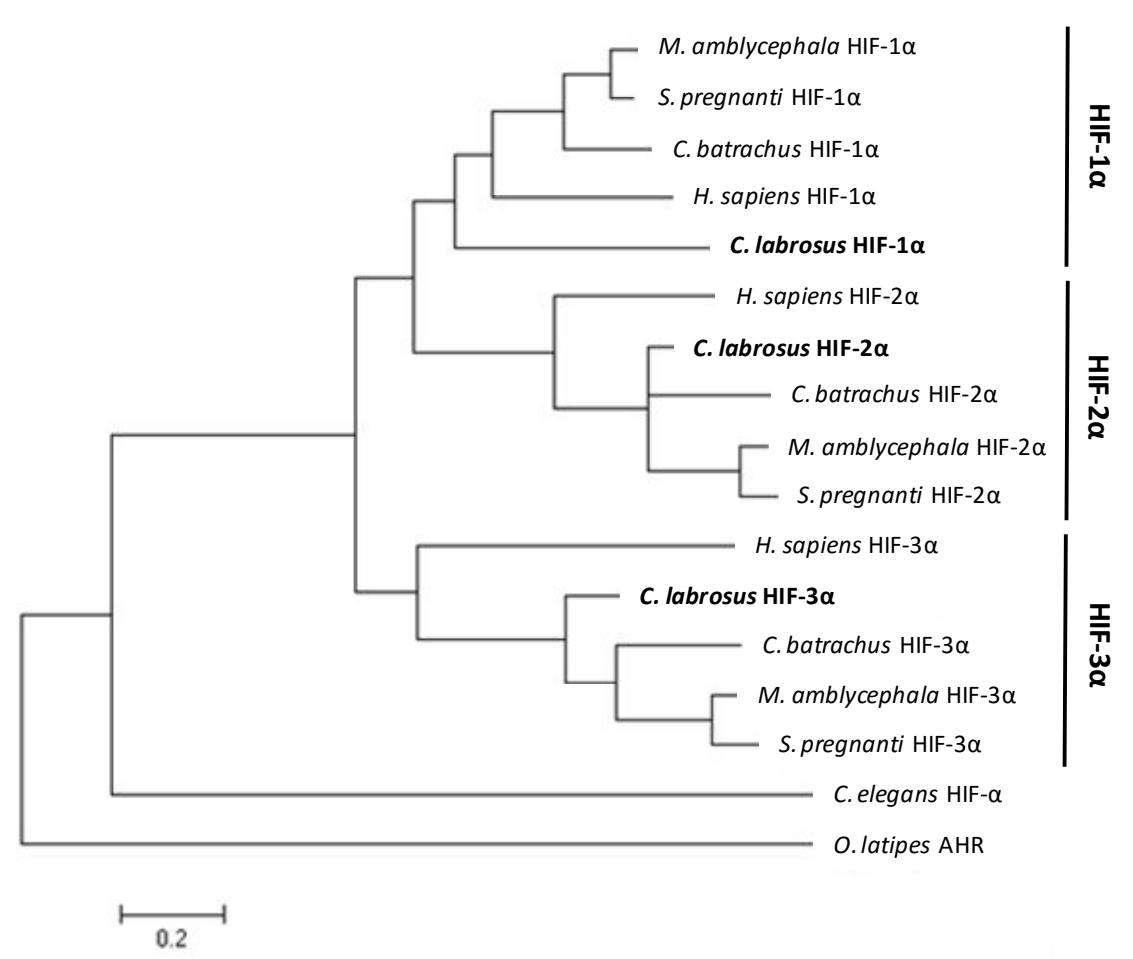

Figure 1. Phylogenetic tree based for the correct unambiguous annotation of the three *C. labrosus hif-α* fragments sequenced. Deduced amino acid sequences belonging to *Homo sapiens* (NP_001521, NP_001421, NP_690007)*, Megalobrama amblycephala* (ADF50043, ADF50044, ADF50045)*, Schizothorax pregnanti* (AFU07561, AFU07569, AFU07576)*, Clarias batrachus* (AGT15726, AGT15727, AGT15728) and *Chelon labrosus* (JF732801, KM402135, KM402136) *hif-α*s were aligned using MUSCLE software in the MEGA 6 program. The phylogenetic analysis was carried out based on the maximum likelihood method. Amino acid sequences belonging to *Caenorhabditis elegans* HIF-*α* and *Oryzias latipes* AHR were included as outgroups (NP_001023893 and NP_001098148, respectively).

3.3. Histopathology and aneurysm affection in gills

Morphology of gills was assessed after 14 days of experiment. Several histopathological alterations were observed (shortening of the secondary lamellae, increased thickness of the primary lamellae epithelium, lifting of the respiratory epithelium and fusion of the adjacent secondary lamellae) but the most conspicuous lesion was the presence of aneurysms (Figure 2). Fish under hypoxic conditions were the most deeply affected ones, in fact, all animals under hypoxia alone suffered from

severe aneurysms. Extent of this lesion was only significantly higher than in the case of the animals exposed to B[a]P alone (Figure 2).

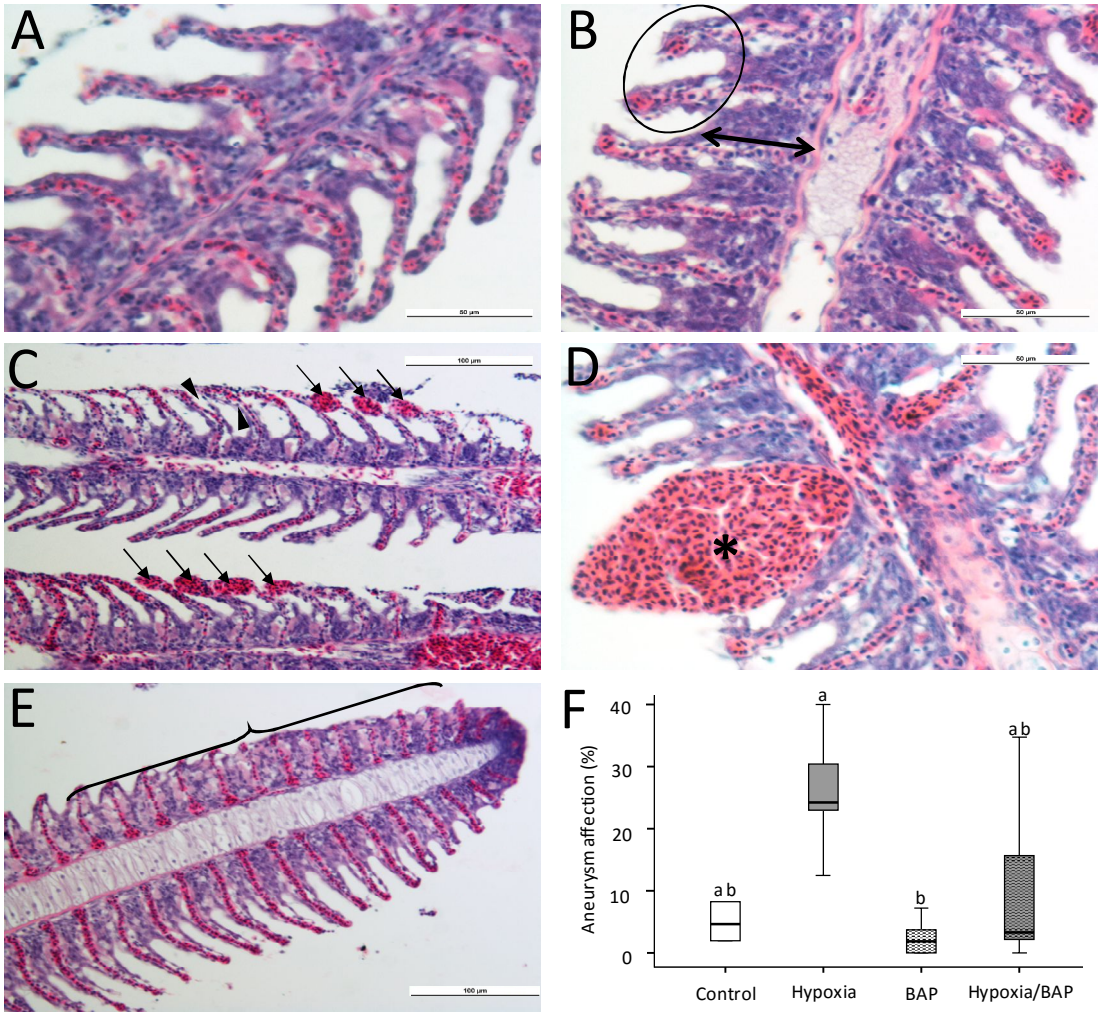

Figure 2. Micrographs of hematoxylin/eosin stained *C. labrosus* gills after 14 days of experiment. (A) Gills of a control animal. (B) Gills showing increased thickness of the primary lamellae epithelium (double-headed arrow) and shortening of the secondary lamellae (black circle) under hypoxia. (C) Gills showing lifting of the respiratory epithelium (arrowheads) and mild aneurysm (single-headed arrows) in the hypoxia group. (D) Gills showing a severe aneurysm (*) in the hypoxia group. (E) Gills showing fusion of the adjacent secondary lamellae ({) in a fish exposed to B[a]P under hypoxia. (F) Extent of aneurysm affection in gills of *C. labrosus* under control and hypoxic conditions in absence and presence of B[a]P for 14 days. Box plots represent the 25th, 50th and 75th percentiles, while whiskers represent the standard deviation. Significant differences (p<0.05) between treatments (control, hypoxia, B[a]P, and hypoxia/B[a]P) are identified by different letters. Statistical differences were based on the Kruskal-Wallis one-way analysis of variance followed by the Dunn´s post-hoc test.

3.4. Transcription levels of selected target genes in liver

Hypoxia did not significantly regulate transcription levels of *hif-1α, 2α* and *3α*. No time-dependent variations were detected either. The same occurred with exposure to B[a]P

alone. In contrast, exposure to B[a]P under hypoxic conditions modified transcription levels for the three *hif-α* genes. After 1 and 7 days of exposure to B[a]P under hypoxia *hif-1α* showed the lowest transcription levels, these being significantly lower than those measured in the B[a]P and in the hypoxia treatments, respectively. Hypoxia-B[a]P treatment resulted in a time-dependent down-regulation of *hif-2α* mRNA levels which turned to be significantly lower than those measured under control and hypoxic conditions at day 7. Transcription levels of *hif-3α* in the hypoxia-B[a]P group decreased from day 1 to day 7, when the combined treatment resulted in significantly lower values than those measured in the control and in the hypoxia treatments (Figure 3).

Regarding *arnt*, transcription levels in the hypoxia and in the B[a]P groups decreased in a time-dependent manner. Such trend was even deeper in the hypoxia-B[a]P group, with significantly lower values than the B[a]P group at day 1, than control and hypoxia groups at day 7 and than control group at day 14. Transcription levels of *vegf* peaked at day 7 for all treatments, especially for the hypoxia and the hypoxia-B[a]P groups, with significantly higher values than at days 1 and 14 respectively. *cyp1a1* was significantly up-regulated at day 1 in both B[a]P exposure groups. Time-dependent decrease in mRNA levels occurred in both B[a]P-containing groups, and only hypoxia-B[a]P showed *cyp1a1* up-regulation at days 7 and 14 in comparison to B[a]P-free groups (Figure 4).

Among genes measured as markers of glycolysis and gluconeogenesis (Figure 5) only minor fluctuations in transcription levels were recorded after maintaining fish under hypoxic conditions or after exposure to B[a]P. However, mRNA levels of *aldolase* in the hypoxia-B[a]P group were significantly lower than those measured in the hypoxia and the control groups at days 7 and 14, respectively. The hypoxia-B[a]P combination resulted in significantly lower transcription levels of *gapdh* in comparison to the hypoxia group at day 7. Finally, *pepck* was significantly up-regulated in the hypoxia-B[a]P group when compared to the control and hypoxia groups at day 1. Such up-regulation disappeared from the combined treatment at days 7 and 14.

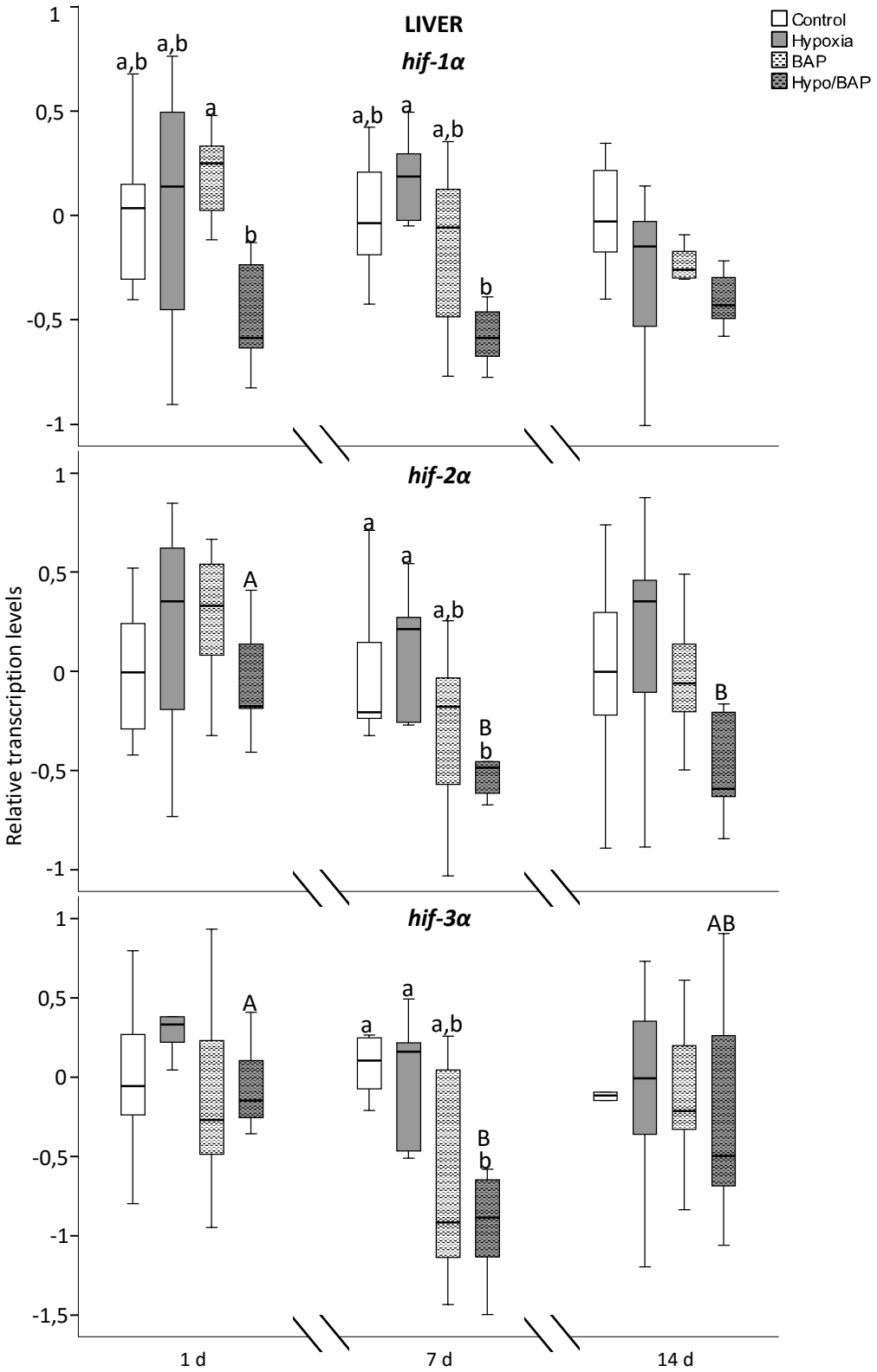

Figure 3. Relative transcription levels of *hif-1α*, *hif-2α* and *hif-3α* in liver of *C. labrosus* under control and hypoxic conditions in absence and presence of B[a]P. Box plots represent the 25th, 50th and 75th percentiles, while whiskers represent the standard deviation. Capital letters show significant differences (p<0.05) between time points (1, 7 and 14 days) within the same treatment. Significant differences between treatments (control, hypoxia, B[a]P, and hypoxia/B[a]P) for the same time point are identified by different lower case letters. Statistical differences were based on the Kruskal-Wallis one-way analysis of variance followed by the Dunn´s post-hoc test.

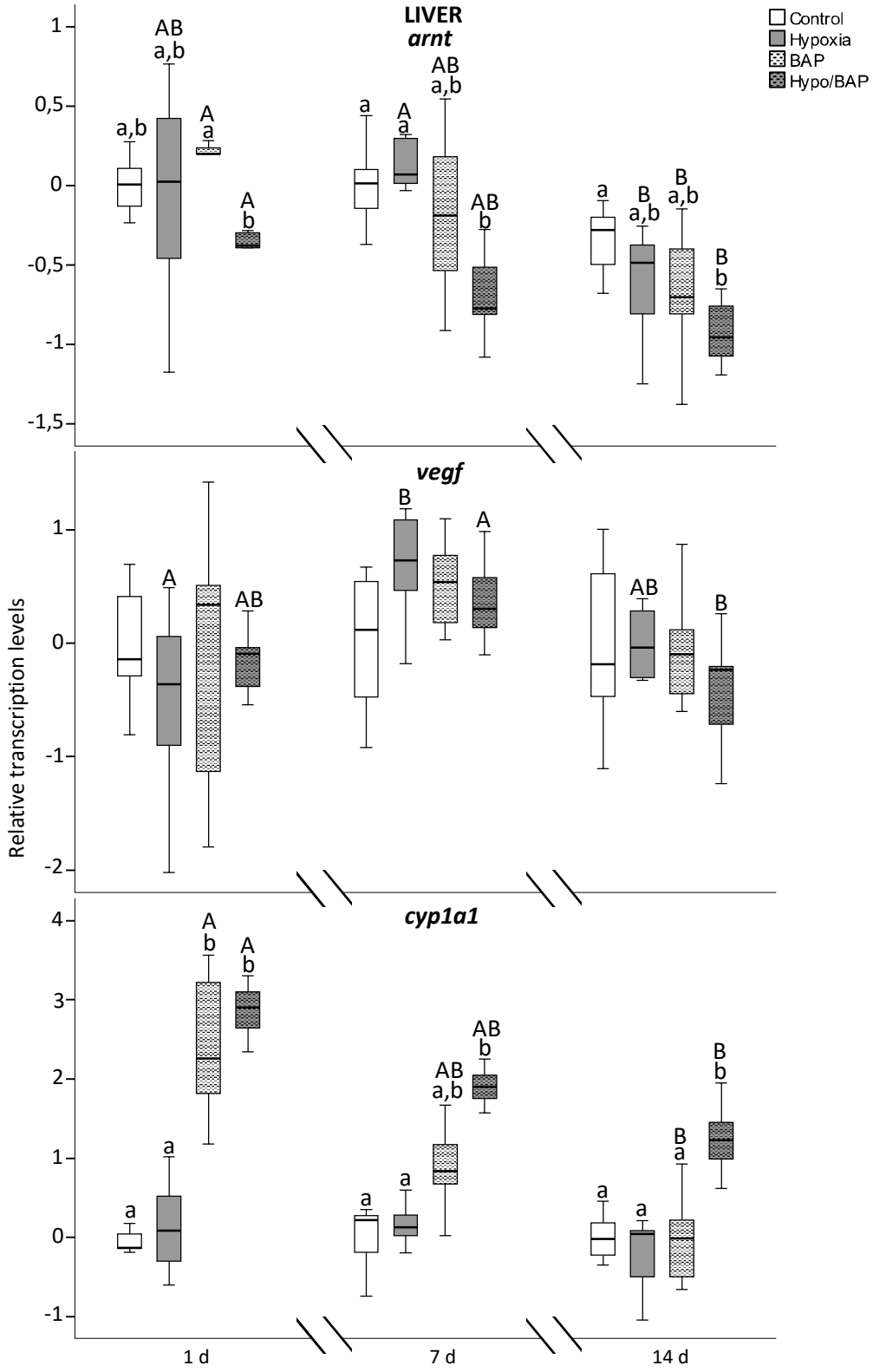

Figure 4. Relative transcription levels of *arnt*, *vegf* and *cyp1a1* in liver of *C. labrosus* under control and hypoxic conditions in absence and presence of B[a]P. Box plots represent the 25th, 50th and 75th percentiles, while whiskers represent the standard deviation. Capital letters show significant differences (p<0.05) between time points (1, 7 and 14 days) within the same treatment. Significant differences between treatments (control, hypoxia, B[a]P, and hypoxia/B[a]P) for the same time point are identified by different lower case letters. Statistical differences were based on the Kruskal-Wallis one-way analysis of variance followed by the Dunn´s post-hoc test.

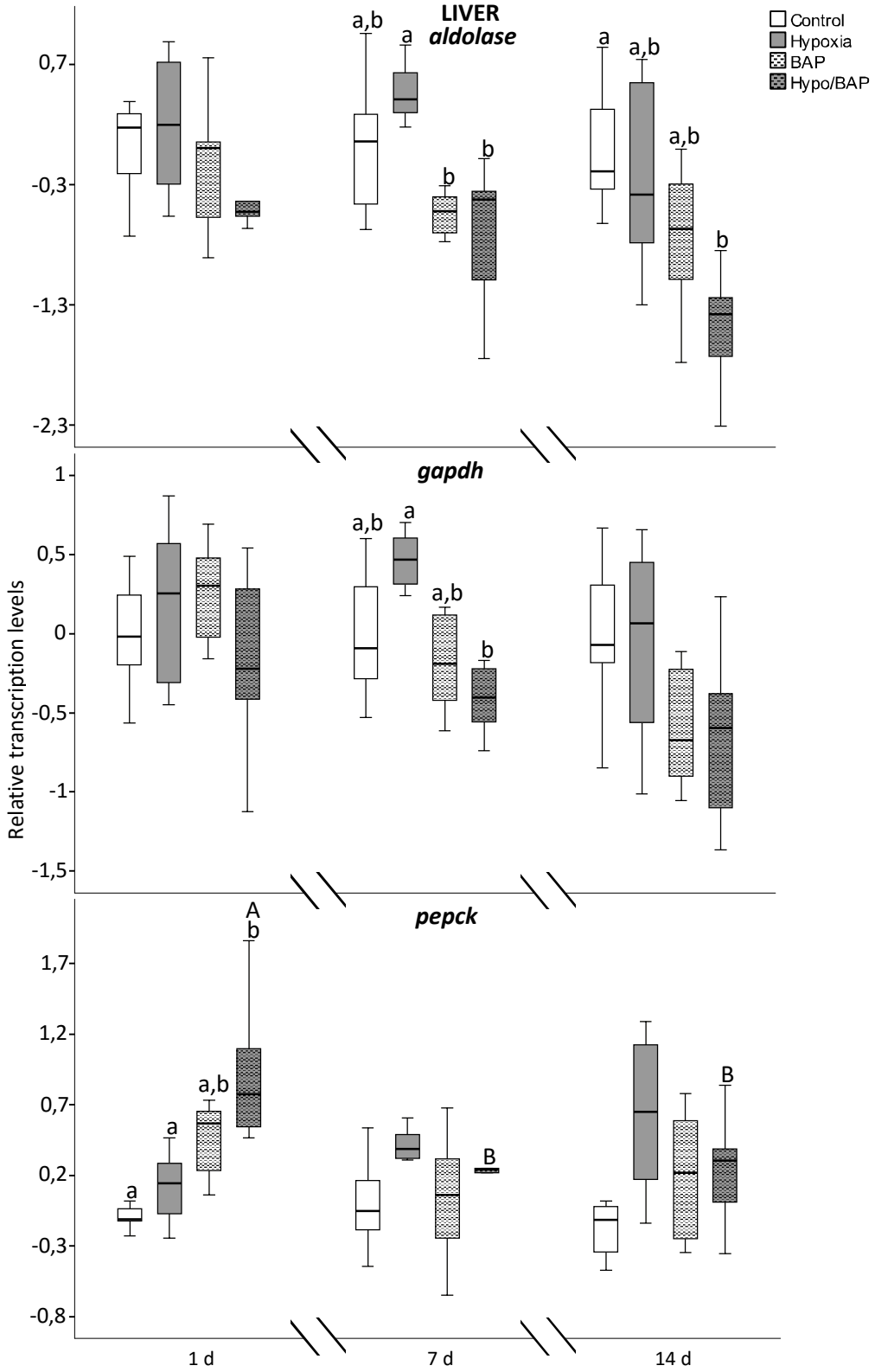

Figure 5. Relative transcription levels of *aldolase*, *gapdh* and *pepck* in liver of *C. labrosus* under control and hypoxic conditions in absence and presence of B[a]P. Box plots represent the 25th, 50th and 75th percentiles, while whiskers represent the standard deviation. Capital letters show significant differences (p<0.05) between time points (1, 7 and 14 days) within the same treatment. Significant differences (p<0.05) between treatments (control, hypoxia, B[a]P, and hypoxia/B[a]P) for the same time point are identified by different lower case letters. Statistical differences were based on the Kruskal-Wallis one-way analysis of variance followed by the Dunn´s post-hoc test.

3.5. Transcription levels of selected target genes in gills

Hypoxia did not significantly modify the transcription levels of any of the studied genes in gills in comparison with the rest of the treatments. Measured *hif-1α* transcription levels were higher in the B[a]P group than in the hypoxia group at days 1 and 7. Significantly higher mRNA levels were measured in the B[a]P group than in the hypoxia-B[a]P group at day 7, when a generalized down-regulation was recorded. Transcription levels of *hif-2α* tended to increase with exposure time in the B[a]P treatment, resulting in a significant up-regulation when compared to control levels at day 14. In the case of *hif-3α*, a general reduction of transcription levels was detected at day 7 which was especially marked in both groups under hypoxia. Exposure to B[a]P caused no changes in the transcription levels of any of the 3 *hif-α* paralogs under hypoxic conditions (Figure 6).

While B[a]P caused no alterations, a time-dependent decrease of *arnt* mRNA levels was observed in the hypoxia-B[a]P group, which was significantly down-regulated at day 14 in comparison to the control and the B[a]P groups. Hypoxia up-regulated *vegf* in fish exposed to B[a]P at day 1, mRNA levels being higher than in the group exposed to B[a]P alone. Presence of B[a]P significantly induced the transcription of *cyp1a1*; this effect was slightly accentuated in the combined hypoxia-B[a]P treatment, where mRNA levels were significantly higher than those of control and hypoxia groups during all the experiment (Figure 7).

In general*, aldolase* did not show any alterations in transcription levels, with the only exception of a significant difference observed between days 7 and 14 in the hypoxia group. Transcription levels of *gapdh* reached their lowest at day 7 for the combined hypoxia-B[a]P treatment, levels being significantly lower than those measured at day 1. No differences with respect to controls were observed in any of the experimental groups. Finally, *pepck* was significantly up-regulated in the combined hypoxia-B[a]P group in comparison to control and hypoxia groups at days 7 and 14 (Figure 8).

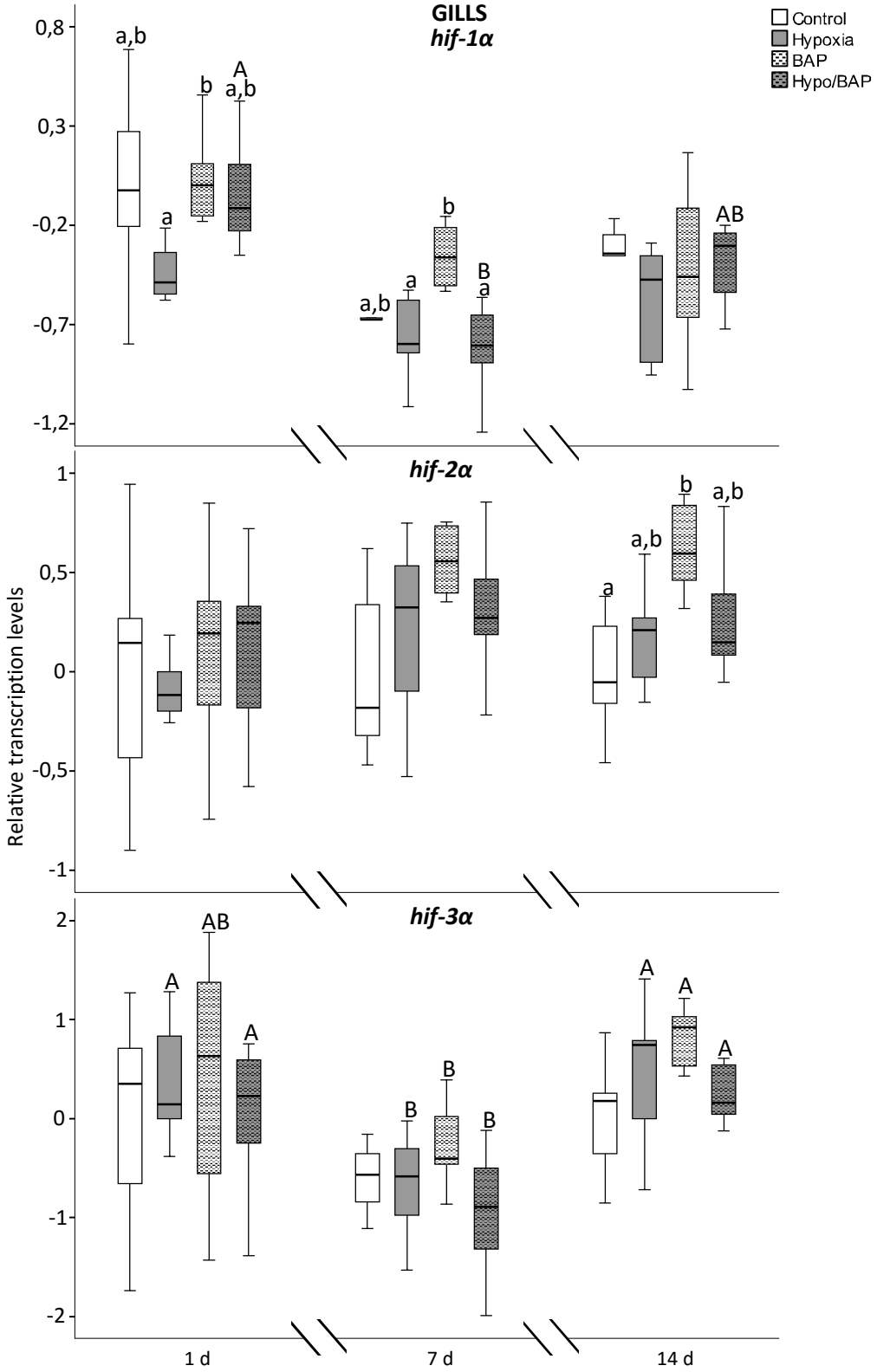

Figure 6. Relative transcription levels of *hif-1α*, *hif-2α* and *hif-3α* in gills of *C. labrosus* under control and hypoxic conditions in absence and presence of B[a]P. Box plots represent the 25th, 50th and 75th percentiles, while whiskers represent the standard deviation. Capital letters show significant differences (p<0.05) between time points (1, 7 and 14 days) within the same treatment. Significant differences (p<0.05) between treatments (control, hypoxia, B[a]P, and hypoxia/B[a]P) for the same time point are identified by different lower case letters. Statistical differences were based on the Kruskal-Wallis one-way analysis of variance followed by the Dunn´s post-hoc test.

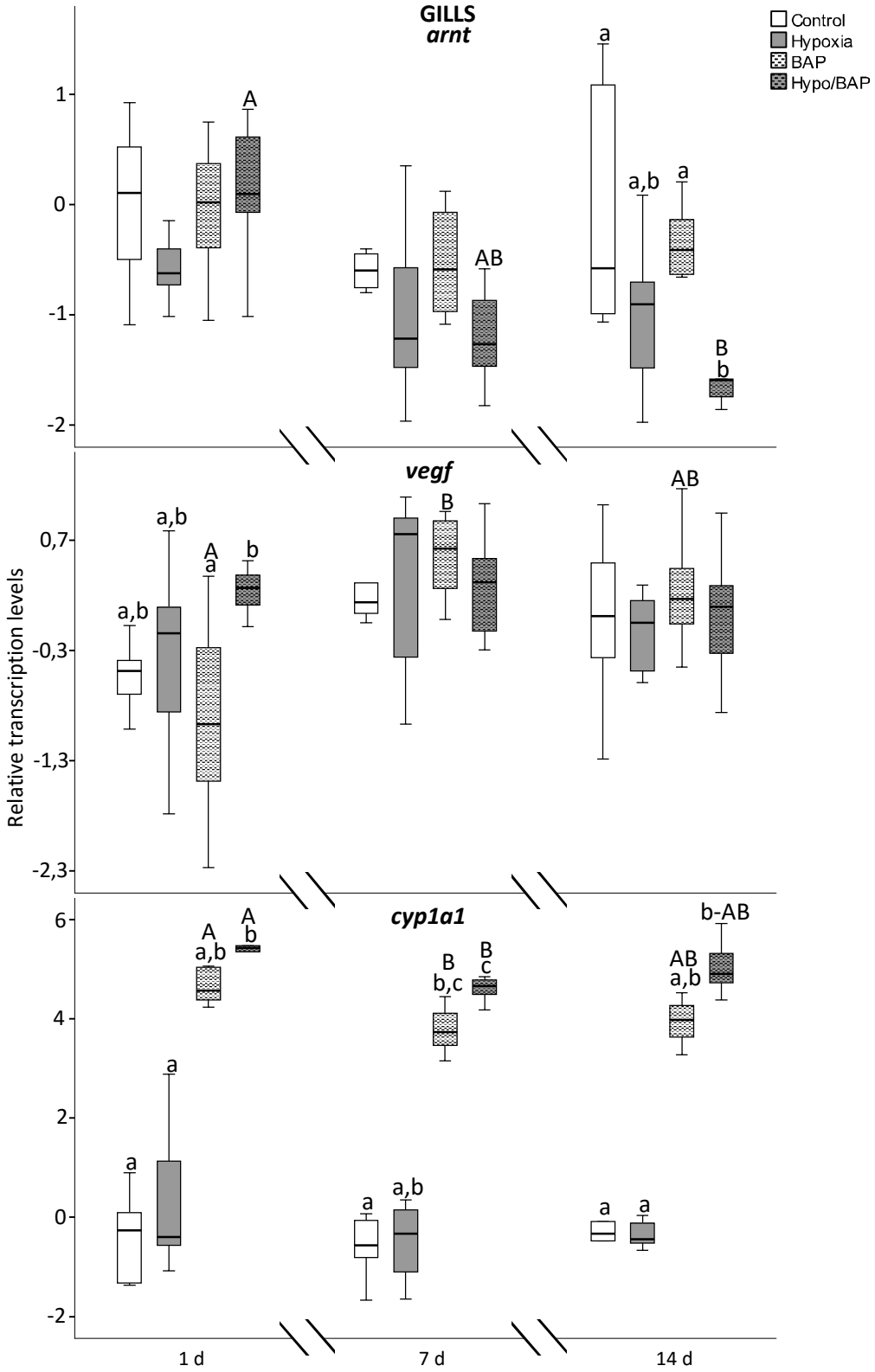

Figure 7. Relative transcription levels of *arnt*, *vegf* and *cyp1a1* in gills of *C. labrosus* under control and hypoxic conditions in absence and presence of B[a]P. Box plots represent the 25th, 50th and 75th percentiles, while whiskers represent the standard deviation. Capital letters show significant differences (p<0.05) between time points (1, 7 and 14 days) within the same treatment. Significant differences (p<0.05) between treatments (control, hypoxia, B[a]P, and hypoxia/B[a]P) for the same time point are identified by different lower case letters. Statistical differences were based on the Kruskal-Wallis one-way analysis of variance followed by the Dunn´s post-hoc test.

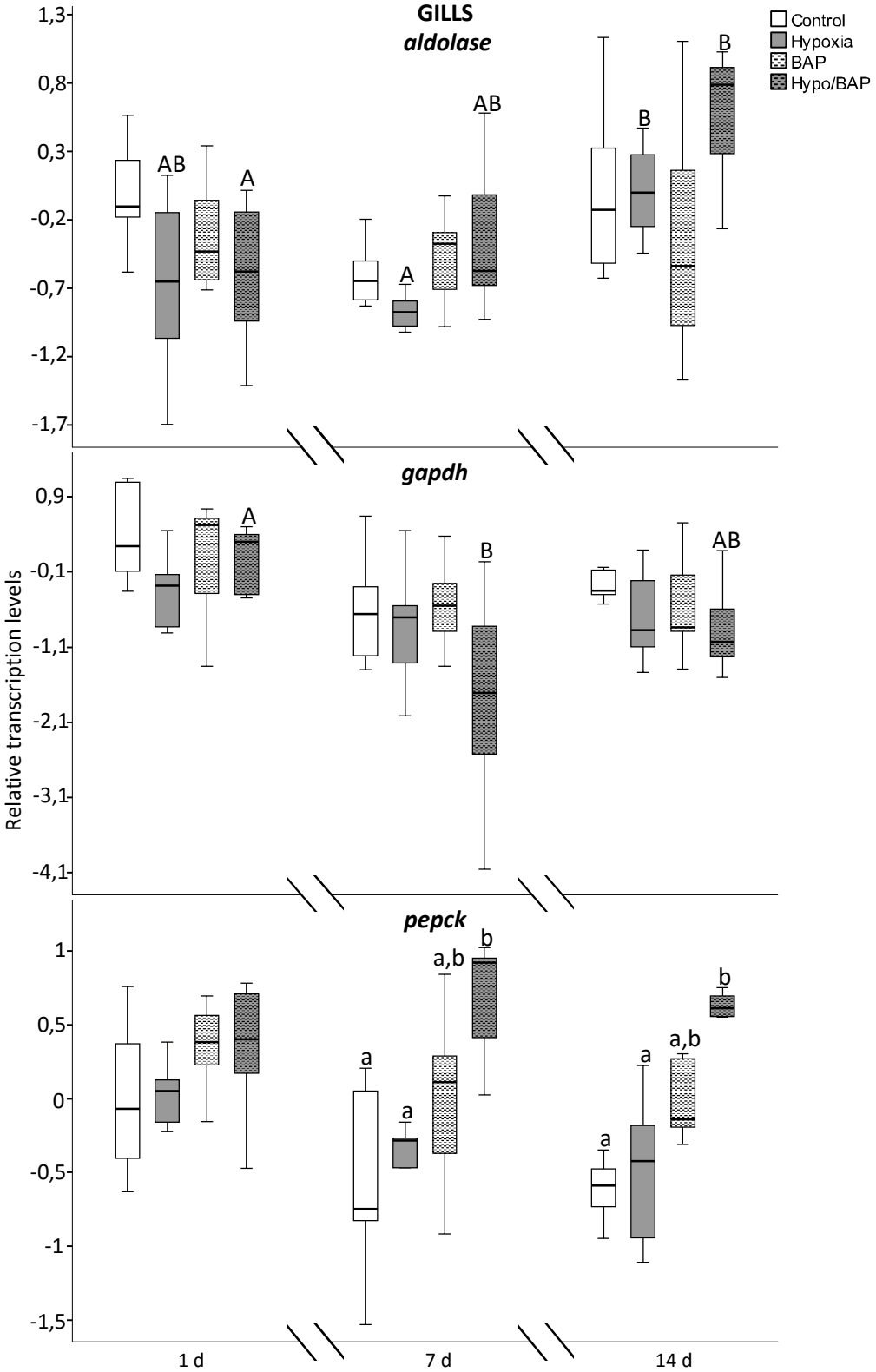

Figure 8. Relative transcription levels of *aldolase*, *gapdh* and *pepck* in gills of *C. labrosus* under control and hypoxic conditions in absence and presence of B[a]P. Box plots represent the 25th, 50th and 75th percentiles, while whiskers represent the standard deviation. Capital letters show significant differences (p<0.05) between time points (1, 7 and 14 days) within the same treatment. Significant differences (p<0.05) between treatments (control, hypoxia, B[a]P, and hypoxia/B[a]P) for the same time point are identified by different lower case letters. Statistical differences were based on the Kruskal-Wallis one-way analysis of variance followed by the Dunn´s post-hoc test.

4. Discussion

Juveniles of the sentinel fish species *C. labrosus* were studied in a laboratory experiment to assess the possibilities that a cross-talk/interference could be established at the transcriptional level between hypoxia adaptive and xenobiotic detoxification pathways. After 1, 7 and 14 days under hypoxic conditions, exposure to B[a]P or exposure to B[a]P under hypoxic conditions, transcriptional levels of key selected genes participating in both molecular routes were quantified in gills and liver. No B[a]P- or hypoxia-related alterations were detected in *hif-αs* and *arnt* transcription levels; in contrast, exposure to B[a]P under hypoxia resulted in a significant down-regulation of the 3 *hif-α* genes in liver. A time-dependent down-regulation of *arnt* was also recorded for the combined treatment in both tissues. The B[a]P-hypoxia combination only elicited a transient up-regulation of *vegf* in gills, while *cyp1a1* was up-regulated in presence of B[a]P both under normoxic and under hypoxic conditions in both tissues. Glycolysis and gluconeogenesis marker genes showed different trends in gills and liver in regard to the combined hypoxi-B[a]P treatment.

Mullets under hypoxic conditions showed reduced mobility in comparison to their counterparts under normoxia. Previous studies carried out under diverse hypoxic conditions have shown a reduction of the swimming activity in different fish species such as *Ammodytes tobianus* (Behrens & Steffensen 2006) or *Gadus morhua* (Skjæraasen et al. 2008). This behavior has been proposed to be part of an energy saving strategy (Fischer et al. 1992), since swimming constitutes a major part of the energy budget in fish (Claireaux & Lefrançois 2007). Evidences of the opposite strategy have been also reported for example in *Clupea harengus*, which increased its swimming activity under hypoxia. In this case, this was interpreted to be consequence of an avoidance behavior (Herbert & Steffensen 2006). Complex strategies combining avoidance and energy saving mechanisms have also been described in other fish species (Dalla Via et al. 1998; Herbert & Steffensen 2005).

On the other hand, several studies have described fish displaying aquatic surface respiration under hypoxia, both in field and in laboratory conditions (Mcneil & Closs 2007; Sloman et al. 2008; Richards 2011). Fish can breathe directly the water layer in

close contact with the atmosphere, to increase oxygen availability (Kramer & McClure 1982). Mullets are known to perform aquatic surface respiration (Shingles et al. 2005) but in our experiment such behavior was not observed. Under these experimental conditions, the atmosphere in contact with the first layer of water was poor in oxygen due to the low atmospheric renovation happening in the water tank, which was sealed.

During conditions of oxygen scarcity, fish are regarded to alter their gill ventilation rate, increasing vascularization and irrigation accordingly (Wu 2002; Nikinmaa & Rees 2005; Shingles et al. 2005). In situations of altered blood flow through the gills, blood vessels can be damaged (Stentiford et al. 2003). Damage to pillar cells can result in an abnormal blood flow into the secondary lamellae, leading to the formation of aneurysms (Martinez et al. 2004; Elshaer & Bakry 2013; Devi & Mishra 2013; Hassaninezhad et al. 2014). In the present study, the presence of this severe lesion, that is difficult to revert, was more evident in mullets under hypoxia and can be considered a side effect of the mechanisms (enhanced arterial blood pressure and synthesis of erythrocytes) triggered in response to hypoxia (Valenzuela et al. 2005; Perry & Desforges 2006). Exposure to B[a]P under hypoxia on the other hand, resulted in a reduction of the severity and extent of aneurysms, which might indicate that the xenobiotic somehow reduces the possibilities to adapt to hypoxia through blood flow increase in mullets.

In adaptation to hypoxia in vertebrates, HIF-α proteins play a central role. *hif-1α* (and the other 2 paralogs also) is constitutively expressed under normoxia, and protein levels are post-translationally controlled through oxygen concentration-driven proteolytic degradation (Loenarz et al. 2011). Results obtained with *Oncorhynchus mykiss* hepatocyte cultures (Soitamo et al. 2001) and in liver of *Gasterosteus aculeatus* (Leveelahti et al. 2011) or *Perca fluviatilis* (Rimoldi et al. 2012) under different hypoxic conditions revealed no alteration in the *hif-1α* mRNA levels, pointing the post-translational regulation as the main mechanism to increase HIF-1α activity under hypoxia in fish. However, transcriptional up-regulation under hypoxic conditions in fish has also been described in other studies (Rahman & Thomas 2007; Terova et al. 2008; Borley et al. 2010; Kopp et al. 2011; Rimoldi et al. 2012), remarking the importance of the transcriptional regulation of *hif-1α* as a master gene in this adaptive pathway. *hif-*

2α shares the general mechanisms of regulation explained for *hif-1α* but a distinctive fine-tune modulation could be expected, since both mediate non-overlapping biological responses (Majmundar et al. 2010). Long-term hypoxia caused a similar up-regulation of both paralogs in ovaries of *Micropogonias undulatus*, while short-term hypoxia (3 days) did not enhance *hif-2α* mRNA levels (Rahman & Thomas 2007). In contrast, Shen and colleagues (2010) found *hif-2α* up-regulation, but not of *hif-1α,* in liver and brain of *Megalobrama amblycephala* after 4 h of hypoxia. Regarding *hif-3α*, it was initially described as a repressor of the hypoxia-inducible gene expression in vertebrates (Hara et al. 2001), but a recent study in *Danio rerio* embryos described *hif-3α* as a transcription factor with its own battery of hypoxia responsive genes that only partially overlaps with that of *hif-1α* (Zhang et al. 2014). Besides, *hif-3α* has been described in *Ictalurus punctatus* as the most transcriptionally sensitive of the 3 paralogs under acute hypoxia, due to its quick and deep up-regulation in different tissues (Geng et al. 2014). However, the transcriptional regulation of these 3 paralogs in the present study was unaffected by mild hypoxic conditions alone in both analyzed tissues, suggesting that adaptation to such specific conditions in mullets relies mostly in post-translational regulation of *hif-α*s.

Exposure to B[a]P alone up-regulated *hif-1α* and *hif-2α* in gills, suggesting the presence of an inter-relation between the presence of this xenobiotic and the hypoxia adaptive pathway. Hypoxia-mimicking effects have been attributed to situations of high cellular levels of ROS production (Vuori et al. 2004), as it could occur in presence of xenobiotics such as B[a]P. This putative production of ROS would have been more potent in activating the transcription of HIF-*α* genes than hypoxia itself.

Finally, a transcriptional down-regulation was detected in liver for the 3 genes when fish were exposed to B[a]P under hypoxic conditions. This effect should be understood as a cause of constraints in adaptation possibilities for mullets that should depend on the hypoxia adaptive pathway under low oxygen availability. The accumulation of both types of stresses might be causing such down-regulation of *hif-α*s.

vegf is one of the master genes regulating angiogenesis in vertebrates (including fish) and it is usually transcriptionally up-regulated under hypoxia by HIF-α proteins (Zhu et

al., 2013). In this way, *vegf* has been described to be up-regulated in liver of *E. coioides* under hypoxia, and co-exposure to B[a]P further enhanced such up-regulation (Yu et al. 2008). This would suggest that competition for ARNT does not happen at the level of *vegf* gene regulation. Something similar was proposed in embryos of *Cyprinodon variegatus*, since the transcription levels of *vegf* were unaffected by pyrene exposure during development (Hendon et al. 2008). In our study, up-regulation of *vegf* was detected in gills of mullets exposed to the combined hypoxia-B[a]P treatment at day 1. Such response did not happen in the hypoxia, despite the severe disruption of the secondary lamellae detected in gills at day 14, nor in the B[a]P exposure groups. We could hypothesize that the low oxygen availability together with the increased ROS production elicited by B[a]P exposure could have synergized to result in the transcriptional up-regulation of *vegf*.

Hypoxic conditions are known to cause a generalized metabolic depression in vertebrates which has also been described in fish (Olsvik et al. 2013). In such circumstances fish, but also mammals and insects, have been described to increase the carbon flow through the glycolytic route, which is oxygen-independent (Webster 2003). In addition, the gluconeogenic route is also enhanced under hypoxia as it has been reported for instance in liver of *Gymnotus carapo* (Moraes et al. 2002). Probably, during reduced oxygen availability the liver supplies glucose for the whole organism as described in *Gillichtys mirabilis* (Gracey et al. 2001). Seemingly, the glycolytic enzymes respond differently to such demand. In *Fundulus grandis,* for instance, hepatic aldolase activity decreased while GAPDH activity significantly increased under hypoxia (Martínez et al. 2006). Hypoxia also elicited the transcriptional down-regulation of *aldolase* in gills of *Oryzias latipes* (Zhang et al. 2012). In the present experiment an up-regulation of glycolytic genes was expected under hypoxia but this did not occur, neither in liver or in gills. However, in a scenario of generalized metabolic depression, the fact that mRNA levels of glycolytic enzymes are unaltered under hypoxic conditions would suggests an effort to maintain glycolysis active.

Kraemer & Schulte (2004) described that the presence of PCB-77 (an AHR agonist) inhibited the hepatic induction of the glycolytic enzyme activities under hypoxia in *Fundulus heteroclitus*, suggesting antagonistic interactions between the hypoxia

adaptive and the xenobiotic detoxification pathways. The hepatic down-regulation of *aldolase* and *gapdh* resulting of the exposure to B[a]P under hypoxia in mullets might be the consequence of a similar interaction, leading to the misregulation of the glycolytic route. On the other hand, up-regulation of *pepck* was observed after hypoxia-B[a]P exposure in liver and especially in gills. Thus, it could be hypothesized that these mullets would require more glucose than their counterparts only exposed to B[a]P or only under hypoxia.

ARNT mRNA and protein levels have been described to increase in particular human cell lines cultured under atmospheres poor in oxygen (Mandl & Depping 2014). In fish, ARNT is known to be up-regulated under exposure to AHR agonists (Calò et al. 2014). In mullet liver, *arnt* transcriptional trends were similar to those observed for *hif-α* genes (especially for *hif-1α*), with the combined treatment causing the highest degree of down-regulation. This could point towards the existence of a common regulatory system acting on the transcription of at least *hif-1α* and *arnt* transcription factors in mullet. Accordingly, Wolff and colleagues (2013) proposed a mechanism in which ARNT and HIF-1α would stabilize each other through heterodimerization and avoid degradation. In the present study, the combination of stressors caused by the combination of hypoxia and B[a]P elicited the down-regulation of *arnt* also in mullet gills.

Up-regulation of *cyp1a1* under exposure to B[a]P is well characterized in fish, as observed for example in liver and gills of *C. labrosus* (Bilbao et al. 2010) or in different tissues of fish *Gobicypris rarus* (Yuan et al. 2013). Hypoxia on the other hand, has also been described to modify *cyp1a1* transcription levels. Hypoxic conditions increased the mRNA levels of *cyp1a2* and EROD activity in liver of *G. aculeatus* (Leveelahti et al. 2011), while Rahman & Thomas (2012) described a reduction in *cyp1a1* mRNA and protein levels in liver of *M. undulatus*. The transcriptional up-regulation of *cyp1a1* caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin was attenuated in presence of hypoxic conditions in embryos of *D. rerio* (Prasch et al. 2004), and the same occurred with the EROD activity under exposure to B[a]P in an hypoxic environment (Matson et al. 2008). In the present study, no alterations in the transcription pattern were detected for *cyp1a1* under hypoxia alone, while B[a]P exposure resulted in a significant up-

regulation in both liver and gills. Addition of hypoxic conditions to the exposure tended to up-regulate *cyp1a1* to a higher extent, especially in liver after 14 days, although not significantly. CYP1A1 as a monooxygenase requires molecular oxygen to initiate the detoxification of B[a]P, thus, low oxygen availability could reduce enzyme activity and therefore limit xenobiotic clearance capacity. This observation suggests that mullets exposed to CYP1A1 substrates under hypoxia might have compromised their ability to cope with chemical pollution.

In conclusion we consider that *C. labrosus*, as a hypoxia tolerant species, could be able to deal with mild hypoxic conditions deploying a generalized energy saving mechanism. The combination of B[a]P and low oxygen concentrations reduced the transcription of *hif-αs* and *arnt* (especially in liver), probably leading to difficulties to cope with both stressors at the same time. Furthermore, the addition of hypoxic conditions to B[a]P exposure tended to accentuate the up-regulation of *cyp1a1* especially in liver, while B[a]P exposure alone produced an up-regulation *hif-2α* in gills. Both results may reflect the potential importance of the molecular interaction occurring between both adaptive pathways which seems to transcend the mere competition for ARNT. A potential consequence of such interference might be an unbalance in the glucose metabolism proposed for liver and gills of mullets exposed to B[a]P under hypoxic conditions. In the present study, the response to AHR agonists seemed to be favored over adaption to hypoxia in mullets. This observation is also supported by the detected reduced presence of aneurysms in gills in the combined hypoxia-B[a]P group. Further studies are required to improve the knowledge of the molecular scenario affecting hypoxia adaptive and xenobiotic detoxification pathways, which could be of critical importance for the adaptability of coastal and estuarine fish species.

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Chapter 3

Regulation of hypoxia adaptive pathway genes in thicklip grey mullets *Chelon labrosus* caged in a chronically polluted harbour

Parts of this chapter have been presented at:

J. Martos-Bernal, O. Díaz de Cerio, E. Bilbao, I. Cancio. *Regulation of hypoxia adaptive pathway genes in thicklip grey mullets* Chelon labrosus *caged in a chronically polluted harbour* 18th INTERNATIONAL SYMPOSIUM ON POLLUTANT RESPONSES IN MARINE ORGANISMS. Trondheim, Norway. 24th-27th May 2015. Poster.

This chapter is in preparation to be submitted as:

J. Martos-Bernal, O. Díaz de Cerio, E. Bilbao, I. Cancio. *Regulation of hypoxia adaptive pathway genes in thicklip grey mullets* Chelon labrosus *caged in a chronically polluted harbour.* Marine Environmental Research.

Abbreviations:

AHR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
ARNT, HIF-1β	Aryl hydrocarbon receptor nuclear translocator
bHLH-PAS	Basic helix-loop-helix Per/Arnt/Sim family
CYP1A	Cytochrome p450
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HIFs	Hypoxia-inducible factors
IH	Inner harbour
OH	Outer harbour
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
qPCR	Quantitative PCR
TBT	Tributyltin

Abstract

Organisms living in coastal areas disturbed by human activities are likely to be simultaneously affected by different stressors. High levels of chemical pollutants and hypoxic episodes have been described in the Pasaia harbour (43°17´N; 01°55´W). At the molecular level, xenobiotic detoxification and hypoxia adaptive pathways are regulated in vertebrates by aryl hydrocarbon receptor (AhR) and hypoxia-inducible factor-α (HIF-α) respectively. Since both transcription factors need to heterodimerize with the aryl hydrocarbon receptor nuclear translocator (ARNT), the effect that co-occurrence of both stressing situations may have on both adaptive pathways becomes an interesting matter of study.

In the present study, two cages especially designed to house juvenile mullets (*Chelon labrosus*) were placed in the inner (IH) and outer (OH) part of the Pasaia harbour. After 5 and 21 days, liver and gills were sampled to measure transcription levels of *hif-1α*, *hif-2α*, *hif-3α*, *arnt*, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and cytochrome p450 1a1 (*cyp1a1*). Physicochemical conditions of the water column were monitored and availability of chemical compounds was measured in mussels *Mytilus galloprovincialis* caged with the mullets for 21 days.

High bioavailability of metals, PAHs, PCBs, phthalates, and organometallic compounds was detected in mussels at both sites, but especially in IH. However, this difference did not result in a site dependant differential transcription of *cyp1a1*. Hypoxic conditions (2.05-5.39 mg O_2/L) were confirmed for IH (6.79-7.81 mg O_2/L at OH); together with an increasing trend of hepatic and gill *hif-1α* and *arnt* transcription at day 5. Transcription of *hif-3α* was particularly down-regulated at day 21 in IH; when *gapdh* was at its highest in gills, suggesting an enhancement of glycolysis. During the study, *hif-2α* transcription levels remained unaltered. arnt showed a transcriptional pattern very similar to *hif-1α*, which could be considered a consequence of a mechanism to prevent a hypothetical competition between AHR and HIF-1α for ARNT during xenobiotic exposure under hypoxic conditions. Specificity of *hif1a*, *hif2a*, and *hif3a* and their respective down-stream genes requires further research in order to understand the transcriptional response to hypoxia in polluted areas.

Key words: hypoxia, pollutants, *Chelon labrosus*, HIF-αs, ARNT

Resumen

Los organismos que residen en áreas costeras alteradas por las actividades humanas tienden a sufrir los efectos causados por la presencia simultánea de varios tipos de estrés. En el puerto de Pasaia (43°17´N; 01°55´W) se ha descrito la presencia de altas concentraciones de contaminantes, además de episodios de hipoxia. Las rutas moleculares de detoxificación de xenobióticos y de adaptación a hipoxia en vertebrados están reguladas por el receptor de hidrocarburos arilo (AHR) y el factor inducido por hipoxia-α (HIF-α), respectivamente. Dado que ambos factores de transcripción necesitan la heterodimerización con el translocador nuclear del receptor de hidrocarburos arilo (ARNT) para realizar su función, resulta interesante estudiar el efecto que la presencia simultánea de ambos factores estresantes podría tener sobre ambas rutas moleculares. En éste estudio se colocaron dos cajas diseñadas para alojar mubles juveniles (*Chelon labrosus*) en la parte interior (IH) y en la exterior (OH) del puerto de Pasaia. Tras 5 y 21 días se tomaron branquias e hígado de los animales para medir los niveles de transcripción de *hif-1α*, *hif-2α*, *hif-3α*, *arnt*, *gliceraldehído-3-fosfato deshidrogenasa* (*gapdh*) y *citocromo p450 1a1* (*cyp1a1*). Se monitorizaron las condiciones fisicoquímicas de la columna de agua y se midió la biodisponibilidad de compuestos químicos en mejillones *Mytilus galloprovincialis* alojados durante 21 días en cada una de las cajas. En ambos puntos se midieron valores elevados de metales, PAHs, PCBs, ftalatos y compuestos organometálicos, pero especialmente en IH. A pesar de ello, ésta sutil diferencia no causó diferencias en los niveles de transcripción de *cyp1a1* al comparar IH y OH. En el caso de IH, junto a una tendencia a la sobreexpresión hepática y branquial de *hif-1α* y *arnt* tras 5 días de confinamiento, se observaron situaciones de hipoxia (2.05-5.39 mg O_2/L). Esto no sucedió en para OH (6.79-7.81 mg O_2/L). Los niveles de transcripción de *hif-3α* resultaron particularmente bajos tras 21 días en IH; cuando los niveles de *gapdh* alcanzaron su máximo en branquias, sugiriendo un aumento de la glicólisis. Los niveles de transcripción de *hif-2α* no cambiaron durante el estudio. El patrón de transcripción de *arnt* resultó muy similar al de *hif-1α*, lo que podría ser considerado un mecanismo para prevenir la hipotética competición de AHR y HIF-1α por ARNT en presencia de xenobióticos bajo condiciones de hipoxia. Para comprender mejor la respuesta transcripcional a hipoxia en lugares contaminados por compuestos químicos se requiere investigar en mayor profundidad la respuesta específica de *hif1a*, *hif2a*, y *hif3a* y los genes regulados por cada uno de los parálogos de HIF-α.

Palabras clave. hipoxia, contaminantes químicos, *Chelon labrosus*, HIF-αs, ARNT

Laburpena

Giza eraginpean dauden kostaldeko guneetan bizi diren organismoek, estres mota ezberdinak paira ditzakete aldi berean. Pasaiako portuan (43°17´N; 01°55´W) kutsatzaile kimikoen maila altuak eta hipoxia egoerak deskribatu dira. Ornodunetan, molekula mailan, aril hidrokarburoen hartzaileak (AHR) eta hipoxiak induzitutako α-faktoreak (*hif-α*) erregulatzen dituzte xenobiotikoen detoxifikazioa eta hipoxiaren aurreko adaptazio bidezidorrak, hurrenez-hurren. Beraien funtzioa bete ahal izateko, bi transkripzio faktoreok aril hidrokarburoen hartzailearen translokatzaile nuklearrarekin (ARNT) heterodimerizatu behar dutenez, bi estres mota hauen aldibereko agerpenak, bi adaptazio bidezidorretan izan dezakeen efektua ikertzea interesgarria da.

Ikerketa honetan, *Chelon labrosus* korrokoi heldugabeak bi kutxetan mantendu ziren Pasaiako portuaren kanpo (IH) eta barnekaldean (OH). 5 eta 21 egunen ostean, gibel eta zakatzak lagindu ziren *hif-1α*, *hif-2α*, *hif-3α*, *arnt*, *glizeraldehido-3-fosfato deshidrogenasa* (*gapdh*) eta *p450 1a1 zitokromoa* (*cyp1a1*) geneen transkripzio mailak neurtzeko. Horrez gain, ur zutabearen egoera fisiko-kimikoaren jarraipena egin zen. Konposatu kimikoen bioeskuragarritasun maila, korrokoiekin batera kutxetan sarturiko *Mytilus galloprovincialis* muskuiluetan neurtu zen 21 egunean.

Oro har, metal, PAH, PCB, ftalato eta konposatu organometalikoen maila altuak aurkitu ziren muskuiluetan. Maila altuenak, IH-an zeuden muskuiluetan aurkitu ziren. Hala ere, ezberdintasun hauek ez ziren *cyp1a1* transkripzio mailan islatu. IH-n hipoxia egoera egiaztatu zen (2.05-5.39 mg O_2/L), ez ordea OH-n (6.79-7.81 mg O_2/L). Honekin batera, gibel eta zakatzetan *hif-1α* eta *arnt* geneen transkipzio mailek goranzko joera erakutsi zuten. *hif-3α* bereziki azpi-erregulatuta agertu zen 21. egunean IH-n; aldi berean, *gapdh*-ek maila altuenak aurkeztu zituen zakatzetan, glukolisiaren igoera iradokiz. Ikerketan zehar, *hif-2α*-ren transkripzio mailak antzekoak izan ziren. *arnt*-k, *hif-1α*-aren antzeko transkripzio maila aurkeztu zuen. Azken hau, hipoxia pean ematen den xenobiotikoen esposizioak eragin dezakeen AhR eta HIF-1α-en ARNT-rekiko lehia hipotetikoa ekiditeko mekanismoaren ondorioa kontsidera daiteke. *hif-1α, hif-2α* eta *hif-3α*-ren espezifikotasuna eta euren eraginpean dauden geneek ikerketa sakonagoa eskatzen dute, gune kutsatuetan hipoxia pean ematen den transkripzio mailako erantzuna hobeto ulertu ahal izateko.

Hitz gakoak: hipoxia, Kutsatzaileak, *Chelon labrosus*, HIF-1α, ARNT

1. Introduction

Organisms living in anthropized estuaries and coastal areas are likely to be affected by different stressors resulting from diverse human activities. Ecosystems around industrial harbors are under special risk, since activities in such infrastructures tend to produce a wide variety of chemical pollutants that may affect the biota (Pal et al. 2010; Wang et al. 2013; Floehr et al. 2013). Other activities such as intensive farming can additionally produce episodes of water eutrophication that might eventually lead to periods of oxygen scarcity (Zhang et al. 2010).

Diverse xenobiotic compounds such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) can be accumulated in cells where, in the case of vertebrates, they bind to the aryl hydrocarbon receptor (AHR), a member of the basic helix-loop-helix Per/Arnt/Sim (bHLH-PAS) family of transcription factors (Poland et al. 1976). Upon xenobiotic binding, AHR enters the nucleus where heterodimerizes with another member of the bHLH-PAS family, the aryl hydrocarbon receptor nuclear translocator (ARNT), to activate a series of genes that allow xenobiotic detoxification (Hoffman et al. 1991). ARNT has been described also as the heterodimeric partner required by the hypoxia inducible factor-1α (HIF-1α), member of the bHLH-PAS family as well, to trigger adaptive responses to conditions of low oxygen availability (Wang & Semenza 1995). Situations in which the presence of xenobiotics occurs under hypoxic conditions could increase the stress of affected organisms; which in some cases could show constrains for adaptation at the molecular level as ARNT is central to trigger both xenobiotic detoxification and for hypoxia adaptive pathways (Vorrink & Domann 2014).

In recent years, several studies have been conducted to study the interaction between both stress adaptive pathways in fish. Laboratory experiments, both *in vivo* and *in vitro*, have revealed divergent results (Prasch et al. 2004; Kraemer & Schulte 2004; Yu et al. 2008; Fleming et al. 2009). The task of setting common conclusions is made more difficult by differences in the experimental design, fish species sensibility to oxygen or xenobiotic availability and the level of hypoxia/xenobiotic exposure employed. Field approaches have been also carried out to study the biological effects of environmental

hypoxia (Reardon & Chapman 2009; Davies et al. 2011; Thomas & Rahman 2012; Hedges & Abrahams 2015).

In this study, a caging experiment was devised in the highly industrialized estuary of the Oiartzun River. The watershed of this river in the northeast of the Iberian Peninsula has approximately 85 km^2 with 70000 inhabitants, which are mainly concentrated around the estuary that flows into the Bay of Biscay. As the river approaches its mouth the water quality decreases due to the influence of agricultural uses and industrial, harbor urban runoff waters. The ecological estate of the river was evaluated between 2009 and 2013 by the regional water evaluation agency within the water framework directive, being defined as deficient due to all the combined environmental pressures (www.uragentzia.euskadi.eus). Of special concern is the impact caused by the activities around the harbor of Pasaia, a busy port that houses shipyards, metallurgical activity and paper and textile industries among others (Borja et al. 2006; Rodríguez et al. 2010). The deeply modified fjord-like morphology of this estuary produces high water residence times leading to oxygen depletion, something that is favored by eutrophication events (Gorostiaga et al. 2004).

Thicklip grey mullet *Chelon labrosus* was selected to perform the present caging study. *C. labrosus* is usually found in highly polluted and eutrophicated waters and it has been used as a pollution sentinel species to evaluate the health status of coastal and estuarine areas (Baptista et al. 2013; Pujante et al. 2015), including the Bay of Biscay (Puy-Azurmendi et al. 2013; Ortiz-Zarragoitia et al. 2014). For active biomonitoring purposes, *C. labrosus* satisfactorily fulfills the requirements described by Wepener (2013) for a sentinel species: it is representative of the Oiartzun estuary, it is relatively easy to obtain and handle, it has adequate size and lifespan and it is relatively tolerant to the selected stressors, responding rapidly and efficiently to them (Bizarro et al. 2014; Ortiz-Zarragoitia et al. 2014).

In this study, two *ad hoc* designed caging devices were deployed in the harbor of Pasaia; one close to the river mouth and another one in the interior of the harbor, right in the middle of the industrial zone. Juvenile *C. labrosus* were caged in both places, where they were expected to experience different pollution and dissolved

oxygen conditions. Transcription levels of genes related to the hypoxia adaptive pathway (hypoxia inducible factor-1, 2 and 3 alpha – *hif-α*s and aryl hydrocarbon receptor nuclear translocator - *arnt*), to the glucose metabolism (glyceraldehyde-3-phosphate dehydrogenase - *gapdh*) and to the xenobiotic detoxification pathway (cytochrome p450 1a1 - *cyp1a1*) were quantified by quantitative PCR (qPCR).

2. Materials and Methods

2.1. Experimental animals

A total of 48 juvenile (12-20 cm in length) thicklip grey mullets *Chelon labrosus* were captured using a fishing rod in Plentzia (43º19´18.77”N; 1º 55´49.04”W), a relatively non-polluted area (Orbea & Cajaraville 2006). Mullets were maintained in the laboratory in clean seawater from an aquaculture farm pipe (Getaria, 43º18´13.26´´N; 2º 14´21.96´´W) in a 600 L volume tank during 21 days before the beginning of the caging experiment. Water was decanted and sequentially filtered through 100, 50 and 5 µm pore size meshes while receiving ultraviolet treatment. It was stabilized to 18ºC prior to the final filtering step through a 0.45 µm pore size mesh. A 12 hours light/dark cycle was set in the fish tank, with 33.3‰ salinity, 47 mS conductivity and pH 7.8. Residual metabolites (ammonium, nitrite and nitrates) were maintained at 0-0.5 mg/L, 0-0.5 mg/L and 5-10 mg/L respectively using commercial kits (Sera GmbH, Heinsenberg, Germany). Animals were daily fed during this period with dried bread and Vipagran (Sera GmbH, Heinsenberg, Germany). The experimental procedures were performed following the specifications agreed with the Ethics Committee for Animal Experimentation of the University of the Basque Country under approved experimental protocol (CEEA/151/2010/CANCIO).

Mediterranean mussels *Mytilus galloprovincialis* were taken from the aquaculture farm INCUMAR (Industrias y Cultivos Marinos S.L., Comillas, Cantabria, Spain) with the warranty of being free of chemicals. They were housed under the same conditions as mullets and daily fed during the acclimatization period with Sera Marin Coraliquid (Sera GmbH, Heinsenberg, Germany).

2.2. Caging experiment

The cages employed to house the sentinel species were *ad hoc* designed at Azti Tecnalia, their characteristics are detailed in Figure 1. Deployment of cages (Figure 1) took place during the month of July in Pasaia (43º19´00´´N; 1º55´00´´W), a harbor with high pollutant burdens (Borja et al. 2006; Rodríguez et al. 2010).

After the acclimatization period, animals were randomly distributed in two cages (24 mullets and 50 mussels into each one), one installed in the inner part of the harbor (IH, 43º19´18.77”N; 1º55´49.04”W) and the other in the outer part (OH, 43º19´46.77”N; 1º55´22.95”W). Both cages were immersed at 2 m depth at the same time, containing algae and several blocks of fish oil mixed with agar produced in-house to feed the mullets.

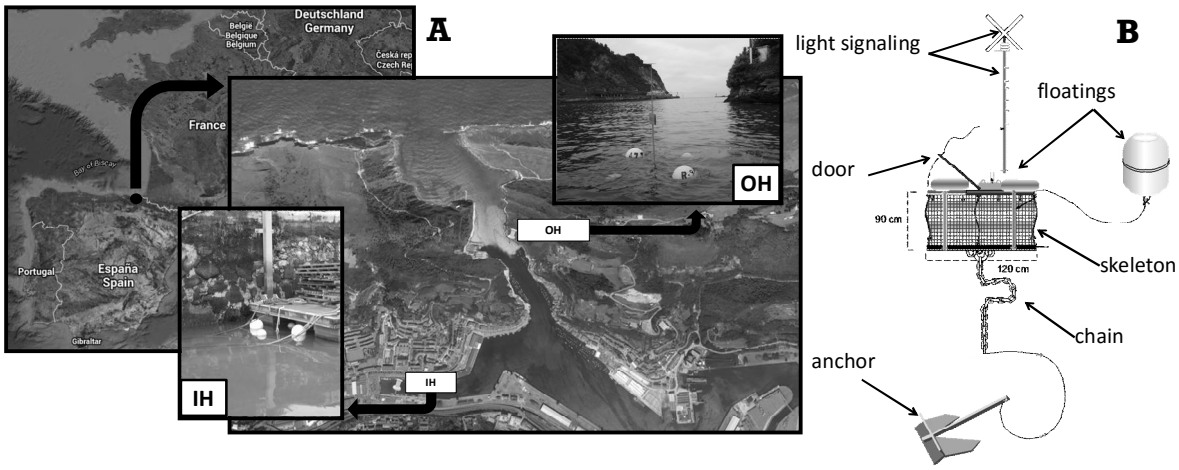

Figure 1. A. Map indicating the location of the Pasaia harbor, close to the mouth of the Oiartzun estuary, and the inner harbor (IH) and outer harbor (OH) sites, where cages were deployed. **B.** Design and specifications of the caging system and its principal components. The volume of the cage was 1.02 m^3.

Oxygen concentration in the water column was measured 7 days after the deployment, at high and at low tide, and taking measurements both in the water surface and in the bottom. At days 14 and 21 measurements were only taken during low tide, and in the water surface.

At least 10 mullets were sampled from each cage 5 and 21 days after deployment. Fish liver and gills were dissected *in situ*, immediately immersed in RNA later (Sigma-

Aldrich, St. Louis, Missouri, USA) and frozen in liquid nitrogen. All the samples were stored at -80ºC until further analysis. At the end of the experiment, all caged mussels were collected and frozen at -20ºC until chemical analyses were performed.

2.3. Chemical analysis on caged mussels

The concentration of chemical compounds was assessed in both sampling sites, measuring chemicals accumulated in whole mussel tissues at the end of the experiment. Total levels of the 16 EPA polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCB), phthalates, butyltin compounds and metals (Al, V, Cr, Mo, Fe, Co, Ni, Cu, Zn, As, Cd, Sn and Pb) were measured in dried flesh of mussels. As described by Bartolomé and colleagues (2010), PAHs, PCBs and phthalates were measured by GC coupled to a 5973N mass spectrometer, butyltin compounds were measured through HP-5MS capillary columns, and metals with an ICP–MS system.

2.4. Total RNA isolation and cDNA synthesis

Fragments of liver and gills (50-100 ng) of mullets were separately homogenized with zirconia/silica beds (Biospec, Bartlesville, USA) in a Precellys® 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) by using the double 6 m/s for 20 s program. Total RNA was extracted following the TRIzol® (Invitrogen, Life Technologies, Carlsbad, USA) method and purified with the MiniElute PCR Purification kit (Qiagen, Hilden, Germany). Final concentration and purity were spectrophotometrically measured (EPOCH microplate, Biotek, Colmar Cedex, France), accepting A260/A280 ratios between 1.8 and 2. cDNA was synthesized with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies) by using random hexamers in the iCycler™ thermocycler (Bio-Rad, San Diego, USA). Synthesized cDNA was stored at -40ºC.

2.5. Quantitative PCR analyses (SYBR®Green)

Transcription levels of *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh,* KP981394*)*, *cytochrome p450 1a1* (*cyp1a1,* DQ438983), *aryl hydrocarbon receptor nuclear translocator* (*arnt,* JF732766) and *hypoxia inducible factor-1, 2* and *3 alpha* (*hif-1α* JF732801*, hif-2α* KM402135 *and hif-3α* KM402136) were measured in a 7300 Real

Time PCR System (Applied Biosystems, Foster City, CA). *β-actin* (AY836368) was used as reference gene, since its transcription levels remained stable during the experiment in both studied tissues. Quantitative PCRs (qPCRs) were carried out in triplicates using 10 µl of SYBR®Green (FastStart Universal SYBR®Green Master, Roche, Indianapolis, USA) into each reaction, in a final volume of 20 µl per well. Sequence of primers used for each amplification and qPCR specifications are shown in Table 1. qPCR conditions were: 50 °C for 2 min, 95 °C for 10 min; 40 cycles at 95°C for 15 s followed by the melting temperature (Table 1) for 30 s. The specificity of each primer set was confirmed by the analysis of the dissociation curve and the presence of a single peak. Relative transcription levels were calculated using the Delta-Delta Ct method corrected for PCR efficiency (modified from Pfaffl 2001). Mean Ct values corresponding to 5 days of deployment in OH were employed as calibrator.

Table 1. Primer sequences and melting temperatures (T) used for the specific amplification of selected target genes by qPCR. The cDNA concentration per tissue used for amplifications is also depicted. Final primer concentration used for the amplification of all gene fragments was 1.25 pmol/µL.

			cDNA (pg/µL)	
Gene (Acc. number)	**Primer sequence (5´-3´)**	**T. (°C)**	**Liver**	**Gills**
gapdh (KP981394)	Fw: CTGGAGAAAGCCGCCAAGTA Rv: CGACCTGATCCTCTGTGTATGC	55,5	71,5	71,5
cyp1α (DQ438983)	Fw: CTGCACCACAAAAGACACATCTC Rv: CCACAGCTCAGGATCATGGTT	55,5	285,5	143
arnt (JF732766)	Fw: GACTTGAAGACAGGAACAGTGAAGAAG Rv: TGCCACATCTCATTCTGCAGAT	55,5	285,5	285,5
hif-1α (JF732801)	Fw: CACAGTTGGACAAAGACGTCTCA Rv: GCTCCCTGTCCCATGATCTC	56	285,5	285,5
hif-2α (KM402135)	Fw: CTGGAAGCGGATTCGGTAAA Rv: TGACGGYGCGTCCTCTGTT	56	285,5	285,5
hif-3α (KM402136)	Fw: CCACGTCCAGGTCCGAGTAA Rv: CCTGCTGGTCAGTGTGCTCTT	59	285,5	285,5
β-actin (AY836368)	Fw: ACCCAGATCATGTTCGAGACCTT Rv: TGACGATACCGGTGGTACGA	57	285,5	285,5

2.6. Statistical analysis

Statistical differences between groups were studied in the SPSS 20.0 software. Normality was assessed by the Kolmogorov-Smirnov test and homogeneity of variances by the Levene test. The significance of the differences detected between

groups was assessed by the one-way ANOVA test complemented with the Tukey post-hoc test (p<0.05).

3. Results

3.1. Dissolved oxygen concentrations

Oxygen concentration measurements taken 7, 14 and 21 days after cage deployment showed lower oxygen availability in IH than in OH (Table 2), where the values were close to saturation (100% oxygen = 7.43±0.4 mg O_2/L). The detailed characterization performed at day 7 recorded a reduced dissolved oxygen concentration at the bottom of the water column. While this reduction was not noticeable at OH (10 m depth), hypoxia (2.05 mg O_2/L) was recorded in the case of IH (5 m depth). Since cages were immersed at 2 m depth in both sites, fish at IH experienced oxygen availability conditions close to hypoxia.

Table 2. Dissolved oxygen concentrations (mg O_2/L) registered in both caging sites, inner harbor (IH) and outer harbor (OH). Exhaustive measurements were taken at day 7 at the surface and close to the bottom of the caging site, while at days 14 and 21 measurements were only taken on the surface water.

Dissolved O2 (mg/L)	**Day 7**				**Day 14**	**Day 21**
Probe location	Surface		Bottom		Surface	Surface
Tidal conditions	High	Low	High	Low	Low	Low
IH	4,17	5,01	2,05	2,9	5,05	5,39
OH	7,37	7,59	7,13	7,01	7,81	6,79

3.2. Bioavailability of pollutants

The xenobiotic concentrations (phthalates, PCBs, PAHs, tributyltin-TBT- and metals) measured in whole mussel body 21 days after deployment of cages showed elevated bioaccumulation levels in both caging sites (Table 3). Extremely high levels of phthalates, PAHs, TBT (the single organometallic compound detected) and metals were present at IH, higher than in OH, while the highest bioavailability of PCBs was measured at OH.

Table 3. Concentration of chemical pollutants (µg/kg) measured in whole mussel dried tissue after 21 days of deployment in the inner harbour (IH) and the outer harbour (OH). TBT was the single organometallic compound found. ND indicates no detected, while UDL indicates under detection limit.

Compounds		IH (µg/kg)	OH (µg/kg)
PHTHALATES	DMP (Dimethyl phthalate)	<UDL	<UDL
	DEP (diethyl phthalate)	150	140
	DBP (di-n-butyl phthalate)	1,43	1,23
	BBP (butyl, benzyl-phthalate)	156	30
	DEHP (bis-2,ethylhexyl-phthalate)	ND	ND
	DOH (di-n-octyl phthalate)	49	45
	TOTAL Σ Phthalates	1,785	1,445
PCB	PCB 18	72	52
	PCB 28 + PCB 31	108	93
	PCB 44	110	131
	PCB 52	215	228
	PCB 101	281	447
	PCB 118	129	203
	PCB 138	520	1,007
	PCB 149	483	829
	PCB 153	391	690
	PCB 180	106	176
	PCB 194	138	93
	TOTAL Σ PCBs	2,553	3,949
PAH	Naphthalene	228	177
	Acenaphthylene	294	222
	Acenaphthene	345	300
	Fluorene	125	65
	Phenantrene	506	324
	Anthracene	173	115
	Fluoranthene	495	293
	Pyrene	679	291
	Benzo[a]anthracene	155	104
	Chrysene	580	352
	Benzo[b]fluoranthene + Benzo[k]fluoranthene	164	133
	Benzo[a]pyrene	205	166
	Indeno pyrene	66	54
	Dibenzo[a,h]anthracene	62	51
	Benzo[g,h,i]perylene	84	59
	TOTAL Σ PAHs	4,161	2,706
TBT	Tributyltin	1,634	1,057
Metals	Al	1,266.44	1,180.84
	V	48.77	26.57
	Cr	200.09	53.91
	Mn	237.27	256.55
	Fe	3,448.43	2,570.72
	Co	10.32	9.79
	Ni	31.79	15.24
	Cu	307.59	170.60
	Zn	7,925.21	5,486.27
	As	442.81	419.02
	Cd	14.74	10.83
	Sn	8.11	7.42
	Pb	120.50	136.96
	TOTAL Σ metals	14,062.07	10,344.72

3.3. Mortality

No mortality was observed among caged mullets or mussels until the 21st day of deployment, when 2 fish were retrieved dead from the cage located in IH.

3.4. Transcription levels of selected target genes

The coefficient of variation in the transcription levels of *β-actin* was calculated both in liver and in gills of caged mullets, and it was lower than 5% in both deployment sites and sampling times. Therefore *β-actin* was considered a suitable reference gene for this study.

Hepatic *gapdh* mRNA levels were stable in fish caged in OH, while mullets deployed in IH showed and increasing trend at day 5 (Figure 2). *cyp1a1* transcription levels were stable in IH and OH during the whole experiment. Regarding *arnt*, its transcription levels were up-regulated in IH after 5 days of deployment, and they were significantly higher than those found after 21 days of deployment in OH. Transcription levels of *hif-1α* at day 5 in IH tended to be higher than the levels found for the rest of the groups, with a stable trend during the caging. In the case of *hif-2α* only minor changes were detected among different groups. Finally, *hif-3α* mRNA levels at IH decreased in a significant way until day 21 of deployment. A similar but more moderate trend was observed in liver of mullets in OH.

gapdh mRNA levels in gills significantly increased from day 5 to day 21 of deployment in IH, while no changes were detected in OH (Figure 3). Slight changes were recorded for *cyp1a1*, with a mild up-regulation at day 21 in both caging sites. The transcriptional trends measured for *arnt* and *hif-1α* genes were almost identical, showing significantly higher levels in IH at day 5 than at day 21 in both deployment sites. mRNA levels of *hif-2α* were unaltered during the whole experiment in both sites. The lowest transcriptional levels were registered for *hif-3α* after 21 days of deployment in IH; these levels being significantly lower than those measured in gills of fish caged in OH at any sampling time.

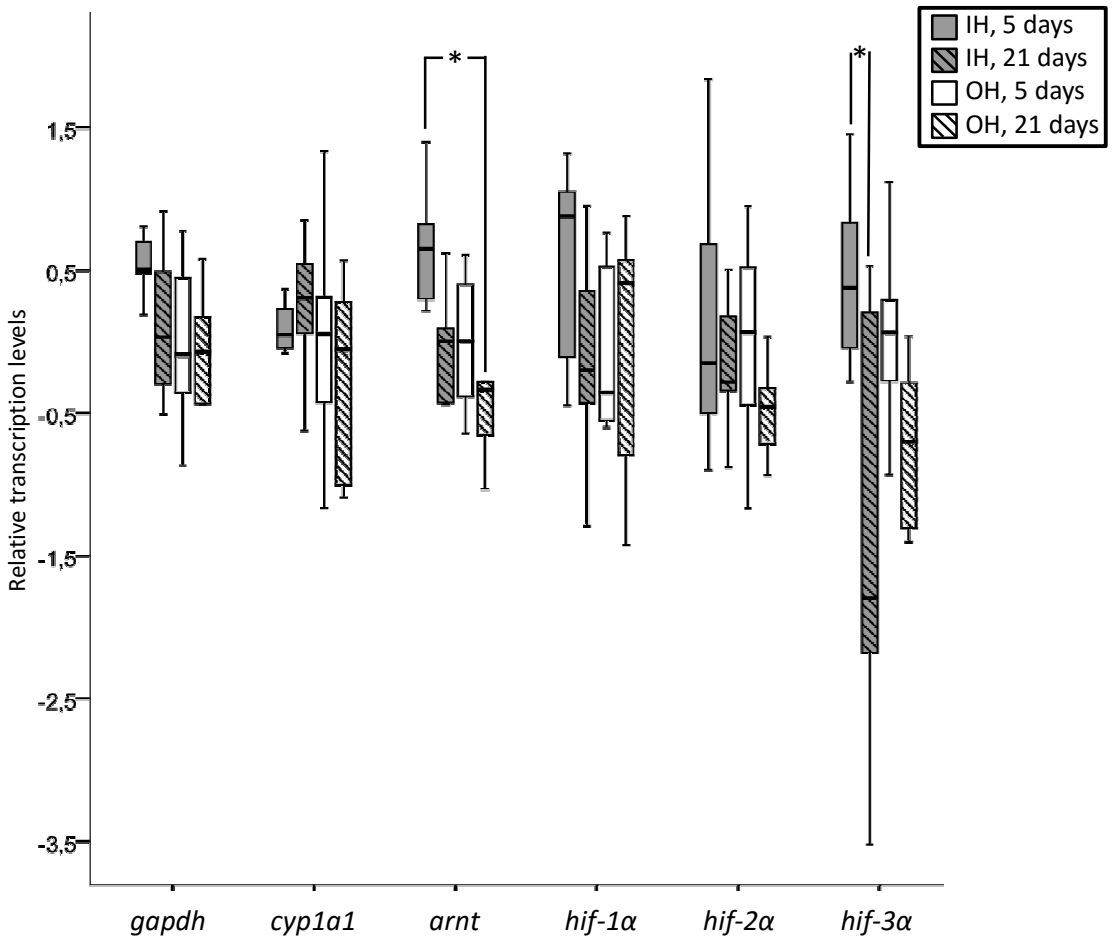

Figure 2. Relative transcription levels of *gapdh, cyp1a, arnt, hif-1α, hif-2α* and *hif-3α* measured in liver of *C. labrosus* caged for 5 and 21 days in the inner (IH) or the outer (OH) part of the Pasaia harbor. Box plots represent the 25th, 50th and 75th percentiles, while whiskers represent the standard deviation. Asterisk indicates significant differences (p<0.05) among groups according to the one-way ANOVA test complemented with the Tukey post-hoc test.

4. Discussion

A caging field experiment was performed with *C. labrosus* juveniles to assess the effect of environmental hypoxia, in an industrial scenario with high levels of chemical toxic compounds, on key genes participating in the hypoxia adaptive and the xenobiotic detoxification pathways. Low levels of dissolved oxygen were quantified in the site presenting the highest bioavailability of chemicals (inner harbor, IH); while the other site (outer harbor, OH) was in normoxia. Oxygen scarcity resulted in an up-regulation trend for *gapdh* in gills after 21 days of deployment. The transcriptional levels of *cyp1a1* (in liver and gills) were similar in both caging sites during the experiment. *arnt* and *hif-1α* transcription factors showed comparable transcriptional trends in both

sites, with a transient up-regulation detected in both tissues in IH at day 5. While *hif-2α* transcription levels remained unaltered during the whole experiment, *hif-3α* mRNA levels were down-regulated in both tissues after 21 days in IH.

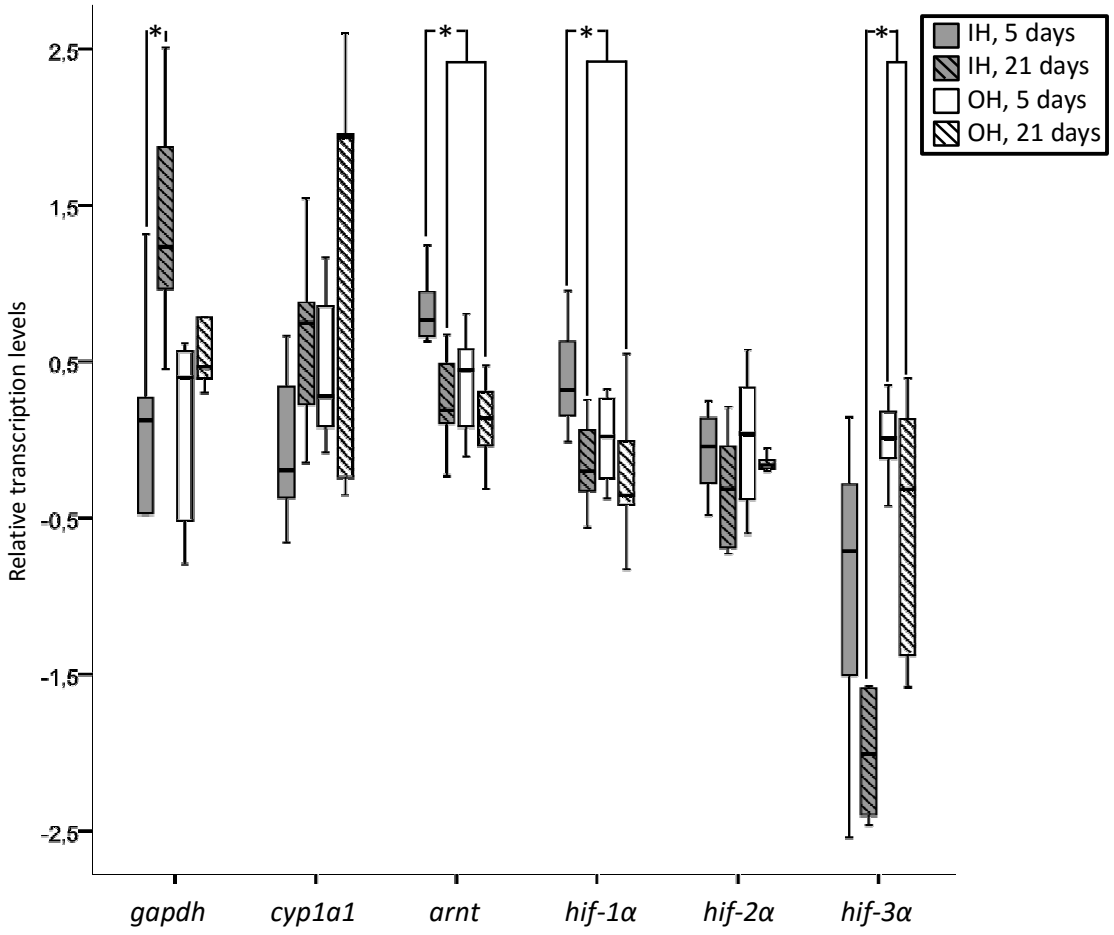

Figure 3. Relative transcription levels of *gapdh, cyp1a, arnt, hif-1α, hif-2α* and *hif-3α* measured in gills of *C. labrosus* caged for 5 and 21 days in the inner (IH) or the outer (OH) part of the Pasaia harbor. Box plots represent the 25th, 50th and 75th percentiles, while whiskers represent the standard deviation. Asterisk indicates significant differences (p<0.05) among groups according to the one-way ANOVA test complemented with the Tukey post-hoc test.

Glycolysis is a cellular catabolic process that produces energy without consuming oxygen, and its regulation in the wide majority of organisms (including fish) seems to be linked to oxygen availability (Webster 2003; Mandic et al. 2014). Enhancement of the glycolytic activity, also at the transcriptional level through up-regulation of genes coding for glycolytic enzymes, has been described in fish under both short- and long-

term hypoxic conditions (Kraemer & Schulte 2004; van der Meer et al. 2005). Our results are in concordance and point towards an enhancement of glycolysis in mullets deployed in a site with moderately low oxygen availability. *gapdh*, which codes for the enzyme catalyzing the 6th step of glycolysis in which the first reductive agent is liberated, was up-regulated in fish caged in IH. Accordingly, in a field study performed in hypoxic lakes, increased glycolytic enzyme activities were observed in white muscle of *Lepomis gibbosus* that disappeared after a 7 days recovery in oxygenated water (Davies et al. 2011). Time differences were detected between tissues in *C. labrosus*; while liver showed just an early up-regulation trend for *gapdh* (day 5), gills displayed a significant up-regulation after long-term deployment (21 days). Enhancement of glycolysis cannot be concluded with the response of a single gene, because the participating enzymes can show disparate responses during hypoxia (Abbaraju & Rees 2012). For example, Martinez and co-workers (2006) described increased hepatic GAPDH activity (among other enzymes) in *Fundulus grandis* after 4 weeks of hypoxia , while the activity of other glycolytic enzymes were unaffected or even decreased. However, the up-regulation of *gapdh* seems to corroborate that mullets caged in IH suffered from limiting cellular levels of oxygen. The present study was based on the work performed by Díaz de Cerio (2012), in which a transcription profile analysis was carried out in the same mullet individuals analyzed hereby. Hepatic transcription levels of *heme oxygenase 1* (*ho1*), a gene whose transcription and activity increase in fish under hypoxia (Wang et al. 2008; Tzaneva & Perry 2014), were significantly up-regulated in fish deployed in IH for 5 days. This also supports the idea that fish deployed in IH were actively responding to the low oxygen availability

Presence of diverse xenobiotics such as PAHs and PCBs is detected in fish cells by AHR (Billiard et al. 2002); which can in turn unchain the biotransformation of such compounds by triggering the transcription of genes such as *cyp1a1*, involved in phase I detoxification metabolism (Hahn et al. 2006). The transcriptional up-regulation of *cyp1a1* has been used as biomarker of exposure to various PAHs, PCBs and dioxins in different fish species (Zhou et al. 2010; Roy et al. 2011; Räsänen et al. 2012; Yuan et al. 2013). Bilbao and colleagues (2010) performed a laboratory experiment where *C. labrosus* waterborne exposed to a heavy fuel oil, similar to the one spilled by the

Prestige tanker, showed a significant hepatic up-regulation of *cyp1a1*. In the Pasaia harbor, chronic pollution and punctual mini-spillages caused by industries and the high maritime traffic produce complex mixtures of chemicals that are accumulated into biota, as measured here in tissues of mussels caged together with mullets. Significant concentrations of PAHs and PCBs were detected both in IH and in OH. No significant differences were recorded in *cyp1a1* transcription levels in liver and gills when comparing mullets of both deployment sites, probably because the sum of bioavailable PAHs and PCBs measured in mussels is similar in both deployment sites: 6714 µg/kg in IH versus 6655 µg/kg in OH. No significant changes with deployment time were detected either. Lisosomal membrane stability measured in the digestive glands of those mussels and PAH-metabolite concentrations identified in the bile of the mullets deployed both IH and OH (Díaz de Cerio 2012) also point towards a significant availability of chemical pollutants in Pasaia harbour. Microarray analysis, performed using a low density toxicologically tailored microarray with only 160 genes, identified a response to xenobiotics signature mainly linked to the regulation of *cyp* genes and of phase II biotransformation genes (Diaz de Cerio 2012). Such signature was more evident when comparing both times of depletion (being more evident at day 21) than comparing IH vs OH.

Our interest is focused in several members of the bHLH/PAS protein family; the ARNT transcription factor is required by HIF-α proteins as heterodimeric partner to trigger the response to hypoxic conditions (Kaelin & Ratcliffe 2008). Besides, ARNT also heterodimerizes with AHR to unchain the response to xenobiotic exposure, including the phase I biotransformation metabolism (Safe et al. 2013). Eventually, this has raised the question of the existence of interferences in the regulation of both pathways (Prasch et al. 2004; Kraemer & Schulte 2004). Different results have been reported in this regard. Yu and collaborators (2008) found benzo[a]pyrene to further enhance the response of the hypoxia responsive element-containing genes in *Epinephelus coioides* under hypoxic conditions, without affecting the xenobiotic responsive element-containing ones. On the other hand, Fleming and co-workers (2009) stated that HIF-1α sequesters ARNT, reducing the activity of AHR in a *Poeciliopsis lucida* fish cell line under exposure to 3 different AHR agonists (PAHs and PCBs) in hypoxic conditions. The

pivotal role of ARNT in this interference was also pointed out in a study performed in human cell cultures in which the response to xenobiotics elicited by the exposure to an AHR agonist decreased during hypoxia. Such decrease was partially reverted by artificial over-expression of ARNT (Vorrink et al. 2014). The results obtained hereby for mullets showed a transient up-regulation of *arnt* in both liver and gills after deployment in IH for 5 days. ARNT expression has been reported to be increased in terms of both, mRNA and protein levels, in human cell lines under hypoxia (Mandl et al. 2013; Wolff et al. 2013). As reviewed by Mandl & Depping (2014), this up-regulation might prevent ARNT from being a limiting factor during oxygen scarcity events. AHR agonists can also elicit *arnt* up-regulation, in this sense PCB-126 has been described to up-regulate hepatic ARNT protein levels in *Sparus aurata* after 12, 24 and 72 h exposure (Calò et al. 2014). Increased *arnt* levels in IH could be justified by the conditions of hypoxia and high bioavailability of xenobiotics and thus, by the need to simultaneously activate the HIF-α- and AHR-mediated pathways.

The hypoxia adaptive pathway is ancient and conserved, and it is triggered after detection of low oxygen availability at the cellular level (Loenarz et al. 2011). For this oxygen level sensing, 3 different paralog genes coding for transcription factor members of the bHLH/PAS family have been described in fish: *hif-1α*, *hif-2α* and *hif-3α* (Zhu et al. 2013). The best known paralog is *hif-1α*, whose regulation occurs mainly at the post-translational level by increasing its protein half-life and activity when oxygen concentrations decrease (Soitamo et al. 2001; Leveelahti et al. 2011). Up-regulation of *hif-1α* was detected in gills of mullets deployed in IH, where mild hypoxia was registered. Previous studies showed transcriptional up-regulation of *hif-1α* in different fish species suffering hypoxic conditions (Rahman & Thomas 2007; Terova et al. 2008; Borley et al. 2010; Kopp et al. 2011). Rimoldi and colleagues (2012) found *hif-1α* to be transcriptionally up-regulated in *Perca fluviatilis* under chronic hypoxia while no transcriptional regulation was observed under acute hypoxia. Up-regulation of mullet *arnt* and *hif-1α* mRNA levels after short term (5 days) caging under environmental hypoxic conditions in presence of xenobiotics reinforces the importance of studying the transcriptional regulation of both transcription factors in achieving homeostasis in *C. labrosus*.

HIF-2α and HIF-3α were initially considered to have functions redundant to those of HIF-1α, but differences found at the structural and functional levels suggest that HIF-2α and HIF-3α present their own regulation processes and control partially different biological responses (Webb et al. 2009; Majmundar et al. 2010; Xiao 2015). Studies in diverse fish species show that the transcriptional regulation of each paralog differs depending on the hypoxic conditions and the tissue examined (Law et al. 2006; Mohindra et al. 2013). Regardless of the deployment site and the exposure time, no transcription level alterations in *hif-2α* were noticed in liver and gills. On the contrary, in *Sebastes schlegelii* maintained under mild hypoxic conditions (4,5 mg O2/L) for 1 h, *hif2α* showed a much more marked transcriptional response in liver and gills than *hif-1α* (Mu et al. 2015). Similarly, liver and brain *hif-2α* mRNA levels were increased in *Megalobrama amblycephala* after 4 h under hypoxic conditions, while *hif-1α* mRNA levels were unaltered (Shen et al. 2010). However, Rahman & Thomas (2007) observed that while long-term hypoxia (1 and 3 weeks) caused comparable transcriptional up-regulation of both paralogs in ovaries of *Micropogonias undulatus*, short-term (12 h and 3 days) hypoxia did not up-regulate *hif-2α*.

hif-3α in vertebrates was initially thought to be a transcription factor acting in a negative feedback loop activated by the HIF1 heterodimer under hypoxic conditions (Hara et al. 2001). Later, a function redundant to that of *hif-1α* was attributed to *hif-3α.* Subsequent studies, like the one performed by Zhang and co-workers (2014) in *Danio rerio*, have reported that *hif-3α* regulates a battery of hypoxia responsive genes only partially overlapping that of *hif-1α*. In our study, *hif-3α* showed a pronounced down-regulation with caging time, in the same sense as the one seen for *hif-1α* and *arnt*. *C. labrosus hif-3α* transcriptional levels were down-regulated (especially in gills) after deployment in IH for 21 days. In gills, transcription levels were always higher in OH than in IH. Such deep down-regulation under hypoxia was unexpected, and was not observed for *hif-1α* and *hif-2α* either. Geng and colleagues (2014) described *hif-3α* as the most sensitive of the 3 paralogs, showing the quickest and most marked transcriptional up-regulation in *Ictalurus punctatus* tissues under acute hypoxic conditions. Such discrepancies depict the complexity of the regulation of each of the 3 *hif-αs*, and thus the importance of taking into account changes at the mRNA levels.

In conclusion, although the altered transcription level of one glycolysis-related gene is not sufficient to claim induction of the whole route, *gapdh* mRNA levels and measured dissolved oxygen concentrations strongly suggest that mullets faced mild hypoxic conditions in IH. Results of *cyp1a1* mRNA levels, and PAHs and PCBs bioavailability measurements, allow thinking that fish deployed in both sites had a similarly active biotransformation metabolism. The similar transcriptional trends of *arnt* and *hif-1α*, showing higher mRNA levels in IH than in OH, could help preventing a hypothetical competition between AHR and HIF-1α for ARNT in the present scenario of xenobiotic exposure under hypoxic conditions. The lack of response of *hif-2α* and the down-regulation of *hif-3α* add complexity to the general response of *hif-α* paralogs to the environmental hypoxic stimulus. Further studies have to be carried out in order to obtain a deeper knowledge about the ARNT cross-talk and the detailed response of the hypoxia adaptive pathway to environmentally relevant hypoxic conditions.

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Chapter 4

Crosstalk between hypoxia adaptive response and xenobiotic detoxification pathways in primary hepatocyte cultures (*Oncorhynchus mykiss*) exposed to benzo[a]pyrene in hyperoxic, normoxic and hypoxic conditions

Parts of this chapter have been presented at:

J. Martos-Bernal, M.T. Hultman, E. Bilbao, K. E. Tollefsen, I. Cancio. *Crosstalk between hypoxia response and xenobiotic detoxification pathways in primary hepatocyte cultures (*Oncorhynchus mykiss*) under exposure to benzo[a]pyrene in hyperoxic, normoxic and hypoxic conditions.* 18th INTERNATIONAL SYMPOSIUM ON POLLUTANT RESPONSES IN MARINE ORGANISMS. Trondheim, Norway. 24th-27th May 2015. Poster.

This chapter is in preparation to be submitted as:

J. Martos-Bernal, M.T. Hultman, E. Bilbao, K. E. Tollefsen, I. Cancio. *Crosstalk between hypoxia adaptive response and xenobiotic detoxification pathways in primary hepatocyte cultures (*Oncorhynchus mykiss*) exposed to benzo[a]pyrene in hyperoxic, normoxic and hypoxic conditions*. Aquatic Toxicology.

Abbreviations:

18SrRNA	18S ribosomal RNA
AB	Alamar Blue
AHR	Aryl hydrocarbon receptor
ARNT, HIF-1β	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine triphosphate
B[a]P	Benzo[a]pyrene
bHLH-PAS	Basic helix-loop-helix Per/Arnt/Sim family
BSA	Bovine serum albumin
CFDA-AM	5-CarboxyFluorescein DiAcetate AcetoxyMethyl ester
CYP1A	Cytochrome p450
DMSO	Dimethyl sulfoxide
EROD	Ethoxyresorufin-O-deethylase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HIFs	Hypoxia-inducible factors
HREs	Hypoxia responsive elements
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PERMANOVA	Permutational multivariate analysis of variance
PHDs	Prolyl hydroxylase domain family of enzymes
PPP	Pentose phosphate pathway
qPCR	Quantitative PCR
ROS	Reactive oxygen species
XREs	Xenobiotic responsive elements

Abstract

Estuarine and coastal waters usually experience hypoxic conditions, a stress situation often aggravated in human-influenced areas by the presence of xenobiotics. Cellular signaling pathways responsible to detect and adapt to low oxygen availability and presence of xenobiotics are centered in two receptor proteins: hypoxia inducible factor-alpha (HIF-α) and aryl hydrocarbon receptor (AHR), respectively. Since both share the aryl hydrocarbon receptor nuclear translocator (ARNT) as heterodimeric partner, a competitive cross-talk between these pathways is foreseen. The aim of this study was to assess the role of HIF-α and AHR mediated signaling at the transcriptional level in *Oncorhynchus mykiss* primary hepatocyte cultures after exposure to the AHR agonist benzo[a]pyrene (B[a]P, 128 nM) under hyperoxic (7±0.5 mg O_2/L), normoxic (1.75±0.25 mg O_2/L) and hypoxic (0.55±0.25 mg O_2/L) conditions. Toxicity endpoints determined included cell viability (metabolic activity and cell membrane stability), anaerobic metabolism (lactate production) and transcriptional changes of *cytochrome p450 1a1 (cyp1a1), glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*)*, ahr2β* and *hif-1α*.

The metabolic activity was stable during the exposure period, while loss of membrane integrity was only observed after 72 h in the group exposed to a combination of B[a]P and hyperoxic conditions. Production of lactate increased over time and was negatively correlated with oxygen concentration, as might be expected in cells increasing the anaerobic metabolism. Co-exposure of B[a]P inhibited the lactate accumulation induced by hypoxia in cells under hypoxic conditions. *cyp1a1* transcription levels increased in presence of B[a]P, although hyperoxia and hypoxia alone also enhanced *cyp1a1* transcription. *gapdh* transcription levels tended to increase after 48-72 h under hypoxia. The slight up-regulation of *ahr2β* after B[a]P exposure under normoxic conditions was partially increased in the B[a]P-hyperoxia group (48 h) but attenuated in the B[a]P-hypoxia group (72 h). *hif-1α* and *ahr2β* showed quite similar transcriptional patterns in all treatments, being significantly down-regulated in the hyperoxic group in comparison to the rest of treatments after 48 h. These results indicate that a transcriptional level interference occurs between the hypoxia adaptive and the xenobiotic detoxification signaling pathways. However, quantification of ARNT transcription levels and post-transcriptional level regulation of studied genes would clarify such interaction.

Keywords: hypoxia, hyperoxia, benzo[a]pyrene, HIF-α, AHR, primary hepatocyte cultures, *Oncorhynchus mykiss*

Resumen

Las aguas costeras y los estuarios tienden a presentar condiciones hipóxicas, situaciones frecuentemente agravadas en las zonas de influencia humana por la presencia de xenobióticos. La rutas moleculares responsables de detectar y responder a la escasez de oxígeno y a la presencia de xenobióticos a nivel celular, se centran en dos receptores: el factor inducido por hipoxia-α (HIF-α) y el receptor de hidrocarburos arilo (AHR). Dado que ambos comparten al translocador nuclear del receptor de hidrocarburos arilo (ARNT) como pareja de heterodimerización, se prevé una competición entre ambas rutas moleculares. El objetivo de éste estudio es la evaluación del papel de las señales mediadas por HIF-α y AHR a nivel transcripcional en cultivos primarios de hepatocitos de *Oncorhynchus mykiss* tras su exposición al agonista de AHR benzo[a]pireno (B[a]P, 128 nM), bajo condiciones hiperóxicas (7±0.5 mg O_2/L), normóxicas (1.75±0.25 mg O_2/L) e hipóxicas (0.55±0.25 mg O_2/L). Los parámetros de toxicidad medidos incluyen el análisis de la viabilidad celular (actividad metabólica y estabilidad de la membrana celular), el metabolismo anaerobio (producción de lactato) y de cambios en el nivel de transcripción de *citocromo p450 1a1 (cyp1a1), gliceraldehído-3-fosfato deshidrogenasa* (*gapdh*)*, ahr2β* y *hif-1α*.
La actividad metabólica se mantuvo estable durante el periodo de exposición, mientras que la membrana celular sufrió una reducción de su estabilidad únicamente en los hepatocitos sometidos a la combinación de hiperoxia y B[a]P durante 72 h. La producción de lactato aumentó con el tiempo de cultivo mostrando una correlación negativa con la cantidad de oxígeno suministrada, como cabría esperar de células que están incrementando su metabolismo anaeróbico. La acumulación de lactato consecuencia de las condiciones de cultivo hipóxicas no sucedió bajo co-exposición a B[a]P. Los niveles de transcripción de *cyp1a1* aumentaron en presencia de B[a]P, aunque las condiciones de hiperoxia e hipoxia también elevaron dichos niveles por sí solas. Bajo condiciones hipóxicas, los niveles de transcripción de *gapdh* tendieron a aumentar. La ligera sobreexpresión de *ahr2β* observada tras la exposición a B[a]P en condiciones de normoxia aumentó en el grupo hiperoxia-B[a]P (48 h), mientras que en el grupo hipoxia-B[a]P se vio atenuada (72 h). *hif-1α* y *ahr2β* mostraron patrones de transcripción bastante similares en todos los tratamientos, resultando en niveles significativamente menores en el grupo bajo hiperoxia en comparación con el resto de tratamientos (48 h). Estos resultados indican la presencia de una interferencia a nivel transcripcional entre las rutas moleculares de adaptación a hipoxia y de detoxificación de xenobióticos. Sin embargo, se requiere el estudio de los niveles de transcripción de *arnt* y de los efectos postranscripcionales sobre los genes antes mencionados para ser capaces de entender dicha interacción.

Palabras clave: hipoxia, hiperoxia, benzo[a]pireno, HIF-α, AHR, cultivo primario de hepatocitos, *Oncorhynchus mykiss*

Laburpena

Itsasertz eta estuarioek baldintza hipoxikoak izateko joera erakusten dute, gainera, sarritan, gizakien eraginpean agertzen diren guneetan ematen den xenobiotikoen agerpenak egoera larritu egiten du. Xenobiotikoen presentzia eta oxigeno eskasia antzeman eta erantzuteko erantzunkizuna duten molekula mailako bidezidorrak bi hartzaile proteikotan oinarritzen dira, batik-bat: hipoxiak induzitutako α-faktorea (HIF-α) eta arilo hidrokarburoen hartzailea (AHR). Heterodimerizazio kide gisa, arilo hidrokarburoen hartzailearen translokatzailea (ARNT) elkarbanatzen dutenez biek, molekula mailako bidezidor bien arteko lehia aurreikus daiteke. Ikerketa honen helburua, transkripzio mailan, HIF-α eta AHR-ak bideratzen dituzten seinaleen papera aztertzea da, horretarako, AHR-ren agonista den benzo(a)pireno pean (B[a]P) 128 nM) baldintza hiperoxiko (7±0.5 mg O_2/L), normoxiko (1.75±0.25 mg O_2/L) zein hipoxikoetan (0.55±0.25 mg O_2/L) mantendutako *Oncorhynchus mykiss* amuarrainaren hepatozitoen kultibo primarioak aztertuz. Neurtutako parametroak, zelulen bideragarritasuna (jarduera metabolikoa eta mintz plasmatikoaren egonkortasuna), metabolismo anaerobioa (laktatoaren ekoizpena) eta *p450 1a1 zitokromoa (cyp1a1), gliceraldehido-3-fosfato deshidrogenasa* (*gapdh*)*, ahr2β* eta *hif-1α geneen* transkripzio mailen aldaketak izan ziren.

Jarduera metabolikoa egonkor mantendu zen esposizio denboran zehar; hiperoxia eta B[a]P pean 72 orduz mantendutako hepatozitoek bakarrik galdu zuten mintz plasmatikoaren egonkortasuna. Laktatoaren ekoizpenak gora egin zuen zelulen hazkuntza-denbora luzatzearekin batera. Aldi berean, zelulak hornitzeko erabilitako oxigeno emariarekin korrelazio negatiboa neurtu zen, metabolismo anaerobioa areagotzen ari diren zelulelan espero bezala. Hipoxiak eragindako laktato metaketa, zelulei B[a]P-a gehitzean inhibitu egin zen. *cyp1a1*-en transkripzio mailek gora egin zuten B[a]P pean, nahiz eta, hipoxia eta hiperoxia baldintzek bakarrik antzeko eragina izan. Hipoxia baldintzetan, *gapdh*-ren transkripzio mailek gora egin zuten. Normoxia baldintzetan B[a]P-ak eragindako *ahr2β*-ren gainerregulazio arina sendotu egin zen hiperoxia-B[a]P taldean (48h) eta hiperoxia-B[a]P taldean (72h) moteldu. *ahr2β* eta *hif-1α-ek* antzeko trankripzio profilak erakutsi zituzten talde guztietan, hiperoxia taldekoak gainontzeko tratamenduetakoekin (48h) alderatuz gero ordea, esangarriki azpierregulatuta agertu ziren. Emaitza hauek, xenobiotikoen detoxifikaziorako eta hipoxia peko adaptaziorako molekula mailako bidezidorren arteko interferentzia adieraz dezakete, maila transkripzionalean. Hala ere, elkarrekintza hau argitzeko, ARNT-ren trankripzio mailak eta aurretik aipatutako geneen transkripzio ondorengo erregulazioa mekanismoak ikertu beharko lirateke.

Hitz gakoak: Hipoxia, hiperoxia, benzo[a]pyreno, *hif-1α*, AHR, hepatozitoen kultibo primarioak, *Oncorhynchus mykiss*

1. Introduction

Estuarine and coastal waters may experience environmental hypoxia, periods of reduced oxygen that occur naturally due to water stratification, upwelling events or other processes (Levin 2002; Helly & Levin 2004). However, frequency, severity and extent of the hypoxic events have progressively increased since the 1960s due to human impact. Predictive models for climate change and human population foresee a deteriorating future scenario due to increased anthropological influence on aquatic water resources (Keeling & Garcia 2002; Bopp et al. 2002; Diaz & Rosenberg 2008; Keeling et al. 2010; Rabalais et al. 2010; Bijma et al. 2013). Water bodies connected to anthropized areas are also likely to be affected simultaneously by toxic chemicals derived from urban and industrial activities (Ravindra et al. 2008; Tobiszewski & Namieśnik 2012). Effects originating from multiple stressors may act in concert an may be underestimated or misdiagnosed when assessing one stressor at a time (Silva et al. 2002), as it is often done.

Cells detect low oxygen conditions and trigger molecular and functional adaptive responses through regulation of their gene expression pattern, metabolism, membrane transport and energy demand (Wu 2002; Nikinmaa & Rees 2005; Semenza 2012). Adaptation to conditions of low oxygen availability in vertebrate cells is regarded to be mainly regulated by the hypoxia inducible factor-1α (HIF-1α), a transcription factor of the basic helix-loop-helix Per/Arnt/Sim (bHLH-PAS) family (Wang & Semenza 1995; Loenarz et al. 2011). Under normoxic conditions HIF-1α is hydroxylated, triggering its ubiquitination and subsequent degradation via the proteasome complex. When oxygen is scarce, such hydroxylation does not occur (Greer et al. 2012) and accumulating HIF-1α protein enters the nucleus where it heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT), member of the same family of transcription regulatory proteins. The HIF-1α/ARNT heterodimer acts on hypoxia responsive elements (HREs) located in the promoter region of specific genes (Kaelin & Ratcliffe 2008) unchaining the specific response to hypoxia. Although three different HIF-α paralogs have been described in vertebrates, HIF-1α is the most studied one (Majmundar et al. 2010; Zhang et al. 2014).

Aryl hydrocarbon receptor (AHR), yet another component of the bHLH-PAS protein family, is in charge of detecting and binding xenobiotics such as polycyclic aromatic hydrocarbons (PAHs), planar polychlorinated biphenyls (PCBs) and dioxins (Zhou et al. 2010). The binding of these xenobiotics results in the activation and translocation of AHR into the nucleus, where heterodimerization with ARNT occurs. As reviewed by Safe and colleagues (2013), the AHR-ARNT complex recognises and binds to xenobiotic responsive elements (XREs) located in the promoter region of genes involved, for instance, in the phase I biotransformation metabolism of xenobiotics (e.g. cytochrome P450 1a, *cyp1a*).

The fact that both HIF-α and AHR-mediated pathways require heterodimerization with ARNT suggests a possible competitive crosstalk when organisms are exposed to xenobiotics under hypoxic conditions, potentially compromising their ability to adapt to changing environmental conditions (Mandl & Depping 2014). However, mechanisms of regulation not based on competition for ARNT have also been proposed. For example, the prolyl hydroxylases (PHDs) in charge of the hydroxylation of HIF-1α are regulated by oxygen availability, the redox state of cells and the availability of intermediaries of the Krebs cycle (Webb et al. 2009). Therefore, an eventual redox unbalance caused by xenobiotics may alter their capacity to hydroxylate HIF-1α.

Interactions between the hypoxia adaptive and the xenobiotic detoxification pathways has been reported in mammalian systems (Allen et al. 2005; Zhang & Walker 2007; Seifert et al. 2008; Vorrink et al. 2014). In fish (*Gasterosteus aculeatus* and *Micropogonias undulatus*) maintained under hypoxic conditions, the xenobiotic detoxification pathway was altered in absence of AHR agonists (Leveelahti et al. 2011; Rahman & Thomas 2012). Furthermore, crosstalk between HIF and AHR pathways has been described in zebrafish (*Danio rerio)* and mummichog (*Fundulus heteroclitus*) exposed to xenobiotics under hypoxic conditions, where interaction between both pathways occurred (Prasch et al. 2004; Kraemer & Schulte 2004). In contrast, Yu and colleagues (2008) found benzo[a]pyrene (B[a]P) to increase the transcription of genes possessing HREs in orange-spotted grouper (*Epinephelus coioides*) during hypoxia, while that of XRE-containing genes remained unchanged. Interestingly, a study carried out in a hepatocellular carcinoma cell line of top minnow (*Poeciliopsis lucida)*

maintained under hypoxia suggested that HIF-1α could sequester ARNT precluding its heterodimerization with AHR in presence of different xenobiotics, but not *vice versa* (Fleming et al. 2009). Crosstalk between HIF-1α- and AHR-mediated pathways in fish is still poorly characterized and studies to reveal possible interactions are necessary.

Bioassays using primary hepatocyte cultures derived from liver from different organisms including fish are widely used in toxicology. Primary fish hepatocyte cultures retain many native functions specific of the liver, such as biotransformation metabolism or partial estrogen responses (Pedersen & Hill 2000; Segner & Cravedi 2000; Hultman et al. 2015), and allow to characterize the mode of action of AHR agonists (e.g. dioxin, anti-dioxin) at the molecular and functional level (Mortensen et al. 2006; Mortensen & Arukwe 2008; Tollefsen et al. 2008; Gräns et al. 2010; Petersen & Tollefsen 2011). Characterization of cross-talks between receptors that act as transcription factors has been previously performed in primary hepatocyte cultures, identifying for instance specific mechanistic properties of the estrogen receptor-AHR interaction (Mortensen & Arukwe 2007; Gräns et al. 2010).

In liver, hepatocytes reside at the end of a portal system where, depending on their location, dissolved oxygen in blood ranges between 11% (periportal zone) and 4% (perivenous zone) approximately (Kietzmann et al. 2006). However, most of the cell cultures are conducted under atmospheric oxygen saturation (i.e. 21% oxygen), a physiological condition seldom experienced by hepatocytes *in vivo* (Godoy et al. 2013). Thus, the study of cell cultures maintained under adequate physiologically realistic dissolved oxygen concentrations is essential to understand responses *in vivo*.

In the present study, *Oncorhynchus mykiss* primary hepatocyte cultures were maintained under different oxygen concentrations in combination with the exposure to the AHR agonist B[a]P, to assess interactions between the hypoxia adaptive and the xenobiotic detoxification pathways. Three different oxygen concentrations were tested: hyperoxia (7±0.5 mg O_2/L, 25%), normoxia (1.75±0.25 mg O_2/L, 5.25%) and hypoxia (0.55±0.25 mg O_2/L, 1.65%). A combination of methods measuring changes in cell viability, accumulation of lactate as proxy of anaerobic metabolism activation and transcription regulation of key genes in the HIF-1α and AHR signalling pathways in fish

hepatocyte primary cultures was performed to characterise the potential interaction of both stressing situations.

2. Materials and Methods

2.1 Hepatocyte isolation and cell culture

Rainbow trouts *Oncorhynchus mykiss* (200–500 g) were obtained from Haadem Fisk AS (Valdres, Norway) and reared at the Institute of Biology of the University of Oslo (Norway) for over 4 weeks prior to the isolation of hepatocytes. Water temperature in tanks was 6 ±2ºC, oxygen saturation 100% and pH 6.6 with a 12 h light/dark regime. Fish were daily fed with commercial pellets (Skretting AS, Stavanger, Norway) corresponding to approximately 0.5% of fish total body weight.

A total of 3 juvenile rainbow trouts were terminated by cephalic concussion and subjected to a 2-step liver perfusion as previously described by Tollefsen et al. (2003). Briefly, each liver was *in situ* perfused (5 mL/min, 10–15 min) with a calcium free cold buffer (NaCl 122 mM, KCl 4.8 mM, $MgSO_4$ 1.2 mM, Na_2HPO_4 11 mM, NaH_2HPO_4 3.3 mM, $NaHCO_3$ 3.7 mM, EGTA 26 µM, 4ºC) to remove blood from the liver and disrupt any possible cell-cell interaction. Digestion of connective tissues and dislodgment of cells were performed using the same perfusion buffer (5 ml/min, 10–15 min, 37ºC) containing $CaCl_2$ (1.5 mM) and collagenase (0.3 mg/mL) but without EGTA. The liver was then dissected out, transferred to a glass beaker and gently disaggregated in ice-cold calcium free buffer supplemented with 0.1% w/v bovine serum albumin (BSA). The obtained suspension was sequentially filtered through two nylon meshes (250 and 100 µm respectively) before centrifugation three times at 500 rpm at 4^{o}C. After the first centrifugation the supernatant was removed and cells were re-suspended in ice-cold calcium free buffer supplemented with 0.1% w/v BSA. During the second and third centrifugations, cells were re-suspended in serum-free L-15 medium containing L-glutamine (0.29 mg/mL), $NaHCO_3$ (4.5 mM), penicillin (100 Units/mL), streptomycin (100 µg/L) and amphotericin (0.25 µg/mL). Cell viability was assessed using a Bürcker chamber and trypan blue:cell suspension mixture (2:1). Only suspensions with cell viability greater than 90 % were accepted for further use. Upon successful isolation,

0.5 million cells/mL were seeded in a final volume of 200 µL/well and 1.25 mL/well in 96 well and 24 well plates, respectively.

2.2 Exposure of cultured hepatocytes

Hepatocyte exposures were carried out using three separate cell suspensions, each one originated from an individual fish. All plates were seeded and pre-cultured for 24 h at 15ºC in specifically designed microincubators (29.3 x 23.2 x 9.1 cm, transparent polycarbonate) receiving ambient atmospheres with relevant dissolved oxygen concentrations for fish livers (Kietzmann & Jungermann 1997; Broughan et al. 2008; Godoy et al. 2013). Oxygen concentration was set on 7±0.5 mg O_2/L for the hyperoxic culture, 1.75±0.25 mg O_2/L for the normoxic culture (eventually referred as control) and 0.55±0.25 mg O_2/L for the hypoxic culture. After pre-culturing for 24h, half of the culture media of each well was replaced with fresh media containing either anhydrous ultrapure dimethyl sulfoxide (DMSO, <1% v/v, Sigma-Aldrich, St. Louis, MI, USA) as control or benzo[a]pyrene (B[a]P, Sigma-Aldrich) dissolved in DMSO for additional 24 or 48h (final concentration in the well: 128 nM). Copper sulphate (Sigma–Aldrich) was freshly prepared in cell media as positive control of cytotoxiciy analysis (final concentration in the wells: 0.078–10 mM). Plates were covered with adhesive gas permeable seals to reduce evaporation and cross-contamination as well as to facilitate efficient gas exchange.

2.3 Cell viability assay

Cell viability was determined measuring cell metabolic activity with alamar blue (AB, BioSource, Nivelles, Belgium) and plasma membrane stability with 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM, Molecular Probes, Leiden, The Netherlands) according to Schreer et al. (2005). Briefly, after complete removal of the culture media, cells were incubated with tris buffer (50 mM, pH 7,5) containing 5% AB and 4 µM CFDA-AM in the dark for 30 min on an orbital shaker (100 rpm). Fluorescence was measured at excitation-emission wavelength pairs of 530-590 nm (AB) and 485-530 nm (CFDA-AM) in a Victor V3 multilabel counter (Perkin Elmer, Waltham, MA, USA). Cell viability was estimated after data normalization using the corresponding oxygen experimental atmosphere (hyperoxia, normoxia or hypoxia) as

control (100 % viability) and maximum cell death (0 % viability) caused by exposure to 0.1 M $CuSO_4$.

2.4 Lactic acid assay

With the aim of assessing the extent of the anaerobic metabolism in hepatocytes under each culture condition lactic acid concentrations were measured in culture media using the L-Lactate Assay Kit (Abcam, Cambridge, UK) according to manufacturer instructions. In brief, culture media was removed from the wells and properly diluted to fit samples within a lactate standard curve. Then, the reaction mix (containing the provided buffer, enzyme mix and probe) was added, and the final mix was incubated in the dark for 30 min on an orbital shaker (100 rpm) at room temperature. Fluorescence was measured in a Victor V3 multilabel counter (Perkin Elmer) with an excitation-emission wavelength pair of 535-590 nm.

2.5 Gene transcription levels

After 48 and 72 h of culture (24 and 48 h of B[a]P treatment, respectively), total RNA was isolated using the RNeasy plus mini kit (Qiagen, Hilden, Germany) with an on-column DNAse treatment. RNA concentration was spectrophotometrically measured (Spectrophotometer ND 1000, Nanodrop technologies Inc., Wilmington, USA) and integrity was assessed using the Agilent BioAnalyzer RNA 6000 nano series kit (Agilent Technologies, USA), only accepting RIN values above 9. 100 ng of RNA were reverse-transcribed into cDNA with the qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) following manufacturer instructions and stored at -80ºC until further use. Transcription levels of *cytochrome p450 1a1* (*cyp1a1*), *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*), *aryl hydrocarbon receptor-2 beta* (*ahr2β*) and *hypoxia inducible factor-1 alpha* (*hif-1α*) were determined by quantitative PCR (qPCR) using *18S ribosomal RNA* (18*S rRNA*) as reference gene. Primer sequences were designed with the Primer 3 software (Table 1). PCRs were carried out in triplicates in a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) using 10 µl of SYBR Green (FastStart Universal SYBR Green Master, Roche, Indianapolis, USA) in a final volume of 20 µl. Optimized primer and cDNA concentrations are shown in Table 1. PCR conditions were set as: 50 °C for 2 min, 95 °C for 10 min; 40 cycles at 95°C for 15 s

followed by each melting temperature for 30 s (Table 1). Specificity of PCRs was confirmed by the presence of a single peak in the dissociation curve analysis that followed each amplification run. Relative transcription values were calculated with the Delta-Delta Ct method after correcting the efficiency of PCRs (modified from Pfaffl 2001).

Table 1. Name and accession number of target genes measured in the present study are shown, followed by the sequence of specific primers, cDNA concentrations and melting temperature used for qPCRs. Final concentration of primers was 700 nM in all the cases.

Gene (Acc. number)	Primer sequence (5´- 3´)	T. (ºC)	cDNA (pg/µl)
18S rRNA (AF243428)	Fw: CGAGCAATAACAGGTCTGTG Rv: GGGCAGGGACTTAATCAA	61.3	421.25
cyp1a1 (U62796)	Fw: GATGTCAGTGGCAGCTTTGA Rv: TCCTGGTCATCATGGCTGTA	66.8	421.25
gapdh (AF027130)	Fw: GGAATCAAAGTCGTTGCCAT Rv: GGATCTCATGGGGCTTCATA	57.8	70.21
ahr2β (DQ358693)	Fw: GCACCCCCAGGACCAGAGT Rv: GTTGTCCTGGATGACGGC	57.8	70.21
hif-1α (NM_001124288)	Fw: CCACCTCATGAAGACCCATCA Rv: TCTCCACCCACACAAAGCCT	61.3	70.21

2.6 Statistical analysis

Significant differences among groups in cell viability, lactic acid concentration and gene transcription levels were set based on the permutational multivariate analysis of variance (PERMANOVA, created by Marti J. Anderson, Department of Statistics, University of Auckland, 2005). In all cases significance was set at $p < 0.05$.

3. Results

3.1 Cell viability assay

Cell metabolic activity (AB) remained stable during the tested 72h (Fig. 1), with values close to 100% in all the experimental groups. No significant differences were found neither between hepatocytes cultured under the same oxygen conditions when B[a]P was added, nor between values of the same group after 48 and 72 h of culture.

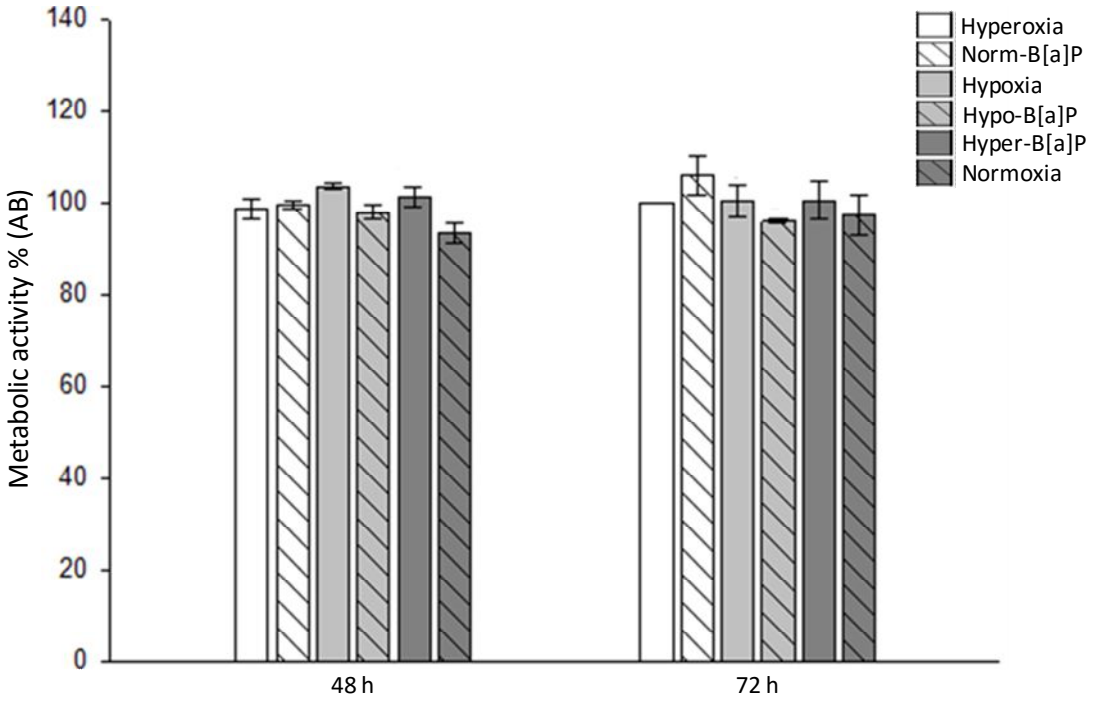

Figure 1. Metabolic activity percentage (mean ± SD) based on the Alamar blue method measured in primary cultures of hepatocytes under hyperoxia, normoxia and hypoxia in absence and presence of benzo[a]pyrene.

Regarding membrane stability measurements (CFDA-AM), most of the groups presented viability values close to 100% after 48 and 72 h of exposure. However, hepatocytes in the hyperoxia-B[a]P group suffered a significant viability reduction from 48 to 72 h (Fig. 2). After 48 h of exposure to hyperoxia-B[a]P, hepatocytes showed significantly higher viability levels than those of the hyperoxic group. However, at 72 h, the viability of hyperoxia-B[a]P treated hepatocytes was significantly lower than that of hyperoxia alone treated cells (Permanova test, $p<0.05$)

3.2 Lactic acid assay

After 48 h, no significant differences in lactic acid concentration were found between treatments (Fig. 3). There was an approximately 2-fold increase in lactic acid levels from 48 to 72 h in all the groups with the exception of the cells exposed to B[a]P under normoxic conditions. After 72 h, oxygen levels and the production of lactate were inversely related in cells not exposed to B[a]P. Co-exposure to B[a]P apparently reversed this effect under normoxic and hypoxic conditions (Permanova test, $p<0.05$).

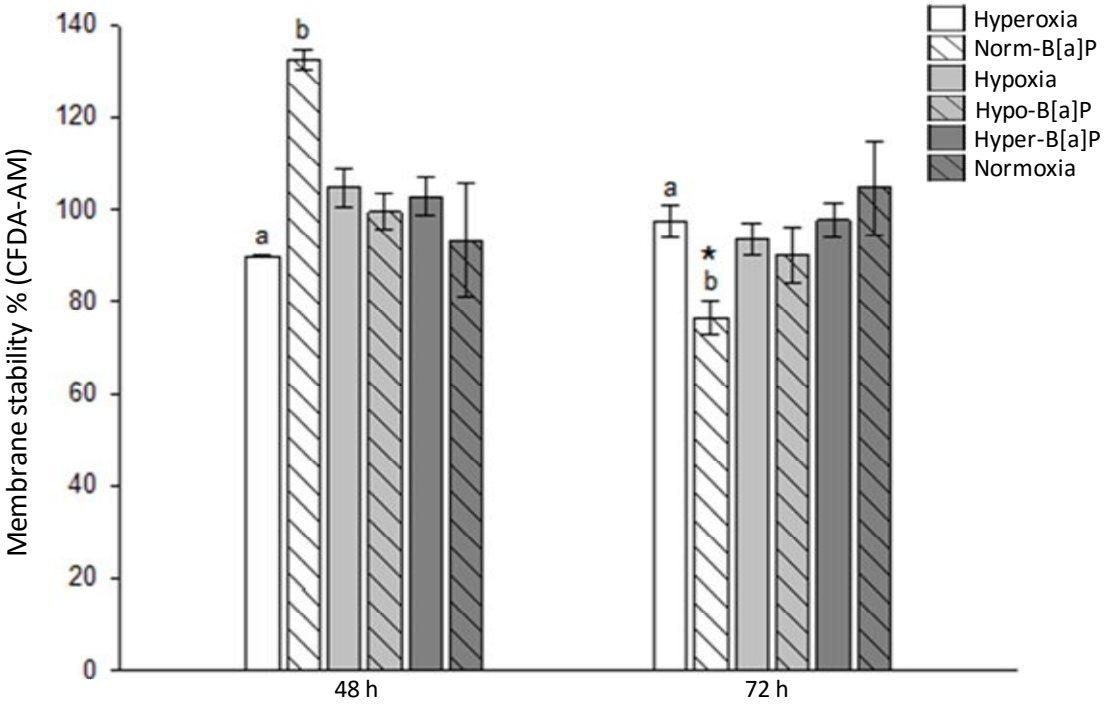

Figure 2. Membrane stability percentage (mean ± SD) based on the CFDA-AM method measured in primary cultures of hepatocytes under hyperoxia, normoxia and hypoxia in absence and presence of benzo[a]pyrene. Asterisks identify significant differences between 24 and 48 h exposure times for the same treatment, and different letters indentify significant differences between treatments at the same exposure time, both based on Permanova (p<0.05).

3.3 Gene transcription levels

hif-1α transcription levels were not altered in hepatocytes maintained under normoxic conditions (control). Hyperoxia increased *hif-1α* transcription levels after 24 h, being this increase significant in comparison to *hif-1α* levels in cells incubated under hypoxia. After 48 h, *hif-1α* transcription levels in the hyperoxia group were significantly lower than in the hypoxia and hypoxia-B[a]P groups, increasing again towards initial values at 72 h. Under hypoxic conditions, *hif-1α* transcription levels were significantly reduced at 72 h in comparison to values at 24 and 48 h (Fig. 4). Addition of B[a]P to normoxic and hypoxic hepatocytes did not alter *hif-1α* transcription levels. However, co-exposure to B[a]P altered the previously described transcriptional trend under hyperoxia, up-regulating *hif-1α* at 48 and 72 h. Hepatocytes under hypoxia maintained their *hif-1α* transcription levels after B[a]P exposure (Fig. 4).

Control conditions did not alter transcription levels of *gapdh* over time. Hyperoxia alone down-regulated *gapdh,* transcription levels being significantly lower than those

in both hypoxia groups at 48 h. Hyperoxic cultures recovered control transcription levels at 72 h. Under hypoxia, *gapdh* showed a trend towards up-regulation at 48 and 72 h (Fig. 4). Addition of B[a]P to hepatocytes in normoxia increased *gapdh* transcription levels at 72 h, with significant up-regulation when compared with normoxia and hyperoxia-B[a]P groups. Similarly, the hypoxia-B[a]P co-exposure recorded a slight increase of *gapdh* transcription levels, which at 72 h resulted significantly higher than *gapdh* transcription levels measured in the normoxia and hyperoxia-B[a]P groups (Fig. 4). On the contrary, *gapdh* transcription levels were unaltered in the hyperoxic group upon B[a]P exposure.

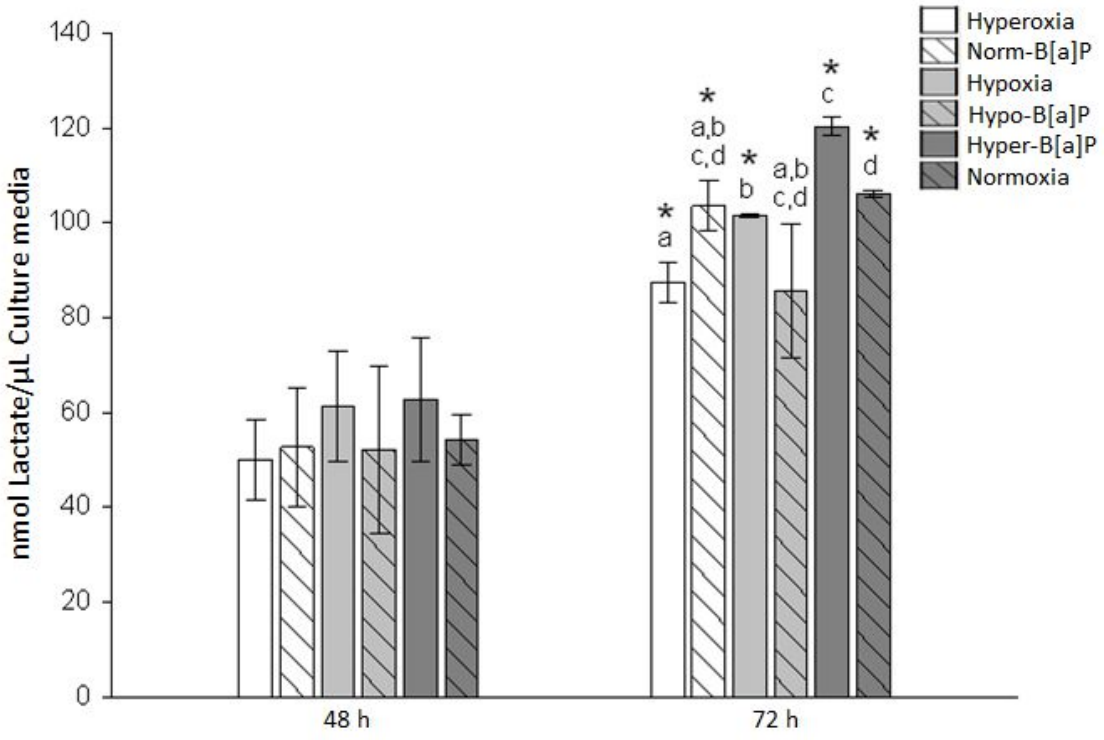

Figure 3. Lactic acid concentration (mean ± SD) in primary cultures of hepatocytes under hyperoxia, normoxia and hypoxia in absence and presence of benzo[a]pyrene. Asterisks identify significant differences between 24 and 48 h exposure times for the same treatment, and different letters indentify significant differences between treatments at the same exposure time, both based on Permanova (p<0.05).

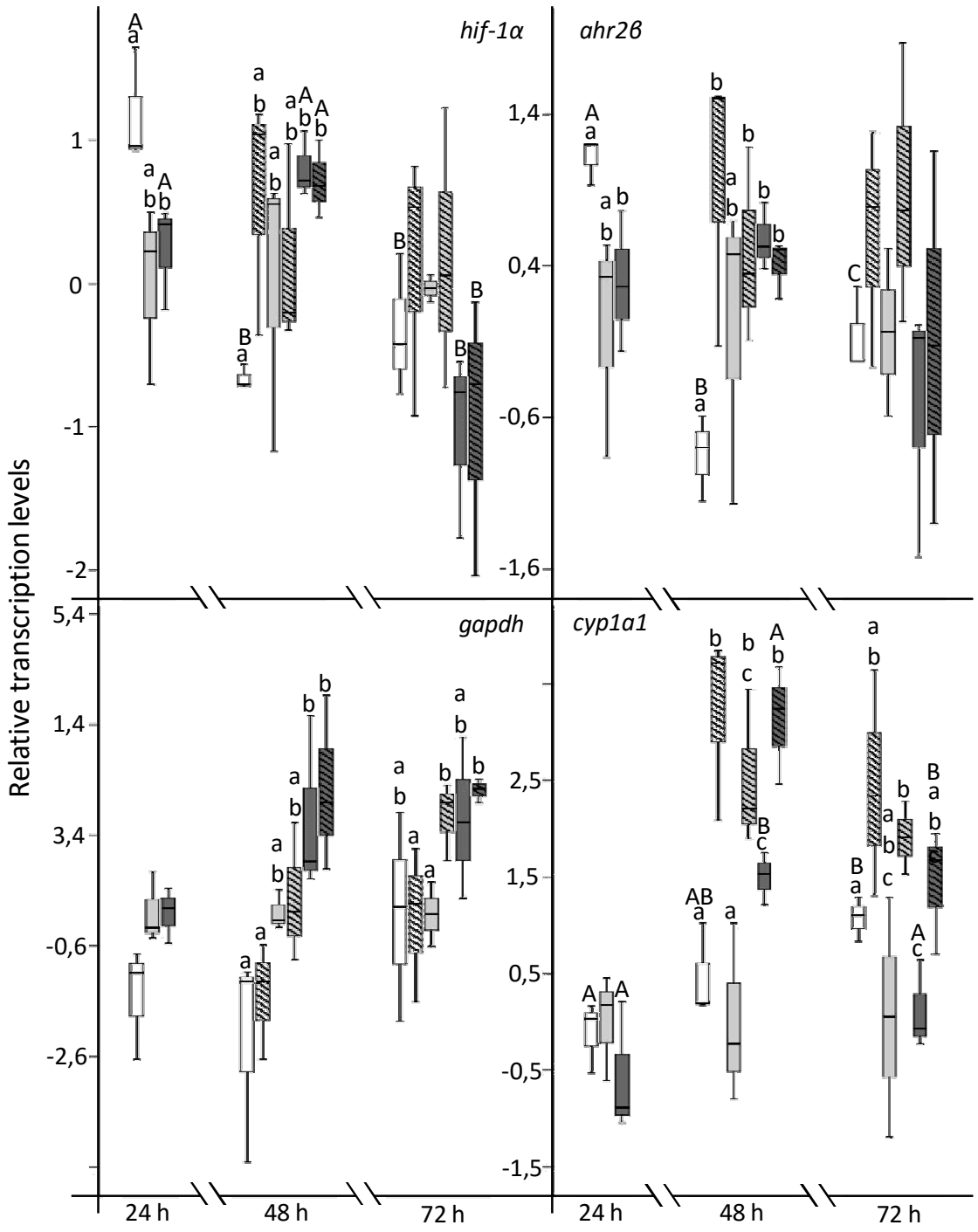

Figure 4. Relative transcription levels of *cyp1a1*, *gapdh*, *ahr2β* and *hif1α* measured in primary cultures of hepatocytes under hyperoxia (□), normoxia (▨) and hypoxia (■) in absence (no fill) and presence (linear pattern) of benzo[a]pyrene. Box plots represent the 25th, 50th and 75th percentiles, while whiskers represent standard deviation. Different lower case letters indicate significant differences between different treatments at the same exposure time, while different capital letters indicate significant differences between exposure times of the same treatment. Both analyses were based on Permanova (p<0.05).

No alterations in *ahr2β* transcription levels were detected with time in normoxia. Hyperoxia elicited an increase in *ahr2β* transcription levels after 24 h, these being significantly higher than levels under hypoxia. *ahr2β* transcription levels of hepatocytes under hyperoxic conditions decreased at 48 h, being significantly lower than in the rest of treatments with the exception of normoxia. At 72h similar transcription levels were measured in hyperoxic and control cells. *ahr2β* transcription levels were slightly altered in hypoxia treated cells, including a down-regulation at 72 h (Fig. 4). Dosage of B[a]P caused a moderate time-dependent increase of *ahr2β* transcription levels in normoxia while the transcriptional trend observed during hyperoxia was altered; causing an up-regulation that peaked at 48 h. The hypoxia-B[a]P co-exposure did not significantly alter the transcription levels of *ahr2β* (Fig. 4).

Under normoxia, *cyp1a1* transcription levels were similar at 24, 48 and 72 h. Under hyperoxia, *cyp1a1* transcription levels peaked at 72 h, when levels were significantly higher than in the hypoxic group. On the other hand, transcript levels peaked after 48 h of hypoxia, being significantly higher than levels measured under normoxia and hyperoxia (Fig. 4). After B[a]P exposure, transcription of *cyp1a1* was up-regulated in all the oxygen concentration groups: values peaked at 48 h (significantly higher for the 3 groups in comparison to normoxia alone) and decreased at 72 h approaching normoxia levels (Fig. 4).

4. Discussion

In the last years, cell cultures have gained consideration as an alternative to *in vivo* studies on animal models, given their advantages. They have reduced variability, the possibility to control the experimental conditions and higher throughput analysis possibilities than *in vivo* exposure experiments in toxicology. In this way, they constitute a consolidated strategy in the context of the "Three Rs: Replacement, Reduction and Refinement" philosophy (Schirmer 2006). Therefore, primary cultures of rainbow trout hepatocytes have been frequently used for *in vitro* studies in ecotoxicology (Rissanen et al. 2006; Tollefsen et al. 2008; Petersen & Tollefsen 2011; Tollefsen et al. 2012). In addition, primary hepatocyte cultures might constitute a suitable model to study interferences between different molecular pathways such as

the one described for the hypoxia adaptive and the xenobiotic biotransformation pathways. In the present study, rainbow trout primary hepatocytes were cultured under 3 different dissolved oxygen concentrations and co-exposed or not with the model AHR agonist B[a]P in order to assess whether interactions occur in the transcriptional regulation of genes participating in the xenobiotic detoxification and the hypoxia adaptive pathways. Cells cultured under normoxic conditions were considered the control of the experiment. Endpoints measured in normoxic hepatocytes did not change significantly over time, with the exception of the lactic acid assay between 48 and 72 h. As expected, a time dependent increase of lactate production was observed in all treatments between 48 to 72 h that was attributed to culture ageing.

4.1 Cell metabolic activity and plasma membrane stability

Cell viability measured as metabolic activity and plasma membrane stability was unaffected among the tested treatments (values close to 100%) with the exception of the hyperoxia-B[a]P treatment, whose plasma membrane stability changed over time (48-72 h). Viability of hepatocytes exposed to hyperoxia-B[a]P for 48h was 132.5% and decreased to 76.5% at 72 h. Even if cell viability greater than 100% has previously been reported in similar primary hepatocyte experiments (Petersen & Tollefsen 2011), such high values are difficult to explain and may be related to the nature of the dye used (Ganassin et al. 2000). In regard to the decreased viability observed in the last sampling time, the properties of the plasma membrane could have been altered due to the intercalation of PAHs in the lipid bilayer (Korchowiec et al. 2008). Therefore, after 72 h, before being metabolized, B[a]P could have increased solubility of the plasma membrane (Liland et al. 2014). Furthermore, increased B[a]P metabolization rate could be envisaged under high oxygen availability, boosting the accumulation of B[a]P-metabolites (Verma et al. 2012) and reactive oxygen species (ROS) derived from their biotransformation metabolism (Jifa et al. 2006; Jebali et al. 2013). B[a]P-metabolites and ROS could have attacked lipids in cell membranes (Collin et al. 2014). Such sequence of events could have occurred in the present experiment, producing the significant cell viability reduction observed after 72 h of exposure to B[a]P under hyperoxic conditions.

4.2 Lactic acid assay

In hypoxic situations, carbohydrates are a fundamental source of energy, since glycolysis can produce ATP in absence of oxygen. A switch from aerobic to anaerobic metabolism has been described in vertebrate cells under hypoxic conditions, with the concomitant increase of lactate production (Krumschnabel et al. 2000; Seagroves et al. 2001; Omlin & Weber 2010; Yan et al. 2010). Accordingly, in the present study, the highest and the lowest lactate production were recorded under hypoxia and hyperoxia respectively after 72 h of culture, suggesting an increased dependence on the anaerobic metabolism in hypoxic hepatocytes.

Hepatocytes exposed to B[a]P under normoxic conditions decreased their lactate production (especially at 72 h). The carbon flow may have possibly suffered a partial redirection from the glucose metabolism to the pentose phosphate pathway (PPP) (Ralser et al. 2007; Semenza 2013), that would produce the required NADPH to activate the CYP P450-mediated biotransformation metabolism of B[a]P (Miwa et al. 1978; Hannemann et al. 2007). Previous *in vivo* studies reported heterogeneous results after fish exposure to different PAHs (Pollino & Holdway 2002; Vieira et al. 2008; Tintos et al. 2008), including enhancement of the anaerobic metabolism that differs from our results. This may reflect processes involving different tissues, such as the lactic acid cycle in which the liver recycles lactate into glucose to re-fuel muscles. This is something that cannot occur in our hepatocyte *in vitro* tests. In the same way, lactate concentration decreased in hypoxic hepatocytes when B[a]P was dosed. Zhao and colleagues (2010) found that the oxidative arm of the PPP was down-regulated by hypoxia in the mouse BaF3 cell line. In the present study, such regulation could be overridden by the presence of B[a]P, following the sequence of events explained for the normoxia-B[a]P group.

Hepatocytes under hyperoxia-B[a]P treatment responded differently, the presence of B[a]P counteracted the repressing effect of high oxygen availability on the anaerobic metabolism. Lactate concentration after 72 h under the hyperoxia-B[a]P treatment was similar to that of normoxia and hypoxia-B[a]P hepatocytes. Thus, the elevated oxygen availability in combination with B[a]P could increase the production of ROS, as

previously discussed for the cell viability test, causing a hypoxia-mimicking effect that would allow such high lactate levels. Increased ROS production has been reported to block the activity of prolyl hydroxylases by altering their redox state, preventing them to hydroxylate HIF-1α. HIF-1α would then be free to act on the HREs of genes coding for anaerobic metabolic enzymes, among others (Pouysségur & Mechta-Grigoriou 2006; Cash et al. 2007).

4.3 Hypoxia adaptive pathway genes

Expression of *hif-1α*, key gene of the hypoxia adaptive pathway, is mainly post-transcriptionally regulated. However, transcriptional regulation has also been reported in fish during hypoxic conditions (Rahman & Thomas 2007; Terova et al. 2008; Rimoldi et al. 2012). In agreement, the present study showed alterations on the transcriptional regulation of *hif-1α*, which was significantly down-regulated after 72 h of exposure to hypoxia. This could be reflecting the existence of a negative feedback loop, something that has been previously described under sustained hypoxic conditions (Webb et al. 2009). A similar effect could be reflected in the study published by Rimoldi and colleagues (2012) in liver of *Perca fluviatilis,* where *hif-1α* transcription levels were higher during acute hypoxia than during chronic hypoxia. On the other hand, alterations in transcription levels of *hif-1α* were also found in hepatocytes under hyperoxia, being especially relevant at 24 and 48 h.

Co-exposure to B[a]P did not change the transcription levels of *hif-1α* in hepatocytes under normoxic or hypoxic conditions. This could be expected from a gene not involved in the xenobiotic detoxification pathway. However, exposure to B[a]P up-regulated *hif-1α* transcription in hepatocytes under hyperoxic conditions (especially at 48 h).

We need to have in mind that the genome duplications occurred in the early evolution of vertebrates resulted in the apparition of three different *hif-α* paralogs (Loenarz et al. 2011) whose functions are only partially overlapped (Majmundar et al. 2010; Zhang et al. 2014). In the present study, transcription levels of *hif-1α* have been measured, however, the study of *hif-2α* and *hif-3α* transcriptional responses might help elucidate molecular level regulation against multiple stressors involving hypoxia.

During oxygen scarcity, a global induction of glycolysis is considered to occur (Kraemer & Schulte 2004; Davies et al. 2011). Thus, in an attempt to assess the regulation of genes involved in the glycolytic pathway, transcription levels of *gapdh* were measured. Accordingly, hepatocyte *gapdh* transcription levels were up-regulated after 48 and 72 h of hypoxia. This is also consistent with the elevated lactate concentrations in this group and suggests increased anaerobic metabolism. The down-regulation of *gapdh* detected after 24 and 48 h under hyperoxia might be caused by the excess of oxygen, which would increase the hydroxylation of HIF-1α by PHDs for degradation (Kaelin 2005). Such decrease on HIF-1α protein levels would reduce the transactivation of hypoxia responsive genes, unchaining the generalized decrease of glycolytic processes in favour of the aerobic metabolism. However, most studies using primary hepatocyte cultures consider this hyperoxic condition as their control. For example, Graven et al. (1994) found lower *gapdh* transcription levels in bovine endothelial cells cultured in a 21% oxygen atmosphere (considered control) than in those cultured in a 3% oxygen atmosphere (considered hypoxic).

When normoxic hepatocytes were exposed to B[a]P, *gapdh* was up-regulated at 72 h. An overall induction of glycolysis has been previously observed in *Oncorhynchus mykiss* injected with B[a]P, and was attributed to increased energy achievement (Tintos et al. 2008). Hepatocytes under hypoxia-B[a]P displayed *gapdh* transcription levels similar to those described in cells under hypoxia alone. These trends are not in line with an enhancement of PPP in detriment of the glycolysis. However, results reported by Agarwal et al. (2012) in mouse lungs strengthen the idea of a (at least partial) post-translational regulation of GAPDH. This could explain present *gapdh* transcription levels, which would be overridden by post-translational changes of the glycolytic enzyme, allowing for the observed decrease in lactate concentrations. Therefore, alterations caused in the response to hypoxia by co-exposure to B[a]P (such as the reduced production of lactate) would be explained in our study by means independent of competition for ARNT. The transcriptional trend of *gapdh* was similar in hepatocytes under hyperoxia and hyperoxia-B[a]P treatments. Such transcriptional trend could appear as opposed to the slight increase of lactate production attributed to a hypoxia-mimicking process. Therefore, it could be defended that *gadph* was regulated at the

post-transcriptional level (as it has been previously discussed) or that its transcriptional response might have been delayed; being more appropriate to compare the transcriptional levels of the hypoxia-B[a]P group at 72 h with the levels measured under hypoxia alone at 24 and 48 h.

4.4 Xenobiotic detoxification pathway genes

In order to assess changes in the main regulatory genes coding for proteins of the xenobiotic biotransformation metabolism, *cyp1a1* and *ahr2β* transcription levels were measured by qPCR. Transcriptional regulation of *ahr2* has been reported in teleost fish species after exposure to xenobiotics (Hahn et al. 2005; Valdehita et al. 2012). In the present study, only minor *ahr2β* transcription level fluctuations were found in trout hepatocytes under hypoxia. In opposition, significant changes were found after hyperoxia treatment. Transcription of *ahr2β* was initially up-regulated (24 h), down-regulated at 48 h and reached levels close to normoxia at 72 h.

B[a]P dosage under normoxia elicited an up-regulation trend of *ahr2β* transcription levels at 72 h. This transcription factor is known to be transcriptionally up-regulated in presence of PAHs (Hansson & Hahn 2008). In agreement, hepatocyte *ahr2β* transcription levels were increased 48 and 72 h after exposure to B[a]P under hyperoxia. In contrast, hypoxia-B[a]P and the simple hypoxia treatment showed similar transcriptional trends, where oxygen deprivation apparently blocked the up-regulation of *ahr2β* under B[a]P exposure. According to Hansson & Hahn (2008), *ahr2α* is regarded to give a deeper answer to PAHs than *ahr2β*, and this fact might possibly explain the weak transcriptional responses found for *ahr2β* in the present study. Unfortunately, measurement of *ahr2 α* transcription levels was not successful.

To assess the transcriptional regulation elicited by AHR, *cyp1a1* was selected as target gene. Induction of EROD activity has been extensively reported upon exposure of primary hepatocyte cultures to B[a]P (Behrens et al. 2001; Ferreira et al. 2014). Multiple examples of *cyp1a1* up-regulation in liver of fish exposed to xenobiotics in general and B[a]P in particular exist in the literature (Zhou et al. 2010; Bilbao et al. 2010; Roy et al. 2011; Räsänen et al. 2012; Yuan et al. 2013). However, the transcriptional increase measured in hepatocytes cultured under hyperoxic and

hypoxic conditions was unexpected. Anyway, similar results were found for liver *cyp1a2* transcription levels in *Gasterosteus aculeatus* (Leveelahti et al. 2011) and for zebrafish embryos *cyp1a* (Prasch et al 2004) under hypoxia. Apparently, hypoxic conditions were able to elicit *cyp1a1* up-regulation in absence of AHR-agonists.

As expected, exposure to B[a]P caused the up-regulation of *cyp1a* at 48 h in all cases. Fleming et al. (2009), hypothesized a cross-talk in which HIF-1α would take ARNT from AHR (but not *vice versa*) limiting the activation of the xenobiotic response pathway in *Fundulus heteroclitus*. Alternatively, in a study with mouse primary hepatocytes, hypoxia limited the up-regulation of *cyp1a* caused by an AHR agonist, but authors concluded that this was due to reasons different from the competition for ARNT (Allen et al. 2005). On the contrary, oxygen deprivation did not compromise the transcriptional activation of the xenobiotic response pathway in our study, suggesting that the hypoxia adaptive pathway was not sequestering all available ARNT. Several studies discard ARNT as a rate limiting molecule in this interference (reviewed in Mandl & Depping 2014). Therefore, competition for ARNT could result critical if it is constitutively expressed at limiting levels, but for cells that can up-regulate its expression under hypoxic conditions such competition would be avoided (Vorrink et al. 2014).

4.5 Parallel regulation of transcription factors

It was observed that *ahr2β* and *hif-1α* transcription factors shared almost identical transcriptional trends when cells were cultured under hyperoxia, both in presence or absence of B[a]P. Besides, the up-regulation measured after B[a]P exposure under hyperoxic conditions was expected for *ahr2β* (Hansson & Hahn 2008) but not for a gene such as *hif-1α*, unrelated to the xenobiotic detoxification pathway. B[a]P exposure under hypoxia did not alter the transcription levels of *ahr2β* and *hif-1α* when compared to the levels found during hypoxia alone. The up-regulation of *ahr2β* after B[a]P exposure observed in normoxic and hyperoxic conditions was blocked under hypoxia.

Such results suggest the presence of a common upstream mechanism which modifies the transcription pattern of *ahr2β* and *hif-1α*. Apparently, hepatocytes under

hyperoxia prioritize the transcriptional responsiveness of *ahr2β* and *hif-1α* to B[a]P, while under hypoxia the response pattern observed under low oxygen availability prevails. This allows to think about a regulatory mechanism shared by both adaptive pathways different from the heterodimerization to ARNT (Pollenz et al. 1999; Prasch et al. 2004).

4.5 Conclusion

Results of the present study reveal the possibility of a common regulatory mechanism affecting the responses of the hypoxia adaptive and the xenobiotic detoxification pathways. This mechanism would be different from competition for ARNT, which seems to be insufficient to explain all the transcriptional fluctuations identified in key genes of both pathways. An upstream regulatory mechanism affecting AHR2β and HIF-1α could explain the results obtained after exposure to the simple and combined treatments, but further research is required to expand the knowledge about the hypoxia-xenobiotic metabolism interference mechanisms.

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SUMMARY AND GENERAL DISCUSSION

The driving objective of the present work was to contribute to the study of the possible interplay involving the bHLH-PAS transcription factor family members HIF-αs, AHR and ARNT in aquatic animals. To do so, four experiments were designed, in which mullets *Chelon labrosus*, mussels *Mytilus galloprovincialis* and freshly isolated *Oncorhynchus mykiss* hepatocytes were studied under diverse experimental conditions involving different concentrations of dissolved oxygen and exposure to chemical pollutants that function as AHR agonists. Overall, various endpoints were measured, being the transcriptional regulation of selected genes the focus of analysis. Such selected genes included the transcription factors themselves and several genes involved in the adaptive response that they trigger. However, only fragmentary gene sequence information was available for the studied species, mullets and mussels, so sequencing analysis had to be run first. The main issues discussed in each of the 4 individual experiments are presented here, followed by a general discussion to summarize the main conclusions of this PhD work.

In an attempt to gain knowledge regarding the hypoxia adaptive pathway at the molecular level in bivalves, an experiment with mussels *Mytilus galloprovincialis* was devised. After acclimatization, Mediterranean mussels were placed in 4 different tanks where 3 treatments and a control group would be held. Mussels were thus maintained under hypoxic conditions (2.5 mg O_2/L), under B[a]P (0.4 mg/L) exposure and under B[a]P exposure in hypoxia for 1, 7 and 14 days. Several hypoxia adaptive pathway-related genes (as known for vertebrates) were selected as targets for transcriptional quantification in digestive gland of mussels, namely: the genes coding for the HIF-α transcription factor and energy supply related COX-4, HK, ALDOLASE and PEPCK, which in vertebrates are known to be regulated by HIF-αs. We first sequenced fragments of their mRNAs, which allowed performing qPCR analysis for all the target genes except for *aldolase*. Further, the tissue specific transcription pattern of *hif-α* was characterized in unexposed mussels. *hif-α* was found to be transcribed in all studied tissues (gills, muscle, digestive gland and mantle) and specially in gills, as it was observed for other molluscs in previous studies (Piontkivska et al. 2011; Kawabe & Yokoyama 2012; Cai et al. 2014). Transcription levels of *hif-α* in digestive gland showed a decreasing trend during hypoxia. We hypothesized this was consequence of a

negative feedback loop aimed to protect the animal from re-oxygenation that is to be expected in the intertidal zone, where this mussel species reside. In general, *hk* and *pepck* (related to the glucose metabolism) maintained unaltered transcription levels. This could be reflecting an effort of mussels to maintain their glycolytic activity while a general metabolism repression strategy is deployed to cope with oxygen scarcity (Ivanina et al. 2010). Dosage of B[a]P under hypoxic conditions modified such trends but not in a significant way.

Based on the previous experience, another *in vivo* laboratory study was planned using *Chelon labrosus* mullets, a pollution sentinel species that thrives in highly euthrophized areas and is employed in the monitoring of the Basque coast. The structure of the previous *in vivo* laboratory study with mussels was followed, placing juvenile fish in 4 different tanks. The animals were maintained under hypoxic conditions (2.5 mg O_2/L), under exposure to B[a]P (0.4 mg/L) and under B[a]P exposure in hypoxic conditions for 1, 7 and 14 days. During the experiment, mullets were observed in case any behavioral change could occur due to the different treatments. Transcription levels of genes coding for HIF-1α, HIF-2α, HIF-3α and ARNT transcription factors and for VEGF, ALDOLASE, GAPDH, PEPCK (related to the hypoxia adaptive pathway) and CYP1A1 (related to the xenobiotic detoxification pathway) were quantified in each time point in liver and gills. At the end of the experiment, a histopathological assessment was performed in gills, an organ regarded to specially suffer under hypoxia (Wu 2002; Nikinmaa & Rees 2005; Shingles et al. 2005). The evaluation of fish behavior suggested that mullets deprived from oxygen (in presence or absence of B[a]P) displayed an energy saving behavior since their activity was conspicuously reduced (Behrens & Steffensen 2006; Skjæraasen et al. 2008). Exposure to B[a]P under hypoxic conditions caused a reduction of the transcriptional levels of the three *hif-α* paralogs in liver and the *arnt* gene in both liver and gills, displaying a trend that could compromise the ability of the animal to adapt when both stresses coexist. A common transcriptional pattern could be observed for the studied transcription factors, which suggests the presence of a common upstream mechanism regulating their transcription. The transcription levels of glycolytic enzymes were not altered by oxygen scarcity alone, neither in liver nor in gills. That might illustrate the effort of fish to maintain the levels

of transcription of such genes stable, even in a scenario of generalized metabolic repression (Olsvik et al. 2013). However, co-exposure to B[a]P under hypoxic conditions tended to down-regulate *aldolase* in liver and up-regulate *pepck* in both tissues; so the combination of stressors might be causing a glucose unbalance in these animals. The fact that the up-regulation of *cyp1a1* caused by B[a]P exposure was accentuated under hypoxic conditions underpins the hypothesis for the presence of an interplay between the signaling pathways of both adaptive routes. Surprisingly, the metabolism of xenobiotics seemed to be enhanced under hypoxia while the adaptive response to oxygen scarcity tended to be hampered, suggesting that the interference could transcend the mere competition for ARNT. Accordingly, the increase in gill aneurysms detected in hypoxic fish (considered a collateral effect of the adaption to oxygen scarcity) was reduced in presence of B[a]P, supporting the hypothesis that the adaptive response to hypoxia could have been obstructed by exposure to AHR agonists.

The skills and experience obtained in both previous *in vivo* lab experiments were then applied in an *in vivo* field study. To do so, the Pasaia harbor in the estuary of the Oiartzun River (in the Basque coast) was selected as study area. Two groups of juvenile mullets *C. labrosus* were housed for 5 and 21 days in separate *ad hoc* designed cages deployed in the Pasaia harbor, which is regarded to be chronically polluted and to suffer periodical events of oxygen scarcity (Gorostiaga et al. 2004; Borja et al. 2006; Rodríguez et al. 2010). The precise sites in which each cage was deployed were named inner harbor (IH) and outer harbor (OH). Transcriptional levels of genes coding for HIF-1α, HIF-2α, HIF-3α and ARNT transcription factors, GAPDH (related to the hypoxia adaptive pathway) and CYP1A1 (related to the xenobiotic detoxification pathway) were quantified in both time points in liver and gills. Relatively low levels of dissolved oxygen were detected after 7, 14 and 21 days of deployment in IH, while normoxic conditions were measured in OH. Bioavailability of pollutants was measured in whole soft tissue of mussels caged together with the mullets for 21 days, showing higher chemical levels in IH than in OH, but similar levels of combined PAHs and PCBs in both places. Exposure to chemical pollutants under oxygen scarcity conditions (in IH) for 5 days increased the transcription levels of *hif-1α* in mullet liver and, especially, in gills.

Transcription levels of *hif-3α* were reduced in both tissues after 21 days of deployment in IH. Surprisingly, gills of fish caged in IH displayed lower transcription levels of *hif-3α* than those of fish caged in OH during the whole experiment. *arnt* transcription levels on the other hand, were increased in both analyzed tissues after 5 days of deployment in IH. This could have possibly prevented ARNT from becoming a limiting factor in response to xenobiotic exposure under low oxygen conditions. Thus, a shared general transcription pattern could be noticed for *hif-1α, hif-3α* and *arnt* genes, suggesting the presence of a common regulatory mechanism for the 3 transcription factors. *gapdh* was up-regulated in mullet liver and gills after 5 and 21 days of deployment respectively in IH, probably indicating that glycolysis was enhanced (Nikinmaa & Rees 2005). Finally, transcription levels of *cyp1a1* were similar in both caging sites (with comparable levels of PAHs and PCBs), suggesting that oxygen scarcity did not alter the xenobiotic metabolism pathway in IH and that ARNT was not a limiting factor for AHR.

In the last chapter of the PhD thesis, primary hepatocyte cultures were employed to study the interplay between both adaptive pathways in cells isolated from liver of *Oncorhynchus mykiss* trout. It was devised as an approach in line with the "three Rs principle" (Russell & Burch 1959) allowing to test *in vitro* with a higher throughput the endpoints that have been previously used in the *in vivo* experiments. Freshly isolated hepatocytes were cultured in three different oxygen atmospheres: hyperoxic (7±0.5 mg O_2/L), normoxic (1.75±0.25 mg O_2/L) and hypoxic (0.55±0.25 mg O_2/L). The first atmosphere corresponds to the one normally used in studies using trout hepatocyte cultures, the second one resembled the conditions found in normoxic liver *in vivo* (Kietzmann et al. 2006; Godoy et al. 2013) and the last one tried to imitate conditions in the liver cells of fish living in hypoxic waters. Benzo[a]pyrene (B[a]P, 128 nM) was dosed to generate 6 experimental groups, with 3 different oxygen conditions in presence and absence of this AHR agonist. First, the cell viability was measured in terms of metabolic activity and membrane stability. Lactate production by hepatocytes was also assessed, as an indicator of the extent of anaerobic metabolism within the cells. Finally, transcriptional levels of genes coding for HIF-1α and AHR2β and their downstream genes *gapdh* and *cyp1a1* were quantified. Membrane stability measurements for the hyperoxia-B[a]P combined treatment yielded highly variable

results including loss of viability in cultures, which questions the suitability of using saturating oxygen concentrations to test the effects of exposure to chemical compounds in fish hepatocyte cultures. Lactate measurements for the B[a]P-free experimental groups showed that the amount of this metabolite increased as the oxygen concentration decreased. This would point out to an increased reliance of cells in anaerobic metabolism (Krumschnabel et al. 2000; Seagroves et al. 2001; Omlin & Weber 2010; Yan et al. 2010). However, exposure to B[a]P reduced the accumulation of lactate found under normoxic and hypoxic atmospheres at 72 h, maybe as a consequence of the increased activity of the pentose phosphate pathway elicited as a mechanism to assist B[a]P biotransformation (Miwa et al. 1978; Ralser et al. 2007; Hannemann et al. 2007; Semenza 2013). The traffic of metabolites such as lactate between liver and the rest of the organs given *in vivo* cannot occur in vitro; which may lead to unexpected responses in complex situations such as the exposure to B[a]P. After 72 h under hypoxic conditions, the transcription levels of *hif-1α* in cultured primary hepatocytes were reduced, probably in response to a negative feedback loop (Webb et al. 2009). On the other hand, a mild up-regulation of *gapdh* was detected in the normoxia-B[a]P treatment, perhaps as a strategy to produce extra energy to fuel the detoxification of the xenobiotic (Tintos et al. 2008). Such transcriptional trend was not blocked in the hypoxia-B[a]P treatment, suggesting the absence of competition for ARNT. Moreover, the transcription levels of *ahr2β* tended to be up-regulated under exposure to B[a]P, as described in previous studies (Billiard et al. 2002; Hansson & Hahn 2008). Such trend was blocked under hypoxic conditions, suggesting the existence of an upstream mechanism to favor the response to oxygen scarcity. Nevertheless, *cyp1a1* transcription levels were also up-regulated by B[a]P (Bilbao et al. 2010; Yuan et al. 2013) even under hypoxia (actually, hypoxia alone tended to up-regulate this gene), supporting again the possibility pointed out before of an absence of competition between HIF-αs and AHR for ARNT under such conditions. Interestingly, *hif-1α* and *ahr2β* displayed similar transcription profiles in most of the treatments, especially under hyperoxia. Such results allowed us to suggest the presence of an upstream regulatory mechanism shared by both transcription factors.

Regarding the suitability of using primary *O. mykiss* hepatocyte cultures to study mechanisms of action of exposure to xenobiotics and hypoxia and the relevance of the results obtained we can extract some conclusions. Lactate, measured as an indicator of changes in the anaerobic metabolism, indicated that cells increased their reliance in anaerobic metabolism during hypoxia, but B[a]P co-exposure caused responses that have not been previously observed in *in vivo* experiments in fish (Pollino & Holdway 2002; Vieira et al. 2008; Tintos et al. 2008). Apparently, the absence in culture conditions of the complex interrelations occurring between different organs imposes the need to analyze results with care. The hyperoxic atmosphere normally applied in previous studies using such cultures does not appear logical to us. For instance, the oscillations observed in terms of membrane stability suggest that excessive oxygen alters the behavior of hepatocyte cell membranes, increasing the response to B[a]P and its metabolites (Verma et al. 2012). Among the 4 chapters of this work, results suggesting hypoxia-mimicking events were only found during exposure to B[a]P under hyperoxic conditions in rainbow trout hepatocytes. Probably, the enhancement of B[a]P metabolism in such oxygen-rich situation resulted in an increased ROS production that affected PHDs and, therefore, stabilized HIF-αs (Jaakkola et al. 2001; Pouysségur & Mechta-Grigoriou 2006). Thus, we suggest that oxygen atmospheres closer to the physiological ones (between 11 and 4 % of oxygen) should be used in such primary cultures to obtain more mechanistically realistic results, especially regarding the toxicity of certain chemical compounds.

At the beginning of this PhD, it was hypothesized that fish and mussels under hypoxic conditions would deploy general energy saving strategies. However, the genes coding for glucose metabolism-related enzymes maintained their transcription levels similar to those found in the control groups in most of the cases. Both aquatic animals seemed to make an effort in maintaining the transcription of such genes, essential for survival in conditions of oxygen deprivation, in a theoretical scenario of general metabolic repression in which the transcription of non-essential genes would be down-regulated (Mustroph et al. 2010; Richards 2011). The fact that *O. mykiss* is an aquatic species far less used to cope with oxygen deprivation conditions than mullets and

mussels could explain the tendency of *gapdh* towards up-regulation during hypoxia in our *in vitro* study (Hochachka & Somero 2002).

A negative feedback loop has been proposed acting on the transcription levels of *hif-αs* when hypoxic conditions are sustained in time (Webb et al. 2009). Among the experiments carried out in the present work, the down-regulation of *hif-α* in the digestive gland of *M. galloprovincialis* subjected to hypoxia was the clearest evidence of such phenomenon. This has to be taken with care as we do not know yet whether HIF-α plays a role in adaptation to hypoxia in molluscs. In any case, these mussels residing in the intertidal zones are forced to possess efficient mechanisms to cope with hypoxia and the consequent the re-oxygenation occurring twice a day with the tidal regime in the Basque coast. In fish, mullets specially, transcriptional down-regulation of *hif-αs* was mainly observed after co-exposure to B[a]P under hypoxic conditions. The absence of this trend in fish under hypoxia alone leads us to hypothesize that such down-regulation was not due to the previously explained feedback loop, but to the increased stress.

Previous studies have described *hif-3α* as the fastest and more responsive of the three vertebrate *hif-α* paralogs in terms of transcriptional regulation under hypoxic stimuli (Geng 2014). Accordingly, in the lab experiment performed with mullets, *hif-3α* was the most severely down-regulated of the 3 *hif-α* paralogs in both analyzed tissues after exposure to B[a]P under hypoxic conditions. Nevertheless, the down-regulation of *hif-3α* gene recorded for *C. labrosus* in the field experiment under low oxygen availability and in presence of diverse pollutants was even deeper than that observed in the lab experiment, especially in gills. However, the sense of the response is opposed to the classically described up-regulation in hypoxic conditions (Geng 2014). Previous studies informed about the ability of certain *hif-3α* splice variants to work as inhibitors of the hypoxic response (Hara 2001). We suggest that *hif-3α* was not working as an inhibitor of the response to hypoxia in the mullet lab study, even if the hypoxic response was addressed as partially repressed after B[a]P exposure under hypoxia, since the deep transcriptional down-regulation detected for this gene is opposed to such idea.

In the 3 experiments performed with fish species, the transcription levels of genes coding for proteins regarded to be regulated by HIF-αs under hypoxic conditions were unaltered or they were slightly up-regulated upon co-exposure to xenobiotics (with the exception of *aldolase* in liver of mullets during the lab experiment). Moreover, the transcription levels of the gene coding for CYP1A1, that depends on AHR activation, were maintained in the presence of chemical pollutants (*in vivo* field experiment) or were even up-regulated during B[a]P exposure (in vitro and in vivo lab experiments) in combination with hypoxic conditions. During co-occurrence of both stresses, both, hypoxia adaptive and xenobiotic detoxification pathways require ARNT as heterodimerization partner for HIF-αs and AHR (Vorrink & Domann 2014). Thus, the fact that apparently none of both pathways was negatively affected at the transcriptional level allowed us to hypothesize that the interplay between them was not elicited by competition for ARNT. This was so in our studies, even with the transcriptional down-regulation of *arnt* observed under exposure to AHR agonists in hypoxia. Perhaps, the ARNT pool in cells was large enough to allow simultaneous heterodimerization with HIF-αs and AHR. For instance, particular human cell lines have been shown to increase their ARNT mRNA and protein levels during events of oxygen scarcity (Mandl & Depping 2014); whereas fish exposed to AHR agonists increased their hepatic ARNT protein levels (Calò 2014).

Even if competition for ARNT is not the main cause, it seems that the hypoxia adaptive and xenobiotic detoxification pathways interplay at the transcriptional level. During the *in vitro* experiment performed with *O. mykiss* hepatocytes, the transcriptional levels measured for *hif-1α* and *ahr2β* followed quite similar trends in all treatment groups (especially in the hyperoxic one). Thus, we suggest the presence of an upstream regulatory mechanism simultaneously acting on the transcriptional regulation of both transcription factors. In the case of the *in vivo* lab experiment, *C. labrosus* hepatic *hif-α*s (*hif-1α* especially) and *arnt* transcription levels were similar in all the treatment groups. These were even more marked after exposure to B[a]P under hypoxic conditions. Thus, the hypothetic upstream regulatory mechanism suggested seems also to act on *arnt*, perhaps also affecting other bHLH-PAS transcription factors. Accordingly, during the field experiment, *hif-1α*, *hif-3α* to some extent and *arnt* also

displayed similar transcriptional patterns in liver and gills of grey mullets deployed in a site with situations of periodic low oxygen availability in the presence of chemical pollutants. Such unexpected results were obtained by using different methodologies (*in vivo* and *in vitro* approaches), different degrees of exposure to the stressors (laboratory and field exposures) and even different fish species. The transcriptional trends towards down-regulation upon exposure to xenobiotics under hypoxic conditions could condition the ability of fish to adapt and survive in hypoxic polluted scenarios. In the lab mullet experiment, this was true at least for the hypoxia adaptive pathway; reflecting the importance of increasing our knowledge about the mechanisms ruling the regulation of such inter-related pathways.

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CONCLUSION AND THESIS

CONCLUSIONS

1. Fragments of genes related to the hypoxia adaptive pathways of *Chelon labrosus* (*hif-1α*, *hif-2α*, *hif-3α*, *vegf*, *aldolase* and *gapdh*) and *Mytilus galloprovincialis* (*hif-α*, *cox-4*, *hk*, *aldolase* and *pepck*) were successfully sequenced, improving the gene sequence knowledge regarding both pollution sentinel species.
2. *hif-α* is most strongly transcribed in the gills of *Mytilus galloprovincialis* mussels, although it is transcribed in all studied organs. Little transcription level alterations are recorded in *hif-α* and glucose metabolism genes in digestive gland of mussels under hypoxia or exposure to benzo[a]pyrene. Although interference between hypoxia adaptive and xenobiotic detoxification pathways is suggested, further studies are required for its confirmation in molluscs.
3. Juvenile *C. labrosus* fish seemed to respond with an energy saving strategy after facing mild hypoxic conditions in a laboratory study. Dosage of benzo[a]pyrene under such conditions favored the transcription of detoxification metabolism genes over those of adaptation to hypoxia.
4. Juvenile *C. labrosus* caged in a polluted harbour under environmental hypoxic conditions showed an increasing trend in the transcription levels of glycolysis-related genes, while the transcription of xenobiotic detoxification-related genes was unaltered.
5. Hyperoxic atmospheres commonly used in the culture of *Oncorhynchus mykiss* primary hepatocytes seem to alter the behavior of hepatocyte cell membranes, exacerbating the reactivity of B[a]P and its metabolites. Atmospheres more similar to those recorded under normal physiological conditions should be used in *in vitro* experiments to obtain realistic results, especially regarding the toxicity of certain chemical compounds.
6. The trends recorded in the transcription levels of genes regulated by HIF-αs and AHR strongly suggest that interferences between the hypoxia adaptive and the xenobiotic detoxification pathways occur without competition for ARNT when *O. mykiss* hepatocytes and *C. labrosus* fish are exposed to benzo[a]pyrene under hypoxic conditions.

7. The studied bHLH-PAS family transcription factors show similar transcriptional patterns under different treatments (hypoxia, benzo[a]pyrene exposure and their combination), suggesting the presence of a common upstream mechanism regulating their transcription. This results of special interest, since such negative trends could alter the fitness of fish living in polluted hypoxic waters.

THESIS

Fish exposure to xenobiotics under hypoxic conditions results in alterations of the transcriptional control of genes participating in the hypoxia adaptive and xenobiotic detoxification pathways by means different from competition for ARNT, involving a transcriptional regulation mechanism apparently shared by *hif-αs, ahr* and *arnt*. In mussels, further studies are needed to clarify the presence and nature of such interferences.

ACKNOWLEDGEMENTS

I would like to thank:

- All the members of the "Cell Biology in Environmental Toxicology" group in the facilities of Leioa and Plentzia. Teamwork is fundamental for science, and in this team we definitely know how to put such theory into practice.
- Professor Ionan Marigomez for offering me the possibility to join the team by running for a PhD thesis when the Master thesis was all I had in mind.
- Professor Miren P. Cajaraville for accepting me as a member of the "Cell Biology in Environmental Toxicology" group.
- My directors, Dr. Ibon Cancio and Dr. Eider Bilbao (“El jefe” and “La jefa”) for your guidance during these 5 years. I especially appreciate the freedom and trust you have granted me, which allowed me concluding a work that became into something really personal.
- Dr. Knut-Erik Tollefsen for hosting me in the “Ecotoxicology” section of the Norwegian Institute for Water Research (NIVA) in Oslo. I would like to acknowledge also Karina Petersen and Maria Thérése Hultman, who guided me in the lab during my stay.
- Dr. Oihane Díaz de Cerio for giving me the original idea of the 3rd chapter of this thesis and easing my work to the extent that I could finish it within the adequate timing.
- Dr. Urtzi Izaguirre for his invaluable help in the planning and consecution of the *in vivo* lab experiments. His fishing, technical, scientific and diplomatic skills, among others, make him transcend the mere figure of a research Doctor.