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Environment and early life stages in fish : developmental plasticity responds to seawater changes in oxygen and temperature

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Préparée à l'Institut Français de Recherche
pour l'Exploitation de la Mer

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**Environment and early life stages
in fish: developmental plasticity
responds to seawater changes in
oxygen and temperature**

Thèse soutenue le 20 Décembre 2017

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“One of the biggest obstacles to making a start on climate change is that it has become a cliché before it has even been understood”

Tim Flannery

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List of abbreviations

ATP	Adenosine triphosphate
CrP	Creatine phosphate
Dph	Days Post Hatching
EGLN	EGL-Nine homolog family
GTP	Guanosine triphosphate
Hb	Hemoglobin
HIF-1	Hypoxia-inducible factor 1
HRE	Hypoxia-responsive element
PHD	Proline hydroxylases
TGD	Teleost-specific duplication
WGD	Whole-genome duplication

Chapter 1

General Introduction



1. Global climate change

The Earth's climate has changed throughout history (Zachos, 2001), and will likely continue to change (Crowley and Hyde, 2008), over multiple time scales. The current warming trend is of particular significance because most of it is extremely likely to be the result of human activity. The anthropogenic greenhouse gas emissions (primarily CO₂, NO₂, methane and nitrous oxide) have increased since the preindustrial era causing a greater retention of heat from the sun, resulting in global warming (Figure 1). Depending on the magnitude of future CO₂ emissions, the average surface temperature is projected to increase of 0.3-4.8°C for the end of the century (IPCC, 2013). The potential future consequences of global climate change across the globe include devastating results in the form of floods and severe storms, more frequent droughts, loss of plant and animal life and a greater risk of human casualties (Diffenbaugh and Field, 2013; Fuller et al., 2010; Hollowed et al., 2013; Thomas et al., 2004; Wheeler and von Braun, 2013).

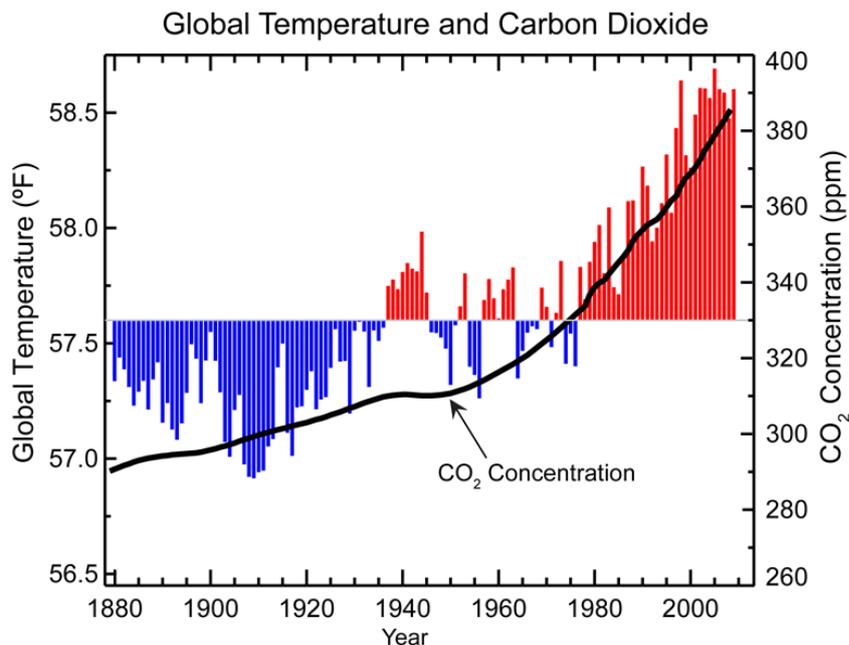


Figure 1. Global temperature and carbon dioxide from 1880 to 2012. Global annual average temperature (as measured over both land and oceans) has increased by more than 1.5°F (0.8°C) since 1880. Red bars show temperatures above the long-term average, and blue bars indicate temperatures below the long-term average. The black line shows atmospheric carbon dioxide (CO₂) concentration in parts per million (ppm). While there is a clear long-term global warming trend, some years do not show a temperature increase relative to the previous year, and some years show greater changes than others. These year-to-year fluctuations in temperature are due to natural processes, such as the effects of El Niños, La Niñas, and volcanic eruptions (adapted from Karl et al. 2009).

1.1 The Impact of Climate Change on the Oceans: warming temperature induced hypoxic waters

Over the last years, atmospheric energy has been taken up by the oceans, with over 60% of the increased energy in the climate system stored in the upper 700 m (IPCC, 2013). This has led to warming of surface waters at average rates of $>0.1^{\circ}\text{C}$ per decade in the upper 75 m and 0.015°C per decade at 700 m depth (IPCC, 2013). This results in shifts in heat storage, changes in strength and direction of currents and heat transport, warming of adjacent land masses, rising sea levels, deoxygenation and ocean acidification, among others (Hoegh-Guldberg and Bruno, 2010) (Table 1).

A serious consequence of global warming is a decrease in the dissolved oxygen (O_2) content (hypoxia) of the world's oceans. Aquatic hypoxia occurs when the rate of O_2 consumption by organisms exceeds O_2 production rates or the capacity of O_2 to diffuse in from the air (Graham, 1990). Even if the sensitivity to low dissolved O_2 depends on living organisms, environmental hypoxia has been general defined when the dissolved O_2 is below 30% air saturation (Levin et al., 2009; Zhang et al., 2010;) ranging from acute (short-term) to chronic (long-term). Episodes of moderate hypoxia have also been described at 40% air saturation (Haselmair et al., 2010). The loss of dissolved O_2 is exacerbated in warmer waters because the solubility of O_2 decreases as temperature increases [2°C rise in temperature reduces the O_2 content at saturation by around 5% (Benson and Krause, 1984; Peck and Uglow, 1990). Additionally, global warming may enhance stratification by heating surface waters, which in turn will reduce the O_2 supply to the ocean interior (Keeling and Garcia, 2002; Matear et al., 2000; Plattner et al., 2001). Warmer sea waters also increase O_2 consumption for an organism, decreasing dissolved O_2 in the water (Pörtner, 2010). Other anthropogenic causes of environmental hypoxia include eutrophication produced by nutrient input into aquatic systems (Bricker et al., 2008). The presence of excess nutrients, primarily nitrogen and phosphorus, promote algal growth. As algae die, they are subjected to oxidative decay by microorganisms which consume O_2 levels (Justić et al., 1993). Consequently, although hypoxia may result from natural causes (e.g. stratification, ice-cover, tidal action and fluctuations of photosynthesis) the incidences and severity of aquatic hypoxia will increase in the future as global temperatures rise and as urbanization and pollution continue (Diaz, 2001).

Ocean warming effect	Current response
Changes in heat storage (Ocean Heat Content, OHC)	<ul style="list-style-type: none"> - Rising water temperatures at all depths - Shallowing of the pycnocline - Intensification of El Niño (ENSO) events
Changes in the strength/position of currents and heat transport	<ul style="list-style-type: none"> - Increased poleward heat transport in western boundary currents of the subtropical gyres
Warming of adjacent land masses	<ul style="list-style-type: none"> - Warmer land surface temperatures - Melting permafrost - Retreating mountain glaciers - Increased extent and magnitude of forest fires
Rising sea levels due heat expansion of water and melting ice sheets and glaciers	<ul style="list-style-type: none"> - Permanent land inundation, coastal erosion, loss of some coral atolls and islands
Negative feedback on the ocean carbon sink	<ul style="list-style-type: none"> - Higher sea surface temperature reduces pCO₂ uptake from the atmosphere - Higher global sea surface temperature and land surface temperature enhance the rate of increase in atmospheric CO₂
Deoxygenation	<ul style="list-style-type: none"> - Reduced oxygen solubility in warmer water - Reduced penetration of oxygen into deeper water due to enhanced stratification
Ocean Acidification	<ul style="list-style-type: none"> - Reduction of the production of dimethylsulphide by phytoplankton

Table 1. Principal abiotic changes associated with ocean warming.

1.2 Sensitivity of shallow coastal areas to climate change: nurseries areas for many marine species

Shallow coastal areas with restricted water circulation are particularly subject to hypoxia and warming temperatures. Last decades was observed an increase in the frequency, duration, severity and extent of hypoxia events in coastal and estuarine waters due to adjacent human activities. Indeed, eutrophication fuelled by riverine nutrient run-off results in bloom of phytoplankton which, when dying, induces increased microbial activity that consumes dissolved O₂ (Meire et al., 2013; Rabalais et al., 2009; Zhang et al., 2010). Extremely hypoxic (anoxic) zones concern several coastal area throughout the world (Figure 2) (Diaz and Rosenberg, 2008; Zhang et al., 2010).

Coastal waters are amongst the most biologically productive and valuable ecosystems in the world (Able, 2005; Beck et al., 2001; Gillanders et al., 2003). Hypoxia exacerbated by warmer conditions may have major consequences on these ecosystems, causing mass mortality of living organisms, loss of habitat, changes in trophic patterns, increased susceptibility to predation, susceptibility to infection and changes in food resources (Naqvi et al., 2000; Mallin et al., 2006). Coastal ecosystems are particularly characterized by the presence of a high abundance and diversity of marine fish and therefore are considered as major fishery areas of strong economic interest (Diaz and Rosenberg, 2008; Elliot and Dewailly, 1995). The nearshore areas serve specially as nurseries for numerous fish species with complex life cycle in which adults spawn offshore, larvae are passively transported to coastal waters, where they develop and grow during juvenile stages, and then move to adult habitats offshore (Franco et al., 2008). Early life fish inhabit these estuaries, generally throughout Spring and Summer, benefiting from suitable conditions for growth, such as high food

availability, water temperature and refuge from predators until recruiting to adult populations in the marine environment (Beck et al., 2001). The recruitment level of fish from juvenile grounds (i.e. the number of individuals of a specific year class that survive to attain sexual maturity and join the reproductive component of a population), is strongly determined by the quality of the nursery (Gibson, 1994).

Among the different organisms of coastal ecosystem, fish are considered amongst the most sensitive to environmental constraints and particularly hypoxia events (Vaquer-Sunyer and Duarte, 2008). In the context of climate change, **understanding how hypoxia in warming coastal waters affects individual organisms is therefore particularly crucial** to forecast possible effects on juvenile recruitment which determine fish population structure and dynamics (van der Veer, 2000).

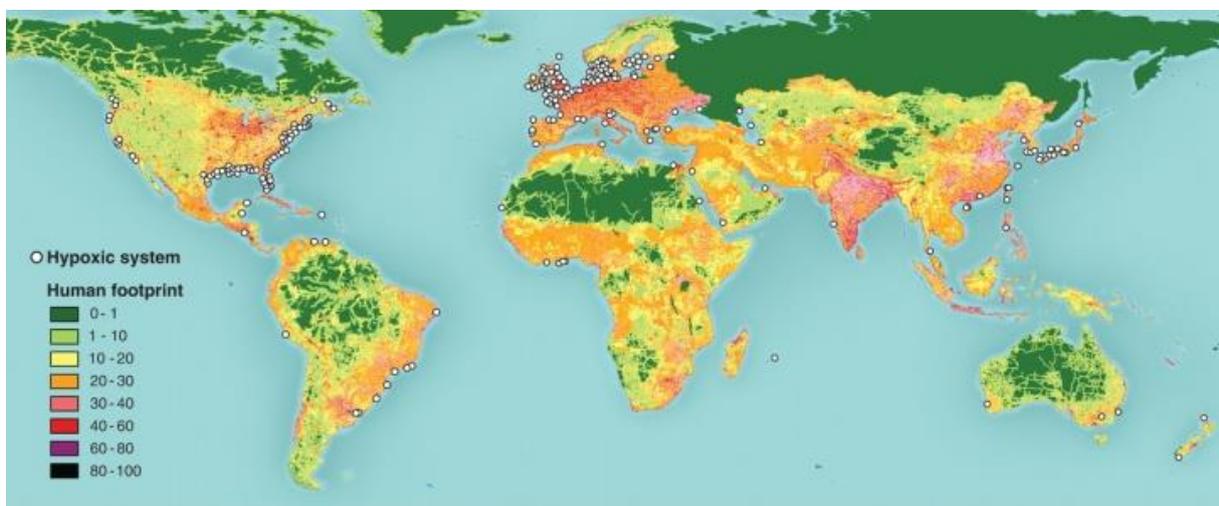
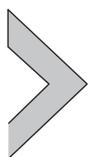


Figure 2. Global distribution of hypoxic systems that have been associated to eutrophic areas. Their distribution matches the global human footprint (the normalized human influence is expressed as a percent) (Diaz and Rosenberg, 2008).



2. Physiological significance of environmental acclimation in fish: focus on hypoxic constraint in warming coastal waters

For an organism, exposure to an environmental condition perceived as a constraint can lead to different possible outcomes. Fish may escape to a more suitable habitat, acclimate to the changes through physiological and/or behavioral response or not survive.

Escaping hypoxic waters and setting up acclimation process first requires for fish to sense changes in the ambient O₂ content. This is made possible since fish has developed a complex array of O₂ chemoreceptors in the gills (Randall, 1982). After transmission of the hypoxic signal to the brain, organism can initiate behavioral and physiological response. To escape a harsh environment a fish requires locomotive ability and the opportunity to migrate to new suitable habitats. This option may represent an impossible task in the case of extended hypoxic zones in coastal area, especially for fish with reduced active swimming capacity (such as early life fish). Therefore, it is important **to deeply understand the physiological response of fish exposed to coastal hypoxic environment in a warming world** to predict future fish distributions and population dynamics and to implement successful conservation strategies (Chown and Gaston, 2008; Wikelski and Cooke, 2006).

The physiological response of organisms including fish to environmental stress is largely related to maintaining the energy homeostasis (Sokolova and Lannig, 2008; Sokolova et al., 2012). Indeed, the capacity of fish to tolerate environmental constraints such as hypoxia and/or warming temperature lies particularly in their ability to maintain the production of energy required to sustain their key physiological functions (e.g growth, reproduction, feeding...) (Sandersfeld et al., 2015; Weber et al., 2016). This acclimation depends on the capacity of fish to supply O₂ to tissues and regulate its metabolism. Genetic factors combined to phenotypic plasticity contribute to the ability of fish to acclimate, which also depends on species, life stages, social status and life history.

2.1 Contribution of genomics and phenotypic plasticity in environmental acclimation of fish

Evidence suggests that phenotypic innovation during evolution was mainly supported by successive round of whole-genome duplications (WGDs) that provided new gene material that evolved distinct physiological and biochemical functions (Braasch et al., 2009; Braasch et al., 2010; Dehal and Boore, 2005; Douard et al., 2008; Sato et al., 2009). In fish, two rounds of WGD that occurred in the common ancestor of vertebrates and additionally, a third teleost-specific WGD event (TGD) occurred in the class of the Actinopterygii about 350 million years ago. This TGD is thought to have provided raw materials for the physiological, morphological, and behavioral diversification of teleost fish, likely facilitating their adaptation into diverse aquatic environments across the planet (Braasch et al., 2010; Douard et al., 2008; Opazo et al., 2012; Sato et al., 2009). Indeed, TGD events may create distinct protein isoforms with different functional properties and/or differentially regulated by environment (phenotypic plasticity), thus providing a means to fine tune the acclimation response.

Phenotypic plasticity is defined as the process through which a genotype gives rise to different phenotypes under different conditions (Garland and Kelly, 2006). It includes phenology (changes in the timing of events), morphology (e.g., color patterns, body shape, and size), physiology (regulation of physiological functions), or behavior. Phenotypic plasticity interacts with genetic factors to provide a range of dynamic physiological processes bringing the phenotype closer to a functional optimum in a given environment.

2.2 Dependence of fish energy homeostasis on oxygen availability and temperature

The approximate constancy of the internal environment is known as “homeostasis” (Cannon, 1932) and is central to sustain essential processes such as an appropriate amount of energy for cell’s function in all living organisms. Adenosine triphosphate (ATP) is the “energy currency” of the cell (Khakh and Burnstock, 2010); hydrolysis of its terminal phosphate group releases free energy that can be coupled to numerous exergonic reactions. In fish as in other vertebrates, ATP is synthesized by two types of cellular respiration. Aerobic respiration is based on the utilization of O₂ as terminal electron acceptor producing cellular ATP via oxidative phosphorylation in mitochondria (Maina, 2002; Spees et al., 2006). It is more efficient than anaerobic respiration processes, glycolysis and substrate-level phosphorylation, which does not require O₂ to produce ATP (Livingstone, 1991; Muller et al., 2012). To maintain energy homeostasis, organisms including fish require a coordinated regulation of energetical substrate intake (nutrient intake), O₂ supply (extraction and transport) and energy expenditure (van de Pol et al., 2017). Thus, to ensure maximum fitness of an organism in its environment, ATP supply via aerobic and anaerobic metabolism must be sufficient to cover the maintenance costs as well as activity of the main physiological functions. This involves trade-offs in energy allocation between the different physiological processes/functions (whereby maintenance costs take priority) (Sokolova and Lannig, 2008).

Despite the idyllic picture of energy homeostasis, successful survival in the real world actually involves dealing with environmental stresses that disrupt the energy balance of an organism. In condition of hypoxia, the fish has to face a decrease in O₂ availability which can potentially limit production of energy and then its metabolic rates (metabolic rate defined as the turnover of ATP) (Richards et al., 2009). In contrary, because fish are ectotherm their metabolic rate increases exponentially with rising temperature as a result of thermodynamic effects on biochemical reactions, with an associated increase in O₂ demand and energy (nutrient) intake (Schulte, 2015). Therefore, combination of low O₂ and high temperature is exceptionally challenging since warming temperature increased metabolic demand for O₂ when hypoxia limits the availability of environmental O₂ (McBryan et al., 2013). It is admitted that the effects of hypoxia on the fish worsens as the

temperature increases (McBryan et al., 2016). Thus, hypoxia that has no or moderate effects at low temperature may be lethal at higher temperatures (Schurmann and Steffensen, 1992). In the same way, the balance between the supply and the demand of O₂ is also suggested to determine the ability of fish to cope with changes in temperature (concept called OCLTT for Oxygen and Capacity limitation of Thermal Tolerance; Portner, 2001).

To guarantee a homeostatic supply of energy under environmental stressors such as hypoxia, multiple compensatory mechanisms have evolved in fish. In general, **fish respond to hypoxic constraint by adjusting physiological, behavioral, biochemical and molecular responses allowing (i) to enhance O₂ uptake from water and O₂ transport to the tissues (Mandic et al., 2009; Wells, 2009), and/or (ii) to limit energy consumption (metabolic depression) (Wang et al., 2009; Wu, 2009; Lewis et al., 2007) and shift aerobic to anaerobic metabolism (Gracey et al., 2001; Richards, 2009; Martinez et al., 2006).**

2.3 Regulation of oxygen uptake and transport under hypoxic conditions

Acquisition of molecular O₂ from the external fluid media (water and air) and the discharge of CO₂ into the same milieu is the primary role of respiration (Dejours, 1981; Graham, 1990; Schmidt-Nielsen, 1997). Although the morphologies of gas exchangers differ greatly among organisms, certain shared structural and functional features exist. For example, in all cases, the transfer of O₂ occurs through a cascade of tissue barriers and compartments by diffusion down a partial pressure gradient, which drops to about zero at the mitochondrial level (Dejours, 1981). In the multicellular organisms, species have developed structures to extract O₂ from their environment as lungs in mammals (van Tuyl and Post, 2000), gills in most of the fish (Mandic, 2009) and tracheae in insects (Franz-Guess and Starck, 2016) and to provide O₂ to all body tissues by O₂-transport proteins such as hemoglobins (Hb), hemerythrins and hemocyanins (Mairbäurl and Weber, 2012; Terwilliger, 1998). Under hypoxic constraint, both O₂ uptake and transport have been shown to be regulated. Even if several marine fish species exhibit bimodal respiration (O₂ uptake from water and air) (Graham, 1994; Lefevre et al., 2011), we focus our attention in the present work on water breathers.

2.3.1 Oxygen uptake: generalities and regulation under hypoxic conditions

Because oxygen has considerably low solubility and diffusivity in water, fish have evolved a branchial system to extract efficiently dissolved O₂ from water using “countercurrent oxygen exchange” (Figure 3; Evans et al., 2005). It includes gills which are covered by an opercular flap. Operculum provides the main support for the protection of the gill and is vital for O₂ extraction. Operculum opens as the mouth closes, causing the pressure inside the fish to drop. Water then flows

from the buccal cavity towards the lower pressure inside the opercular cavity and later across the fish's gill. Gills are conforming by gill filaments with lamellae that provide a large surface for gas exchange. As water flows over the gill filaments, blood inside the capillaries picks up the dissolved O_2 . The blood in the capillaries flows in a direction opposite to the flow of water around the gill filaments, i.e. countercurrent O_2 exchange, allowing more O_2 to be picked up from the water (Figure 3). This flow pattern ensures that as the blood progresses through the gills and gains O_2 from the water, it encounters increasingly fresh water with a higher O_2 concentration that is able to continuously deliver O_2 into the blood. Exchange of O_2 from water to blood is dependent upon diffusion. Then, O_2 binds to the respiratory pigment, Hb, encapsulated in the red blood cells and is transported to the tissues by cardiovascular convection where it diffuses into all the body cells (Pittman, 2011).

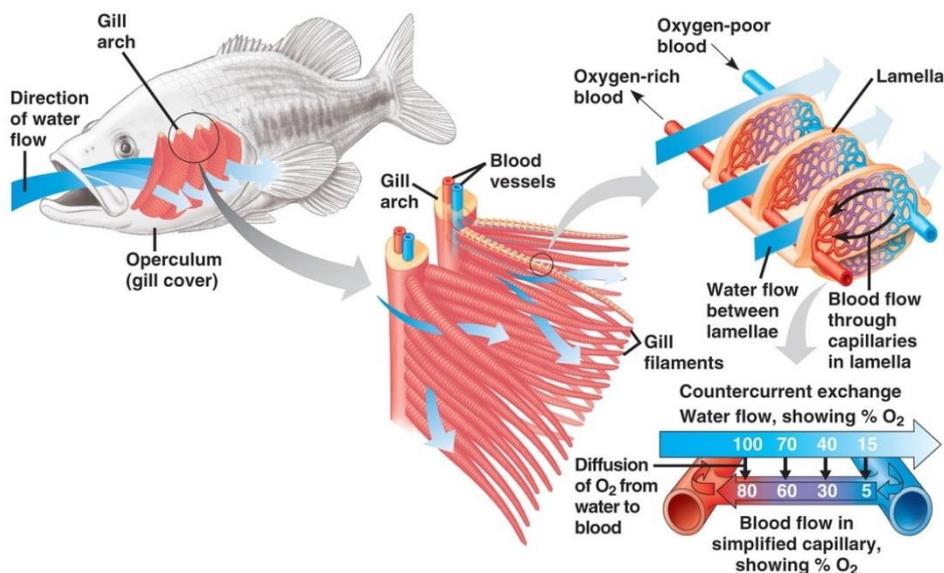


Figure 3. A simplified schematic of the oxygen extraction in fish. Fish extract dissolved oxygen molecules from the water through the gills. Gill filaments have lamellae which increase the surface area available for diffusion. As water passes over the gill filaments, blood inside the capillaries picks up the dissolved oxygen follow the countercurrent principle (image source: Pearson Education).

The maintenance of O_2 delivery from the hypoxic environment over the gills may be achieved by ventilatory response. The ventilatory response is probably the first physiological response of fish to hypoxia. Even if this response depends on species and on the duration and intensity of the constraint, it is generally admitted that fish exhibit increasing ventilatory frequency and/or amplitude

in low O₂ environment (Perry et al., 2009; Steffensen et al., 1982) (Figure 4). Such responses occur once O₂ chemoreceptors located in the neuroepithelial cells of the gill filament detect lower O₂ concentration (Perry et al., 2009). The increase in ventilatory rates under hypoxic condition requires energy and therefore cannot be maintained as hypoxia is prolonged. Other responses may be then initiated to enhance O₂ uptake, particularly by increasing gills surface area (Nilsson, 2007; Sollid and Nilsson, 2006). Indeed, the surface area available for O₂ uptake from gills filament can be enlarged by recruitment of additional gill lamellae (Chapman and Hulen, 2001) or by reducing the inter-lamellar cell mass to increase the functional surface area of lamellae (Figure 5 A and B; Matey et al., 2008; Nilsson, 2007; Sollid, 2003).

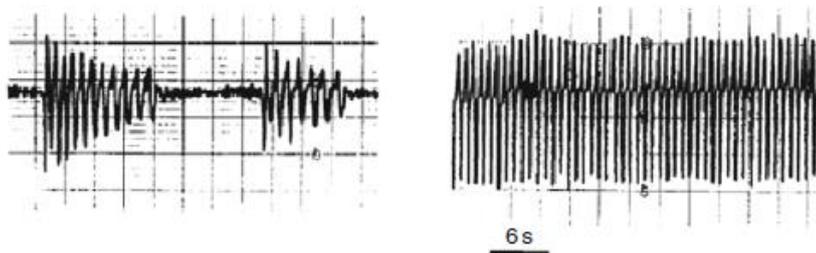


Figure 4. Ventilation under normoxic and hypoxic conditions in the sturgeon (*Acipenser baeri*). Ventilation is episodic under normoxic conditions (left-hand panel) but becomes continuous upon exposure to hypoxic water (right-hand panel; PO₂ = 60 Torr). Note the increase in ventilation amplitude that occurs in addition to the increases in frequency (from Nonnotte et al., 1993).

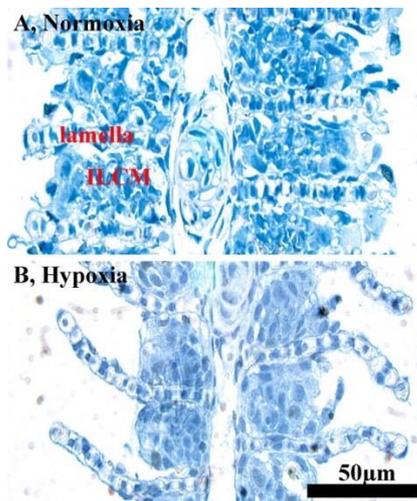


Figure 5 (A,B) Light micrographs of gills from crucian carp kept in normoxic (A) or hypoxic (B) water at 8°C. Note that the lamellae are present in both conditions but that a regression of the interlamellar cell mass (ILCM) during hypoxia makes the lamellae protrude, thereby greatly increasing the respiratory surface area. A filament arteriole (with blood clots) is seen running vertically in the center of each micrograph. Scale bar, 50 μm (from Sollid et al., 2003).

2.3.2 Oxygen transport: generalities and regulation under hypoxic conditions

In vertebrates, including fish, Hb is a tetrameric molecule, composed of two α -like and two β -like polypeptide chains, each containing a porphyrin ring with an iron (Fe^{2+})-containing haeme center, which is the site of O_2 binding (Fago et al., 1997; Royer et al., 2005; Weber and Fago, 2004). In fish, the head (pronephros), trunk (mesonephros) kidney and spleen are the primary blood formation (erythropoietic) organs even if specificity exists in terms of erythropoietic activity (Esteban et al., 2000; Lu et al., 2011; Meseguer, 1990; Rombout et al., 2005; Willett et al., 1999; Witeska, 2013). Spleen and kidney may also act as blood reservoirs, supplying erythrocytes into blood circulation in case of increased O_2 demand (Lai et al., 2006; Rombout et al., 2005; Witeska, 2013). Teleost fish species have the characteristic of expressing several $\text{Hb}\alpha$ and $\text{Hb}\beta$ genes which are grouped into two unlinked clusters labeled “MN” (gene cluster flanked by the mpg and nprl3 genes) and “LA” (cluster flanked by the lcmt1 and aqp8 genes) located on two separate chromosomes resulting from the TGD (Figure 6) (Hardison, 2008; Opazo et al., 2012). These gene products lead to diversified Hb isoforms likely associated to different functional properties. This diversity is suggested to allow teleost to cope with a variable O_2 demand and/or availability throughout the different ontogenetic stages and environmental conditions (Opazo et al., 2012; Weber and Jensen, 1988).

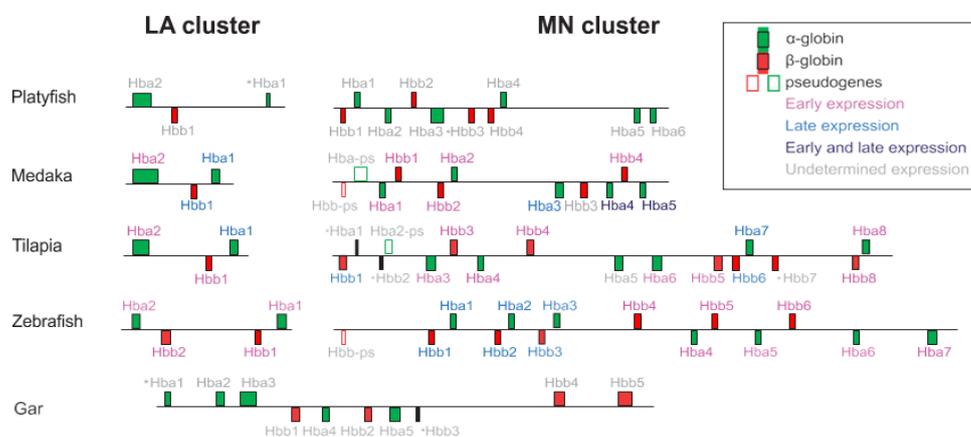


Figure 6. Genomic structure of the MN and LA globin gene clusters of some teleost fish. Genes in the forward orientation are shown on top of the chromosome, whereas genes in the reverse orientation are shown below. Gene labels are color coded based on the timing of their expression (adapted from Opazo et al. 2012).

Hemoglobin proteins exist in equilibrium between two alternative structures the T (“tense” deoxygenated) state and the R (“relaxed” oxygenated) state (Hundahl et al., 2003; Tsuneshige et al., 2002; Weber and Fago, 2004). The O₂ binding properties of Hb are depicted by the O₂ equilibrium curve (OEC), which describes the relationship between partial pressure of O₂ (PO₂) and the degree of Hb saturation with O₂ (Figure 7) (Wells, 2009). The OEC shape is determined by the degree of cooperative binding between Hb subunits, which is indicated by the Hill coefficient (nH). The position of the OEC relative to PO₂ is often used to determine Hb-O₂ affinity, characterized by one point, the PO₂ at which 50% of Hb is oxygenated (P50). A low P50 indicates a higher Hb-O₂ affinity, which favours O₂ loading in the gills. A high P50 indicates a lower Hb-O₂ affinity, which favours unloading of O₂ in the tissue. P50 is known to be influenced by a number of physiological parameters including temperature, pH and phosphate compounds within red blood cells. For example, decreasing pH can affect O₂ affinity in fish in two ways. The first is the Bohr effect where protons bind to Hb and induce a conformational change that reduces the O₂ affinity of Hb, shifting the OEC to the right (Bohr et al., 1904). The Bohr effect facilitates O₂ unloading at the tissues, where pH is lowered due to accumulated lactic acid from anaerobic metabolism and dissolved carbon dioxide from respiration (Figure 7) (Wells, 2009). Furthermore, the Bohr effect also allows O₂ loading at the gills where CO₂ excretion increases pH (Souza and Bonilla-Rodriguez, 2007).

The pH also affects O₂ affinity via the Root effect, which is only observed in fishes (Brittain, 1987), where low pH reduce the maximum carrying capacity of Hb for O₂ (Figure 7) (Fago et al. 1993, 1997). This allows for rapid O₂ unloading to specialized tissues such as the swim bladder and in the choroid rete of the eye, which facilitates buoyancy and vision, respectively (Pelster and Randall, 1998; Waser and Heisler, 2005). The presence of organic phosphates (i.e. adenosine triphosphate (ATP) and guanosine triphosphate (GTP)) may also influence haemoglobin-O₂ affinity. These phosphates can bind to specific sites within the Hb tetramers and serve to stabilize their structure in the low affinity conformation (Jensen, 2004). A decrease in the concentration of ATP and GTP therefore results in increased haemoglobin-O₂ affinity (Val, 2000).

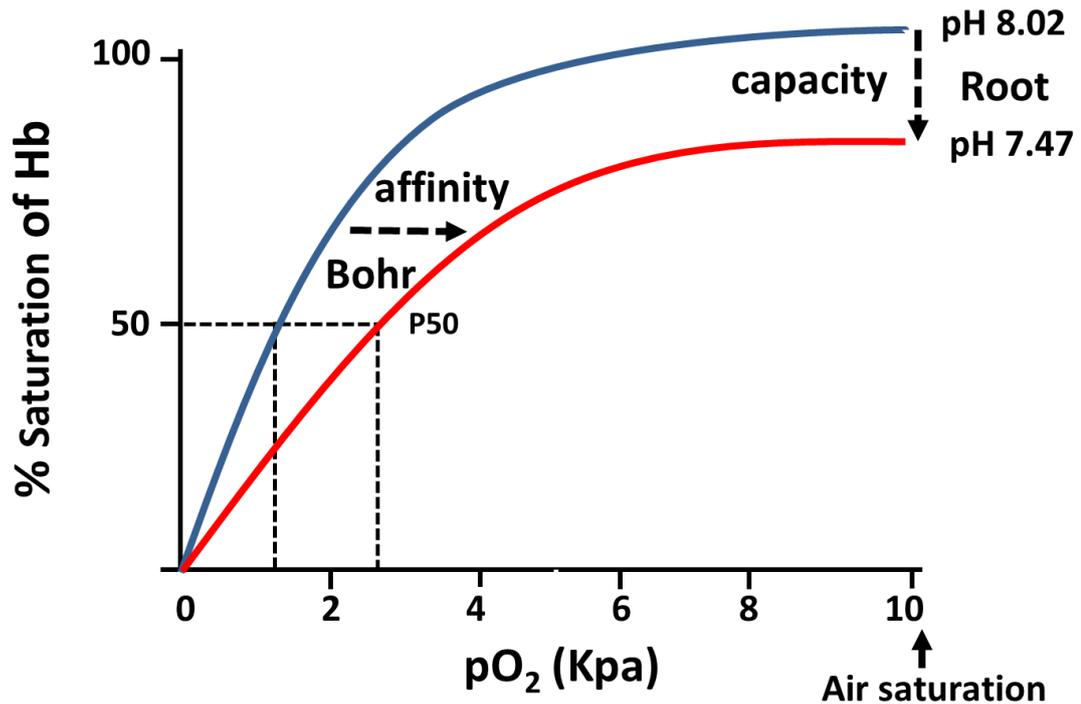


Figure 7. A theoretical oxygen equilibrium curve (OEC) depicting the relationship between hemoglobin-oxygen saturation (%Hb-O₂) and blood partial pressure for O₂ (PO₂; Kpa). A reduced pH results in a Bohr effect (low affinity) rightward shift and Root effect (low carry-capacity) rightward and downward shift. Thin black drop lines depict P50 values, the PO₂ at which 50% of Hb is saturated with O₂, for each of the two OECs.

Under hypoxic conditions, changes occur at the level of the erythrocyte to increase blood O₂ carrying capacity and Hb-O₂ affinity (Wells, 2009). Hypoxia exposure triggers a stress response in fishes, which releases catecholamines into the circulation (Randall and Perry, 1992; Perry and Gilmour, 1996; Wells, 2009). Teleost fishes are unique in having beta-adrenergic receptors on the erythrocyte membrane (Nikinmaa et al., 2001). These receptors bind to epinephrine and norepinephrine, which ultimately raises erythrocyte intracellular pH, thereby increasing Hb-O₂ (Nikinmaa, 1983). Catecholamines binding to the erythrocyte membrane also provoke erythrocytic swelling (Holk and Lykkeboe, 1995; Nikinmaa 2001). The erythrocytic swelling decreases the organic phosphates concentrations (mainly ATP and GTP) due to the dilution of erythrocyte contents, rising blood O₂ affinity (Nikinmaa 2001; Val, 2000). In response to chronic hypoxia exposure, an increase in haematocrit has been observed in rainbow trout (*Oncorhynchus mykiss*) as the result of hormonal stimulation of the kidney by erythropoietin (Lai et al., 2006). A rise in concentration of red blood cells enhances the O₂ carrying capacity of the blood, but due to the resulting increase in blood viscosity, it comes at the cost of more energetically expensive cardiac pumping (Wells, 2009). Accordingly,

chronic hypoxic exposure do not produce any increase in haematocrit in either turbot (*Scophthalmus maximus*) or European seabass (*Dicentrarchus labrax*) indicating that these species employ more energetically favourable strategies for enhancing blood O₂ carrying capacity (e.g. by increasing Hb-O₂ affinity; Pichavant et al., 2003).

Hypoxia is known to regulate in fish the amount of Hb proteins and/or Hb transcripts levels produced by erythropoietic tissues. However, any common pattern emerged from the different data obtained in different studies (table 2). Therefore, while there is no doubt that Hb plays an essential role in overcoming the effects of reduced O₂ availability in teleost fish, their response depend on the fish species, the degree of stress and ontogenetic stage considered. This may reveal the different strategies of fish for coping with O₂ deprivation.

	<i>Fish species</i>	<i>Stage</i>	<i>Hypoxia duration</i>	<i>Hypoxia intensity</i>	<i>Protein/mRNA level quantity</i>	<i>Reference</i>
Hb protein	<i>Poecilia latipinna</i>	Adult	6 weeks	PO ₂ = ~2.7 kPa	Increase	Timmerman and Chapman 2004
	<i>Carassius auratus</i>	Adult	- 48 h - 8 h	- PO ₂ = ~6.7 kPa - PO ₂ = ~1.7 kPa	Unchanged	Roesner et al. 2008
	<i>Oncorhynchus mykiss</i>	Larval	47 days	30% air saturation	Decrease	Bianchini and Wright, 2013
Hb transcript	<i>Danio rerio</i>	Embryonic	24h	5% air saturation	Decrease	Ton et al. 2003
	<i>Danio rerio</i>	Adult	48h	- PO ₂ = ~8.6 kPa) - PO ₂ = ~4.1 kPa	Decrease	Roesner et al. 2006
	<i>Carassius auratus</i>	Adult	- 48 h - 8 h	- PO ₂ = ~6.7 kPa - PO ₂ = ~1.7 kPa	Unchanged	Roesner et al. 2008
	<i>Poecilia latipinna</i>	Adult	6 weeks	PO ₂ = ~2.7 kPa	Increase	Timmerman and Chapman 2004
	<i>Oryzias latipes</i>	Adult	- 24 h - 48h - 2h	- PO ₂ = 2 kPa - PO ₂ = 4 kPa - PO ₂ = 0.5 kPa	Increase	Wawrowski et al. 2011

Table 2. Hb protein and transcript levels in response to hypoxia in teleost fish species.

2.4 Regulation of energy metabolism under hypoxic conditions

Metabolism is usually referred to as the chemical processes that occur within a living organism, enabling it to grow and reproduce, maintain and regenerate biological structures, and interact with its environment (Seebacher, 2009; McCarthy, 2001; Wikelski et al., 2003). In O₂-limited hypoxic environments, duration of survival requires a well-coordinated response to defend against the metabolic consequences of low O₂ supply to the mitochondria, which limits aerobic ATP production. Under these O₂ limiting conditions fish may adjust its metabolism by (1) reducing metabolic demands (metabolic depression); and (2) switching from aerobic to anaerobic metabolism. By combining these regulations animal may maintain energy balance under hypoxic exposure within a tolerance range (Gracey et al., 2001; Lewis et al., 2007; Martínez et al., 2006; Richards et al., 2009; Wang et al., 2009; Wu, 2009).

2.4.1 Hypoxia-induced metabolic rate depression

A potential strategy to prolong survival in hypoxia is the depression of metabolic rate which reduces the ATP demand of cells (Storey and Storey, 1990; Jibb et al., 2008). Metabolic rate is depressed via a variety of behavioral, physiological and biochemical rearrangements. Behaviourally, metabolic rate can be reduced through reductions in locomotor activity, feeding, mating and courtship behavior. Reductions in these behaviors are typical of hypoxia-exposed fishes (McKenzie et al., 1995; Nilsson et al., 1993; Wang et al., 2009; Wu, 2009). For example, Nilsson et al. (1993) observed that the crucian carp (*Carassius carassius*), an anoxia-tolerant species, responded to anoxia by rapidly decreasing their locomotor activity. This was estimated to translate into a 35-40% reduction in muscle energy consumption. In addition, reducing skeletal energy demands enables the organisms to shift metabolic resources from muscle to other organs that are more crucial to survival (Nilsson et al. 1993). Physiologically, metabolic rate can be decreased through reductions in growth, digestion, specific dynamic action, gonad development and gametogenesis (Fitzgibbon et al., 2007; Wang et al., 2009). Biochemically, metabolic rate can be further depressed through reductions in the major cellular ATP-consuming pathways, including protein turnover and the maintenance of membrane ion gradients. The suppression of protein synthesis has been described in both isolated hepatocytes and fish *in vivo* in species ranging from the crucian carp (Smith et al., 1996), goldfish (*Carassius auratus*) (Jibb and Richards, 2008), to the Amazonian oscar (*Astronotus ocellatus*; Lewis et al., 2007). The contributions that these processes make to the total cellular energy demand and the degree to which they are down-regulated likely vary across tissues and species (Bickler and Buck, 2007; Hylland et al., 1997; Vornanen et al., 2009).

2.4.2 Aerobic and anaerobic metabolism: generalities and regulation under hypoxic conditions

In vertebrates, including fish, the Krebs (or tricarboxylic acid) cycle, in conjunction with oxidative phosphorylation, provides the majority of energy used by aerobic catabolism when the O₂ supply is sufficient (Akram, 2014; Blomstrand et al. 1997; Karasov, 1986). Firstly, fish mainly used organic fuels such as lipids and proteins to produce acetyl coenzyme A (acetyl CoA). Secondly, the acetyl groups enter the Krebs cycle which degrades them to yield energy-rich hydrogen atoms and to release CO₂, the final oxidation product of organic fuels (Campbell and Reece, 2005). The hydrogen atoms generated by the Krebs cycle are transferred via NADH and FADH₂ through a series of membrane proteins (referred to as the electron-transport chain) to generate a proton gradient across the membrane. The resulting proton gradient is used to phosphorylate ADP and create ATP that is referred to as oxidative phosphorylation. O₂ is required for the Krebs cycle as the electron acceptor at the end of the electron-transport chain, necessary to regenerate NAD⁺ and FAD (Campbell and Reece, 2005).

When dissolved O₂ decreases, the mitochondria are limited in their capacity to generate ATP due to the lack of O₂ available to act as the terminal electron acceptor in the electron transport chain. Under this threshold, vertebrates including fish can activate O₂-independent pathways of metabolism, such as anaerobic glycolysis, and deactivate aerobic pathways, such as lipid oxidation, the TCA cycle, and the electron transport chain (Richards, 2009). Fishes can use two pathways to generate ATP anaerobically: substrate-level phosphorylation via creatine phosphate (CrP) and anaerobic glycolysis. Substrate-level phosphorylation occurs in the cytosol of certain tissues (e.g., muscle) and involves the rapid, direct transfer of phosphate from CrP to ADP. Because the cell's CrP reserves are small, CrP can be quickly depleted, forcing it to rely on glycolysis for the anaerobic supply of ATP (Wang and Richards, 2011). Glycolysis is the pathway by which glucose is converted into pyruvate, producing ATP by substrate-level phosphorylation (Figure 8). Once free glucose is used, glucose requirements are satisfied by glycogen depletion to glucose (glycogenolysis) (Figure 8).

Tissue-specific response to hypoxia exposure has been mentioned in several studies. In Gulf killifish (*Fundulus grandis*) submitted to prolonged hypoxia, glycolysis and glycogen metabolism were strongly suppressed in skeletal muscle, while in the liver glycolysis and carbohydrate oxidation were activated (Martínez et al., 2006). Metabolic regulation induced by hypoxia in the liver may be associated to the mobilization, transport and utilization of substrates (primarily glycogen), since hepatic tissue contains the largest store of glycogen used as a fuel for anaerobic glycolysis (Richards, 2009). Because hypoxia exposure suppresses the appetite, feed intake and digestive functions of

fishes (Wang et al., 2009), species with larger glycogen stores (e.g., goldfish, carp, tilapia) can fuel glycolysis for longer time periods than species with smaller glycogen stores (e.g., rainbow trout), and this prolongs their hypoxic/anoxic survival time (Richards, 2009). Liver glycogen can also be used by other tissues to support glycolytic ATP production during hypoxia exposure; however, it requires that the glucose liberated from liver glycogen must be transported through tissues. Glucose can also be supplied by *de novo* glucose synthesis through gluconeogenesis (from lactate, glycerol and some amino acids). Gluconeogenesis has been shown to be additionally activated during hypoxia in the liver of some hypoxia-tolerant species, such as burrow-dwelling goby (*Gillichthys mirabilis*) and *F. grandis* (Gracey et al., 2001; Gracey et al., 2011; Martínez et al., 2006). In particular, Gracey et al. 2011, showed in *G. mirabilis* exposed to hypoxia, that glucose can be generated from glycerol derived from triglycerides (Gracey et al., 2011).

Even if the use of anaerobic metabolism in hypoxia may help organisms to make up aerobic energy shortfalls, the efficiency of ATP production by glycolysis is lower than by aerobic metabolism (Vazquez et al., 2010). Furthermore, anaerobic glycolysis can only be supported during temporal sets due to the exhaustion of endogenous carbohydrates and metabolic acidosis resulting from the accumulation of lactate, the end product of glycolytic reactions (Kieffer, 2000; Bickler and Buck, 2007). Metabolic acidosis can cause detrimental effects, particularly affecting the respiratory and cardiovascular systems (Kojima et al., 2004). Some tolerant fish species as goldfish and carp have the ability to counteract the effects of metabolic acidosis converting lactate to ethanol, which is eliminated across the gills (Bickler and Buck, 2007).

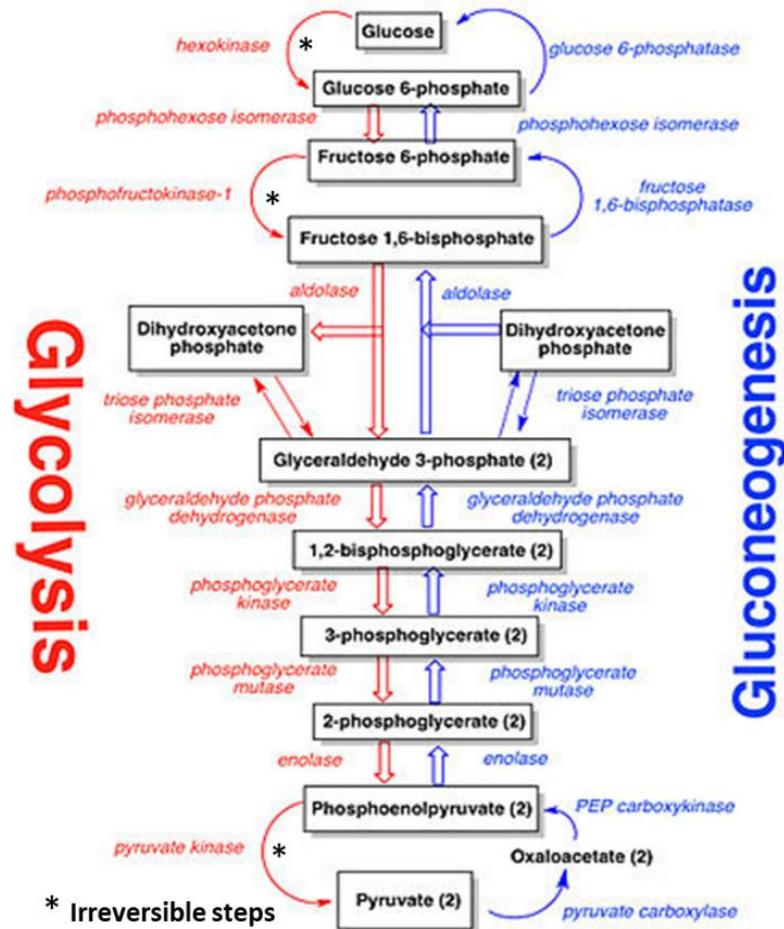


Figure 8. Representation of the pathways of glucose phosphorylation (anaerobic glycolysis) and glucose production (gluconeogenesis). Irreversible steps were marked with an asterisk.

2.5 Molecular pathways in cellular response to hypoxia

Part of the organismal response (plasticity) to O_2 limiting conditions depends on changes in the levels or types of proteins produced, which ultimately dictates cell and tissue function. In vertebrates, including fish, changes in gene expression are critical to hypoxia tolerance and hypoxic survival. Regulation of the genes that are induced by hypoxia is mediated mainly by hypoxia-inducible factor 1- (HIF-1) pathway (Nikinmaa and Rees, 2005; Wu, 2002; Soitamo et al. 2001). HIF-1 is a heterodimeric transcription factor that is highly conserved from mammals (Kaelin and Ratcliffe, 2008; Lendahl et al, 2009; Semenza, 1999), fishes (Nikinmaa and Rees, 2005; Richards, 2009) to invertebrates (Gorr et al, 2006; Zhang et al., 2009). In higher metazoans, HIF-1 consists in three α -subunits (HIF-1 α , HIF-2 α or HIF-3 α) and the β -subunit (HIF-1 β) (Kaelin and Ratcliffe, 2008). HIF-1 β is a constitutive nuclear protein, which also participates in the cellular response to environmental toxins such as aryl hydrocarbons, whereas HIF- α is specific to the response to hypoxia (Hoffman et al.,

1991). Under normoxic conditions, HIF α proteins are very unstable due to hydroxylation by a family of proline hydroxylases (PHD) (also termed EGL-Nine homolog (EGLN) family) (Figure 9) (Pescador et al., 2005; Schofield and Ratcliffe, 2004). Three HIF prolyl hydroxylases (PHD1, PHD2, and PHD3) have been identified in vertebrates (Myllyharju, 2009). Although initial analysis has established that each of them have the ability to hydroxylate HIF- α polypeptides (Lieb et al., 2002; Cioffi et al., 2003), the precise function of each PHD isoform is still under debated. Since PHDs require O₂ to catalyze HIF hydroxylation, this reaction does not efficiently occur under low O₂ tension. Therefore, under hypoxia, HIF α is less degraded and can regulate target gene transcription by binding to specific hypoxia-responsive elements (HREs) in their promoter regions (Figure 9) (Fedele et al, 2002; Kaluz et al., 2008). In mammals, several HIF target genes have been characterized, including genes involved in red blood cell production, vascularization, apoptosis, and iron, catecholamine, and carbohydrate metabolism (Bracken et al., 2003; Semenza and Wang, 1992; Semenza, 2000; Wenger, 2000). However, the target genes important for survival in hypoxia in teleost fish are less documented. Kajimura et al. (2006) identified insulin-like growth factor binding protein-1, a gene involve in growth and development arrest, as a HIF-1 target in zebrafish.

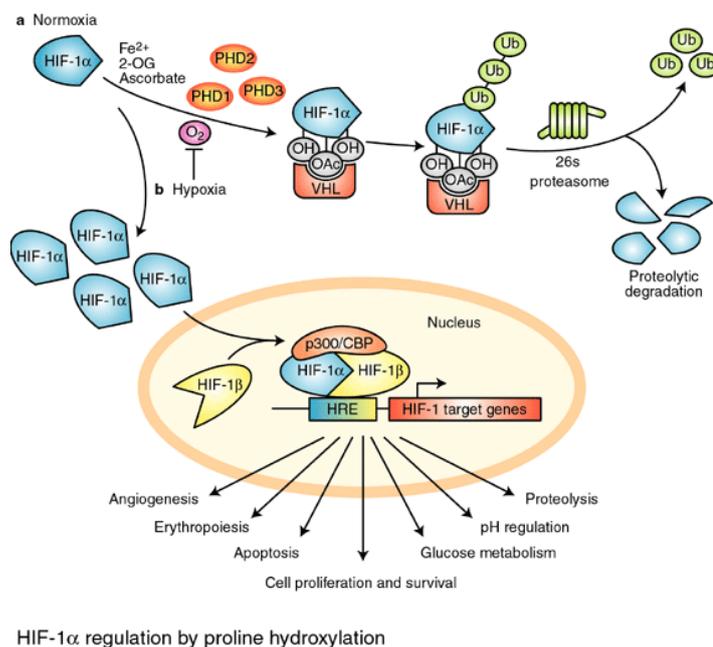


Figure 9. HIF-1 α regulation by proline hydroxylation. (a) In normoxia, hypoxia-inducible factor (HIF)-1 α is hydroxylated by proline hydroxylases (PHD1, 2 and 3) in the presence of O₂, Fe²⁺, 2-oxoglutarate (2-OG) and ascorbate. (b) In response to hypoxia, proline hydroxylation is inhibited, which leads to HIF-1 α accumulation and translocation to the nucleus. There, HIF-1 α dimerises with HIF-1 β , binds to hypoxia-response elements (HREs) within the promoters of target genes (image source: Carroll and Ashcroft (2005), *Expert Reviews in Molecular Medicine*).

Hypoxia-induced regulation of gene expression in fish has been confirmed by numerous studies using dedicated (PCR) or transcriptomic approach (e.g. microarray) (Ton et al., 2003; Gracey et al. 2001; van der Meer et al. 2005). These studies revealed that metabolic switch from aerobic to anaerobic may be regulated at molecular level. For instance, in zebrafish embryos, Ton et al. (2003) showed a decrease in the expression of mRNA coding for genes involved in the Krebs's cycle (i.e. succinate dehydrogenase, malate dehydrogenase, and citrate synthase) and an increase in expression of genes involved in glycolysis (i.e. phosphoglycerate mutase, phosphoglycerate kinase, enolase, aldolase). Similarly, in gills of zebrafish exposed to hypoxia, the levels of mRNA for proteins involved in the Krebs cycle and electron transfer chain were all decreased signifying an overall decline in mitochondrial ATP production (van der Meer et al., 2005). Simultaneously, increases in mRNA coding for proteins involved in glycolytic ATP production were noted in the gill during hypoxia exposure, including increases in glycogen phosphorylase and aldolase (van der Meer et al., 2005).

2.6 Variability of acclimation of fish against hypoxic constraint

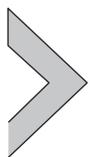
The physiological and biochemical response of an organism to hypoxia does not depend exclusively from O₂ levels since environmental hypoxia is likely to interact in a variety of ways with other stressors (temperature, pollution, acidification....). As mentioned previously for temperature, co-exposure to hypoxia and other environmental stressor may represent a particularly challenging physiological constraint for fish and thus it can condition its response. Response (capacity to acclimate or not) of fish to low dissolved O₂ also depends on the duration and the intensity of hypoxia event.

The effects of low O₂ concentration vary widely depending on fish species. Species like rainbow trout and tuna, that extensively rely on aerobic metabolism for rapid and sustained swimming, are moderately to extremely sensitive to hypoxia (Gamperl et al. 2001; Bushnell et al. 1990), whereas carp, eels, and hagfish can perform well at low O₂ levels (Axelsson et al. 1990; Bailey et al. 1999; Faust et al. 2004). The crucian carp and common goldfish are considered among the most hypoxia tolerant freshwater fish species, which can endure months of hypoxia at low temperature (Nilsson and Renshaw, 2004; Lutz and Nilsson, 2004; Stecyk et. al, 2004). Hypoxia tolerance of both *Carassius* rely on large muscle and liver glycogen reserves, reduced metabolism, and avoidance of lactic acidosis by converting lactate to ethanol and CO₂ (Nilsson and Renshaw, 2004; Lutz and Nilsson, 2004; Stecyk et. al, 2004).

Species exhibit also pronounced intraspecific variations in hypoxia tolerance (Joyce et al., 2016; Roze et al., 2013) depending on different factors including genetic basis, social status, sex and life history. For example, Thomas and Gilmour (2012) showed that subordinate trouts (*Oncorhynchus*

mykiss) were less effective than dominant individuals in coping with acute hypoxia, experiencing higher ventilation but failing to defend blood O₂ content. There is also evidence that individual variations in hypoxia tolerance may depend on sex. Robertson et al. (2014) reported that male zebrafish (*Danio rerio*) have a higher hypoxia tolerance (i.e. lower Pcrit) than females. Previous exposure (preconditioning) to sublethal levels of low O₂ has also been shown to prime subsequent hypoxia tolerance of individuals to hypoxic constraint in a number of vertebrates species including the epaulette shark (Rytkönen et al. 2012). Rytkönen and collaborators discovered that hypoxia preconditioning amplifies transcriptional responses of shark subsequently exposed to hypoxic condition. In mammals, previous experience of low O₂ confers tissue protection in subsequent hypoxic situation with HIF pathway playing a central role in these protective responses (Eckle et al., 2008).

Although the ontogenetic period of the greatest sensitivity to environmental constraints, including hypoxia, varies among fish species, it is generally accepted that the early life stages are the most vulnerable (Hassell et al., 2008; Portner and Farrell, 2008; Shang and Wu, 2004). Yet, as previously mentioned in the introduction section (paragraph 1.2), fish at early life stages may be exposed to hypoxia in warming coastal waters. To date, **several studies have investigated the response of early life fish to hypoxic and temperature constraints** due to the fact that their sensitivity may have strong ecological consequences on juvenile recruitment with impacts on fish population structure and dynamics.



3. Sensitivity of early life stages of fish to hypoxic and temperature constraints

By contrast to juvenile and adult fishes that may escape stressors by moving to a different area, the limited swimming capacities of embryos and larvae force them to face environmental constraints (Balfour, 1999). Depending on the intensity and the duration of the constraint and on the developmental window at which it occurs, hypoxia and thermal events may disrupt energy metabolism and biological processes involved in organogenesis and morphogenesis of early life organisms. This could have short term impact on fish fitness. Moreover, since early life is a critical stage for developmental plasticity, O₂ and temperature conditions during this period may induce long term impact on fish phenotype.

3.1 Short term impact of early exposure to hypoxia exacerbated by warm temperature

Low dissolved O₂ likely represent one of the major stress for organisms in development since they have to sustain high O₂ requirements for energy-linked functions related to developmental processes as cell proliferation, differentiation, migration and apoptosis, while facing an overall decrease in ambient O₂ (Nilsson and Östlund-Nilsson 2008; Wu et al. 2009). Warming waters may exacerbate the hypoxic constraint since it increases metabolism and, therefore, the O₂ demand by fish (Pörtner 2002 and Barnes et al., 2011). To face such environmental constraint, embryos of zebrafish (Padilla and Roth, 2001) and annual killifish (*Austrofundulus limnaeus*) (Podrabsky et al., 2007) have the capacity to entrance in developmental arrest (i.e. diapause) that allows them to drastically reduce their demand of energy. However, to our knowledge this saving strategy concerns only few freshwater fish species. Energy saving strategy for marine fish species, which consists in reducing O₂ consumption, is more problematic. For instance, by decreasing their swimming activity, larvae are more vulnerable to predation (Breitburg et al., 1994; Shoji et al., 2005). It is difficult to combine cellular processes involved in development (e.g. proliferation, differentiation, migration, growth) that require high energy, with energy saving strategy. When a mismatch between the capacity to supply O₂ and the O₂ demand occurs, fish may mobilize anaerobic metabolism to provide additional energy (Pörtner and Lannig 2009). Nevertheless, the compensatory anaerobic capacity of embryos and larvae appears particularly limited (Ninness et al. 2006; Wieser, 1995). Part of the explanation for this is that smaller fish have poor glycogen stores that provide the fuel for anaerobic metabolism and that their capacity to tolerate accumulation of toxic anaerobic end products is also limited (Almeida-Val et al. 2000; Nilsson and Ostlund-Nilsson 2008).

When metabolic depression and energy produced by anaerobic metabolism does not allow organism to cover the needs of ontogenic processes, developmental disturbance may occur, resulting in malformations (ocular, spinal and craniofacial defects), lower fitness and high mortality rates at larval and juvenile stages, e.g., brown trout (*Salmo trutta*) (Massa et al., 1999; Roussel, 2007); black bream (*Acanthopagrus butcheri*) (Hassell et al. 2008); zebrafish (Padilla and Roth, 2001; Shang and Wu, 2004); and dogfish (*Scyliorhinus canicula*) (Diez and Davenport, 1990). Low tolerance to hypoxia has been observed over the period of metamorphosis in sea bream (*Pagrus major*) (Ishibashi et al., 2005) and Japanese flounder (*Paralichthys olivaceu*) (Ishibashi et al., 2007). It is supposed that this sensitivity is due to the increase in metabolic rates just before and just after the flexion stage, which is a period of dramatic molecular, physiological, and behavioural changes for marine fish species.

Depending on the duration and the intensity of the constraint, exposure of early life fish to hypoxia in warming waters may induce immediate or short term deleterious effects resulting in death or decreasing fitness. Such short term detrimental effects of early hypoxia exposure may reduce juvenile recruitment of fish population as it has been shown for black bream (*Acanthopagrus butcheri*) in south-eastern Australia (Nicholson et al., 2008). In addition to these short term effects, **early exposure to hypoxic and thermal constraints may produce carry-over effects in the subsequent life-history trajectory even when immediate effects on mortality are not visible.**

3.2 Long-term effects of an early-life hypoxia and thermal event resulting from developmental plasticity on fish

In addition to generate short term impact at embryonic and larval stages, early environmental conditions may induce developmental plasticity and result in profound consequences in their subsequent life-history trajectory. Developmental plasticity is a form of phenotypic plasticity where responsiveness to input occur during the period of the initial growth (Nettle and Bateson, 2015). Developmental plasticity may be often (Piersma and Drent, 2003) but not always (Champagne and Meaney, 2007; Fischer et al., 2015) irreversible, meaning that plastic adjustments experienced during key periods of development may result in permanent changes (Ho and Burggren, 2012; Naguib et al., 2006). Developmental plasticity has been shown to imply both beneficial and deleterious long-lasting effects on teleost fish species. Beneficial developmental plasticity may allow an individual to adapt its phenotype in response to environmental cues and to prepare it to cope better with similar environmental conditions in the future. This form of developmental plasticity is known as “the Predictive Adaptive Response” and it confers adaptive advantages for an organism (Bateson et al., 2014).

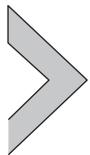
Information related to developmental plasticity following early exposure to hypoxia and temperature in fish is quite scarce. Adaptive developmental plasticity has been found in African cichlids (*Pseudocrenilabrus multicolor victoriae*) reared in hypoxia, since subsequent adults exhibit increased gill surface area and filament length after early preconditioning (Chapman et al, 2000). Barrionuevo and collaborators (2010) showed that adult zebrafish surviving moderated hypoxia exposure at early life stages exhibited higher tolerance to hypoxia due to a better aptitude to regulate aerobic and anaerobic capacities when exposed to acute low O₂ environment. Although little is known about the mechanisms underlying developmental plasticity in marine fish species, there is no reason to be different from genetic, physiological and epigenetic factors described in other organisms (Beldade et al., 2011). In recent studies, it has been determined that the HIF-1 response plays an important role in the developmental plasticity of embryos that are subjected to

hypoxia. Robertson et al. (2014) utilized critical developmental windows of zebrafish to link the induction of the HIF-1 cascade with increased proportion of adult males in the population which are more tolerant to hypoxia than females.

Elevated rearing temperature in embryos and larval fishes has also been showed to induce beneficial effects in subsequent life stages. Scott and Johnston (2012) have shown that swimming performance in adult zebrafish is improved when embryos are incubated at elevated temperatures. The higher swimming performance is explained by differences in the abundance and proportion of fiber types in the muscle induced by increased rearing temperature. In marine fish species, Zambonino-infante et al. (2013) showed that sole (*Solea solea*) juveniles that had experienced elevated temperatures during larval stage exhibited higher tolerance to acute hypoxia, likely through long-term programming of metabolic pathways.

Nevertheless, if the early environment is severe, or the signal is not transmitted faithfully, the organism will receive cues that are not representative of the actual environment, and thus, it will make incorrect predictions. The developmental plasticity therefore appears deleterious: the adult phenotype simply represents the cost of getting a bad start in life. Concerning these adverse effects, it has been shown that zebrafish developing larvae that survive an exposure to hypoxia can experience altered cardiovascular response (Bagatto, 2005) to future hypoxia, slower swim performance (Widmer et al., 2006) and avoidance reaction to aggression (Marks et al., 2005). Previous studies on marine fish species, the European sea bass, revealed that exposure to moderate hypoxia at the larval stage had a negative impact on subsequent juvenile growth rate (Vanderplancke et al., 2015b; Zambonino-Infante et al., 2017). This negative effect on growth was associated with a long-lasting down-regulation of genes involved in energy consuming metabolic pathways in the liver and in a specific change in the efficiency of protein digestive functions. More recently, data obtained in the Atlantic salmon (*Salmo salar*) showed that the capacity of hypoxia tolerance was reduced when fish were exposed to early-life hypoxic event at larval stage (Wood et al., 2017). What is less documented, however, is the detrimental carry-over effects of temperature on the phenotype of developing fish. An accelerating growth trajectory (growth compensation) resulted from atypical temperatures during early life stage was associated in sticklebacks to alteration of swimming activity, modification of sexual maturation and decline of life span (Lee et al., 2010; Lee et al., 2012). Therefore, exposure to hypoxic and thermic conditions during critical developmental windows in fish may induce lifelong morphological or physiological effects that could have an impact on fitness. Nevertheless, the information related to developmental plasticity associated to the interaction of these two stressors remains unknown in teleost fish.

While short and long term responses of early life fish to warm temperature and hypoxia used as single stressors have been investigated, the impact of these co-occurring environmental constraints are less known. Particularly, it is increasingly important to understand whether early ecologically relevant exposure to moderate constraints inducing no apparent short term detrimental effects may have long term positive or negative effect on the fitness of juveniles. This problematic could be particularly important for fish species endangered and those whose stocks are fallen last decades.



4. Biological model: European sea bass (*Dicentrarchus labrax*, Linnaeus, 1758)

Commonly known as European sea bass, *D. labrax* belongs to the class Osteichthyes, subclass Actinopterygii, order Perciformes and family Moronidae. It is an Atlantic species normally found from Norway to Morocco, Canary Islands and Senegal, as well as in all the Mediterranean coasts and in the Black Sea (Freyhof and Kottelat, 2008). The European sea bass inhabits coastal waters; however, it can also enter brackish waters in estuarine areas and coastal lagoons, and occasionally rivers. It has therefore been considered as an eurytherm and euryhalin fish capable to tolerate a wide range of temperatures (2 °C to 32 °C) (Hidalgo and Alliot, 1988) and salinities (0.5 ‰ to 40 ‰) (Eroldogan, 2004). Since 2005, seabass stocks are threatened by overfishing and pollution inducing an overall decline of their populations (FAO, 2015). Nevertheless, the impact of climate change in nurseries areas that can affect juvenile recruitment contributing to the decline of sea bass stock is still largely unknown.

The sea bass life cycle can be split into the following phases: eggs and larvae, juvenile, adolescent and adult (Figure 10) (Dando and Demir, 1985). Seabass eggs are pelagic and take between two and five days to hatch (Kennedy and Fitzmaurice, 1972). At approximately 5 days post hatching (dph) the still immobile larvae open their mouth. Upon the opening of the mouth all four pairs of gill arches have been formed enabling the gradual transition from a cutaneous to branchial respiration in sea bass larvae (Varsamos et al. 2002). At this stage (6 dph) the gall bladder, liver and pancreas are already differentiated enabling early sea bass larvae to ingest, digest and assimilate the first exogenous food (Kamaci et al. 2010). In European sea bass, larvae will reach the coast at approximately flexion stage around 22-25 (dph) (Dufour et al., 2009; Jennings and Pawson, 1992). Juveniles are gregarious, especially during the seasonal migrations and they can stay in estuaries and coastal habitats for at least the first three years of their lives (Jennings and Pawson, 1992). Adult sea

bass exhibit sexual growth dimorphism and they reach sexual maturity at four/five years in males and at six/seven years in females in the Atlantic Ocean (Kennedy and Fitzmaurice, 1972).

The European sea bass displays a wide range of characteristics to its use as a suitable marine fish model of the temperate latitudes. First, as an appreciated commercial species, the domestic rearing conditions of European sea bass are completely controlled, facilitating its maintenance in the laboratory. Also, an extensive bibliography exists on these species providing a general overview of their physiology. Previous studies in European sea bass have focused on various topics ranging from ecology (Handelsman et al. 2010; Pope et al., 2014; Bento et al. 2016), nutrition (Kousoulaki et al. 2015; Parpoura and Alexis, 2001) to genetics (Ciftci et al. 2002; Moreno et al., 2016). In addition, the recent published European sea bass genome sequence is a valuable source of information for transcriptional studies (Tine et al., 2014).

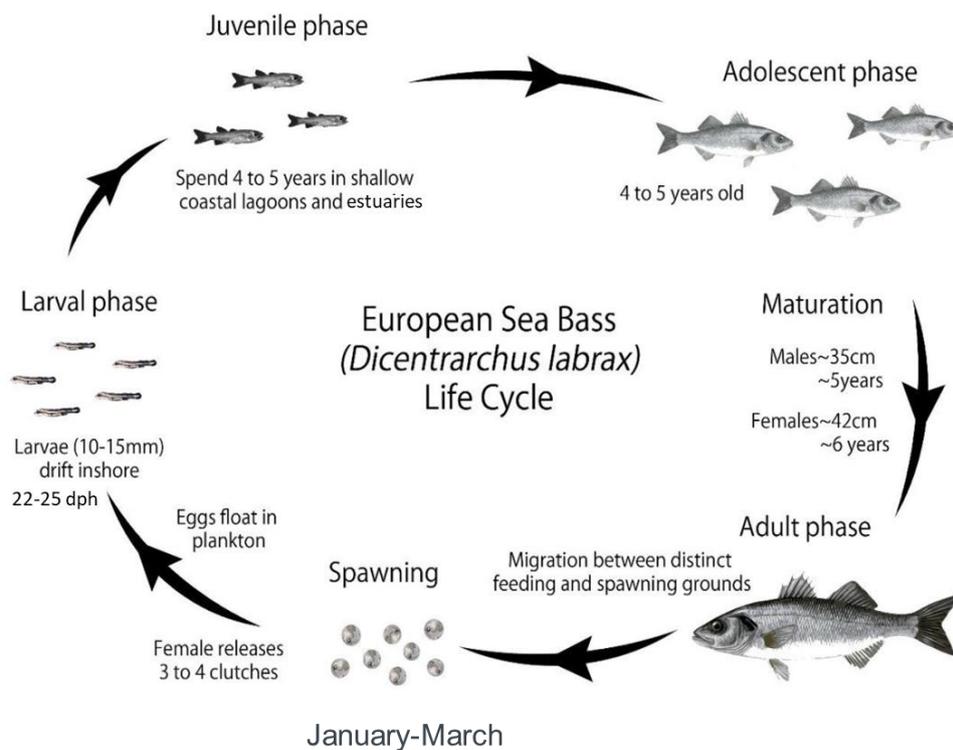


Figure 10. Life cycle of European sea bass (*Dicentrarchus labrax*) (from Dando and Demir 1985; Pawson et al. 1987; Pawson, et al. 2007).



5. Thesis goals and hypotheses

As previously mentioned, high temperature and low O₂ concentration (hypoxia) are critical co-occurring stressors that can have synergistic negative effects on organisms in coastal marine ecosystems. Such environmental changes can particularly affect marine fish larvae that are likely to be present in coastal nursery at the end of their development. It is now well established that the regulations implemented by organisms to cope with their environment during the early stages of life can imprint their physiological functions in the long-term and change their life trajectory. While the immediate effects of thermal and oxygenation conditions have been investigated in marine fish larvae (Vanderplancke et al. 2015a; Shang and Wu, 2004; Herbing et al. 2002; Landsman et al. 2011), the long term implications of early exposure to these constraints in an ecological context are poorly documented. In particular, it would be of great interest to determine whether early exposure to ecologically relevant combinations of hypoxia and temperature leads to fish individual phenotypes, able to better cope with these constraints at later life stages.

In this context, the main issues of the present thesis are:

1. Does early exposure to moderate environmental constraints (hypoxia at different temperatures) during the last stages of larval development impact physiological traits of the future adult?
2. Does it contribute to improve their future ability to cope with these specific environmental constraints?

In order to assess these questions, our experimental protocol was designed to mimic a realistic field condition using a combination of warm temperature and moderate hypoxia that European sea bass larvae may experience along the European West coast of Atlantic Ocean (Copernicus Marine environment monitoring service, <http://marine.copernicus.eu>). The experimental treatments were applied at the end of the larval phase of European sea bass, corresponding to the temporal window likely to be encountered in the coastal zones (Figure 11) (Dufour et al., 2009; Jennings and Pawson, 1992). The temperatures tested here, 15°C and 20°C, therefore fall within the likely seasonal thermal amplitude in coastal temperate sea water, from winter to summer. Moderate hypoxia levels (40% air saturation) were previously tested in our experimental structures to ensure that it did not cause any remarkable mortality (Vanderplancke et al., 2015a). Indeed, we were interested in the effect of phenotypic plasticity instead of selection on subsequent phenotypes of fish previously exposed to hypoxic and warming temperature.

In the present study, European sea bass (*Dicentrarchus labrax*) larvae were purchased from a commercial hatchery (Aquastream, Ploemeur, France). Their progenitors were selected (five generation of selection for productivity traits) from a wild population. To assess the first question of this thesis, one sexually immature population of European sea bass juveniles were submitted to hypoxic challenge tests (HCT) over one year (**Chapter 2**). HCT was used in order to evaluate the global physiological status of juveniles since the tolerance of fish to HCT gives an integrative evaluation of biological processes related to O₂ extraction and transport, cardiovascular functions and energy metabolism. The overall appearance of operculum, which contributes to water flow across the gills lamellae and allows O₂ extraction, was also examined.

In parallel, our second question was approached in other immature population of sea bass juveniles exposed to chronic moderate hypoxia (40% air saturation) at two different temperatures (15°C and 20°C) in order to determine whether hypoxic adaptability could be modulated by early life environment. Characterization of physiological traits related to O₂ transport (**Chapter 3**) and metabolism (**Chapter 4**) was performed.

The first part of **Chapter 3** reported on the first characterization of the European sea bass hemoglobin (Hb) genes, including genomic organization, phylogeny and spatio-temporal gene expression. Since Hbs are involved in O₂ transport, understanding changes in Hb gene expression could give new insights related to the tolerance to the lack of O₂. The second part of the chapter assessed the regulation by moderate hypoxia of Hb gene expression in whole larvae and in the hematopoietic tissues (head kidney and spleen) of juveniles. Above all, the hypothesis that hypoxia exposure at the larval stage could induce a long-term effect on the regulation of Hb gene expression was tested.

Finally, the last chapter (**Chapter 4**) assessed whether the metabolic response of juveniles to hypoxia could be modulated by O₂ and temperature conditions experienced at early life stages. Growth rate and metabolic parameters related to carbohydrate and lipid metabolism in the liver, which has a pivotal role in energy metabolism, were investigated at the juvenile stage under normoxic and chronic hypoxic conditions.

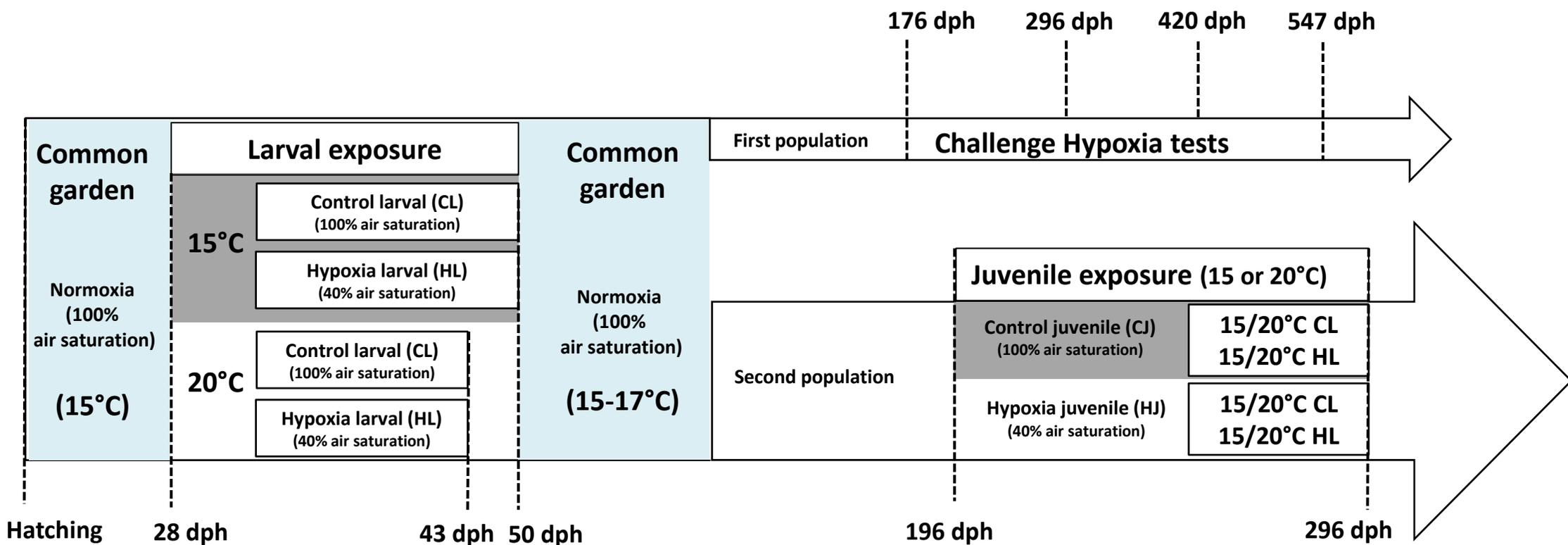


Figure 11. Schema of the experimental protocol. Sea bass larvae were exposed to each combination of oxygenation level [40% air saturation: hypoxia larval group (HL); and 100% saturation: control larval group (CL)] and temperature (15°C and 20°C) from 28 to 43dph (20°C) or 50 dph (15°C). Following the larval exposure, fish experienced a four-five months period of common garden (15-17°C, 100% air saturation). Afterward, one population of sea bass juveniles was submitted to hypoxic challenge tests (HCT) over one year (including 100 juveniles for each larval group). From 196 to 296 dph a second population of sea bass juveniles was chronic exposed to moderate hypoxia (40% air saturation) at 15°C or 20°C. Juveniles were divided into two tanks, including 30 juveniles from each larval group. Water oxygenation in one of the tanks was reduced to 40% air saturation (hypoxia juvenile group, HJ), while it was maintained at 100% saturation in the other (control juvenile group, CJ).

Chapter 2

*Adverse carry-over effects are observed in European sea bass (*Dicentrarchus labrax*) juveniles when exposed to moderate hypoxia at larval period*



The objective of this chapter was to evaluate the potential impact of larval exposure to hypoxia and warming on the capacity of juvenile fish to acquire O₂ from its environment. First, the global physiological status of juveniles was assessed over one year through hypoxia challenge tests that give an integrative evaluation of biological processes related to oxygen extraction/transport and energy metabolism. The water temperature was intentionally not controlled and it therefore followed the natural thermal cycle (10-17°C). Subsequently, our analysis focused on the overall appearance of the operculum that contributes to water flow across the gills lamellae and allows O₂ extraction from the environment. Analysis of branchial parameters (e.g. gill surface, cellular composition and diffusion distance) are in progress (collaboration with Isabelle Leguen, INRA) since gills represent the main structure involved in O₂ extraction together with operculum.

1 **Adverse carry-over effects are observed in European sea bass (*Dicentrarchus labrax*)**
2 **juveniles when exposed to moderate hypoxia at larval period.**

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8

9 **Abstract**

10 Environmental conditions, to which organisms are exposed during key periods of early development,
11 may cause possible adaptive responses with consequences in their subsequent life-history trajectory.
12 The objective of this study was to investigate whether ecologically relevant combinations of hypoxia
13 (40% and 100% air saturation) and temperature (15° and 20°C), occurring during the larval period of
14 European sea bass larvae (*Dicentrarchus labrax*), could have long-lasting impacts on the physiology of
15 resulting juveniles. Hypoxic challenge tests were performed over one year to give an integrative
16 evaluation of physiological performance. We revealed that juvenile performance was negatively
17 impacted by hypoxia but not by the thermal conditions experienced at larval stage. This impact was
18 related to the prevalence of opercular abnormalities. The present study indicates that exposure to a
19 moderate hypoxia event during larval stage may have adverse carry-over effects, which could
20 compromise fitness and population recruitment success.

21

22 Key words: hypoxia, temperature, developmental plasticity, opercular malformation, European sea
23 bass

24 **Abbreviations:** days post hatching (dph); hypoxic challenge test (HCT)

25 **1. Introduction**

26 Environmental conditions encountered by living organisms during early life stages can affect
27 their development and have long lasting effects on morphology, physiological and behavioral traits
28 (Jonsson and Jonsson, 2014; Lindström, 1999; Monaghan, 2008; Rödel and Monclús, 2011). Lasting
29 modifications of traits are related to developmental plasticity which may be adaptive or not. Indeed,
30 adverse environmental factors may disrupt development of individuals to produce non-adaptive
31 outcomes affecting their fitness (Bateson et al., 2014; Lindström, 1999). In contrast, adaptive
32 developmental plasticity define the ability of developing organisms to fine tune their phenotypes in
33 response to the environmental conditions encountered, in order to produce animals able to better
34 tolerate similar conditions at later life stages (Bateson et al., 2014).

35 Coastal marine areas are particularly prone to spatial and temporal fluctuations in terms of
36 environmental conditions. These fluctuations are intensifying as a result of the multifaceted on-going
37 global change, which is reflected by several episodes of variable magnitude combining warming and
38 hypoxia (Díaz and Rosenberg, 2011; Levin and Breitburg, 2015). Marine coastal and estuarine
39 ecosystems serve as nursery for a large number of fish species which are thus likely to be exposed to
40 periods of low dissolved oxygen (O₂) and high temperature at a particularly sensitive life stages
41 (Korwin-Kossakowski, 2008).

42 Short term effects of temperature and hypoxia on fish performance have been intensively
43 investigated at early life stages. Within a species thermal tolerance range, temperature controls
44 growth rate following bell shaped curve (O'Connor et al., 2007). Beyond thermal tolerance limits of
45 species, temperature may induce developmental abnormalities with sub-lethal or lethal
46 consequences (Rombough, 1997). For its part, hypoxia exposure can result in hatch defects,
47 metabolic depression and skeletal deformities which may compromise the growth and survival of
48 early life fish (Hassell et al., 2008a; Mejri et al., 2012; Nicholson et al., 2008; Vanderplancke et al.,
49 2015b; Wu, 2009). Temperature and oxygen conditions encountered during early life stages have
50 also been shown to induce long term effect on fish performance. Temperature may have effects on
51 developmental trajectories in fish including modification of growth, sex
52 determination/differentiation, age at maturity (Ali et al., 2003; Baroiller et al., 2009; Jonsson and
53 Jonsson, 2014). In zebrafish (*Danio rerio*), developing larvae that survive an exposure to hypoxia can
54 display altered cardiovascular response to future hypoxia (Bagatto, 2005), reduced swimming
55 capacity (Widmer et al., 2006) and altered avoidance reaction to aggression (Marks et al., 2005). Data
56 obtained in zebrafish suggest also that early hypoxia exposure may increase the subsequent hypoxia

57 tolerance at adult stage (Robertson et al., 2014). Such long term beneficial effects on hypoxia
58 tolerance have only been shown in freshwater fish model species. In contrast, recent data obtained
59 in Atlantic salmon (*Salmo salar*) indicated that fish exposed to hypoxia during early life stages tends
60 to exhibit lower hypoxia tolerance compared with those raised in normoxia (Wood et al., 2017).
61 Understanding the long term effects of these environmental constraints on fish physiological
62 performance is essential to better predict potential impacts on juvenile recruitment, particularly in
63 temperate marine fish species. Indeed, temperate coastal areas mostly experience moderate
64 environmental episodes, which may not induce immediate visible effects in terms of mortality.

65 European sea bass (*Dicentrarchus labrax*) larvae generally enter shallow coastal areas after the
66 flexion stage and could therefore be exposed to environmental fluctuations, while their larval
67 development is not totally completed (Dufour et al., 2009). Previous studies on European sea bass
68 revealed that exposure to moderate hypoxia at the larval stage could induce long lasting effects on
69 metabolic parameters and on digestive and hemoglobin systems (Cadiz et al., 2017b; Cadiz et al.,
70 2017c; Vanderplancke et al., 2015a; Zambonino-Infante et al., 2017). However, the global impact of
71 early exposure to realistic environmental fluctuations, such as combined temperature and hypoxia,
72 on the overall individual fitness of sea bass remains poorly understood. The present study aims to
73 acquire data related to hypoxia tolerance in European sea bass juveniles exposed to the combination
74 of different environmentally realistic oxygen (40% and 100% air saturation) and temperature (15°C
75 and 20°C) conditions at larval stage. Following the larval period, fish experienced a five-month period
76 of common garden (15-17°C, 100% air saturation). Then, fish tolerance to hypoxic challenge test
77 (HCT) was investigated four times at regular time intervals over one year to evaluate the global
78 physiological status of juveniles. HCT was used since the performance of fish to resist to HCT depends
79 on the ability of individuals to regulate physiological processes related to oxygen extraction and
80 transport, cardiovascular functions and energy metabolism (Roze et al., 2013). The operculum, which
81 contributes to water flow across the gills lamellae and allows O₂ extraction, was also examined. The
82 individuals' responses to the challenge tests were examined in relation to their early-life
83 environment and to the presence of opercular deformities.

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88 **2. Materials and methods**

89 **2.1 Animal rearing and treatments**

90 *D. labrax* larvae were reared under normal oxygen conditions in 12 tanks at $15 \pm 0.4^\circ\text{C}$ water
91 temperature and $35 \pm 0.2\text{‰}$ salinity. They were fed daily with *Artemia* according to Zambonino et al.
92 (1996) until the end of larval development. Water temperature was progressively increased from
93 15°C to 20°C in six tanks between 23 and 28 days post hatching (dph) for acclimation purposes. From
94 28 dph, three replicate tanks of larvae were exposed to each combination of oxygenation level [40%
95 air saturation ($2.95 \text{ mg O}_2 \text{ L}^{-1}$): hypoxia larval group; and 100% saturation ($7.35 \text{ mg O}_2 \text{ L}^{-1}$): control
96 larval group] and temperature (15°C and 20°C). Hypoxic conditions were created by bubbling N_2 in a
97 gas equilibration column placed upstream of the experimental tank. Dissolved O_2 was monitored
98 daily using an Odeon oxygen meter (ODEON Classic OPTOD; Caudan, France). Other water quality
99 parameters (salinity, temperature and pH) were also checked daily in each tank during the
100 experiment. It had previously been determined that the level of hypoxia used did not induce
101 mortality; thus, genetic selection during larval exposure was avoided. Larvae were returned to
102 normal oxygen conditions (100% air saturation) at 43 dph ($745^\circ\text{C}\cdot\text{day}$), for larvae exposed at 20°C ,
103 and at 50 dph ($750^\circ\text{C}\cdot\text{day}$), for larvae exposed to 15°C , to ensure that larval treatments were applied
104 at the same stage of development. Replicate tanks were then pooled into one 1 m^3 -tank per
105 treatment and kept under normoxic conditions at $15\text{-}17^\circ\text{C}$. At this stage, the fish were fed with a
106 commercial diet (NeoSupra; Coopérative Le Guessant, Lamballe, Côtes-d'Armor, France). At 152
107 dph, 100 fish from each of the treatment tanks were selected and tagged subcutaneously to identify
108 the early stage treatments (Passive Integrated Transponder; PIT-tag) for individual identification. The
109 fish were then fed with NeoGrower commercial diet (Coopérative Le Guessant; Lamballe, Côtes-
110 d'Armor, France) and pooled in a 4-m^3 tank.

111 **2.2 Hypoxia challenge test (HCT)**

112 Four standardized hypoxia challenge tests were conducted over one year (at 176, 296, 420 and
113 547 dph) according to the protocol described in Roze et al (2013). To avoid unnecessary stress and
114 additional sources of variation, challenge tests were conducted in the fish rearing tank and 48 h prior
115 to challenge, individuals were unfed. Water salinity did not change significantly over the duration of
116 the experiment. Water temperature was intentionally not controlled and it therefore followed the
117 natural seasonal cycle (17° at 176 dph; 10° at 296 dph; 13°C at 420 dph and 17° at 547 dph).

118 HCT consisted in a rapid decrease in water oxygenation (from nearly 100% to 20% air saturation
119 in about 1 h) followed by a much slower descent (approximately 2% air saturation per hour) until the
120 experiment ended. Ambient oxygenation was controlled by bubbling nitrogen in the input of a
121 submersible pump placed in the tank. The water oxygen level was monitored using a calibrated
122 Odeon oxygen meter. As individual fish lost their ability to maintain equilibrium, they were quickly
123 removed from the experimental arena, identified (tag reading) and placed in a fully aerated recovery
124 tank. The corresponding time (time to loss of equilibrium; TLOE) and oxygen level (incipient lethal of
125 oxygen saturation; ILOS) were also recorded. The HCT ended when the last fish was recovered.

126 **2.3 Identification of opercular deformities**

127 Two weeks after the last hypoxia challenge test, juveniles from experimental groups were
128 slightly anesthetized (Tricaine methane-sulfonate 20 mg L⁻¹). Then, individuals were examined
129 macroscopically to identify unilateral and bilateral opercular deformities. Special attention was paid
130 to partial or total lack of the operculum.

131 **2.4 Data analysis and statistics**

132 The resistance of sea bass juveniles to HCT was measured as the fraction of individuals without
133 equilibrium loss as a function of time under hypoxic conditions using the Kaplan-Meier estimator of
134 survival time (time to loss of equilibrium, referred to as resistance time thereafter, being equivalent
135 to time to death; (Kaplan and Meier, 1958).

136 Firstly, the effects of early-life hypoxic and warming exposure on juvenile resistance to HCT were
137 examined by the (Peto and Peto, 1972) modification of the log-rank test, which is a non-parametric
138 test allowing to compare survival (here resistance) time distribution between samples. Tests were
139 stratified according to dates to account for temporal variability in global resistance time distribution
140 across the four HCTs. The effects of early-life temperature and oxygen level were tested both jointly
141 (i.e. differences between cross-factorial treatments) and independently (i.e. differences between the
142 two modalities of each factor separately). As the test consists in across sample comparison,
143 interactions could not be tested.

144 Secondly, the effect of opercular deformities on juvenile tolerance to hypoxia challenge was
145 estimated by parametric survival regression models where the hypoxia resistance time follows a
146 parametric distribution. Various classical resistance time distributions were tested and the AIC
147 (Akaike Information Criterion) was used to compare the performance of the corresponding models

148 and choose one distribution. Models included opercular malformation as a factor (with three
149 modalities corresponding to the number of opercules affected: no, unilateral or bilateral deformity)
150 to test its effect on hypoxia resistance, date as a strata to account for temporal variability, and their
151 interaction. Significance of the effects was tested by likelihood ratio tests between nested models
152 respecting marginality of the effects (type II tests; (Fox and Weisberg, 2011). Post-hoc pairwise
153 comparisons between opercular deformity modalities at each date were performed using
154 simultaneous tests for general linear hypotheses in parametric models (Hothorn et al., 2008). Based
155 on our AIC analysis, parametric model with a Weibull distribution was the most appropriate to
156 describe the data. Hypotheses underlying Weibull survival regression models were checked (Fox and
157 Weisberg, 2011).

158 Finally, the incidence of opercular malformation in relation to early-life hypoxic and warming
159 exposure was assessed by multinomial regression models that allow modelling categorical variables
160 with more than two modalities as response variables. More precisely, we used these models to
161 analyse how the probability being undeformed, unilaterally or bilaterally deformed was influenced by
162 early-life temperature as a continuous variable, early-life oxygen level as a categorical variable and
163 their interaction. Significance of the effects was again tested by likelihood ratio type II tests.

164 Throughout analyses, differences were considered significant at the 95% confidence level (p-
165 value < 0.05). All statistical analyses were performed using R software (R Core Team 2016). Survival
166 analyses were performed using the 'survival' package (Therneau and Grambsch, 2000; Therneau,
167 2015), post-hoc pairwise comparisons with the 'multcomp' package, Weibull survival regression
168 diagnostics with the package 'SurvRegCensCov' (Hubeaux and Rufibach, 2015), and multinomial
169 regressions with the package 'nnet' (Venables and Ripley, 2002). Weight data were log-transformed
170 to fit a normal distribution and they were checked for normality (Shapiro test) and equality of
171 variances (Levene test). Two-way ANOVAs were used to determine the effects of larval treatments
172 (oxygen and temperature) on larval and juvenile body mass. Tukey's test (p < 0.05) was performed
173 for post-hoc comparisons.

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177

178 **3. Results**

179 **3.1 Effect of early-life hypoxic and warming exposure on larval and juveniles weight**

180 Whereas weights of larvae were similar among experimental groups at the beginning of the
 181 exposure period ($\approx 1.8\text{g}$, data not shown), they were significantly different at the end of the exposure
 182 period (43/50 dph) (table 1). The highest weights were observed for larvae reared at 20°C (2 times
 183 higher than at 15°C; $p < 1 \cdot 10^{-6}$) and those reared in normoxia exhibited significantly higher weight
 184 than those exposed to hypoxia (1.3 times higher; $p < 1 \cdot 10^{-5}$). Sixteen months after the end of the
 185 exposure period (at 549 dph), juveniles previously exposed to 20°C still exhibited the highest weights
 186 (1.05 higher than 15°C Normoxia; $p=0.015$). There was no significant difference in juveniles' weight
 187 related to previous exposure to hypoxia.

188 *Table 1. Results of two-way ANOVA for effects of larval treatments (Temperature (Temp.) and*
 189 *Hypoxia (Hyp.)) on larval (43/50 dph) and juvenile (549 dph) body mass. Df means degrees of*
 190 *freedom. N for each group= 100 juveniles. Significant differences between groups are identified with*
 191 *different letters (Tukey's test).*

	15°C Normoxia	15°C Hypoxia	20°C Normoxia	20°C Hypoxia	Df	Temp.	Hyp.	Temp. x Hyp.
43/50 dph (mg)	17.57b	13.86a	35.51d	31.95c	1	F=514.68 $p < 1 \cdot 10^{-6}$	F=75.46 $p < 1 \cdot 10^{-5}$	F=0.55 $p=0.48$
549 dph (g)	143.6a	143.9a	148.5b	151.7b	1	F=9.4 $p=0.015$	F=0.7 $p=0.43$	F=0.5 $p=0.51$

192

193 **3.2 Effect of early-life hypoxic and warming exposure on juvenile tolerance to hypoxia challenge**
 194 **test**

195 Despite the variability of the response over the four challenge tests, juveniles that experienced
 196 early-life hypoxia event showed lower resistance to hypoxia than individuals from the control group
 197 (log-rank test: $p = 1.5 \cdot 10^{-5}$, table 2; Fig.1). In contrast, the four challenge tests did not reveal any
 198 effect of temperature on the ability of fish to tolerate hypoxia; $p = 0.07$ (table 2; Fig.2).

199 *Table 2. Effects of temperature and oxygen conditions at larval stage upon resistance time to the*
 200 *hypoxia challenge tests. Statistical differences of resistance time related to temperature and oxygen*
 201 *conditions were evaluated using Peto & Peto modification of the log-rank test. Df means degrees of*
 202 *freedom.*

	Chisq	Df	p- value
Oxygen	18.7	1	1.5*10 ⁻⁵
Temperature	7.5	1	0.07

203

204 **3.3 Effect of opercular deformities on juvenile tolerance to hypoxia challenge test**

205 Juveniles with opercular deformities exhibited a significant decrease in resistance time to hypoxia
 206 challenge test compared to undeformed fish (significant opercular deformity effect, table 3; Fig.3)
 207 but this effect varied according to the date considered (significant interaction between opercular
 208 deformity and date, table 3). However, post-hoc pairwise comparisons between opercular deformity
 209 modalities (no vs unilateral, no vs bilateral, and unilateral vs bilateral deformity) at each date were all
 210 significant and showed that deformity always reduces resistance time, and that resistance time is
 211 lowest when fish exhibit bilateral deformities (table 4; Fig. 3). More precisely, fish with unilateral
 212 opercular deformities exhibited a decrease in resistance time compared to undeformed fish ranging
 213 from 18% to 30% depending on dates (table 4) while those with bilateral opercular deformities has a
 214 resistance time decreased by 34% to 56% according to the date.

215 *Table 3. Results of the Weibull regression model on the effect of date and opercular deformity on*
 216 *resistance time to hypoxia challenge test. Df means degrees of freedom.*

	Chisq	Df	p- value
Strata (date)	844	3	<1*10 ⁻⁴
Opercular deformity	244	2	<1*10 ⁻⁴
Strata (date)*Deformity	657	6	<1*10 ⁻⁴

217

218 *Table 4. Effects of opercular deformities upon resistance time to the hypoxia challenge test at the*
 219 *different dates. Data are given in percentage of resistance time relative to undeformed individuals*
 220 *(columns unilateral and bilateral). Post-hoc pairwise comparisons of resistance time between no,*
 221 *unilateral and bilateral opercular deformities were evaluated using simultaneous tests for general*
 222 *linear hypotheses in parametric models.*

	unilateral	bilateral	undeformed vs. unilateral	undeformed vs. bilateral	unilateral vs. bilateral
176 dph	21%	39%	$p < 1 \cdot 10^{-4}$	$p < 1 \cdot 10^{-4}$	$p < 1 \cdot 10^{-4}$
296 dph	19%	43%	$p < 1 \cdot 10^{-4}$	$p < 1 \cdot 10^{-4}$	$p < 1 \cdot 10^{-4}$
420 dph	18%	34%	$p < 1 \cdot 10^{-4}$	$p < 1 \cdot 10^{-4}$	$p < 1 \cdot 10^{-4}$
549 dph	30%	56%	$p < 1 \cdot 10^{-4}$	$p < 1 \cdot 10^{-4}$	$p = 0.04$

223

224 **3.4 Effect of early-life hypoxic and warming exposure on the presence of opercular malformations**

225 According to the multinomial regression model on opercular deformities, hypoxia exposure at the
 226 larval stage strongly increased the risk of developing an opercular deformity at juvenile stage (Chisq =
 227 69, df =2, $p = 1.2 \cdot 10^{-15}$). The relative risk of a unilateral and a bilateral opercular malformation (8.7%
 228 and 1.5% of the total population, respectively) was 4.47 or 5.83 times higher, respectively, when
 229 juveniles experienced an early-life hypoxia event compared to those reared in normoxia. Early
 230 temperature condition also significantly influenced the prevalence of opercular malformation (Chisq
 231 = 13, df = 2, $p = 0.0017$). However, the effect was inconsistent between unilateral and bilateral
 232 opercular malformation: while the relative risk of unilateral malformations at 20°C was 1.2 fold
 233 higher than at 15°C, it was 5 fold lower for the relative risk of bilateral malformations.

234

235

236 **4. Discussion**

237 European sea bass larvae are likely to be exposed to environmental fluctuations when they
238 enter shallow coastal and estuarine waters just after the flexion stage. In this context, the present
239 study evaluated whether thermic and oxygen conditions encountered during critical windows of sea
240 bass larval development could have long-lasting impacts on physiological performance, particularly
241 related to hypoxia tolerance. Present data reveal compensatory growth following larval exposure to
242 moderate hypoxia and lower temperature. This result is in good agreement with known phase of
243 accelerated growth when favourable conditions are restored after a period of growth depression in
244 fish (Ali et al., 2003). Moreover, our data revealed that early exposure to moderate hypoxia from day
245 28 to day 45/50 post hatching had a negative effect on the subsequent capacity of fish to tolerate a
246 hypoxic constraint. This effect is explained by an increase in the prevalence of opercular
247 malformation. Even if early exposure to moderate hypoxia did not induce any significant mortality,
248 we assumed that it produces adverse carry-over effects which are likely to affect fish fitness.

249 Our data revealed that European sea bass juveniles previously exposed to chronic moderate
250 hypoxic water showed lower tolerance compared to control fish. This long term impact was globally
251 observed throughout the four hypoxia challenges regularly performed for one year at juvenile stage
252 despite the variability of response observed among dates. This variability is likely due to variations of
253 factors related to hypoxia challenge procedure (water temperature, repeatability of the procedure)
254 and phenotypic shuffling among tests (Killen et al., 2016). Early-life exposure to low dissolved oxygen
255 at larval stage has modified developmental trajectories of European sea bass producing phenotypes
256 that were not adapted to hypoxia. Adaptive plasticity may prime an organism exposed to
257 environmental constraints during early life stage to develop phenotypes that will allow coping better
258 with similar situations later in life (Sheriff and Love, 2013). In contrast, early exposure to constraint
259 may also have detrimental effects on developmental process with lifelong morphological or
260 physiological impairment and, ultimately, negative impact on fitness (Hassell et al., 2008b; Lupien et
261 al., 2009). Our results indicating negative effect of early exposure to hypoxia on subsequent hypoxia
262 tolerance are consistent with recent data obtained in Atlantic salmon, *Salmo salar*, (Wood et al.,
263 2017) but contrasts with the adaptive developmental plasticity revealed in zebrafish adults that
264 benefited from exposure to low dissolved oxygen at embryonic stage (Robertson et al., 2014).
265 Responses to early-life exposure to hypoxia appear likely to be highly species-specific and may also
266 depend on the nature of the early hypoxic constraint (e.g. intensity, duration, developmental
267 window).

268 The mechanisms underlying the developmental plastic responses to hypoxia are largely
269 unknown. In zebrafish, adaptive developmental plasticity of hypoxia tolerance resulted in a
270 modification of the sex ratio in favor of males which exhibit a lower critical oxygen tension and
271 higher hypoxia tolerance compared to females (Robertson et al., 2014). In the present study, hypoxia
272 tolerance test was performed on immature European sea bass juvenile. Therefore, the potential link
273 between early-life exposure of European sea bass larvae to hypoxia and the sex
274 determination/differentiation processes and its potential consequence with later lower hypoxia
275 tolerance cannot be evaluated. Hypoxia tolerance depends partly at least upon the capacity of fish to
276 acquire oxygen from its environment (Mandic et al., 2009). According to that, our data provide
277 evidence that hypoxia tolerance was particularly low in juvenile fish exhibiting apparent opercular
278 malformation. In particular, we demonstrated that the lowest tolerance to hypoxia, the highest the
279 opercular malformation (bilateral > unilateral). It is admitted that opercular malformation can
280 negatively affect biological performance of fish and reduce their resistance to oxygen drops
281 (Koumoundouros et al., 1997). In the present work, deformities consist in partial or total open
282 operculum. Such deformities likely impair the pump function of opercular cavity and water flux
283 across gills lamellae resulting in lower oxygen extraction capacities. Interestingly, our data revealed
284 that prevalence of opercular malformation was significantly higher in juveniles previously exposed to
285 moderate hypoxia. This effect, which concerned 10% of the population, explained the lower hypoxia
286 tolerance observed in juveniles previously exposed to hypoxia. Indeed, no more significant impact of
287 early hypoxia exposure on hypoxia tolerance was observed when considering juveniles without
288 apparent opercular malformation. That means that compensatory growth, revealed for juveniles that
289 were exposed to hypoxia and/or 15°C at larval stage, did not influence hypoxia tolerance.

290 Molecular and cellular processes underlying hypoxia-induced opercular deformities during sea
291 bass larvae development still need to be deciphered. However, a possible alteration of the
292 ossification process in the operculum induced by hyperventilation during hypoxia exposure can be
293 hypothesized. Previous work performed on the gilthead sea bream (*Sparus aurata*) suggested that an
294 increase in ventilation frequency during a specific developmental window corresponding to the
295 operculum ossification (i.e. 400°C.days) resulted in operculum deformities (Beraldo et al., 2003). The
296 hypothesis is reinforced by the fact that ossification of the operculum occurred at around 500°C-day
297 in European sea bass (Darias et al., 2010), which corresponds to the larvae exposure to hypoxia in the
298 present study.

299 Our results also showed an impact of warmer temperature at larval stage on the incidence of
300 opercular deformities in sea bass juveniles. This effect depends on the number of deformities

301 considered. While we could hypothesize that the increase of unilateral deformity prevalence may
302 likely be explained by an increase in the ventilation frequency due to the higher metabolism resulting
303 from warmer temperatures, the decrease of bilateral deformities with warmer temperature remains
304 difficult to explain. However, this last result must be considered with caution as only 6 individuals out
305 of 325 exhibited bilateral opercular malformations and more confidence can be put in the result on
306 unilateral deformities.

307 In conclusion, we assume that exposure to moderate hypoxia and to a lesser extent to warm
308 temperature in European sea bass larvae impact the fitness of part of the future juveniles. Indeed, it
309 has to be pointed out that, in addition to the adverse effect on hypoxia tolerance, opercular
310 malformations may also predispose gills to pathological infections, which could induce delay in
311 growth and high mortality rates in fish juveniles as a consequence of parasite infestation (Abdel et
312 al., 2004). Impact on opercular formation associated to decrease in hypoxia tolerance confirms that
313 developmental, morphological and physiological responses to the early-life environment do not
314 always allow a beneficial adaptive tuning of physiological functions, which should produce animals
315 best suited for the environment they are likely to find later as juveniles or adults. Nevertheless, we
316 cannot exclude other potential effects of early oxygen and temperature conditions on key
317 physiological function (e.g. reproduction) not investigated in the present study. Physiological trade
318 off associated with compensatory growth could particularly be addressed. Future research will help
319 to better characterize the long term impact of early hypoxic and thermal conditions on components
320 of fitness which could influence population recruitment success.

321

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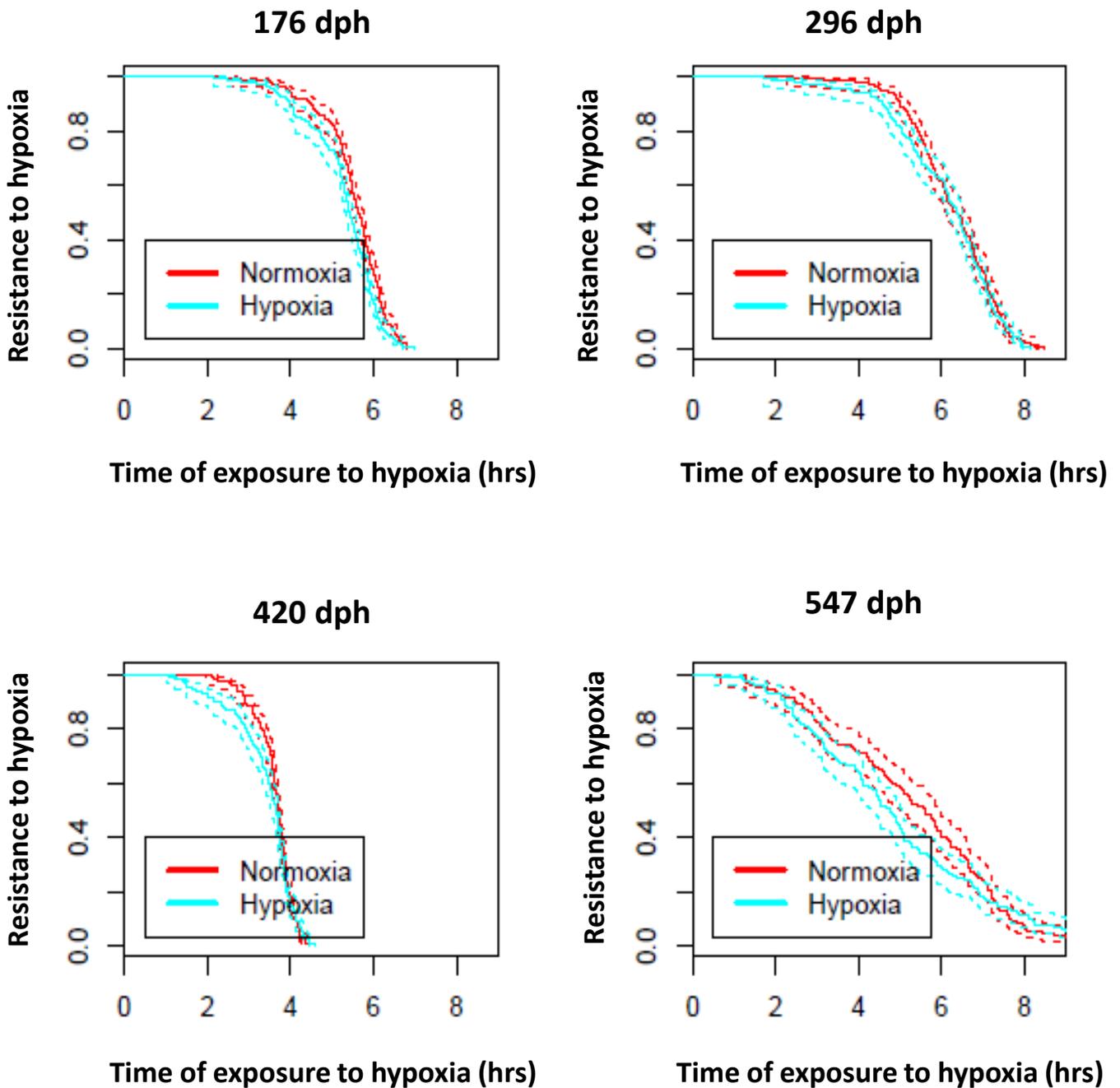


Figure 1. Kaplan-Meier probability plot of tolerance time during a Hypoxia Challenge in juveniles early exposed during larval stage to normoxia (red lines) or hypoxia (blue lines). Continuous lines represent the percentage of individuals that resisted hypoxia up to the time point considered (also called the Kaplan-Meier estimator in survival analysis) and discontinue lines represent the associated 95% confidence intervals. The resistance to hypoxia represents the percentage of individual that resisted hypoxia up to the time point considered. A total of four standardized hypoxia challenge tests were conducted over one year (at 176, 296, 420 and 547 dph).

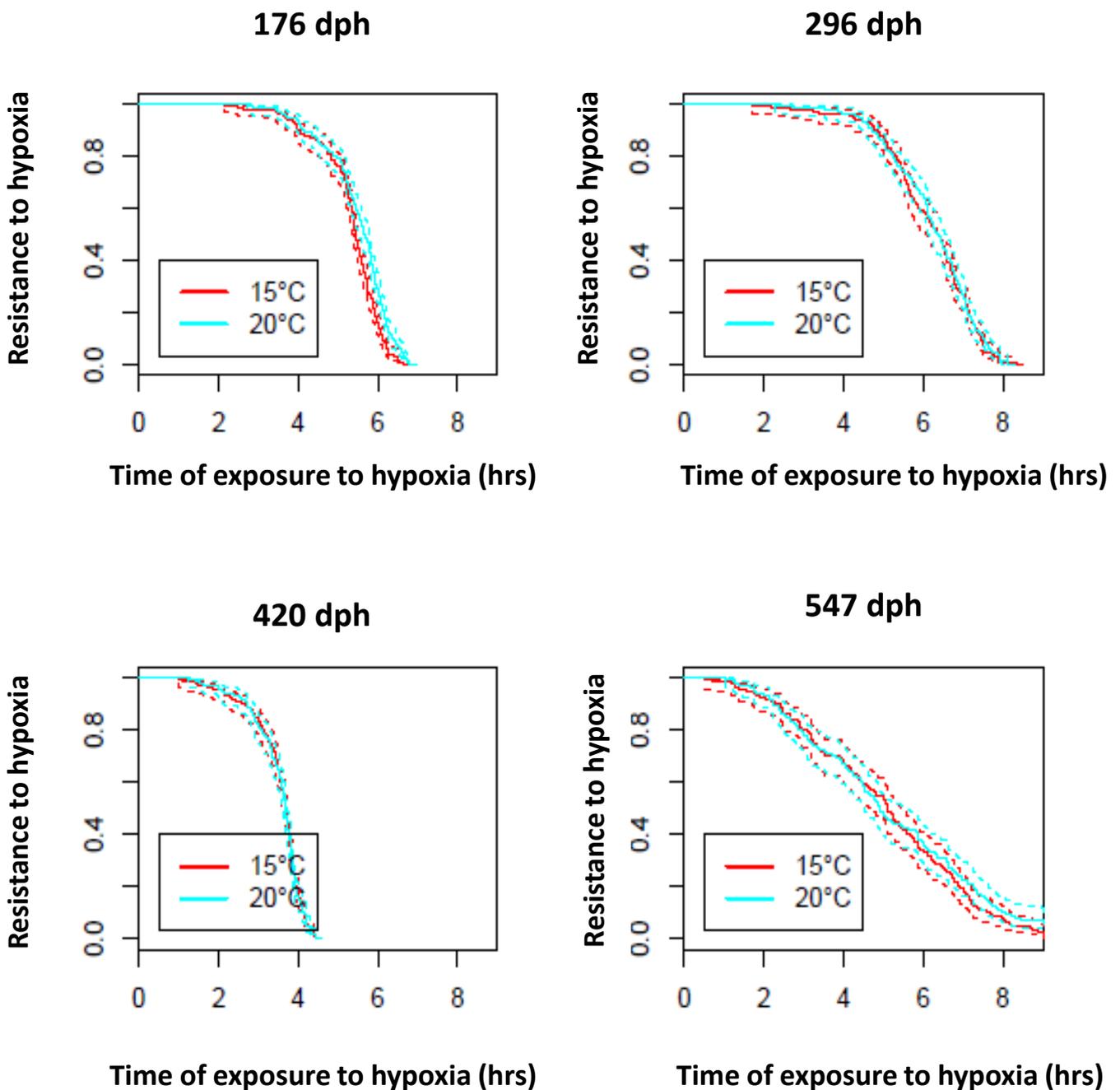


Figure 2. Kaplan-Meier probability plot of tolerance time during a Hypoxia Challenge in juveniles early exposed during larval stage to 15°C (red lines) or 20°C (blue lines). Continuous lines represent the percentage of individuals that resisted hypoxia up to the time point considered (also called the Kaplan-Meier estimator in survival analysis) and discontinue lines represent the associated 95% confidence intervals. The resistance to hypoxia represents the percentage of individual that resisted hypoxia up to the time point considered. A total of four standardized hypoxia challenge tests were conducted over one year (at 176, 296, 420 and 547 dph).

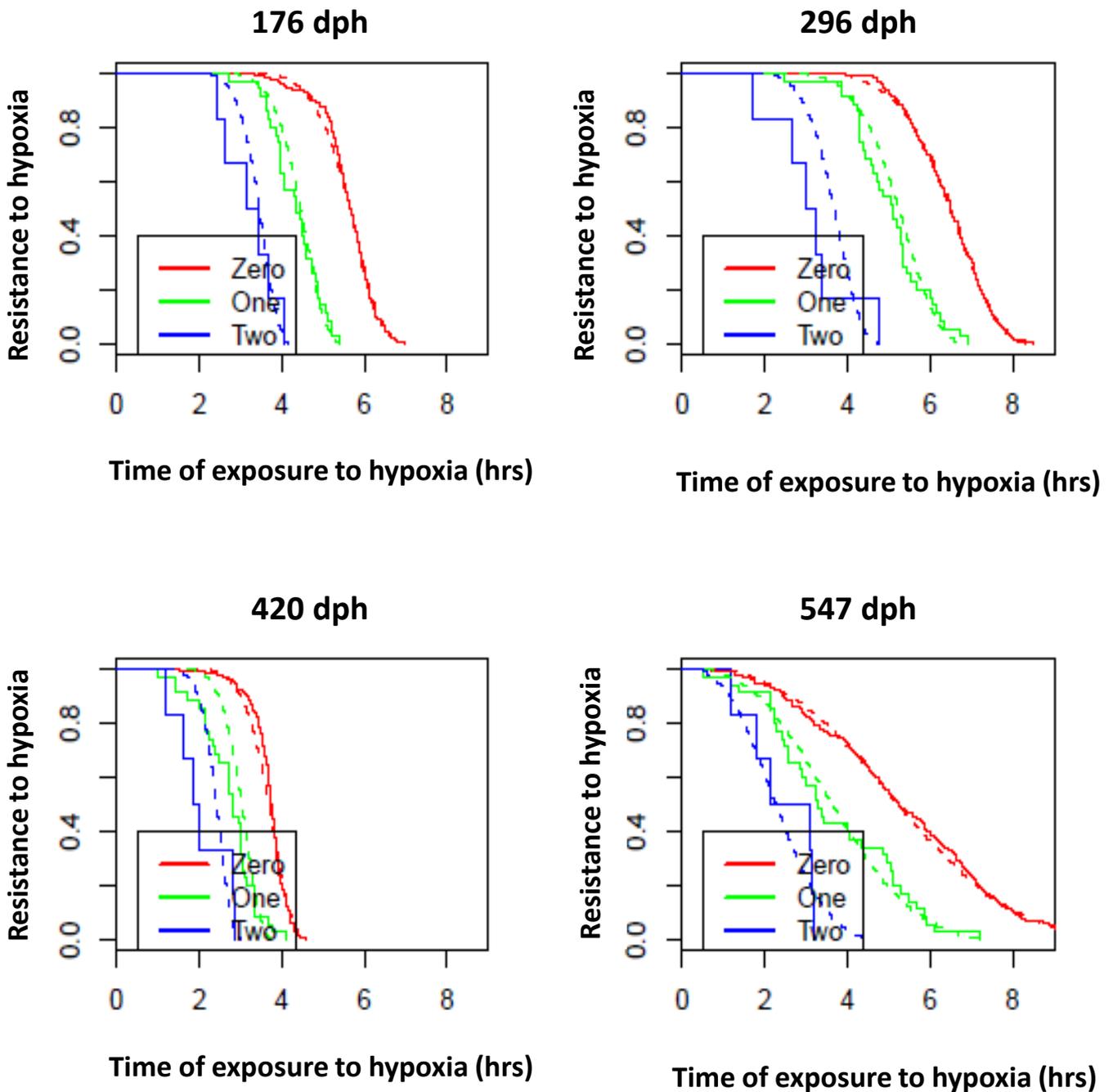
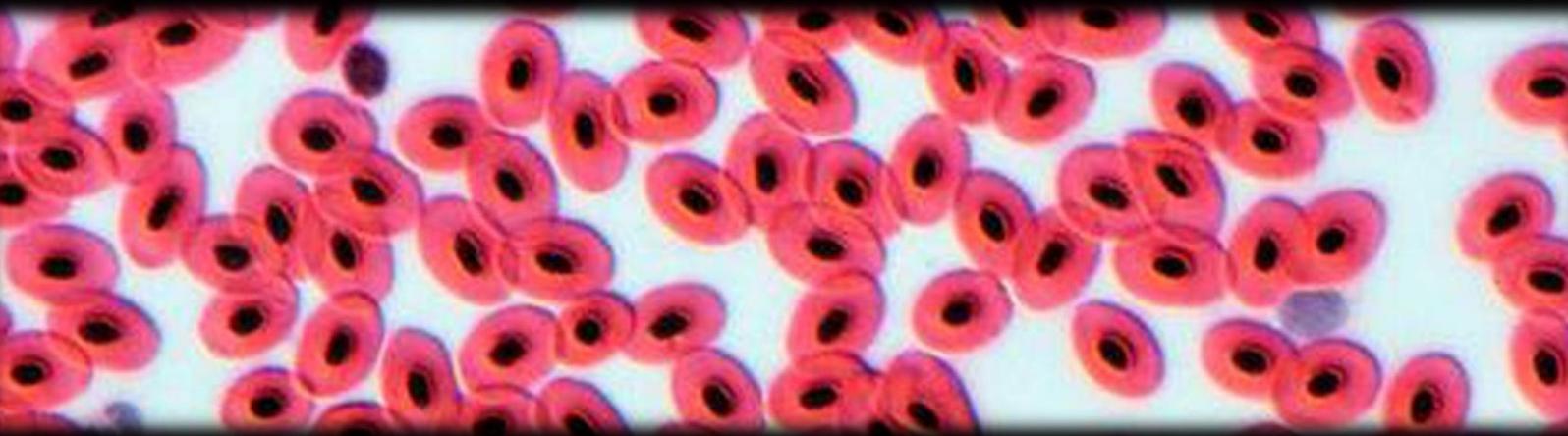


Figure 3. Kaplan-Meier probability plot of tolerance time during a Hypoxia Challenge on the basis of the incidence of opercular deformities in sea bass juveniles: zero (red line), one-unilateral (green line) and two-bilateral (blue line). Continuous lines represent the percentage of individuals that resisted hypoxia up to the time point considered (also called the Kaplan-Meier estimator in survival analysis) and discontinue lines represent the associated 95% confidence intervals. The resistance to hypoxia represents the percentage of individual that resisted hypoxia up to the time point considered. A total of four standardized hypoxia challenge tests were conducted over one year (at 176, 296, 420 and 547 dph).

Chapter 3

Characterization of hemoglobin system in European sea bass (Dicentrarchus labrax) and its regulation under moderate hypoxia at larval and juvenile stage



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The objective of this chapter was to evaluate the potential impact of larval exposure to hypoxia and warming on the capacity for oxygen transport in European sea bass through the study of hemoglobin (Hb) system.

The first part of the chapter reported on the characterization of the European sea bass Hb genes. The genomic organization and structure analysis of Hb gene clusters was clarified. Phylogenetic analysis was performed based on the deduced Hb amino acid sequences of the European sea bass and other teleost and non-teleost species. The amino acid residues involved in Hb oxygen binding, particularly in the Bohr and Root effects, were examined by a comparative analysis of Hb protein sequences. Finally, the expression pattern of Hb genes from embryonic to adult stages and in different adult tissues was evaluated by qPCR. This study was done in collaboration with Erick Desmarais (University of Montpellier, France) and Oivind Andersen (Nofima, Norway).

In the second part of this chapter we investigated the regulation by early environmental constraints of Hb gene expression in whole larvae and in the hematopoietic tissues (head kidney and spleen) of juveniles. Above all, the hypothesis that hypoxia at the larval stage could induce a long-term effect on the regulation of Hb gene expression was tested. The results presented in this chapter were obtained in fish reared at 15°C during both larval and juvenile stages. Data obtained for fish reared at 20°C will be discussed in the general discussion. Special attention was also paid to the expression of Prolyl Hydroxylase Domain-Containing Protein 3 (PHD-3), which is an actor of HIF-1 signaling pathway.



Genomic organization and spatio-temporal expression of the hemoglobin genes in European sea bass (*Dicentrarchus labrax*)

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Abstract Hemoglobins (Hb) play a critical role in satisfying the oxygen demand of vertebrate aerobic metabolism. The present study reports the characterization of the European sea bass (*Dicentrarchus labrax*) Hb genes, including genomic organization, phylogeny, and spatio-temporal gene expression. These Hb genes are divided into two unlinked clusters, the “MN” cluster containing eleven genes (five Hb α genes named MN-*Hba1-5* and six Hb β genes named MN-*Hb β 1-6*) and the “LA” cluster consisting of three genes (two Hb α genes named LA-*Hba1-2* and one Hb β gene named LA-*Hb β 1*). Comparative analysis of Hb amino acid sequences indicates that most of the important amino acid residues involved in hemoglobin-oxygen binding, particularly in the Bohr and Root effects, are generally well conserved, except in MN-*Hb β 3*. Six genes were mainly expressed during early life (MN-*Hba3-5*, MN-*Hb β 4-6*),

while the others were predominantly expressed at juvenile–adult stages. Adult fish expressed Hb genes at high levels in the head kidney and spleen; the main organs involved in blood formation. The Hb genes expressed in non-hematopoietic organs (intestine, gills, heart, brain, and liver) may facilitate oxygen homeostasis or be involved in antimicrobial defense. Stage- and tissue-specific gene expression patterns, together with the sequence features of the different Hb proteins, suggest a broad range of roles in European sea bass.

Introduction

Teleost species inhabit contrasting marine and freshwater environments across the planet and face a wide variety of environmental conditions in terms of water temperature, salinity, and dissolved oxygen concentration (Helfman et al. 1997; von der Heyden et al. 2015). Specifically, several fish species have offshore spawning and larval migration toward coastal areas. As a consequence, genetic adaptation or physiological acclimatization has been observed, especially to adjust oxygen demand (metabolism) and supply (oxygen uptake and transport) to environmental oxygen availability at the different stages of their life cycle (Ishibashi et al. 2007; McKenzie et al. 2008). Several authors have suggested that the ability of fish to colonize a wide range of habitats has evolved together with the molecular and functional modulation of the hemoglobin system (Weber 2000; Verde et al. 2002, 2006, 2008; Marinakis et al. 2003). Hemoglobin (Hb) in vertebrates, including fish, consists of a tetrameric molecule composed of twin α and β globin subunits ($\alpha_2\beta_2$), each of which contain a heme group that can reversibly bind oxygen. While genes coding for α and β globins in

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mammals are clustered on one chromosome, teleost α and β globin genes (Hb α and Hb β genes) are grouped within two clusters labeled “MN” and “LA” located on two separate chromosomes resulting from the teleost-specific genome duplication (TGD) (Hardison 2008; Opazo et al. 2013). The cluster names are derived from their flanking genes: N-methylpurine-DNA glycosylase (*mpg*) and nitrogen permease regulator-like 3 (*nprl3*) for cluster “MN”, and Leucine carboxyl methyltransferase 1 (*lcmt1*) and Aquaporin-8 (*aqp8*) for cluster “LA”. Both physical extent and gene content of the teleost globins have been altered by gene loss and by chromosomal or tandem duplications (Opazo et al. 2013) and the number of Hb genes varies from a single pseudogene in icefishes (Near et al. 2006) to 36 genes (including nine pseudogenes) in Atlantic salmon (Quinn et al. 2010).

The multiplicity of Hb genes in many fish species is considered as an advantage that allows them to cope with a variable oxygen demand throughout ontogenic development as well as with temporal and spatial alterations in environmental oxygen availability (Perutz 1983; Rutjes et al. 2007). The strong adaptability has been shown to involve differential expression of Hb genes throughout the fish life cycle (Maruyama et al. 2004; Tiedke et al. 2011) and differential regulation under hypoxia or extreme conditions (Campo et al. 2008; Feng et al. 2014). Furthermore, the diversified Hb forms may exhibit different functional properties in terms of oxygen affinity and responsiveness to regulation by phosphates (ATP, GTP), temperature, and pH (Bohr and Root effects) (Weber 1990).

European sea bass is an economically important farmed species with a natural distribution ranging in North Atlantic from the south of Norway (60°N) to the west coast of Morocco (30°N) and throughout the Mediterranean and Black Sea (Tortonese 1986; Perez-Ruzafa and Marcos 2014). The metabolic scope was shown to be susceptible to reduced oxygen availability and elevated temperatures, and hypoxia caused morphological changes and altered expression of genes coding for oxygen-dependent molecules in the gills of European sea bass (Claireaux and Lagardère 1999; Rinaldi et al. 2005). Multiple Hbs proteins have been documented by gel electrophoresis, and a partial globin with antimicrobial properties has been reported in European sea bass (Perez and Maclean 1976; Terova et al. 2011). The recently published European sea bass genome sequence is a valuable source of information to investigate the Hb genes (Tine et al. 2014). Starting from this genome assembly, the objectives of the present study were to clarify the organization of the genomic “MN” and “LA” clusters in European sea bass, to identify Hb genes and the predicted globin proteins expressed from embryonic to adult stages in this species.

Materials and methods

Genomic organization and structure analysis of Hb gene clusters

The first genome assembly of European sea bass was produced based on high throughput sequencing and physical maps (Tine et al. 2014). This assembly was first annotated by homology at the protein level with five closely related published genomes and then by mapping of an annotated gene model constructed from RNA-Seq experiments. It contains 19 annotations for Hb genes localized in different linkage groups (LG), including two clusters, one composed of three Hb genes in LG1B and the other composed of eight Hb genes in LG8. These clusters harbored the typical flanking genes of Hb clusters (i.e., *lcmt1* and *aqp8* for LG1B and *mpg* and *nprl3* for LG8), and probably corresponded to the LA and MN clusters, respectively (Hardison 2008). However, eight globin homolog genes were also detected in several unordered genomic contigs (UN), suggesting a potential incomplete or imperfect reconstitution of the genomic clusters in the published assembly. This is not surprising given the perfect homology that some exons of different Hb genes can display and the chromosomal clustering of these genes. In an attempt to improve the original assembly of the Hb cluster regions, especially with the goal of integrating the unplaced contigs containing Hb genes, we used an iterative process of assembly, based on sequential homology searches among long sequences of the plasmids used for building the initial physical map of the sea bass genome (<http://seabass.mpipz.de/index.html?org=European+seabass&db=dicLab1&hgsid=3779>). We first built a blast database containing the original Sanger sequences of the plasmids (500–1000 bp). We then blasted this database with the sequences of the flanking genes *kank2* and *aqp8* to retrieve homologous but overlapping plasmids to walk inward the Hb clusters. Once the first plasmids were merged into the gene sequences, we used this growing assembly as the query for another round of homology search, until we reached the other end of the cluster, i.e., the flanking gene on the other side of the Hb clusters in other species. The rationale for this approach was to benefit from the larger length of these sequences compared with those of NGS reads for resolving assembly ambiguities. Indeed, some parts of the Hb genes can be strictly identical sometimes over more than 200 bp but never on longer stretch of sequence. Moreover, the plasmids contained genomic inserts up to several Kb long and were sequenced from both ends. We first built a (frame) scaffold of overlapping plasmid sequences by taking into account the pairing information between the two sequence ends of each cloned genomic fragment insert (sometimes spanning a 5 Kb region of genome). We then plot the whole genome shotgun

(WGS) short reads on that frame with BWA-MEM algorithm (<http://bio-bwa.sourceforge.net/>). The alignment of short reads was then manually curated with Geneious 9.1.7 over the two Hb clusters to remove inconsistently mapped pairs of reads, i.e., those without the two mates matching in this genomic region. Finally, we reconstituted a consensus sequence based on the short reads and compare it to that obtained from the plasmids sequences only. This approach made it possible to reorganize the two Hb clusters (LA and MN cluster sequences available in Supplementary file 1). Predicted exon–intron structures of genes were investigated using the Genscan software (<http://genes.mit.edu/GENSCAN.html>) (Burge and Karlin 1997). The sequences of the Hb genes have been published in GenBank under accession numbers KX196178–KX196191. To validate the Hb gene succession in the MN Cluster, PCR primers were designed at different locations in this cluster and allowed to confirm the predicted amplicon sizes on genomic DNA (Supplementary file 2). Our LA cluster assembly was similar to that resulting from the automatic LG1B assembly published in the databases (<http://seabass.mpiz.de/cgi-bin/hgGateway>). Then, based on the transcript sequences predicted from this new assembly, we designed primers (supplementary file 2) and amplified cDNA that we cloned and sequenced (GenBank accession numbers: KY425658–KY425671), thereby confirming the biological existence of the new gene sequences we built.

Sequence alignment and phylogenetic analysis

Hb α and Hb β amino acid sequences inferred by the translation of the European sea bass Hb cDNAs cloned in the present study were aligned using ClustalW along with sequences listed in a previous work (supplementary file 3) (Opazo et al. 2013). Human and elasmobranch species were included as outgroup for alignment. To reconstruct the Hb phylogenies, the best model of evolution was assessed from this protein sequence alignment by means of the ProtTest 2.4 program (Abascal et al. 2005). Based on the Akaike information criterion (AIC), LG model was chosen as transition probability matrices (Le and Gascuel 2008), calculated as distance of amino acid substitution per site. We reconstructed Hb phylogeny including Hb α and Hb β genes using maximum likelihood and Bayesian approaches. Maximum Likelihood phylogeny inference as implemented in MEGA v. 6 (Tamura et al. 2013) was used with 1000 replicates for the bootstrap test. The proportion of invariant sites was evaluated during the analysis, and the range of variation rate across site was determined by a gamma distribution with four categories. A Bayesian Markov Chain Monte Carlo (MCMC) analysis was performed using the MrBayes software (<http://mrbayes.sourceforge.net/>) for testing evolutionary hypotheses in which the tree was

weighted proportionally to their posterior probability. Two independent assays were performed for Hb alignment: LG was used as the substitution matrix, the site heterogeneity model was also a gamma distribution with four categories, and the number of MCMC replicates was 10^6 . The final results were summarized in the best tree after discarding the first 25% of them. All phylogenetic trees were viewed by the FigTree v.1.4.3 software (<http://tree.bio.ed.ac.uk/software/figtree/>).

Animals

Eggs, juveniles (3 month post hatching) and adults (18 month post hatching) of European sea bass were obtained from a commercial hatchery (Aquastream, Ploemeur, France). Eggs were transferred to the wet laboratories in one 60-l incubator with open flow and aerated sea water kept at $15 \pm 0.4^\circ\text{C}$. After hatching, larvae were fed daily with *Artemia* until the end of larval development (Zambonino-Infante et al. 1996). To investigate the gene expression pattern during embryonic and larval development, pools (in triplicate) of eggs and larvae between 20 and 50 mg (containing five-to-several hundred individuals depending on developmental stage) were sampled at two embryonic (12 and 60 h post-fertilization) and six larval stages (5, 13, 19, 26, 33, and 45 day post hatching). Juvenile ($n=3$ per triplicate) and adult fishes ($n=3$) were killed by lethal doses of anesthesia (Eugenol 0.05%, Sigma–Aldrich Saint-Louis, Missouri, USA). Adult tissues (brain, gills, spleen, head kidney, heart, intestine, and liver) were immediately dissected. Blood was quickly removed to reduce red blood cell contamination from tissues by rinsing with saline solution (NaCl 0.01%). The pools of eggs and larvae as well as whole juvenile and adult tissues were transferred into microtubes containing *RNAlater* (Qiagen, Hilden, Germany) and placed at 4°C until total RNA extraction. Present work was performed in accordance with French and European policies and guidelines of the French Animal Care Committee (ACCF). No ethical approval for this study was necessary, since fish were reared in optimal conditions until they were sacrificed. Fish were killed with overdose of Eugenol (500 mg/L) by prolonged immersion. Fish were left in the solution for 10 min at least following cessation of opercular movements. Fish were next decapitated to ensure brain death.

RNA extraction and cDNA synthesis

Total RNA for analysis of gene expression was extracted from the samples using Extract-all reagent (Eurobio; Courtaboeuf, Essonne, France) combined with the Zymo Direct-zol™ RNA MiniPrep Kit, following recommendations from the supplier. Genomic DNA was removed using

the DNA-free Kit (MoBio Laboratories Inc.; Carlsbad, CA, USA). The quantity, purity, and quality of RNA were assessed using an ND-1000 NanoDrop® spectrophotometer (Thermo Scientific Inc.; Waltham, MA, USA) and by electrophoresis using an Agilent Bioanalyser 2100 (Agilent Technologies Inc.; Santa Clara, CA, USA). Only samples with an RNA integrity number (RIN) greater than 7 were measured using real-time quantitative PCR (qPCR). RNA samples were stored at -80°C until use.

Synthesis of cDNA was carried out using 0.5 μg of DNase-treated total RNA with an iScript™ cDNA Synthesis kit (Bio-Rad Laboratories Inc.; Hercules, CA, USA). Total reaction was carried out in a final volume of 20 μL containing 5 μL of sample, 4 μL 5 \times iScript™ Reaction Mix, containing oligo(dT), random primers and RNaseA inhibitor, 1 μL iScript™ Reverse transcriptase, and 10 μL RNase/DNase free water. The cDNA synthesis reaction was incubated for 5 min at 25°C followed by 30 min at 42°C and terminated by incubation for 5 min at 85°C to inactivate the enzyme. Reverse transcription (RT) was performed using a Thermo-cycler TC-152 (Techne Barloworld Scientific; Stone, Staffordshire, UK). cDNA was stored at -20°C until use. RT negative controls were performed on each sample.

Quantitative real-time RT-PCR analysis

The analysis of Hb gene expression at different developmental stages and in different tissues of European sea bass was carried out by quantitative RT-PCR using the primers given in Table 1. Standard curves were estimated

for each primer pair using serial dilutions (from 1/10 to 1/270) of a pool of cDNA. Efficiencies of qPCR for each pair of primers ranged from 95 to 100% with $R^2 > 0.99$. Primers were designed using Primer 3 plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>).

Expression of the Hb genes was quantified using the iCycler MyiQ™ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories Inc.). Each sample was run in triplicate in a final well volume of 15 μL containing 5 μL cDNA (1/30 dilution) and 10 μL of reaction mix, composed of 0.5 μL of each primer (10 mM), 1.5 μL RNase/DNase free water, and 7.5 μL iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc.) containing antibody-mediated hot-started iTaq DNA polymerase, dNTPs, MgCl_2 , SYBR® Green I dye, enhancers, stabilizers, and fluorescein. Negative controls (non-template control) were systematically included in each plate. RT negative controls were also used as template to ensure the absence of residual DNA contamination. The qPCR profiles contained an initial activation step at 95°C for 2 min, followed by 39 cycles: 5 s at 95°C and 20 s at annealing temperature (60°C for LA-*Hba1*, LA-*Hba2*, LA-*Hbβ1*, MN-*Hba1*, MN-*Hba2*, MN-*Hbβ1*, MN-*Hbβ2*, MN-*Hbβ3*, MN-*Hbβ4*, and reference genes; 62°C for MN-*Hbβ5* and MN-*Hbβ6*; and 70°C for MN-*Hba3*, MN-*Hba4*, and MN-*Hba5*). Annealing temperatures for each couple of primers were fixed by testing PCR condition on plasmids containing the different Hb cDNAs. Following this procedure, we determined qPCR condition allowing specific amplification of each Hb cDNA subtype. After the amplification phase, a melting curve

Table 1 Specific primers used for real-time amplification of Hb and reference (18 S and EF1 α) genes

Gene	Accession numbers	Forward primer sequences (5'–3')	Reverse primer sequences (5'–3')
LA- <i>Hba1</i>	KX196178	CAGTGGGACAGGATCTTGAAGT	GGTGATGGGTGGAATCAATC
LA- <i>Hba2</i>	KX196180	TTTCCCATGAGAGAGCAGGT	TCAGATGCGCTTCTTAGGATGT
MN- <i>Hba1</i>	KX196190	GGCCAGGATGCTGACTGTA	CCAGCAAGGTCATCCATCTT
MN- <i>Hba2</i>	KX196188	CCTGCCAACTTCAAGATCTG	TTTCTCAGACAAGGCACGAG
MN- <i>Hba3</i>	KX196184	ACAGACAAGATGACCAGTCTCACT	GCCAATGTCCTCTGCCTTC
MN- <i>Hba4</i>	KX196183	ACAGACAAGATGACCAGTCTCACT	GCCAATGTCCTCTGCCTTT
MN- <i>Hba5</i>	KX196181	ACAGACAAGATGACCAGTCTCACA	GCCAATGTCCTCTGCCTTC
LA- <i>Hbβ1</i>	KX196179	CCGACAACCTTCAAACCTGCT	CCTGCGTCTCTGGTGTGAAG
MN- <i>Hbβ1</i>	KX196191	TGATTTGAGCAAAGCTCTGAA	CATGGACGACATCAAGAACG
MN- <i>Hbβ2</i>	KX196189	GTCAGCCAGCAGCTGAAAT	GCAGCTCTTCCAGGTGTCT
MN- <i>Hbβ3</i>	KX196187	CAGAAGCTTTGGCAAGAGTG	GCTGTACTTTGGCGTTACC
MN- <i>Hbβ4</i>	KX196186	GTCGTTTACCCTGGACTCA	GTTTTGCGACCATCGGATTT
MN- <i>Hbβ5</i>	KX196185	ACCATCCAGGACATCTTCTCT	GTTTTGCGACCAACGGATTC
MN- <i>Hbβ6</i>	KX196182	ACCATCCAGGACATTTTCTCC	GTTTTGCGACCAACGGATTC
18 S	AY831388.1	TGGTTCCTTTGATCGCTCTT	AGCAGCTCGTTGGCATGTAT
Ef1 α	AJ866727.1	GCTTCGAGGAAATCACCAAG	CAACCTTCCATCCCTTGAAC

Genbank Accession numbers are also given

was performed to confirm the amplification of a single product in each reaction.

For each sample, the corresponding Cq (Quantification cycle) value was determined automatically using “Gene Expression Module” of CFX Manager software (Bio-Rad Laboratories Inc.). Cq is the cycle number required to yield a detectable fluorescence signal. The relative quantity of messenger was normalized with the $\Delta\Delta C_t$ method using the same CFX Manager software. Reference genes were used to correct for loading differences or other sampling variations present in each sample. The 18 S gene was chosen as reference gene for eggs, larvae, and juvenile tissues, while elongation factor 1-alpha (*ef1a*) and 18 S were used as reference genes in adult tissues. These reference genes were used, since they did not show any significant variation of expression between samples (relative standard deviation <5% among samples). Significant differences of expression among developmental stages and among tissues were analyzed by one-way ANOVA using Tukey’HSD as post-hoc test ($p < 0.05$). Expression data were Log transformed to fit a normal distribution. Heat map was used to illustrate relative expression of each Hb gene during the fish development and among tissues. To this end, normalized data were subjected to hierarchical clustering (distance metric selection: Pearson noncentered) and a heat map was generated using MeV software (Multi Experiment Viewer; <http://www.tm4.org>).

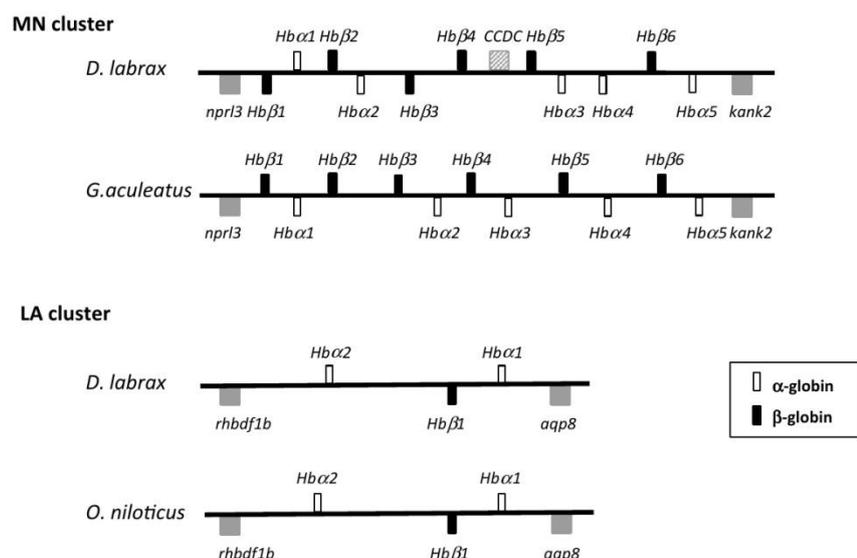
Results

Genomic organization of hemoglobin gene clusters

The MN and LA clusters in European sea bass were shown to contain a total of seven Hb α and seven Hb β genes (Fig. 1). As shown in stickleback (*Gasterosteus aculeatus*), the MN cluster included five Hb α genes annotated MN-*Hba1* to MN-*Hba5* and six Hb β genes annotated MN-*Hbb1* to MN-*Hbb6* following the suggested nomenclature (Opazo et al. 2013). The cluster consisted of about 42 kb from the first codon of MN-*Hba5* to the stop codon of MN-*Hbb1*. Whereas stickleback MN-Hb α and β genes were found on reverse and forward strands, respectively, these genes were indifferently located on either strands in European sea bass (Fig. 1). A gene exhibiting high homology with the CCDC (coiled-coil domain-containing protein) gene family was predicted on the reverse strand between MN-*Hbb4* and MN-*Hbb5*.

The LA cluster included two Hb α and one Hb β genes, annotated LA-*Hba1*, LA-*Hba2*, and LA-*Hbb1* (Fig. 1). These three genes spanned a sequence length of about 14 kb from the first codon of LA-*Hba2* to the stop codon of LA-*Hba1*. The Hb α genes of the LA cluster were encoded by one strand, whereas *Hbb1* was encoded by the reverse strand. LA-*Hba1* and LA-*Hbb1* were organized head to head, whereas LA-*Hba2* and LA-*Hbb1* were arranged tail to tail. The organization of the LA cluster was conserved between the European sea bass and tilapia (*Oreochromis niloticus*).

Fig. 1 Genomic organization of the European sea bass MN and LA clusters. Genomic structures of the *D. labrax* MN and LA clusters are compared with *G. aculeatus* and *O. niloticus*, respectively. The genes in the forward and the reverse orientations are shown boxed above and below the line, respectively. Hb α and Hb β genes are represented by white and black boxes, respectively. Flanking genes are shown in grey and the inserted gene is shaded



Structure of European sea bass hemoglobin genes

The complete gene sequences and deduced coding sequences were confirmed by cDNA cloning. The European sea bass Hb α and Hb β genes typically consist of three exons separated by two introns (Table 2). While all the Hb β genes coded for proteins of 147 aa, the size of the proteins encoded by Hb α genes ranged between 141 and 144 aa.

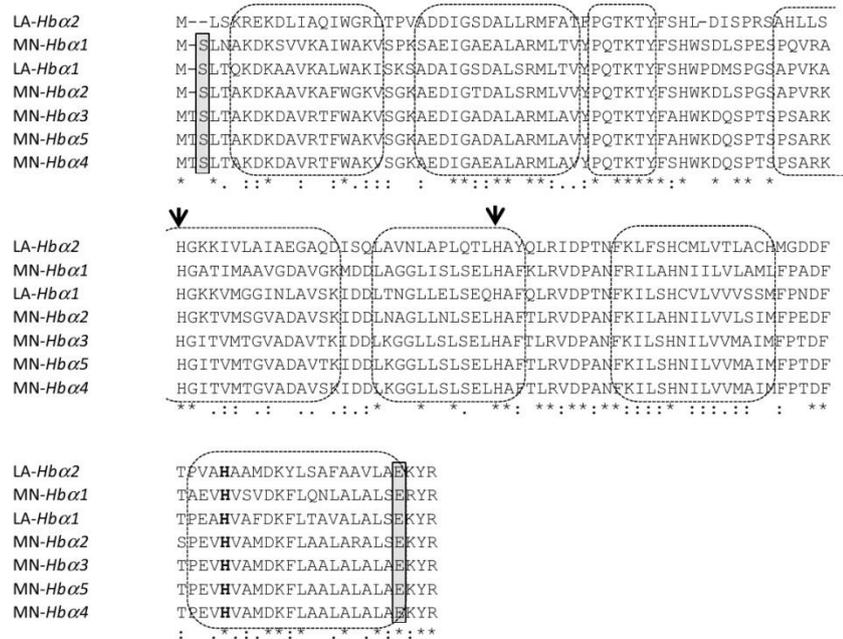
Analysis of putative key residues in European sea bass Hb

The histidine (His) residues deemed to be implicated in oxygen binding were conserved in the predicted Hb α and Hb β globins (Figs. 2, 3). The His residue at position 122 of Hb α globins seems to be involved in the Bohr effect (Bellelli and Brunori 2011) and was found in all Hb α sequences of European sea bass. Similarly, the C-terminal His residue of Hb β responsible for the Bohr

Table 2 Lengths in base pairs (bp) of exons, introns, predicted coding (CDS), and amino acid (AA) sequences for the European sea bass Hb α and Hb β genes

Genes	Length (bp)						
	Exon 1	Intron 1	Exon 2	Intron 2	Exon 3	CDS	AA
LA-Hba1	95	629	208	355	129	432	143
LA-Hba2	92	1103	205	1010	129	426	141
MN-Hba1	95	107	208	176	129	432	143
MN-Hba2	95	105	208	506	129	432	143
MN-Hba3	98	422	208	180	129	435	144
MN-Hba4	98	110	208	184	129	435	144
MN-Hba5	98	348	208	180	129	435	144
LA-Hbb1	92	91	223	246	129	444	147
MN-Hbb1	92	142	223	94	129	444	147
MN-Hbb2	92	110	223	97	129	444	147
MN-Hbb3	92	737	223	106	129	444	147
MN-Hbb4	92	143	223	99	129	444	147
MN-Hbb5	92	146	223	99	129	444	147
MN-Hbb6	92	145	223	99	129	444	147

Fig. 2 Amino acid sequence alignment of European sea bass Hb α . The amino acid similarities between all proteins are indicated as follows: *asterisks* indicate identical amino acids, *colons* or *dots* indicate similar amino acids and *empty spaces* represent absence or a low degree of similarity. Predicted alpha-helices are shown in *dashed boxes*. Histidine residues included in oxygen binding are indicated by *arrows*. Amino acids potentially involved in the Bohr effect are boxed in *grey*. The residues responsible for the Bohr effect are indicated in *bold*



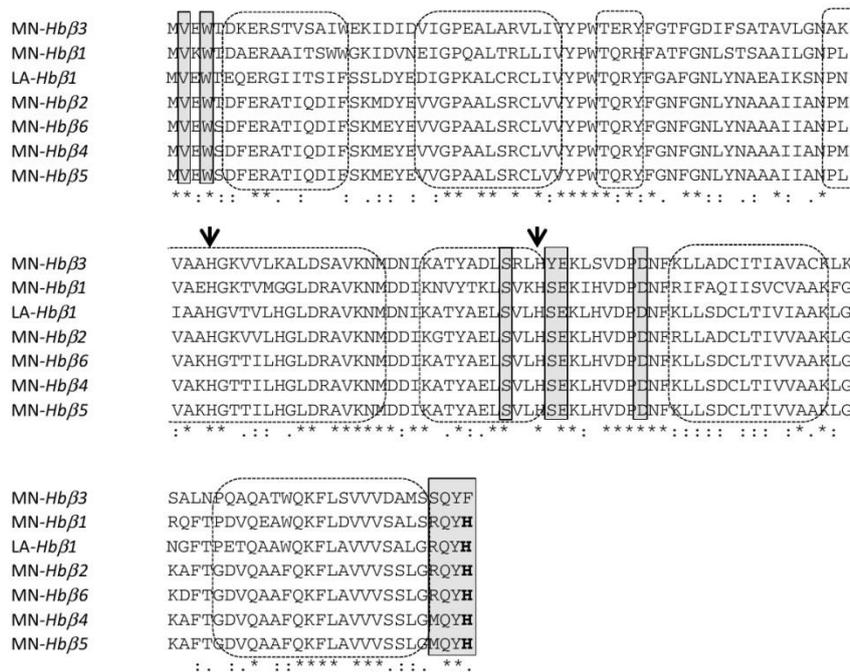


Fig. 3 Amino acid sequence alignment of European sea bass Hb β . The amino acid similarities between all proteins are given as follows: *asterisks* indicate identical amino acids, *colons* or *dots* indicate similar amino acids, and *empty spaces* represent absence or low degree of similarity. Predicted alpha-helices are shown in *dashed boxes*. His-

tidine residues included in oxygen binding are indicated by *arrows*. Amino acids potentially involved in the root effect are boxed in *grey*. The C-terminal His residue responsible for the Bohr effect is indicated in *bold*

effect was also present in all Hb β sequences, except for MN-Hb β 3, where it is substituted by a Phe residue. European sea bass Hb α and Hb β genes possessed all the putative residues responsible for the Root effect, such as Val2 β , Ser2 α , Trp4 β , Ser90 β , Ser94 β , Glu95 β , Asp95 α , Asp101 β , Glu140 α , Arg144 β , Gln145 β , Tyr146 β , and His147 β (Bonaventura et al. 2004), except MN-Hb β 3 which was devoid of Ser94 β and Arg144 β .

Phylogenetic analysis of European sea bass Hb

Phylogenetic analysis was performed based on the deduced Hb α and Hb β amino acid sequences of the European sea bass and other teleost and non-teleost species (Supplementary file 3). The data set was used to calculate the best Hb tree using maximum likelihood (ML) approach and the Bayesian MCMC methods. Since the two methods led to similar phylogenies, we only present the tree obtained with the Bayesian approach (Figs. 4, 5), where the nodes appeared to be more robust, i.e., posterior probabilities above 0.5. The phylogenetic analysis shed light on the ortho- versus paralogy relationships of the *D. labrax* genes with those of other species. The tree allowed to group Hb α

in many clades, two of them composed with genes from the LA cluster. Hb β are grouped in four major clades including three clades related to the MN cluster and one related to the LA cluster.

Analysis of the MN clades revealed that European sea bass MN-Hb α 3–5 on one hand and MN-Hb β 2 and MN-Hb β 4–6 on the other clustered together in the MN-Hb α and MN-Hb β clades, respectively, that is consistent with the high coding sequence similarities (Supplementary file 4).

Expression pattern of Hb genes during developmental stages

The temporal expression patterns of the Hb genes varied considerably, but some common features can be noted (Fig. 6; Supplementary file 5). During embryogenesis (12 and 60 h post-fertilization; hpf), relative expression levels of both Hb α and Hb β genes were very low or not detected (Cq > 30) (Supplementary file 6). Several genes (notably LA-Hb α 2, MN-Hb α 2, MN-Hb β 2, MN-Hb α 3, MN-Hb β 5, MN-Hb β 4, and MN-Hb α 5) exhibited a substantial increase in expression between 60 hpf and 5 day post hatching

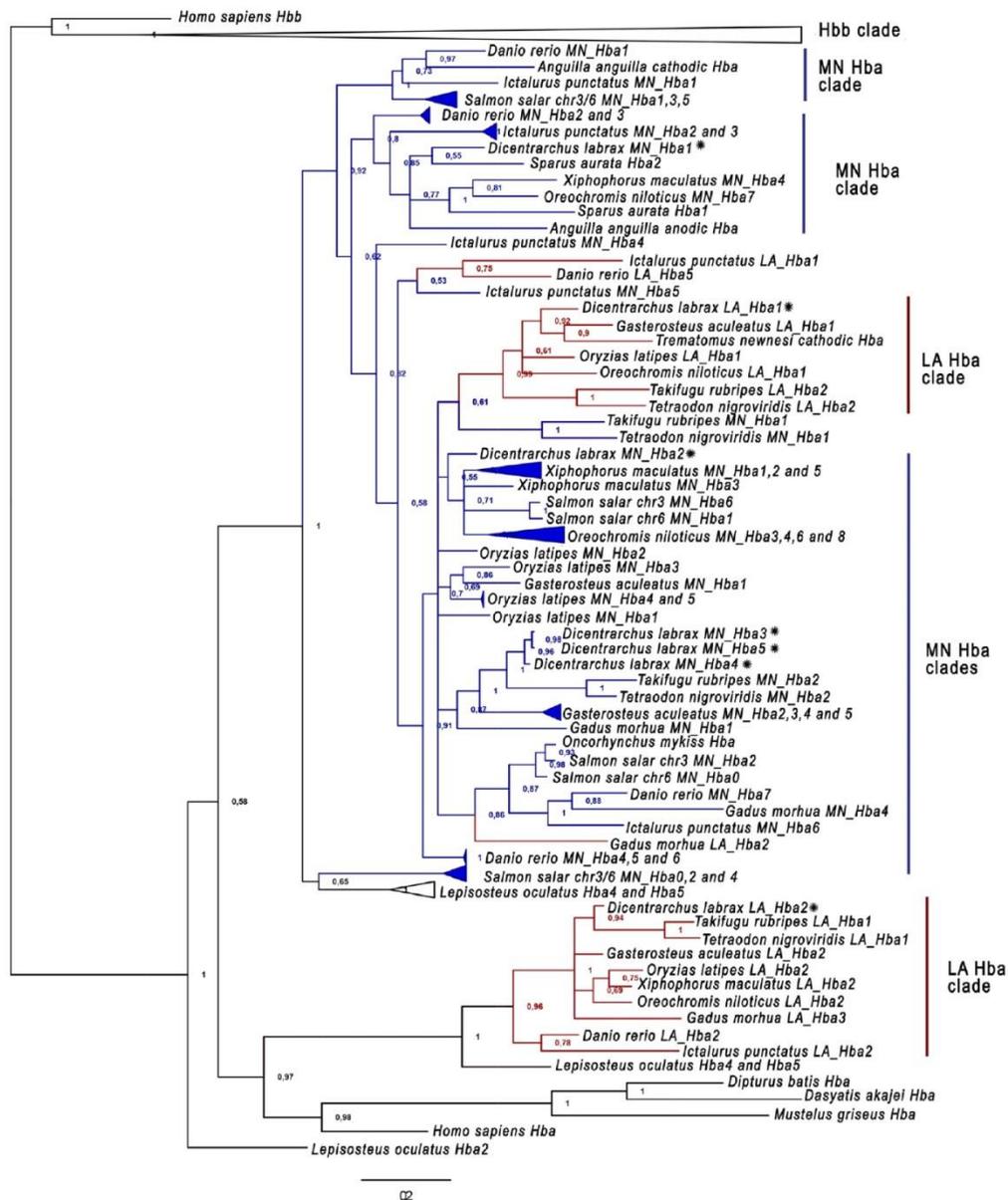


Fig. 4 Bayesian phylogenetic tree of Hb α sequences. Phylogenetic analysis was performed on both Hb α and Hb β following the method detailed in “Materials and methods”, but only Hb α sequences are presented here. European sea bass Hb α genes are marked with asterisks. Hb α amino acid sequences from other species were issued from the translation of nucleotide sequences listed in a previous work by

Opazo and collaborators (10). Monophyletic clades are represented by a triangle. Clades containing Hb β genes of European sea bass and other species have been collapsed in an Hb β clade. Branches are color coded according to the location of the genes: MN-linked Hbs are shown in blue and LA-linked Hbs are shown in red

(dph). The expression levels of several genes (LA-Hb α 2, LA-Hb β 1, MN-Hb β 1, MN-Hb α 2, MN-Hb β 4, MN-Hb α 5, MN-Hb α 4, and MN-Hb β 6) increased exponentially between 5 and 45 dph, with a key step between 26 and 45

dph. After 45 dph, expression increased substantially for some genes (LA-Hb β 1, MN-Hb α 2, LA-Hb α 1, MN-Hb α 1, MN-Hb β 2, and MN-Hb α 2) during the juvenile stage, while it dropped significantly for others (MN-Hb β 4, MN-Hb α 5,

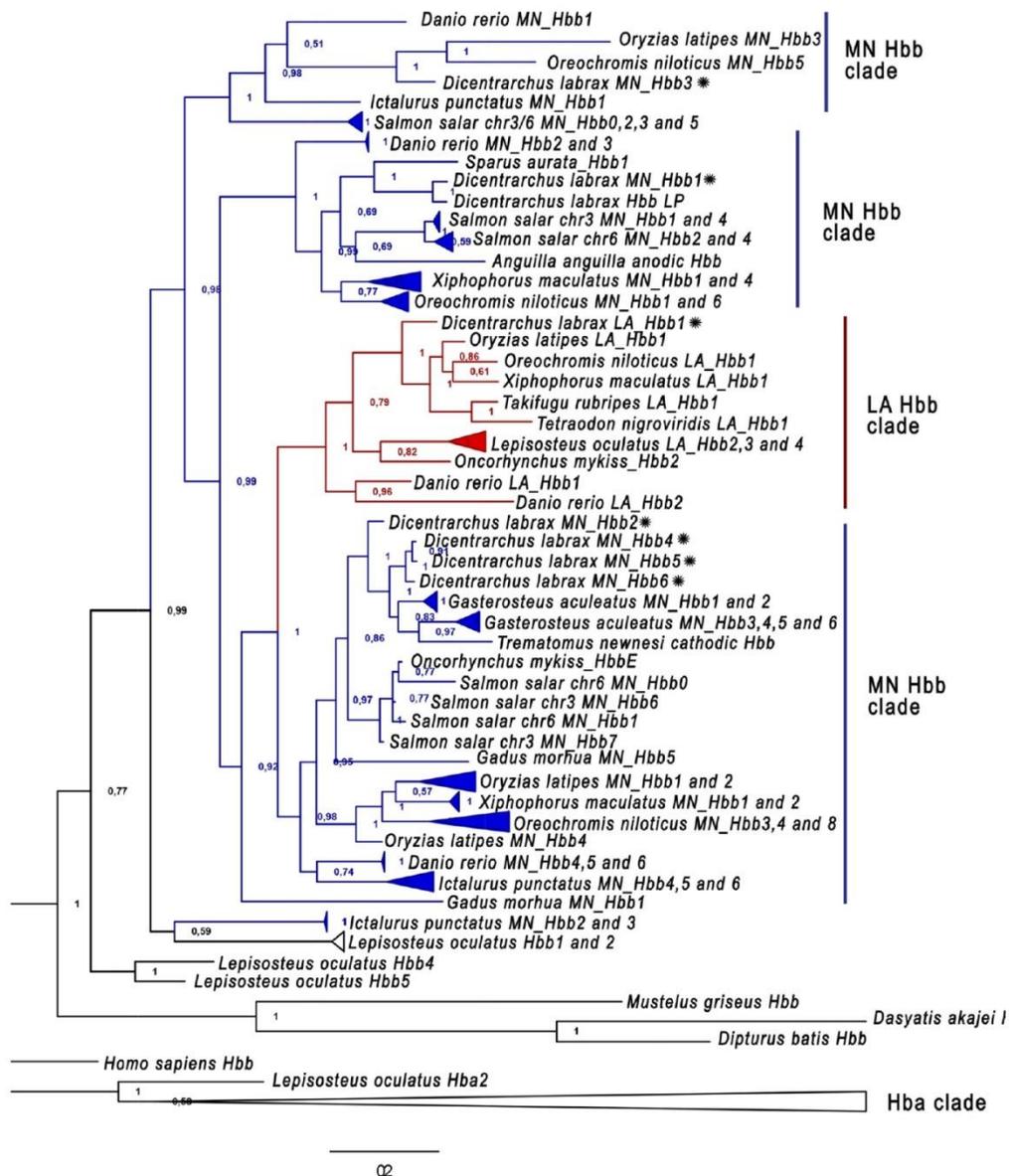


Fig. 5 Bayesian phylogenetic tree of Hb β sequences. Phylogenetic analysis was performed on both Hb α and Hb β following the method detailed in “Materials and methods”, but only Hb β sequences are presented here. European sea bass Hb β genes are marked with asterisks. Hb β amino acid sequences from other species were issued from the translation of nucleotide sequences listed in a previous work by

Opazo and collaborators (10). Monophyletic clades are represented by a triangle. Clades containing Hb α genes of European sea bass and other species have been collapsed in an Hb α clade. Branches are color coded according to the location of the genes: MN-linked Hbs are shown in blue and LA-linked Hbs are shown in red

MN-Hb α 4, and MN-Hb β 6 genes). MN-Hb β 3 was very weakly expressed (Cq > 35) at all the developmental stages investigated.

Tissue expression pattern of Hb genes

Based on the order of Cq value magnitude, MN-Hb α 1 and MN-Hb β 1 exhibited the highest expression levels in the adult fish, irrespective of the tissue considered

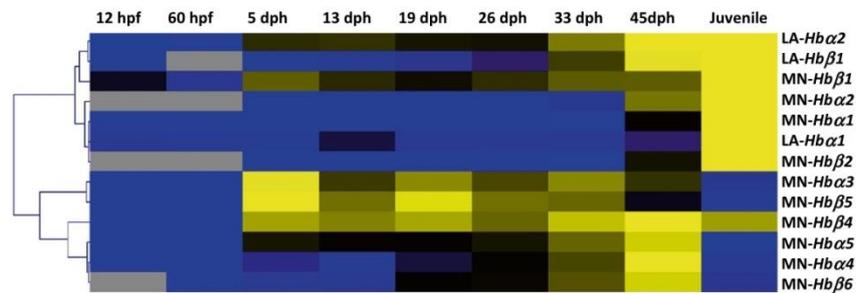


Fig. 6 Relative expression profiles of Hb α and β genes during development of European sea bass. The relative expression profiles of Hb α and β genes during development of European sea bass were determined using qPCR. MN-Hb β 3 expression profile is not shown since Cq values were up to 35 from 12 hpf until juvenile stage. Following normalization with 18 S rRNA levels, relative expression of each

gene throughout the life stages (shown in Supplementary file 5) was subjected to hierarchical clustering and a heat map was generated. Relative expression level of each gene is depicted by a color scale: blue and yellow indicating respective low and high relative expression levels among developmental stages. Hpf hours post-fertilization, dph days post hatching

(Supplementary file 8). In contrast, genes MN-Hb α 3, MN-Hb α 4, and MN-Hb α 5, as well as MN-Hb β 4, MN-Hb β 5, and MN-Hb β 6, were expressed at very low levels in the different adult tissues investigated. MN-Hb α 1, MN-Hb β 1, and MN-Hb β 3 showed the highest expression levels in the head kidney and lesser levels in the spleen (Fig. 7; Supplementary file 7). MN-Hb α 2 and MN-Hb β 2 genes showed the highest transcript levels in spleen. Several Hb genes were expressed at low levels in other non-hematopoietic tissues, such as brain, heart, intestine, and gills.

Discussion

To gain knowledge about Hb genes in the European sea bass, we assembled and confirm by transcript sequencing 14 Hb genes that we assigned onto two genomic LA and MN clusters localized, respectively, on the linking

group (LG) 1B (Sequence ID: emb|CBXY010008361.1) and LG 8 (Sequence IDs: emb|CBXY010016043.1-emb|CBXY010016046.1) of the first assembly of the European sea bass genome (Tine et al. 2014).

Three genes (2 Hb α and 1 Hb β) were located adjacent to the conserved *aqp8* and *lcmt1* genes of the LA cluster in teleosts (Opazo et al. 2013). As observed in platyfish, medaka and tilapia, the two Hb α genes were separated by the Hb β gene located on the opposite strand (Opazo et al. 2013). The similar organization of this cluster suggests that the LA α - and β -Hb genes of European sea bass are 1:1 orthologs with those in platyfish, medaka, and tilapia, in agreement with the phylogenetic analysis which grouped the three LA genes within the respective LA clades of these species. The LA clades also included orthologous Hb genes from green spotted puffer and fugu, wherein these genes have been reversed in the LA cluster. We noted that the ortholog of sea bass LA-Hb β 1 seems to have been lost in stickleback, which yet exhibits high overall orthologous

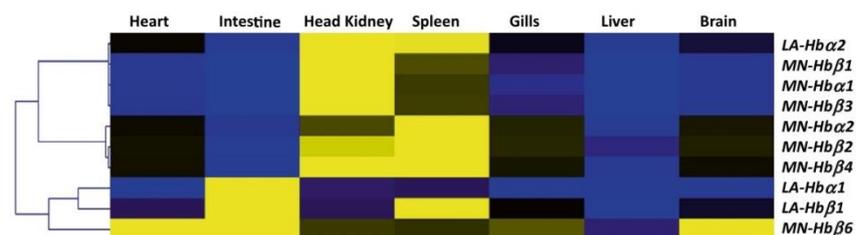


Fig. 7 Expression pattern of Hb α and β genes in different tissues of adult European sea bass. The relative expression levels of Hb α and β genes were determined in different tissues of European sea bass using qPCR. MN-Hb α 3, MN-Hb α 4, MN-Hb α 5, and MN-Hb β 5 expression profiles are not shown, since Cq values were up to 33 among the tissues. Following normalization with 18 S and Ef1a levels, rel-

ative expression of each gene (shown in Supplementary file 7) was subjected to hierarchical clustering and a heat map was generated. Expression level of each gene is depicted by a color scale: blue and yellow indicating respective low and high relative expression levels among the different tissues

sequence homologies with European sea bass (Tine et al. 2014). Our data also revealed that 11 Hb genes (five Hb α and six Hb β) were flanked by *npr13* and *kank2* genes similar to the MN cluster in teleosts. Intriguingly, a *CCDC106-like* gene was found inserted between MN-*Hb β 4* and MN-*Hb β 5*, and the specific functional role of this potential p53-interacting partner (Zhou et al. 2010) warrants further studies. It is accepted that distinct organization between teleost species in the LA and, especially, MN clusters results from lineage-specific changes in gene content via repeated duplication and deletion events (Opazo et al. 2013). Our phylogenetic analysis suggests that two gene groups (MN-*Hba3*, MN-*Hba4*, MN-*Hba5* and MN-*Hb β 2*, MN-*Hb β 4*, MN-*Hb β 5*, MN-*Hb β 6*) each consist of close paralogous copies and are probably the results of recent duplication events that occurred in the Moronidae lineage. In comparison, MN-*Hba4* and MN-*Hb β 2* branch deeper in the phylogenetic trees, suggesting that they might be ancestral with regard to their respective paralog copies located at the tips of their branch. In any case, from an evolutionary point of view, the MN cluster seems to be largely more dynamic than the LA one, which was suggested to have been lost in Atlantic salmon (Quinn et al. 2010).

The LA-Hb genes of European sea bass did not show expression patterns specific to the early life stage, contrary to the embryonic Hb genes in medaka, zebrafish, and tilapia (Maruyama et al. 2004; Tiedke et al. 2011; Opazo et al. 2013). However, the three MN-*Hba3–5* genes and three MN-*Hb β 4–6* genes exhibited high larval expression compared to juvenile and adult levels. Intriguingly, the expression patterns throughout development were similar in the closely linked MN-*Hba4*, MN-*Hba5*, and MN-*Hb β 6* on one hand and in the neighbour genes MN-*Hba3* and MN-*Hb β 5* on the other hand, suggesting that these Hb α and Hb β genes likely display common elements or/and mechanisms of regulation. With the new assembly we built, it will be now possible to investigate further the existence of such regulation elements. The common feature of the Hb genes significantly expressed just after hatching was the increased expression from 60 to 5 dph. These data suggest that around the 60 hpf stage oxygen supplied by simple diffusion throughout the embryonic tissues may be sufficient to fulfill physiological requirements in European sea bass. The absence of significant Hb gene expression in fertilized eggs of sea bass was consistent with data obtained in another seawater species, the Atlantic cod, but contrasted with the expression profiles of embryonic genes observed in medaka and zebrafish (Maruyama et al. 2004; Wetten et al. 2010; Tiedke et al. 2011). It has been shown that the expression of embryonic Hb genes in zebrafish starts with primitive erythropoiesis, which takes place in the intermediate cell mass around 15 hpf (Kulkeaw and Sugiyama 2012). The stage at which intermediate cell mass develops is not

known in European sea bass; however, our data suggested that primitive erythropoiesis resulting in matured erythrocytes that are replete with Hb occurs after 60 hpf. Moreover, European sea bass Hb genes that did not show high mRNA levels during the first stage of larval development (LA-*Hba1*, LA-*Hba2*, MN-*Hba1*, MN-*Hba2*, LA-*Hb β 1*, MN-*Hb β 1*, MN-*Hb β 2* and MN-*Hb β 3*) showed a progressive increase in gene expression from 26 dph to juvenile/adult stage. Most of these “late expressed” Hb genes exhibited a significant rise in expression between 26 and 33 dph. Interestingly, this developmental window was previously associated with a switch of global transcriptomic profile related to a key physiological step in the development of European sea bass larvae (Darias et al. 2008). This increase in Hb gene expression was likely related to the transition from primitive to definitive erythropoiesis, which is associated with the emergence of the hematopoietic function in the kidney and spleen during larval development of teleosts (Brownlie and Zon 1999). Indeed, erythropoietic activity has been detected in the spleen of European sea bass before 40 dph (Quesada et al. 1994). In zebrafish, the switch from primitive to definitive erythropoiesis has been shown to start around 10 dph (Tiedke et al. 2011). The earlier expression of Hb embryonic genes, as well as the earlier period of transition between embryonic and adult Hb expression observed in zebrafish, can be explained by faster developmental processes in this species.

The present investigation revealed that head kidney and, to a lesser extent, spleen are the major tissues in which most of European sea bass Hb genes were expressed at adult stage. Consistently, spleen, kidney (mesonephros), and head kidney (pronephros) are the sites of erythropoiesis in European sea bass (Esteban et al. 1989; Quesada et al. 1994) and other teleosts species, such as catfish (*Ictalurus punctatus*) (Feng et al. 2014) and euryhaline flounder (*Platichthys flesus*) (Lu et al. 2011). MN-*Hba1*, MN-*Hb β 1* and to a lesser extent LA-*Hba2*, MN-*Hb β 3*, MN-*Hba2*, and MN-*Hb β 2* were the most predominantly expressed genes at the adult stage. The expression of three different Hb α and three different Hb β genes in adult European sea bass is consistent with the identification by gel electrophoresis of five major Hb tetramers which likely comprise different combinations of these subunits (Perez and Maclean 1976). Most of the European sea bass Hb genes exhibited moderate expression level in non-hematopoietic tissues, such as brain, heart, liver, intestine, and gill. Consistently, expression of Hb in several vertebrates has been found in many non-erythroid cells (Saha et al. 2014), in which it may facilitate tissue oxygen transport or increase cellular oxygenation (Nishi et al. 2008; Biagioli et al. 2009; Tezel et al. 2010). In channel catfish, the expression of Hb genes in the intestine is associated with the respiratory function of this tissue (Feng et al. 2014). Moreover, antimicrobial

activity has also been attributed to Hb β in gills and skin of channel catfish exposed to parasite (Ullal et al. 2008). Such antimicrobial activity is suggested to be also associated with the increase of Hb β -like (highly similar to the present MN-*Hb β 1* cDNA sequence) expression in the epithelium of European sea bass submitted to an acute stress (Terova et al. 2011).

Compared to mammals, the functional property of fish Hbs known as Root effect is a decrease in both Hb-oxygen affinity and cooperativity at low blood pH. Even if the Root effect cannot be based solely on the presence or absence of residues, Asp95 α and Asp101 β , that are considered as the minimal structural requirement for the Root effect (Mazzarella et al. 2006), were conserved in the different European sea bass Hb genes. Moreover, Ser94 β , Arg144 β , and His147 β also known to be important amino acid residues involved in Hb-oxygen binding and particularly in the Root effect (Mylvaganam et al. 1996; Bonaventura et al. 2004) were conserved in the different European sea bass Hb β genes except for MN-*Hb β 3*. The substitution of key functional residues is partially found in *Hb β 1*, *Hb β 4*, and *Hb β 5* of catfish (Feng et al. 2014) and in trout Hb I (Gabbianelli et al. 2004). Functional characterization of the European sea bass Hb proteins related to water temperature, oxygen affinity, and pH sensitivity will be necessary to determine potential specific functional properties.

Conclusion

Even though the composition the Hb tetramers in European sea bass Hb and their functional properties are not known, present data support the evidence that European sea bass is capable of producing different types of Hb with potential different functional properties over their life cycle. The positive relation between the diversity of Hb genes expressed in fish species and the diversity of their living environments suggests an important role played by these oxygen binding proteins in the ability to cope with environmental constraints. This appears to be particularly relevant in the European sea bass, which inhabits coastal nurseries with large fluctuations in environmental conditions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Early exposure to chronic hypoxia induces short and long-term regulation of hemoglobin gene expression in European sea bass (*Dicentrarchus labrax*)

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Key-words: hypoxia, hemoglobin, early exposure, European sea bass

Summary statement: This paper gives new insights related to hemoglobin gene regulation by chronic moderate hypoxia at different ontogenetic stages in European sea bass (*Dicentrarchus labrax*).

Summary

European sea bass (*Dicentrarchus labrax*) inhabits coastal waters and may be exposed to hypoxia at different life stages, requiring physiological and behavioral adaptation. In the present study, we attempted to determine whether regulation of hemoglobin (Hb) gene expression plays a role in the physiological response to chronic moderate hypoxia in whole larvae and hematopoietic tissues (head kidney and spleen) of juveniles. We also tested the hypothesis that hypoxia exposure at the larval stage could induce a long-term effect on the regulation of Hb gene expression. For this purpose, *D. labrax* were exposed to a non-lethal hypoxic condition (40% air saturation) at the larval stage from 28 to 50 days post hatching (dph) and/or at the juvenile stage from 196 to 296 dph. Data obtained on larvae indicate that hypoxia induced a subtype-specific regulation of Hb gene expression, with significant decrease of MN-Hb α 3, MN-Hb β 4 and MN-Hb β 5 and increase of MN-Hb α 2, LA-Hb α 1 and LA-Hb β 1 transcript levels. Hypoxia did not induce regulation of Hb gene expression in juveniles, except in the head kidney for those that experienced hypoxia at the larval stage. The latter exhibited a significant hypoxia-induced stimulation of MN-Hb α 2, LA-Hb α 1 and LA-Hb β 1 gene expression, associated with stimulation of the *PHD-3* gene involved in the hypoxia-inducible factor oxygen-sensing pathway. We conclude that subtype- and stage-specific regulation of Hb gene expression plays a role in the physiological response of *D. labrax* to cope with hypoxia and that early exposure to low oxygen concentration has a long-term effect on this response.

List of abbreviations used:

Aqp8	Aquaporin-8
CJ	Control juvenile group
CL	Control larval group
Cq	Quantification cycle
Dph	Days Post Hatching
<i>Ef1α</i>	Elongation Factor-1, isoform alpha
Hb	Hemoglobin
HIF	Hypoxia Inducible Factor
HJ	Hypoxia juvenile group
HL	Hypoxia larval group
HRE	Hypoxia responsive elements
Lcmt1	Leucine carboxyl methyltransferase 1
Mpg	N-methylpurine-DNA glycosylase
Nprl3	Nitrogen permease regulator-like 3
<i>PHD-3</i>	Prolyl Hydroxylase Domain-Containing Protein 3
PIT	Passive Integrated Transponder
qPCR	Quantitative Polymerase Chain Reaction
RGR	Relative growth rate
RT	Reverse Transcription

Introduction

In the context of global change, low dissolved oxygen (O₂) concentration (hypoxia) has become a major problem for marine fish species (Breitburg et al., 2009; Roessig et al., 2004; Somero, 2012). Under the combined effects of eutrophication and global warming, hypoxic conditions are especially prevalent in estuarine and coastal regions, towards which fish larvae may drift due to tidal currents (Pihl et al., 1992). Oxygen depletions are likely to affect survival and the success of larval development, which is considered to be the most sensitive stage in the fish life cycle (Ishibashi et al., 2007; Levin et al., 2009). Hypoxia is characterized by the degree of O₂ depletion and the exposition period, with different impacts on species and individuals depending on their coping capacities (Ekau et al., 2010). Fish acclimatization to hypoxia consists of behavioral, physiological, biochemical and molecular adjustments (Ekau et al., 2010; Richards, 2011; Zhu et al., 2013). Physiological responses to low-oxygen conditions include modifications of the respiratory cascade to enhance O₂ extraction and transport (Nilsson, 2007), as well as metabolic regulation in order to limit O₂ demands (Hopkins and Powell, 2001). In fish, as in other vertebrates, hemoglobins (Hb) are involved in O₂ transport from respiratory organs to tissues, and contribute to overcoming the effects of reduced oxygen availability (Gollock et al., 2006; Richards, 2011). Hb molecules consist of two alpha- and two beta-globin subunits and each subunit contains a heme group responsible for the binding of O₂. Teleost fish species have the characteristic of expressing several Hb α and Hb β genes which are grouped into two unlinked clusters labeled "MN" (gene cluster flanked by the *mpg* and *npr13* genes) and "LA" (cluster flanked by the *lcmt1* and *aqp8* genes) located on two separate chromosomes resulting from the teleost-specific genome duplication (Hardison 2008; Opazo et al. 2013). The gene products lead to multiple Hb isoforms adapted to different metabolic demands and/or environmental O₂ availability throughout the different ontogenic stages (Weber and Jensen, 1988; Opazo et al., 2012a). No common pattern emerged from studies that investigated the effect of hypoxia on Hb proteins and transcripts levels (Lai et al., 2006; Roesner et al., 2006; Roesner et al., 2008; Val et al., 2015; Wawrowski et al., 2011; Zi-sheng et al., 2011). The hypoxia response of Hb seems to depend on the fish species, the degree of stress and ontogenetic stage.

The physiological response of fish to hypoxia can also depend on their previous environmental experience (Ho and Burggren, 2012; Robertson et al., 2014). This historical dimension of hypoxia tolerance can be related to developmental plasticity, i.e. the ability of organisms to respond to changes in the environment by developing phenotypes that allow them to better cope with these conditions (Bateson et al., 2014). Molecular data obtained in zebrafish (*Danio rerio*) suggest that the developmental plasticity induced by hypoxia may result from a long-lasting regulation of the Hypoxia-Inducible-Factor-1 (HIF-1) pathway (Robertson et al., 2014). In vertebrates including fish, the transcription factor HIF-1 is a molecular oxygen sensor that regulates the expression of target genes in order to ensure survival in hypoxic conditions (Gracey et al., 2001; Nikinmaa and Rees, 2005). Wawrowski et al (2011) suggested that HIF-1 regulates the expression of Hb genes in Japanese medaka (*Oryzias latipes*), by binding to hypoxia responsive elements (HRE). To date, however, involvement of Hb genes in the long-term regulation of the HIF pathway by hypoxia has never been studied.

European sea bass (*D. labrax*) is a species of high value that inhabits coastal nurseries and is therefore liable to be exposed to hypoxic events (Dufour et al., 2009; Jennings and Pawson, 1992). While effects of moderate hypoxia on metabolic and physiological parameters have been investigated in this species at larval and adult stages (Dupont-Prinet et al., 2010; Vanderplancke et al., 2015), the regulation of Hb under low oxygen availability remains unknown. The recent characterization of 14 Hb genes in *D. labrax* constitutes a valuable source of information for the investigation of Hb system regulation at different life stages (Cadiz et al., 2017). Hb genes in *D. labrax* showed stage-specific patterns, with some genes expressed during early life stage (MN-Hb α 3-5 and MN-Hb β 4-6), while others (LA-Hb α 1-2, LA-Hb β 1, MN-Hb α 1-2 and MN-Hb β 1-3) are principally expressed at juvenile or adult stages. Cadiz et al. (2017) also revealed that most of Hb genes were mainly expressed in hematopoietic tissues (head kidney and spleen) at adult stage in *D. labrax*. In this context, the first objective of the present study was to assess the regulation by moderate hypoxia of Hb gene expression in whole larvae and hematopoietic tissues (head kidney and spleen) of juvenile *D. labrax*. The second objective was to determine whether exposure of larval fish to moderate hypoxic conditions could have a long-term impact on hypoxia-induced regulation of Hb gene expression. Special attention was also paid to the expression of Prolyl

Hydroxylase Domain-Containing Protein 3 (*PHD-3* or *Egln3*), which is an actor in the HIF-1 signaling pathway.

Materials and methods

Animal rearing and treatments

D. labrax larvae were reared under normal oxygen conditions in six tanks at $15 \pm 0.4^\circ\text{C}$ water temperature and $35 \pm 0.2\text{‰}$ salinity. They were fed daily with *Artemia* according to Zambonino et al. (1996) until the end of larval development. From 28 to 50 dph, water oxygenation in three of the tanks (hypoxia larval group (HL)) was reduced to approximately 40% air saturation ($2.95 \text{ mg O}_2 \text{ L}^{-1}$), while in the other three tanks (control larval group (CL)) it was maintained at 100% saturation ($7.35 \text{ mg O}_2 \text{ L}^{-1}$). Hypoxia was generated by bubbling N_2 in a gas equilibration column placed upstream of the experimental tank. Dissolved O_2 was monitored daily using an Odeon oxygen meter (ODEON Classic OPTOD; Caudan, France). Other water quality parameters (salinity, temperature and pH) were also checked daily in each tank during the experiment. It had previously been determined that the level of hypoxia did not induce mortality; thus, genetic selection during larval exposure was avoided. It was not possible to evaluate feed ingestion throughout this experimental phase. At the end of hypoxia-exposure (50 dph), larvae were returned to normal oxygen conditions and replicate tanks were pooled in one 1 m^3 -tank per treatment (normoxia; $15 \pm 0.4^\circ\text{C}$). Fish were then fed with a commercial diet (NeoSupra; Coopérative Le Gouessant, Lamballe, Côtes-d'Armor, France). At 166 dph, 60 fish from each of the hypoxia (HL) and control (CL) tanks were selected and tagged subcutaneously (Passive Integrated Transponder; PIT-tag) for individual identification. Fish were fed with NeoGrower commercial diet (Coopérative Le Gouessant; Lamballe, Côtes-d'Armor, France) and pooled in the same 2-m^3 tank. From 196 to 296 dph, fish were separated into two tanks, including 30 juveniles from each larval group in each tank. Water oxygenation in one of the tanks was reduced to 40% air saturation (hypoxia juvenile group (HJ)), while it was maintained at 100% saturation in the other (control juvenile group (CJ)).

Growth monitoring

Larval growth was evaluated on 150 larvae per group (i.e. HL and CL, 50 larvae from each tank) from the beginning (30 dph) to the end of hypoxia exposure (50 dph). Larvae were euthanized with an excess of anesthetic (Tricaine methane-sulfonate 200 mg L⁻¹, Pharmaq; Hampshire, United Kingdom) and transferred to formaldehyde for fixation (4%) until individual weight measurement. Each juvenile from the HJ and CJ groups was weighed at 196 dph and 296 dph after light anesthesia (Tricaine methane-sulfonate 10 mg L⁻¹). Growth was estimated by calculating the relative growth rate (RGR):

$$\frac{(\text{final weight} - \text{initial weight})}{\text{initial weight}}$$

Larval and juvenile sampling

Larvae for RNA extraction were sampled at 50 dph, i.e. just before returning the hypoxic groups to normal oxygenation conditions. Six pools of eight larvae were sampled from each treatment (two pools from each tank), euthanized with an excess of anesthetic (Tricaine methane-sulfonate 200 mg L⁻¹). They were next transferred into Eppendorf reaction tubes containing *RNAlater* (Qiagen; Hilden, Germany) and placed at 4°C for 24 h, then -20°C until total RNA extraction.

Sampling of juveniles was performed at 296 dph on fish that were left undisturbed and unfed for 24 h. Sixteen juveniles from each of the two groups HJ and CJ (including eight fish from each of the larval groups HL and CL) were randomly selected and slightly anesthetized (Tricaine methane-sulfonate 20 mg L⁻¹). Then, 1 mL of blood was drawn from their caudal vein using a heparinized syringe and divided between two tubes for analysis of hematocrit and Hb concentration. The fish were then euthanized with an excess of anesthetic (Tricaine methane-sulfonate 500 mg L⁻¹) and tissues (spleen and head kidney) were immediately dissected. Blood was removed rapidly to reduce red blood cell contamination from tissues by rinsing with saline solution (NaCl 0.01%). Spleen and head kidney were rapidly placed in *RNAlater* (Qiagen) for RNA extraction.

The present work was performed within IFREMER facilities in accordance with French and European policies and guidelines of the French Animal Care Committee (Agreement number: APAFIS#5173).

Hematological parameters

Hematocrit was determined from blood samples after centrifugation at $13,000 \times g$ in capillary tubes. Hemoglobin concentration was measured using the cyanomethemoglobin method. Blood samples were added to Drabkin's reagent (Sigma-Aldrich Co.[®]; St. Louis, Mo, USA) and compared to hemoglobin standards (Pointe Scientific, 199 Inc.[®]; Canton, MI, USA). Optical density was recorded at 540 nm in a spectrophotometer.

RNA extraction and cDNA synthesis

Total RNA for analysis of gene expression was extracted from the samples (whole larvae and tissues from juveniles) using Extract-all reagent (Eurobio; Courtaboeuf, Essonne, France) combined with the Zymo Direct-zol™ RNA MiniPrep Kit, following recommendations from the supplier. Genomic DNA was removed using the DNA-free Kit (MoBio Laboratories Inc.; Carlsbad, CA, USA). The quantity, purity and quality of RNA were assessed using a ND-1000 NanoDrop® spectrophotometer (Thermo Scientific Inc.; Waltham, MA, USA) and by electrophoresis using an Agilent Bioanalyser 2100 (Agilent Technologies Inc.; Santa Clara, CA, USA). All samples showed an RNA integrity number (RIN) higher than 7 and thus could be used for real-time quantitative PCR (qPCR) analysis. RNA samples were stored at -80°C until use.

Synthesis of cDNA was carried out using 500ng of DNase-treated total RNA with an iScript™ cDNA Synthesis kit (Bio-Rad Laboratories Inc.; Hercules, CA, USA). The total reaction was carried out in a final volume of 20 μL containing 5 μL (500 ng) of sample, 4 μL 5x iScript™ Reaction Mix containing oligo (dT), random primers and RNaseA inhibitor, 1 μL iScript™ Reverse transcriptase and 10 μL RNase/DNase free water. The cDNA synthesis reaction was incubated for 5 min at 25°C followed by 30 min at 42°C and terminated by incubation for 5 min at 85°C to inactivate the enzyme. Reverse transcription (RT) was performed using a Thermo-cycler TC-152 (Techne Barloworld Scientific; Stone, Staffordshire, UK). cDNA was stored at -20°C until use. RT negative controls were performed on each sample (same reaction mix except the reverse transcriptase).

Quantitative real-time RT-PCR analysis

The analysis of gene expression in the whole larvae and in the tissues of European sea bass was carried out by qPCR using the primers listed in table 1. All *D. labrax* Hb genes and the *PHD-3* gene were investigated at the larval stage. At the juvenile stage, we focused on genes whose expression was influenced by hypoxia at the larval stage (i.e. *PHD-3*, *LA-Hb α 1*, *LA-Hb β 1*, *MN-Hb α 2*, *MN-Hb α 3*, *MN-Hb β 4* and *MN-Hb β 5*) and genes highly expressed in the head kidney and spleen at the juvenile stage, i.e. *MN-Hb α 1* and *MN-Hb β 1* (Cadiz et al., 2017).

Gene expression was quantified using the iCycler MyiQ™ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories Inc.). Standard curves were estimated for each primer pair using serial dilutions (from 1/10 to 1/270) of a pool of cDNA. Efficiencies of qPCR for each pair of primers ranged from 95 to 100% with $R^2 > 0.99$. Each sample was run in triplicate in a final well volume of 15 μ L containing 5 μ L cDNA (1/30 dilution) and 10 μ L of reaction mix, composed of 0.5 μ L of each primer (10 mM), 1.5 μ L RNase/DNase free water, 7.5 μ L iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc.) containing antibody-mediated hot-started iTaq DNA polymerase, dNTPs, $MgCl_2$, SYBR® Green I dye, enhancers, stabilizers and fluorescein. Negative controls (non-template control) were systematically included in each plate. RT negative controls were also used as template in order to ensure the absence of residual DNA contamination. The qPCR profiles contained an initial activation step at 95°C for 2 min, followed by 39 cycles: 5 s at 95°C and 20 s at annealing temperature (60°C for *PHD-3*, *LA-Hb α 1*, *LA-Hb α 2*, *LA-Hb β 1*, *MN-Hb α 1*, *MN-Hb α 2*, *MN-Hb β 1*, *MN-Hb β 2*, *MN-Hb β 3*, *MN-Hb β 4* and reference genes; 62°C for *MN-Hb β 5* and *MN-Hb β 6*; and 70°C for *MN-Hb α 3*, *MN-Hb α 4* and *MN-Hb α 5*). After the amplification phase, a melting curve was performed to confirm the amplification of a single product in each reaction.

For each sample, the corresponding Cq (Quantification cycle) value was determined automatically using the “Gene Expression Module” of the CFX Manager software (Bio-Rad Laboratories Inc.). Cq is the number of cycles required to yield a detectable fluorescence signal. The relative quantity of messenger was normalized with the $\Delta\Delta C_t$ method using the same CFX Manager software. Reference genes were used to correct for loading differences or other sampling variations present in each sample. The *elongation factor 1-alpha* (*ef1 α*)

gene was chosen as the reference gene for whole larvae, while 28S was used as the reference gene in spleen and head kidney tissues from juvenile fish. These reference genes were used since they did not show any significant variation of expression between samples (relative standard deviation <5% among samples).

Statistical analyses

Statistical analyses were performed using STATISTICA software version 10 (<http://statsoft.fr/>). All data were log-transformed to fit a normal distribution. For all analyses, variables were checked for normality (Shapiro test) and equality of variances (Levene test). Significant differences between O₂ treatments at the larval stage in growth and gene expression were analyzed by one-way ANOVA. Two-way ANOVAs were used to determine the effects of juvenile exposure and O₂ larval treatment on growth, hematological parameters and gene expression. Tukey's test ($p < 0.05$) was used for post hoc comparisons. All figures were drawn using GraphPad Prism[®](v.5.0b) software.

Results

Growth at larval and juvenile stages

Larval growth was monitored from the beginning (23 dph) to the end (50 dph) of hypoxia exposure. Relative growth rates were significantly lower in larvae exposed to the hypoxic treatment (one-way ANOVA: $p = 0.002$) (Table 2). Similarly, juveniles exposed to hypoxia between 196 dph and 259 dph displayed a significantly lower relative growth rate compared to the normoxic treatment (two-way ANOVA: $p = 10^{-6}$). No significant interaction with the conditions encountered during the larval rearing phase was observed (Table 2).

Larval gene expression patterns under moderate hypoxia

The relative levels of 14 Hb transcripts (7 *Hb α* genes and 7 *Hb β* genes) were compared between *D. labrax* larvae exposed or not to moderate hypoxia treatment (40% air saturation) (Fig.1; table S1). The transcript levels of LA-*Hb α 1*, LA-*Hb β 1* and MN-*Hb α 2* genes were significantly higher (7-fold, 6-fold and 14-fold, respectively) in larvae exposed to hypoxia ($p = 2 \times 10^{-5}$, 1×10^{-5} and 1×10^{-6} , respectively). Conversely, the MN-*Hb α 3*, MN-*Hb β 4* and MN-*Hb β 5* transcript levels were reduced (3-fold, 5-fold and 4-fold, respectively) in

larvae under hypoxia ($p = 4 \times 10^{-4}$, 1×10^{-4} and 1×10^{-4} , respectively). Other Hb genes (LA-*Hb* α 2, MN-*Hb* α 4, MN-*Hb* α 5 and MN-*Hb* β 6) did not exhibit any differential expression or were not sufficiently expressed ($Cq > 33$ for MN-*Hb* α 1, MN-*Hb* β 1, MN-*Hb* β 2 and MN-*Hb* β 3) to be analyzed. The expression levels of the *PHD-3* gene, involved in the HIF pathway, was significantly higher (3-fold induction) under the hypoxic than the control condition ($p = 2 \times 10^{-5}$).

Hematological parameters in juvenile fish under moderate hypoxia

At the juvenile stage, the hematocrit was increased under mild hypoxia, while Hb protein levels in the blood remained unchanged (Table 3). Oxygen conditions experienced at the larval stage did not influence these blood parameters (two-way ANOVA; $p = 0.32$).

Gene expression patterns in the head kidney and spleen of juveniles

We showed that moderate hypoxia at the juvenile stage only induced significant up-regulations of LA-*Hb* α 1, LA-*Hb* β 1 and MN-*Hb* α 2 genes in the head kidney of juveniles that have been previously exposed to larval hypoxia (Fig. 2; table S2). Such effects were not observed in the spleen tissue (Fig. 3; table S3). The mRNA levels of MN-*Hb* α 1 and MN-*Hb* β 1 remained unchanged under moderate hypoxia in the head kidney and spleen tissues. The transcript levels of MN-*Hb* α 3, MN-*Hb* β 4 and MN-*Hb* β 5 genes were too low to identify any potential differential expression in the head kidney or spleen tissues. Hypoxia also induced a stimulation of *PHD-3* gene expression, limited to the head kidney of juveniles that had experienced low oxygen concentration at the larval stage (Fig. 2; table S2).

Discussion

As an inhabitant of shallow marine areas, *D. labrax* is expected to be exposed to environmental fluctuations including hypoxia events. In order to cope with oscillations in water P_{O_2} , marine fish species have developed adaptive mechanisms that often depend, to a varying extent, on changes in gene expression (Gracey et al., 2001; van der Meer et al., 2005). Our results show that the regulation of Hb genes in response to moderate chronic hypoxia is specific to the developmental stage considered, with the involvement of particular genes, and also depends on an individual's life history (previous exposure to a hypoxic environment). We recognize that transcriptional changes in Hb gene expression

observed in the present study may not reflect the concentration of the related subunit in Hb tetramers. It is in fact admitted that Hb tetramers assembly is tightly coordinated, since any alpha subunits formed in excess are broken down if not incorporated to alpha-beta dimers (Kong et al., 2004). Therefore, while present gene expression analysis gives precious insights related to mechanisms involved in hypoxia response, it is difficult to make definitive statements about their functional significance.

At the larval stage, the impact of hypoxia on growth reflected a sensitivity of the organism to the oxygen conditions. The highest regulation of Hb gene expression in response to hypoxia was found at this stage. Transcriptional regulation of Hb expression therefore appears to be a crucial element of the response to mild hypoxia during larval development in *D. labrax*. During hypoxia, the levels of regulation reached 14-fold, 7-fold and 6-fold induction for whole-body MN-Hb α 2, LA-Hb α 1 and LA-Hb β 1 transcripts, respectively, while MN-Hb α 3, MN-Hb β 4 and MN-Hb β 5 were down-regulated 3.5-fold, 4.5-fold and 4-fold, respectively. However, the expression abundance of these genes also needs to be taken into account. Since MN-Hb α 3, MN-Hb β 4 and MN-Hb β 5 transcripts are more abundant than MN-Hb α 2, LA-Hb α 1 and LA-Hb β 1 at the larval stage (Cadiz et al., 2017), the net balance of the Hb regulation observed in the present study tends towards a decrease in global Hb gene expression in sea bass larvae exposed to hypoxia. Further analysis will be necessary in order to determine whether such transcriptional regulation is associated with a decrease in Hb concentration. If so, our data would be consistent with the decrease in Hb concentration reported in *O. mykiss* larvae exposed to moderate hypoxia by Bianchini and Wright (2013). The consequences of the stimulation of MN-Hb α 2, LA-Hb α 1 and LA-Hb β 1 gene expressions on the composition of the Hb tetramers and on the O₂ affinity of Hb are unknown in *D. labrax* larvae. Thus, we can only suggest that *D. labrax* larvae may react physiologically to the functional characteristics of MN-Hb α 2, LA-Hb α 1 and LA-Hb β 1 gene products, which might be determinant for survival when faced with the overall decrease in oxygen concentration. As previously suggested in other teleost species, the regulation of Hb genes expression in hypoxia condition may result from the stimulation by the transcription factor HIF-1 (Wawrowski et al., 2011). The absence of functional anti-HIF-1 antibody in *D. labrax* does not allow us to measure HIF content in larvae and thus to really conclude about the role of HIF-1 in the present Hb genes regulation. However, mRNA levels of oxygen

sensor *PHD-3* gene whose expression is known to be stimulated in hypoxia condition notably via HIF-mediated pathway (Aprelikova et al., 2004) were higher in *D. labrax* larvae exposed to hypoxia. This data suggest an activation of the HIF-mediated pathway associated to the regulation of Hb genes in *D. labrax* larvae exposed to chronic hypoxia.

Juvenile sea bass under chronic moderate hypoxia did not exhibit any regulation of Hb gene expression in spleen and head kidney tissues when these juvenile fish had not experienced any hypoxia during their larval stage. Provided that there was no variation of Hb transcripts in blood or non-erythropoietic tissues, this result suggests that the regulation of Hb gene expression was not needed to cope with the moderate hypoxia condition during the juvenile period. In line with this data, Hb protein levels remained unchanged in juveniles under hypoxia despite a significant increase in erythrocyte volume (hematocrit) in the blood. The erythrocyte volume could increase in hypoxic conditions by adrenergic activation of sodium-proton exchange without association of higher Hb protein levels (Nikinmaa, 2001). Although further investigations will be required to evaluate the impact of hypoxia on blood O₂ transport (e.g. blood oxygen affinity), our results are in agreement with previous data obtained in *D. labrax* juveniles, showing that the O₂ carrying capacity remained unchanged under chronic hypoxia similar to our levels (Pichavant et al., 2003). Our data indicated that the Hb gene response of juveniles differed from that of larvae when exposed to similar environmental O₂ conditions. The lack of a requirement to regulate Hb gene expression in juveniles may be interpreted as a lower sensitivity to moderate hypoxia compared to larvae. In fish, as in other vertebrates, early life stages are generally considered to be the most sensitive to environmental constraints, including hypoxia (Ishibashi et al., 2007; Levin et al., 2009). Indeed, the correct progress of developmental processes associated with morphogenesis, organogenesis and maturation of physiological functions is crucial for larval survival (and cannot really be postponed), requires intense aerobic metabolisms and would likely explain the imperative need for Hb regulation during the larval period. The physiological trade-off adopted by juvenile fish is different, since they can more easily restrain their energy expenditure by limiting food ingestion and growth (as observed in the present study and also reported by Pichavant et al. (2001)), and consequently do not really need any significant down-regulation of Hb synthesis when exposed to moderate hypoxia. Furthermore, the apparent lower Hb sensitivity at juvenile

stage may also be explained by a more efficient oxygen extraction/transport system. A comparison of parameters related to feed intake, metabolic rates (standard metabolic rates) and oxygen consumption (critical tensions of oxygen-Pcrit) at different larval and juvenile stages would represent useful information for a better understanding of sensitivity to hypoxia during the *D. labrax* life-cycle.

The major result of the present study is the fact that Hb regulation in the kidney of *D. labrax* juveniles in response to hypoxia was only observed in fish that were exposed to low oxygen concentration during the larval stage. These juveniles exhibited a significant stimulation of *MN-Hb α 2*, *LA-Hb α 1* and *LA-Hb β 1* genes under hypoxia that was not associated with any higher Hb concentration. This can be explained by the fact that the major subtypes of Hb tetramers at the juvenile stage are *MN-Hb α 1* and *MN-Hb β 1* (Cadiz et al., 2017), which were not regulated under moderate hypoxia in head kidney. An alternative explanation could be that such stimulation was only found in the kidney and not in the other major hematopoietic tissue investigated (i.e. the spleen). As mentioned for the larval stage, we cannot really conclude from present data about the involvement of HIF-1 in the regulation of Hb genes at juvenile stage. However, it is interesting to note that the stimulation of Hb genes in hypoxic condition was again associated with the up-regulation of *PHD-3* expression that could suggest an activation of HIF-1-mediated pathway.

The significant hypoxia-induced stimulation of *PHD-3* gene expression may reflect higher hypoxia sensitivity in juveniles that experienced hypoxia at the larval stage. This hypothesis, in contrast to that suggesting beneficial effects of early-life exposition to hypoxia (Robertson et al., 2014), would be in line with previous data obtained in humans demonstrating an increased susceptibility to hypoxia in specific adult muscle related to antecedent intermittent hypoxia exposure during postnatal development (McDonald et al., 2016). Growth data measured under chronic hypoxia at juvenile stage did not allow to conclude about any potential beneficial or negative effects of the early-life exposition to hypoxia. As mentioned above for the larval stage, additional information related to the functional characteristics of the different Hb genes will be necessary in order to decipher the physiological and functional consequences of their long-term regulation in the kidney. Considering that *MN-Hb α 2*, *LA-Hb α 1* and *LA-Hb β 1* could have specific functional

characteristics (e.g. binding capacities, Root and Bohr effects), we cannot rule out the hypothesis that their regulation may be associated with enhanced O₂ transport capacity, allowing fish to cope better with mild hypoxia, as previously suggested by (Wawrowski et al., 2011). Moreover, regulation of LA-Hb α 1 and LA-Hb β 1 expression may impact other cellular/physiological functions than those strictly related to O₂ transport, since these genes were found most abundantly expressed in non-hematopoietic tissues (Cadiz et al., 2017).

Conclusion

The present work confirms that the regulation of Hb gene expression is involved in the molecular response to hypoxia in *D. labrax*. Regulation of Hb expression in response to hypoxia appears to be dependent on developmental stage, and involves different specific Hb genes. The characteristic of this response to hypoxia reinforces the idea of distinct functional properties in Hb subtypes, as already suggested for this species (Cadiz et al., 2017). Further investigations are needed, however, to better understand the physiological consequences of these regulations at different life stages. In particular, the question remains to be clarified of whether the long-term regulation of Hb gene expression induced by early-life exposure to hypoxia actually results in beneficial or negative effects to cope with moderate O₂ deprivation during the juvenile period. Finally, additional investigations will be necessary to decipher the molecular processes (e.g. epigenetic) underlying the developmental plasticity of hemoglobin gene regulation.

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Competing interest

No competing interests declared

Author contributions

D.M. conceived, designated and coordinated the study. J.L.Z.I supervised and interpreted data. P.Q. and L.M. conducted the fish experiment and molecular analyses respectively. L.C. was involved in all experimental and analytical aspects of this study. J.L.Z.I., D.M., A.S. and L.C. contributed to manuscript drafting.

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Tables

Table 1. Specific primers used for real-time amplification of Hb, PHD-3 and reference (28S and EF1 α) genes.

Gene	Accession number on NCBI (*) or unipd (#)	Forward primer sequences (5'-3')	Reverse primer sequences (5'-3')
LA-Hb α 1	KX196178 (*)	CAGTGGGACAGGATCTTGAAGT	GGTGATGGGTGGAATCAATC
LA-Hb α 2	KX196180 (*)	TTCCCATGAGAGAGCAGGT	TCAGATGCGCTTCTTAGGATGT
MN-Hb α 1	KX196190 (*)	GGCCAGGATGCTGACTGTA	CCAGCAAGGTCATCCATCTT
MN-Hb α 2	KX196188 (*)	CCTGCCAACTTCAAGATTCTG	TTTCTCAGACAAGGCACGAG
MN-Hb α 3	KX196184 (*)	ACAGACAAGATGACCAGTCTCACT	GCCAATGTCCTCTGCCTTC
MN-Hb α 4	KX196183 (*)	ACAGACAAGATGACCAGTCTCACT	GCCAATGTCCTCTGCCTTT
MN-Hb α 5	KX196181 (*)	ACAGACAAGATGACCAGTCTCACA	GCCAATGTCCTCTGCCTTC
LA-Hb β 1	KX196179 (*)	CCCGACAACCTCAAAGTCTGCT	CCTGCGTCTCTGGTGTGAAG
MN-Hb β 1	KX196191 (*)	TGATTTGAGCAAAGATCCTGAA	CATGGACGACATCAAGAACG
MN-Hb β 2	KX196189 (*)	GTCAGCCAGCAGCCTGAAAT	GCAGCTCTTCCAGGTGTCT
MN-Hb β 3	KX196187 (*)	CAGAAGCTTTGGCAAGAGTG	GCTGCTACTTTGGCGTTACC
MN-Hb β 4	KX196186 (*)	GTCGTTTACCCTGGACTCA	GTTTTGCGACCATCGGATTT
MN-Hb β 5	KX196185 (*)	ACCATCCAGGACATCTTCTCT	GTTTTGCGACCAACGGATTC
MN-Hb β 6	KX196182 (*)	ACCATCCAGGACATTTTCTCC	GTTTTGCGACCAACGGATTC
PHD-3	DLPD06823 (#)	TCCTACTCCACCAGGTACGC	GCAGTCATGTTTGCTCTCCA
28S	AH011863.2 (*)	CAAGAACATCCAGCTGCTGAC	GGTGATATGTCGGCCATAAA
Ef1 α	AJ866727.1 (*)	CTGGAGGGCAGTGAAAAGAT	CATCAAGAGCCTCCAGCAGT

Table 2. Relative growth rate of larvae and juvenile of *D. labrax* under normoxia and moderate hypoxia.

	Larval phase			Juvenile phase				larval oxygenation x juvenile oxygenation		
	CL	HL	P-value	CJ		HJ			p-value	
Relative growth rate	9.4	5.9	0.002	1.28	1.16	0.96	0.98	0.26	1x10⁻⁶	0.07

Larval growth was monitored for hypoxia (HL) and control (CL) groups. The P-value was calculated from a one-way ANOVA analysis (n for each group = 150 larvae). Growth of juveniles exposed (HJ, n = 60) or not (CJ, n = 60) to hypoxia was evaluated on fish previously exposed (HL, n = 30 in HJ and CJ groups) or not (CL, n = 30 in HJ and CJ groups) to larval hypoxia. The P-value was calculated from a two-way ANOVA analysis.

Table 3. Blood hematocrit (%) and Hb protein (g dL⁻¹) levels under normoxia (CJ) and moderate hypoxia (HJ) in *D. labrax* juveniles previously exposed (HL) or not (CL) to hypoxia.

	CJ		HJ		p-value		
	CL	HL	CL	HL	larval oxygenation	juvenile oxygenation	larval oxygenation × juvenile oxygenation
Hematocrit (%)	27.28	26.28	35.14	37.14	0.73	2x10⁻⁶	0.32
Hb levels (g dL⁻¹)	8.5	8.6	8.4	8.3	0.97	0.59	0.88

The p-value was calculated from a two-way ANOVA analysis (n for each group = 8 juveniles).

Figures

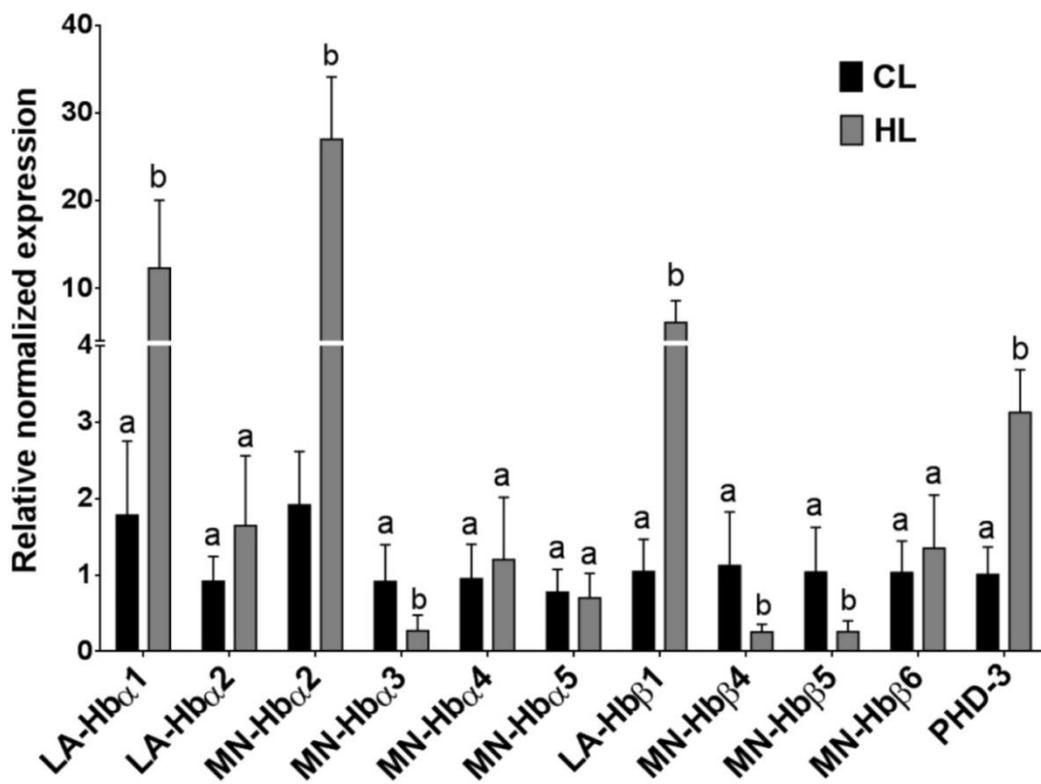


Fig.1. Expression pattern of Hb genes (LA-Hb α 1-2; LA-Hb β 1; MN-Hb α 2-5 and MN-Hb β 4-6) and PHD-3 gene under normoxia (CL) and moderate hypoxia (HL, 40 % air saturation) in the whole larvae (50dph). The Y-axis represents the relative expression normalized by expression levels of Ef1- α . Relative expression levels are expressed relative to normoxia group ($\times 1$). Significant differences for each gene between groups are identified with different letters (one-way ANOVA followed by Tukey's test).

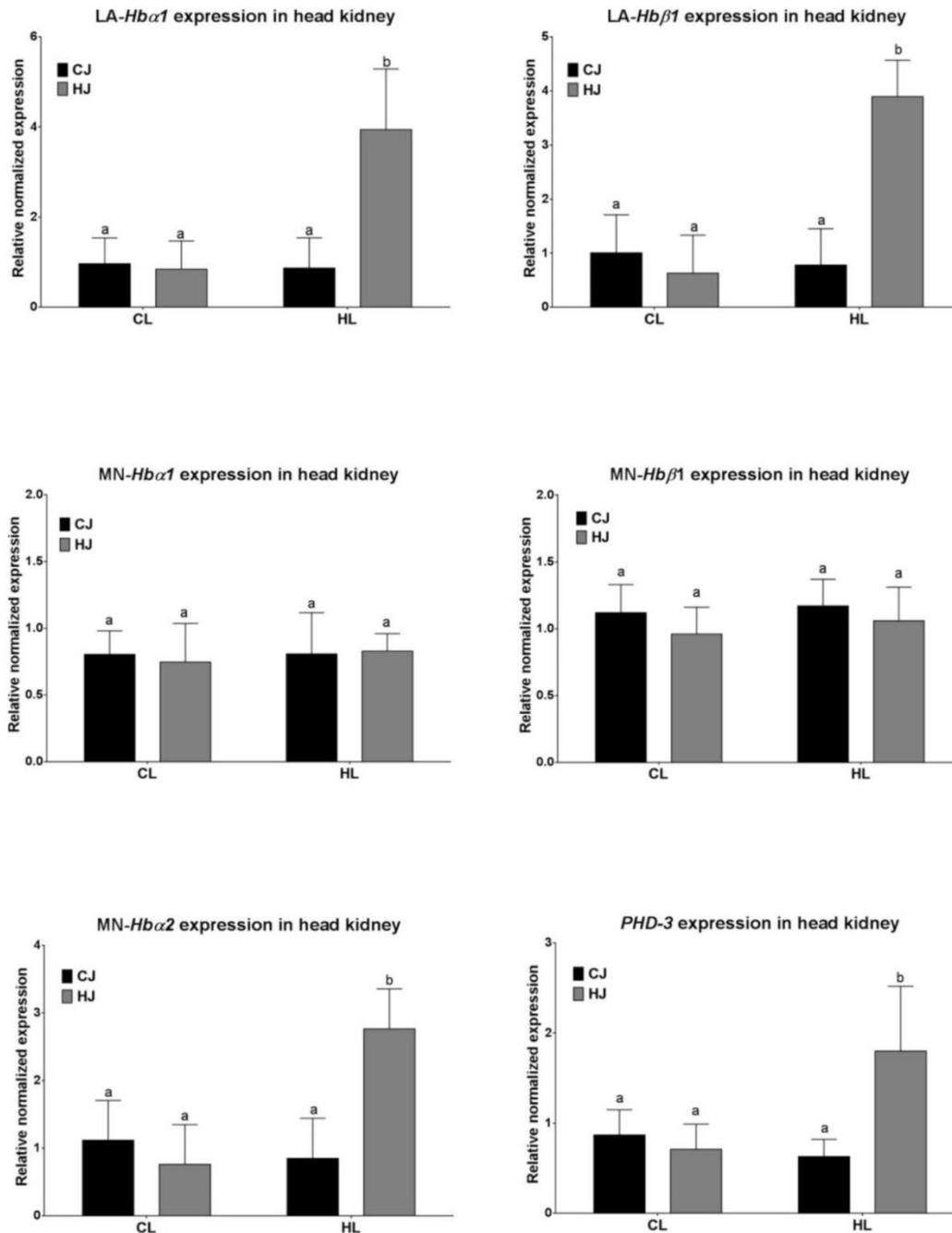


Fig.2. Expression pattern of Hb genes (LA-Hb α 1, LA-Hb β 1, MN-Hb α 1, MN-Hb α 2 and MN-Hb β 1) and the PHD-3 gene under normoxia (CJ) and moderate hypoxia (HJ, 40 % air saturation) in the head kidney of juveniles previously exposed (HL) or not (CL) to larval hypoxia. The Y-axis represents the relative expression normalized by expression levels of 28S.

Relative expression levels are expressed relative to the juvenile control group in normoxia (×1). Significant differences for each gene between groups are identified with different letters (two-way ANOVA followed by Tukey's test).

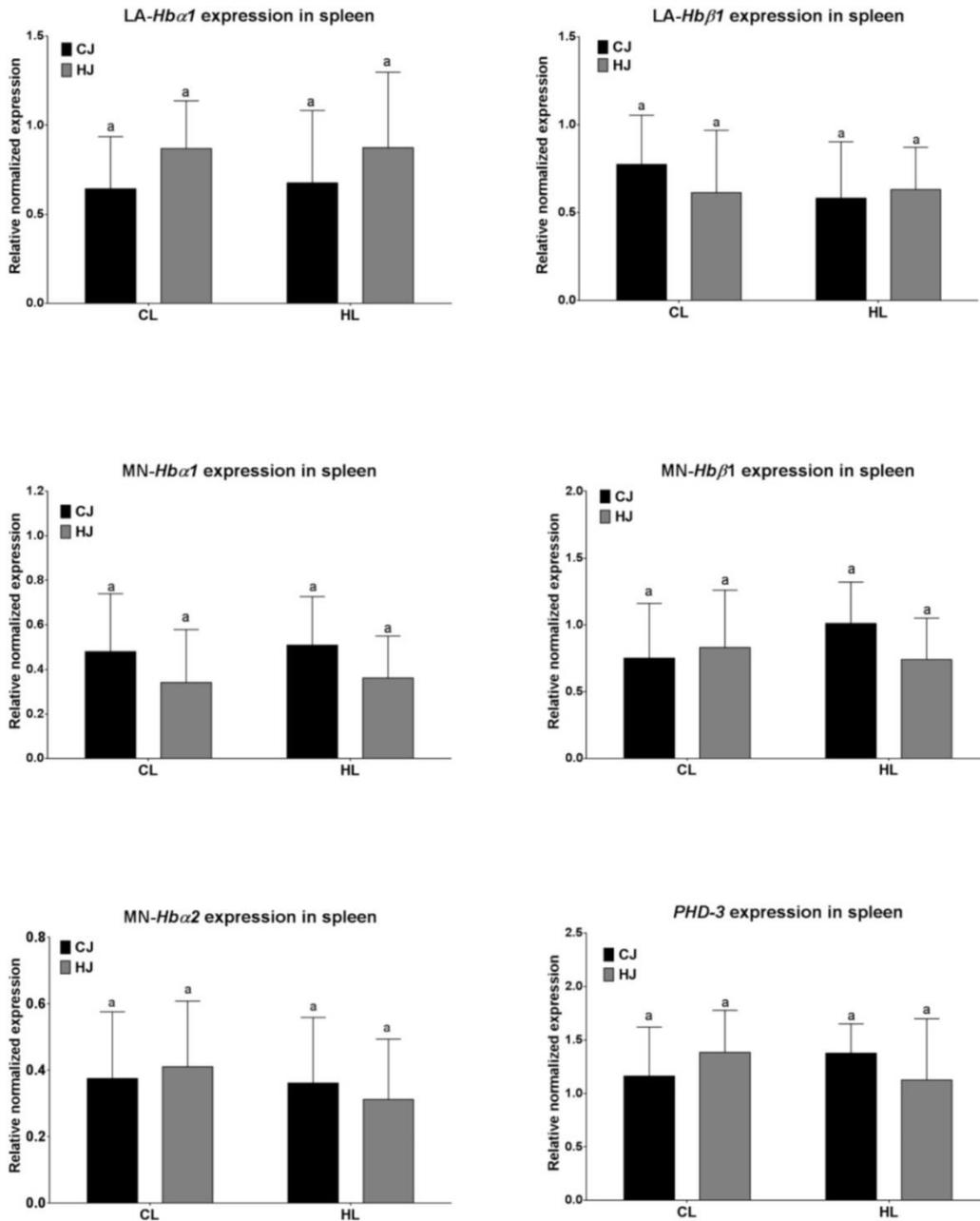


Fig.3. Expression patterns of Hb genes (LA-Hb α 1, LA-Hb β 1, MN-Hb α 1, MN-Hb α 2 and MN-Hb β 1) and the PHD-3 gene under normoxia (CJ) and moderate hypoxia (HJ, 40 % air saturation) in the spleen of juveniles previously exposed (HL) or not (CL) to larval hypoxia. The Y-axis represents relative expression normalized by expression levels of 28S. Relative expression levels are expressed relative to the juvenile control group in normoxia ($\times 1$).

Significant differences for each gene between groups are identified with different letters (two-way ANOVA followed by Tukey's test).

Table S1. Relative expression levels of Hb β - α and PHD-3 genes under normoxia and moderate hypoxia in *D. labrax* whole larvae.

	Genes	CL		HL		p-value	Fold
		Relative expression	Cq	Relative expression	Cq		
Hb	LA-Hb α 1	1.79	30	12.34	28	2×10^{-5}	+7
	LA-Hb α 2	0.92	27	1.65	27	0.07	+2
	MN-Hb α 1	-	33	-	33	-	-
	MN-Hb α 2	1.92	28	27.04	24	1×10^{-6}	+14
	MN-Hb α 3	0.92	21	0.27	24	4×10^{-4}	-3
	MN-Hb α 4	0.95	20	1.20	20	0.44	+1
	MN-Hb α 5	0.78	21	0.70	22	0.61	+1
	LA-Hb β 1	1.05	23	6.12	21	1×10^{-5}	+6
	MN-Hb β 1	-	33	-	33	-	-
	MN-Hb β 2	-	33	-	32	-	-
	MN-Hb β 3	-	35	-	35	-	-
	MN-Hb β 4	1.13	22	0.25	24	1×10^{-4}	-5
	MN-Hb β 5	1.04	21	0.26	23	1×10^{-4}	-4
	MN-Hb β 6	1.03	19	1.36	19	0.24	+1
	HIF pathway	PHD-3	1.01	31	3.13	30	2×10^{-5}

qPCR analysis was performed on RNA from whole sea bass larvae exposed (HL) or not (CL) to moderate hypoxia (40% air saturation). The p-value was calculated from a one-way ANOVA analysis (n for each group = 6 pools of 8 larvae). Quantification cycle (Cq) values are also indicated.

Table S2. Relative expression levels of selected Hb genes (LA-Hb α 1, LA-Hb β 1, MN-Hb α 1, MN-Hb β 1 and MN-Hb α 2) and the PHD-3 gene under normoxia (CJ) and moderate hypoxia (HJ, 40 % air saturation) in the head kidney of juveniles previously exposed (HL) or not (CL) to larval hypoxia.

Genes	CJ head kidney				HJ head kidney				p-value			
	CL		HL		CL		HL		larval ox.	juvenile ox.	larval ox. x juvenile ox.	
	Relative expression	Cq										
Hb	LA-Hb α 1	0.97	22	0.87	22	0.84	22	3.94	20	0.02	0.02	1x10⁻³
	LA-Hb β 1	1.01	20	0.78	20	0.63	20	3.90	18	0.01	0.02	5x10⁻⁴
	MN-Hb α 1	0.80	11	0.81	11	0.75	11	0.83	11	0.58	0.84	0.43
	MN-Hb β 1	1.11	11	1.17	11	0.96	11	1.06	11	0.37	0.12	0.76
	MN-Hb α 2	1.12	22	0.85	22	0.76	22	2.77	20	6x10⁻³	0.01	4x10⁻⁵
HIF pathway	PHD-3	0.87	27	0.63	27	0.71	27	1.80	25	0.02	8x10⁻³	5x10⁻⁵

The P-value was calculated from a two-way ANOVA analysis (n for each group = 8 juveniles).

Ox. means oxygenation. Quantification cycle (Cq) values are also indicated.

Table S3. Relative expression levels of selected Hb genes (LA-Hb α 1, LA-Hb β 1, MN-Hb α 1, MN-Hb β 1 and MN-Hb α 2) and the PHD-3 gene under normoxia (CJ) and moderate hypoxia (HJ, 40 % air saturation) in the spleen of juveniles previously exposed (HL) or not (CL) to larval hypoxia.

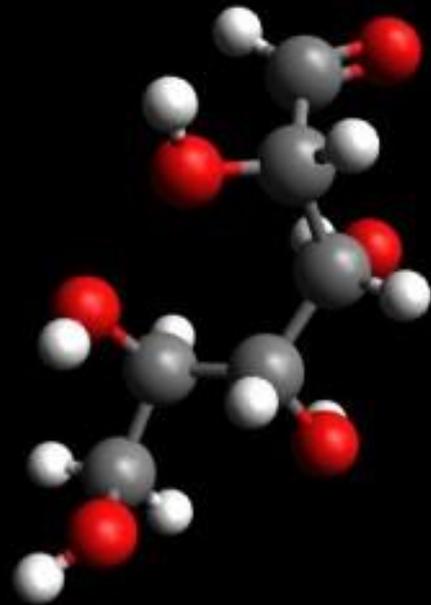
Genes	CJ spleen				HJ spleen				p-value			
	CL		HL		CL		HL		larval ox.	juvenile ox.	larval ox. x juvenile ox.	
	Relative expression	Cq										
Hb	LA-Hb α 1	0.64	19	0.68	19	0.87	18	0.87	18	0.93	0.78	0.95
	LA-Hb β 1	0.77	17	0.58	18	0.61	18	0.63	18	0.66	0.75	0.92
	MN-Hb α 1	0.48	13	0.51	12	0.34	13	0.36	13	0.64	0.40	0.43
	MN-Hb β 1	0.75	13	1.01	12	0.83	13	0.75	13	0.60	0.56	0.32
	MN-Hb α 2	0.38	17	0.36	17	0.41	17	0.31	17	0.49	0.62	0.38
HIF pathway	PHD-3	1.16	24	1.38	24	1.38	24	1.13	24	0.82	0.65	0.12

The P-value was calculated from a two-way ANOVA analysis (n for each group = 8 juveniles).

Ox. means oxygenation. Quantification cycle (Cq) values are also indicated.

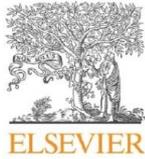
Chapter 4

Metabolic response to hypoxia in European sea bass (Dicentrarchus labrax) displays developmental plasticity



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The objective of this chapter was to evaluate whether the metabolic response of juveniles to hypoxia could be modulated by O₂ and temperature conditions experienced at larval stages. For this purpose, juvenile fish were exposed for three months (from 196 to 296 dph) to the same environmental conditions that they have experienced at larval stage [(40 and 100% air saturation at two different temperatures (15°C and 20°C)]. In the following paper, we only reported the results obtained in juveniles reared at 20°C since they give more relevant information. All the results obtained in this study will nevertheless be discussed in the general discussion part.



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Metabolic response to hypoxia in European sea bass (*Dicentrarchus labrax*) displays developmental plasticity



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ABSTRACT

Several physiological functions in fish are shaped by environmental stimuli received during early life. In particular, early-life hypoxia has been reported to have long-lasting effects on fish metabolism, with potential consequences for fish life history traits. In the present study, we examine whether the synergistic stressors hypoxia (40% and 100% air saturation) and temperature (15° and 20 °C), encountered during early life, could condition later metabolic response in European sea bass (*Dicentrarchus labrax*) juveniles. Growth rate and metabolic parameters related to carbohydrate and lipid metabolism in the liver were investigated at the juvenile stage under normoxic and chronic hypoxic conditions. Juvenile growth rates were significantly lower ($p < 1 \times 10^{-6}$) under hypoxic conditions and were not improved by prior early-life exposure to hypoxia. Growth was 1.3 times higher ($p < 5 \times 10^{-3}$) in juveniles reared at 15 °C during the larval stage than those reared at 20 °C, suggesting that compensatory growth had occurred. Early-life exposure to hypoxia induced higher ($p < 2 \times 10^{-6}$) glycogen stores in juveniles even though there was no apparent regulation of their carbohydrate metabolism. In the liver of juveniles exposed to chronic hypoxia, lower glycogen content combined with stimulation of phosphoenolpyruvate carboxykinase gene expression and higher lactate concentration indicated a stimulation of the anaerobic glycolytic pathway. Furthermore, hypoxia only induced lower ($p < 1 \times 10^{-3}$) lipid content in the liver of juveniles that had experienced 15 °C at the larval stage. The present study provides evidence that environmental conditions experienced during early life shape the metabolic traits of *D. labrax* with potential consequences for juvenile physiological performance.

1. Introduction

Global climate warming and eutrophication caused by nutrient inputs are the two main causes increasing the prevalence of environmental hypoxia in aquatic environments, particularly in shallow marine areas (Diaz, 2001; Diaz and Rosenberg, 2008, 2011; Gillanders et al., 2011). It is known that reduced oxygen availability impacts many behavioral traits, physiological processes and ecological interactions in marine organisms including fish species (Ficke et al., 2007; Levin et al., 2009; Nagelkerken and Munday, 2016; Portner and Farrell, 2008). Oxygen is indeed essential for the supply of metabolic fuel, which in turn determines individual performance for specific energetically expensive processes. More precisely, as the final acceptor of the electron

transport chain in the inner membrane of mitochondria, oxygen is involved in the oxidative phosphorylation that drives the generation of energy in the form of ATP. The acclimation of fish to low environmental O₂ conditions is likely to interact with increased temperature, because both of these stressors affect aerobic metabolism (Anttila et al., 2015; McBryan et al., 2013, 2016; Portner and Farrell, 2008). Raised temperatures force ectothermic fish to increase their metabolism, resulting in higher O₂ demands, while hypoxia limits O₂ supply. Recent data obtained in Atlantic Killifish (*Fundulus heteroclitus*) demonstrated that hypoxia tolerance dramatically reduced with acute temperature increase (McBryan et al., 2016). The inability of fish to provide sufficient energy to meet their need through aerobic metabolism under hypoxia forces them to use anaerobic metabolism. The switch to anaerobic

Abbreviations: Atgl, adipose triglyceride lipase; CJ, control juvenile group; CL, control larval group; Cq, quantification cycle; *Dgat1*, diacylglycerol O-acyltransferase 1; DMSO, dimethyl sulfoxide; Dph, days post hatching; *Ef1a*, elongation factor-1 isoform alpha; *Glut2*, glucose transporter-2; GP, glycogen phosphorylase; *Gs*, glycogen synthase; HIF, hypoxia inducible factor; HJ, hypoxia juvenile group; HL, hypoxia larval group; HIS, hepatosomatic index; NADPH, nicotinamide adenine dinucleotide phosphate; *Pepck*, phosphoenolpyruvate carboxykinase; *PHD-3*, prolyl hydroxylase domain-containing protein 3; *Pk*, pyruvate kinase; PIT, passive integrated transponder; qPCR, quantitative polymerase chain reaction; RGR, relative growth rate; RIN, RNA integrity number; RT, reverse transcription

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respiration is related to the utilization of the glycolytic pathway, in which glucose is converted to pyruvate in the absence of oxygen, producing lactate as the end product (Mandic et al., 2013; Richards, 2011). Glucose can be supplied from glycogen stocks, but also by gluconeogenesis, which can be additionally activated to increase hepatic glucose production during hypoxia in vertebrates, including fish (Gracey et al., 2001, 2011; Martínez et al., 2006). Moreover, glucose has been suggested to be generated from glycerol derived from triglycerides in some hypoxia-tolerant species, such as burrow-dwelling goby (*Gillichthys mirabilis*), when exposed to hypoxia (Gracey et al., 2011). In fish, as in other vertebrates, the hypoxia-inducible factor (HIF) signalling pathway is involved in the physiological response to hypoxia by regulating the transcription of a number of hypoxia-inducible genes, including some involved in metabolic pathways (Wu, 2002).

The adaptive capacity of organisms, including fish, to cope with environmental constraints (e.g., oxygen, temperature, nutrition) depends on species and ontogenic stage, but also on the conditions encountered during early life stages (Geurden et al., 2007; Robertson et al., 2014; Scott and Johnston, 2012). This may be related to developmental plasticity, which consists of the irreversible phenotypic changes to an organism's ontogeny induced by environmental conditions at early life stages (Schaefer and Ryan, 2006; Terblanche and Chown, 2006). Developmental plasticity may offer the ability for an organism exposed to a specific environmental constraint during its early-life period to form phenotypes better able to cope with this environment at later stages (Bateson et al., 2014). Robertson et al. (2014) have shown that exposure of zebrafish (*Danio rerio*) to hypoxia during the embryonic stage affects sex determination in such a way as to produce more males, which are more hypoxia tolerant than females at the adult stage. Developmental plasticity may involve an intricate regulation (programming) of different biological processes including metabolic pathways, which can be detected by modifications in gene expression (Liu et al., 2017). While metabolic programming has been extensively addressed in a number of mammal studies (Fernandez-Twinn and Ozanne, 2010; Hanley et al., 2010; Patel and Srinivasan, 2010), its ecological implications in aquatic organisms are poorly understood (Fuiman and Perez, 2015). In particular, it remains unknown whether temperature and hypoxic conditions encountered by fish at early life stages could influence their subsequent metabolic adaptability in later life to habitats experiencing hypoxia episodes.

The main purpose of the present study was to determine whether the metabolic adaptability of European sea bass (*Dicentrarchus labrax*) juveniles to low oxygen availability could be modulated by larval “life-history”, especially by temperature and hypoxia exposure at early-life stages. European sea bass larvae likely enter shallow coastal areas just after the flexion stage and could therefore be exposed to environmental fluctuations (Dufour et al., 2009; Jennings and Pawson, 1992). Previous studies on European sea bass revealed that exposure to moderate hypoxia at the larval stage had a negative impact on subsequent juvenile growth rate. This negative effect on growth was associated with a long-lasting down-regulation of genes involved in energy consuming metabolic pathways in the liver (Vanderplancke et al., 2015). In the present study, we wanted to go further and obtain a more environmentally realistic assessment of synergistic effects of stressors that fish larvae would be likely to encounter. As hypoxia could often be associated with warming episodes, we evaluated whether a combination of different oxygen concentration (40% and 100% air saturation) and temperature (15 and 20 °C) experienced during early-life could change the subsequent juvenile response to hypoxia, with observable long-lasting regulation of metabolic processes. Experimental conditions tested here mimic field conditions that sea bass is likely to experience along the European West coast of Atlantic Ocean (Copernicus Marine environment monitoring service, <http://marine.copernicus.eu>). Growth rate was used to evaluate the overall physiological status of juveniles. Trypsin expression was measured as an indicator of food consumption and digestion. The capacity for energy production was analysed by

assessing different metabolites (glycogen, glucose, lactate and lipids) and by investigating activity or gene expression of different proteins involved in carbohydrate metabolism [glycogen phosphorylase (GP) activity and gene expression (*gp*); glycogen synthase (*gs*), phosphoenolpyruvate carboxykinase (*pepck*), glucose transporter-2 (*glut2*) and pyruvate kinase (*pk*) gene expression] and lipid metabolism [pancreatic lipase activity; adipose triglyceride lipase (*atgl*), fatty acid transporter (*slc27*) and diacylglycerol O-acyltransferase 1 (*dgat1*) gene expression]. Special interest was also paid to the expression of prolyl hydroxylase domain-containing protein 3 gene (*phd3*), an actor in the HIF signalling pathway.

2. Material and methods

2.1. Animal rearing and treatments

D. labrax larvae were reared under normal oxygen conditions in 12 tanks at 15 ± 0.4 °C water temperature and 35 ± 0.2 ‰ salinity. They were fed daily with *Artemia* according to Zambonino et al. (1996) until the end of larval development. Water temperature was progressively increased from 15 °C to 20 °C in six tanks between 23 and 28 days post hatching (dph) for acclimation purposes. From 28 dph, three replicate tanks of larvae were exposed to each combination of oxygenation level [40% air saturation ($2.95 \text{ mg O}_2 \text{ L}^{-1}$): hypoxia larval group (HL); and 100% saturation ($7.35 \text{ mg O}_2 \text{ L}^{-1}$): control larval group (CL)] and temperature (15 °C and 20 °C). Hypoxic conditions were created by bubbling N₂ in a gas equilibration column placed upstream of the experimental tank. Dissolved O₂ was monitored daily using an Odeon oxygen meter (ODEON Classic OPTOD; Caudan, France). Other water quality parameters (salinity, temperature and pH) were also checked daily in each tank during the experiment. It had previously been determined that the level of hypoxia used did not induce mortality; thus, genetic selection during larval exposure was avoided. Larvae were returned to normal oxygen conditions (100% air saturation) at 43 dph, for larvae exposed at 20 °C (745 °C day), and at 50 dph, for larvae exposed to 15 °C (750 °C day), to ensure that larval treatments were applied at the same stage of development. Replicate tanks were then pooled into one 1 m³-tank per treatment and kept under normoxic conditions at 15–17 °C. At this stage, the fish were fed with a commercial diet (NeoSupra; Coopérative Le Gouessant, Lamballe, Côtes-d'Armor, France). At 166 dph, 60 fish from each of the treatment tanks were selected and tagged subcutaneously to identify the early stage treatments (Passive Integrated Transponder; PIT-tag) for individual identification. The fish were then fed with NeoGrower commercial diet (Coopérative Le Gouessant; Lamballe, Côtes-d'Armor, France) and pooled in a 4-m³ tank. From 196 to 296 dph, fish were separated into two tanks, including 30 juveniles from each larval group in each tank. Water temperature was then progressively increased to 20 °C and water oxygenation in one of the tanks was reduced to 40% air saturation (hypoxia juvenile group, HJ), while it was maintained at 100% saturation in the other (control juvenile group, CJ). Experimental protocol was summarized in Fig. 1.

2.2. Growth monitoring

From each of the HJ and CJ groups, 120 juveniles (including 30 fish from each of the larval groups 15/20 °C HL and 15/20 °C CL) were weighed individually at 196 and 296 dph after light anaesthesia (Tricaine methane-sulfonate 10 mg L^{-1}). Growth was estimated individually by calculating the relative growth rate ($RGR = \frac{\text{final weight} - \text{initial weight}}{\text{initial weight}}$) between 196 and 296 dph.

2.3. Sampling

Sampling of immature juveniles was performed at 296 dph, after

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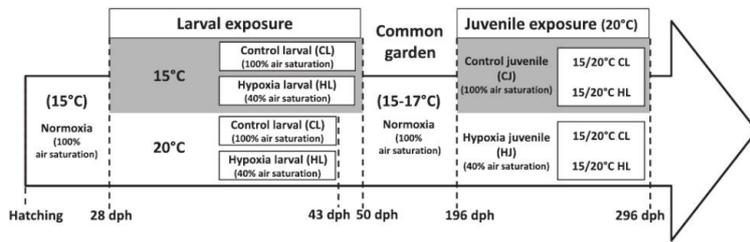


Fig. 1. Schema of the experimental protocol. Sea bass larvae were exposed to each combination of oxygenation level [40% air saturation: hypoxia larval group (HL); and 100% saturation: control larval group (CL)] and temperature (15 °C and 20 °C) from 28 to 43 dph (20 °C) or 50 dph (15 °C). Following the larval exposure, fish experienced a five months period of common garden (15–17 °C, 100% air saturation). From 196 to 296 dph, fish were divided into two tanks, including 30 juveniles from each larval group. Water temperature was 20 °C and water oxygenation in one of the tanks was reduced to 40% air saturation (hypoxia juvenile group, HJ), while it was maintained at 100% saturation in the other (control juvenile group, CJ).

fish had been left undisturbed and unfed for 24 h. From each of the two groups HJ and CJ, 32 juveniles (including eight fish from each of the larval groups 15/20 °C HL and 15/20 °C CL) were randomly selected and euthanized with an excess of anaesthetic (Tricaine methane-sulfonate 500 mg L⁻¹). Tissue including liver and pancreas (pancreatic tissue is diffuse and cannot be separated from the liver) was dissected and weighed to determine the hepatosomatic index (HSI) and immediately frozen in liquid nitrogen.

The present work was performed within IFREMER facilities in accordance with French and European policies and the guidelines of the French Animal Care Committee (Agreement number: APAFIS#5173).

2.4. Hepatosomatic index and metabolite levels

Hepatosomatic index (HSI) was defined by the equation $HSI = \frac{\text{liver weight}}{\text{fish weight}}$. Livers were pooled in duplicate to get enough biological material for determination of metabolite levels. Pools were ground to an ultra-fine powder using a ball mill (Retsch MM400). Liver tissue was homogenized in the following solutions: 0.1 M tri-Sodium citrate and 0.1 M citric acid (for glucose and glycogen measures); 8% PCA and 3 M K₂CO₃ (for lactate measure) and dichloromethane/methanol (2/1) (for lipids measure). Buffer reagents were supplied by Sigma Aldrich® Inc., St. Louis, MO, USA. Glucose and lactate concentrations in liver were assessed using commercial kits (RTU reference no. 61269 from bioMérieux, Marcy l'Etoile, France and LC reference no. 2389 from Randox Laboratories, Crumlin, UK, respectively), while the levels of hepatic glycogen were determined according to the method of Carr and Neff (1984). Extraction of total lipids was done by the Folch method (Folch et al., 1957).

2.5. Enzymatic activity

Glycogen phosphorylase (EC 2.4.1.1.; GP) was measured from liver tissue pooled in duplicate. Fifty mg of powdered and frozen tissue was homogenized with 10 vol. of ice-cold stopping-buffer containing 50 mM imidazole (pH 7.5), 1 mM mercaptoethanol, 50 mM NaF, 4 mM EDTA, 0.5 mM PMSF and 250 mM sucrose. Buffer reagents were supplied by Sigma Aldrich® Inc., St. Louis, MO, USA. The homogenate was centrifuged at 10000g, for 30 min at 4 °C, and the supernatant recovered for enzyme assay. Total GP activity was determined using the microplate reader of a Thermo Scientific Multiskan GO spectrophotometer (ThermoFisher Scientific). Reaction rates of enzymes were determined by the increase or decrease in nicotinamide adenine dinucleotide phosphate (NADPH) absorbance at 340 nm. The reactions proceed at 37 °C. Total GP (a + b) activity was measured in the presence of 2.5 mM adenosine monophosphate. The specific conditions used for GP activity were similar to those previously described in Laiz-carrión et al. (2003) and Polakof et al. (2006).

Lipase (EC 3.1.1.3) was assayed in the liver according to a method slightly modified from Iijima et al. (1998), using *p*-nitrophenyl myristate as substrate (Sigma Aldrich® Inc., St. Louis, MO, USA) dissolved in dimethyl sulfoxide (DMSO, Merck-Schuchardt, Darmstadt, Germany), as this was found to aid substrate solubilisation. Powdered liver tissue (50 mg) was homogenized into 4 vol. (v/w) of 0.01 M Phosphate

Buffered Saline (PBS, Sigma Aldrich® Inc., St. Louis, MO, USA). The homogenate was centrifuged at 10000g for 5 min at 4 °C and the supernatant recovered for enzyme assay. The single path absorption spectra were measured at 30 °C by Evolution 201 UV-Visible spectrometer (Thermo Scientific).

Enzyme activities were expressed as specific activities, i.e., U/mg protein. Protein was determined by the Bradford procedure (Bradford, 1976).

2.6. RNA extraction and cDNA synthesis

Total RNA for analysis of gene expression was extracted from the liver tissue (pooled in duplicate) using Extract-all reagent (Eurobio; Courtabouef, Essonne, France) combined with the Zymo Direct-zol™ RNA MiniPrep Kit, following recommendations from the supplier. Genomic DNA was removed using the DNA-free Kit (MoBio Laboratories Inc.; Carlsbad, CA, USA). The quantity, purity and quality of RNA were assessed using a ND-1000 NanoDrop® spectrophotometer (Thermo Scientific Inc.; Waltham, MA, USA) and by electrophoresis using an Agilent Bioanalyser 2100 (Agilent Technologies Inc.; Santa Clara, CA, USA). All samples had an RNA integrity number (RIN) higher than seven and could thus be used for real-time quantitative PCR (qPCR) analysis. RNA samples were stored at -80 °C until use.

Synthesis of cDNA was carried out using 500 ng of DNase-treated total RNA with an iScript™ cDNA Synthesis kit (Bio-Rad Laboratories Inc.; Hercules, CA, USA). The total reaction was carried out in a final volume of 20 µL containing 5 µL (500 ng) of sample, 4 µL 5 × iScript™ Reaction Mix containing oligo (dT), random primers and RNaseA inhibitor, 1 µL iScript™ Reverse transcriptase and 10 µL RNase/DNase free water. The cDNA synthesis reaction was incubated for 5 min at 25 °C followed by 30 min at 42 °C and terminated by incubation for 5 min at 85 °C to inactivate the enzyme. Reverse transcription (RT) was performed using a Thermo-cycler TC-152 (Techne Barloworld Scientific; Stone, Staffordshire, UK). cDNA was stored at -20 °C until use. RT negative controls were performed on each sample (same reaction mix except for the reverse transcriptase).

2.7. Quantitative real-time RT-PCR analysis

The analysis of gene expression in liver tissue of European sea bass was carried out by qPCR using the primers listed in Table 1. Primers were designed using Primer3plus (<http://primer3plus.com/>), based on cDNA sequences (*trypsin*, *glut2*, *pk*, *atgl*, *phd3* and *ef1a*) available from the NCBI (<https://www.ncbi.nlm.nih.gov/>) and the Sigena website (<http://www.sigena.org/>). For *gp*, *gs*, *pepck*, *slc27* and *dgat1* genes, the cDNA sequences were predicted from the *D. labrax* genome using Genscan software (<http://genes.mit.edu/GENSCAN.html>).

Gene expression was quantified using an iCycler MyiQ™ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories Inc.). Standard curves were estimated for each primer pair using serial dilutions (from 1/10 to 1/270) of a pool of cDNA. qPCR efficiencies of primer pair ranged from 95 to 100%, with R² > 0.99. Each sample was run in triplicate in a final well volume of 15 µL, containing 5 µL cDNA (1/30 dilution) and 10 µL of reaction mix, composed of 0.5 µL of each

Table 1

Forward and Reverse Primer sequences used for quantitative PCR. *Efl1α* was used as housekeeping gene. Accession numbers of cDNA sequences (*trypsin*, *glut2*, *pk*, *atgl*, *phd3* and *ef1a*) available from NCBI (*) and the Sigenae (**) websites. For *gp*, *gs*, *pepck*, *slc27* and *dgat1* genes, the cDNA sequences were predicted from the *D. labrax* genome. Accession numbers of the *D. labrax* contigs available from the NCBI (#).

Gene	Accession numbers	Forward primer sequences (5'-3')	Reverse primer sequences (5'-3')
<i>trypsin</i>	AJ006882.1 (*)	CTCCACTGCTGACAGGAACA	CATGCCAGGGTAGGAGTTGT
<i>glut2</i>	EF014277.2 (*)	CAGCTGATCGTTGCACTCAT	TGTGGCATAGACTGGCTGAG
<i>gp</i>	CBXY010002083.1 (#)	TTCCAGACAAAAGTCGCTGT	GTCTTGTGAGGTCCCATGC
<i>gs</i>	CBXY010017321.1 (#)	GACAAGGAGGCAGGTGAGAG	GAAGACGTGAGCACAGTGGA
<i>pk</i>	KF857578.1 (*)	CAAGGTGGAAAGCCGCAAGGC	GGTCACCCCTGGCAACATCA
<i>pepck</i>	CBXY010006528.1 (#)	TCAGGTGGACCATGTATGT	GTCACCTGGACGCCATATTT
<i>atgl</i>	KF857294.1 (*)	GCTCCTTCTCGGTGCATAA	ATTCGCACAGTCTCTCCAA
<i>slc27</i>	CBXY010013343.1 (#)	GCTTCCCAATTCTGGAATGA	TGTCTCTTTGGTGGGTTG
<i>dgat1</i>	CBXY010017042.1 (#)	CCACAGAGGGGCATGAGTAT	CCATGACCCAGGAGTCTAC
<i>phd3</i>	DLPD06823 (**)	TCCTACTCCACAGGTACGC	GCAGTCATGTTGCTCTCCA
<i>ef1a</i>	AJ866727.1 (*)	CTGGAGGGCAGTGAAAAGAT	CATCAAGAGCCTCCAGCAGT

primer (10 mM), 1.5 μL RNase/DNase free water, 7.5 μL iQ™ SYBR® Green Supermix (Bio–Rad Laboratories Inc.) containing antibody-mediated hot-started iTaq DNA polymerase, dNTPs, MgCl₂, SYBR® Green I dye, enhancers, stabilizers and fluorescein. Negative controls (non-template control) were systematically included in each plate. RT negative controls were also used as template in order to ensure the absence of residual DNA contamination. The qPCR profiles contained an initial activation step at 95 °C for 2 min, followed by 39 cycles: 5 s at 95 °C and 20 s at 60 °C. After the amplification phase, a melting curve was performed to confirm the amplification of a single product in each reaction.

For each sample, the corresponding Cq (Quantification cycle) value was determined automatically using the “Gene Expression Module” of CFX Manager software (Bio–Rad Laboratories Inc.). Cq is the number of cycles required to yield a detectable fluorescence signal. The relative quantity of messenger was normalized with the ΔΔCt method, also using CFX Manager software. Several reference genes were tested to correct for loading differences or other sampling variations present in each sample. The *elongation factor 1-alpha (ef1a)* gene was used as reference gene in the liver tissue of juvenile fish since it did not show any significant variation of expression between samples (relative standard deviation < 5% among samples).

2.8. Statistical analyses

Statistical analyses were performed using STATISTICA software version 10 (<http://statsoft.fr/>). All data were log-transformed to fit a normal distribution. For all analyses, variables were checked for normality (Shapiro test) and equality of variances (Levene test). Three-way ANOVAs were used to determine the effects of larval treatments (oxygen and temperature) on growth, metabolites, enzymatic activities and gene expression in tested juveniles. Tukey’s test ($p < 0.05$) was performed for post-hoc comparisons of metabolite data. All figures were drawn using GraphPad Prism®(v.5.0b) software.

3. Results

3.1. Weight and growth rate

Weight of European sea bass juveniles measured at the beginning (196 dph) and at the end (296 dph) of the hypoxia exposure are shown in Table 2. The weights of juveniles at 196 dph were significantly higher (1.3 times) in fish previously reared at 20 °C during larval stage. At the end of hypoxia exposure (296 dph), the weights of juveniles exposed to moderate hypoxia were significantly lower (1.3 times) and there was no interaction with larval conditions.

The effects of larval and juvenile conditions on relative growth of juveniles are shown in Fig.2A–B. Statistical analysis (Table 3) revealed a significant effect of juvenile stage oxygen conditions on relative

growth rate ($p < 1 \times 10^{-6}$). This effect of juvenile oxygenation on growth depended upon larval oxygenation ($p < 0.02$). The decrease in growth of juveniles under hypoxia is even marked when fish have been exposed to hypoxia at larval stage (Fig.2A). Our data also revealed a significant effect of larval temperature conditions on juvenile growth ($p < 1 \times 10^{-6}$). Overall, juveniles reared at 15 °C during larval development exhibited higher (1.3 times) relative growth rates than those reared at 20 °C (Fig.2B). There was also a significant interaction between temperature and oxygen conditions at the larval stage on relative growth rate of juveniles ($p < 5 \times 10^{-3}$) (Table 3). Whether larval oxygenation affected the growth of juvenile bass depended, in part, on larval temperature: only when the temperature of larval development was 15 °C did larval hypoxia result in a slight reduction in juvenile growth (Fig.2B).

3.2. Hepatosomatic index and hepatic metabolites

The hepatosomatic index (HSI) decreased in juveniles exposed to hypoxia ($p < 2 \times 10^{-6}$) but was not significantly impacted by early life conditions (Fig.3A; Table 3). Similarly, juveniles exposed to moderate hypoxia displayed a significant decrease in hepatic glycogen content compared to the normoxic group ($p < 2 \times 10^{-6}$) (Fig.3B; Table 3). In addition, glycogen levels were significantly higher in the liver of juveniles that had experienced hypoxia during their larval period ($p < 2 \times 10^{-6}$). No significant difference in the hepatic glucose content was observed between the experimental groups (Fig.3C; Table 3). The levels of hepatic lactate were higher in juveniles exposed to hypoxia ($p < 2 \times 10^{-6}$) (Fig.3D; Table 3), but this hypoxia-induced increase in hepatic lactate content was not influenced by early life treatments ($p > 0.45$). Finally, lipid content only decreased under hypoxia in the liver of juveniles that had experienced 15 °C at larval stage (Fig.3E; Table 3).

3.3. Enzymatic activities

Hypoxia exposure at the juvenile stage led to significantly higher specific activities of GP ($p < 2 \times 10^{-6}$) (Table 4). Larval conditions did not significantly influence this effect ($p > 0.26$). Pancreatic lipase enzyme activity was not significantly regulated by either juvenile or larval treatments ($p > 0.21$; Table 4).

3.4. Gene expression

Real-time PCR analysis (Table 4) showed a significant decrease in trypsin expression in juveniles exposed to hypoxia ($p = 7 \times 10^{-6}$). Furthermore, higher trypsin expression was found in the liver of juveniles previously exposed to 15 °C during their larval stage compared to those exposed to 20 °C. Concerning genes involved in glucose metabolism, significant increases in *gs* and *pepck* gene expression were noted in

Table 2

Weight of European sea bass juvenile exposed to moderate hypoxia. Weight was measured at the beginning (initial weight: 196 dph) and at the end (final weight: 296 dph) of hypoxia exposure. Juveniles were exposed (HJ) or not (CJ) to hypoxia. They were previously exposed (HL) or not (CL) to hypoxia at two different temperatures (15 °C and 20 °C) during the larval stage. *N* = 30 juveniles for each combination of larval and juvenile conditions. Standard deviation was indicated for each value. The *p*-values were calculated from a three-way ANOVA analysis. L.Ox.: larval oxygenation; L.T.: larval temperature; J. Ox.: juvenile oxygenation. NS means non-significant. The *p*-values of the interaction between treatments were omitted (*p*-value = NS).

	CJ				HJ				p-Value		
	15°		20°		15°		20°		L. Ox	L. T.	J. Ox.
	CL	HL	CL	HL	CL	HL	CL	HL			
Initial weight	11.71 ± 2.8	12.08 ± 2.6	15.4 ± 3.6	14.07 ± 2.8	11.72 ± 2.2	12.4 ± 2.6	14.82 ± 3.4	14.54 ± 2.9	NS	NS	NS
Final weight	45.89 ± 9.1	46.64 ± 9.6	49.38 ± 9.1	47.91 ± 9.1	35.73 ± 6.7	34.15 ± 7.7	35.68 ± 8.5	34.92 ± 6.5	NS	NS	1 × 10 ⁻⁶

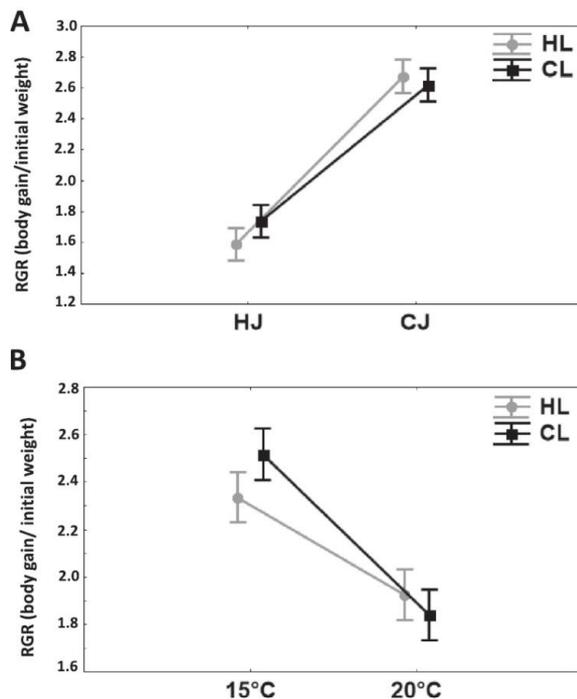


Fig. 2. A–B. Impact of juvenile and larval conditions on juvenile relative growth rates. A. Graph representing significant interaction (see Table 3) between hypoxia exposures at juvenile and larval stages on juvenile relative growth rates. Juveniles reared under moderate hypoxia (HJ, *n* = 120) or normoxia (CJ, *n* = 120) include fish previously exposed (HL, *n* = 120) or not (CL, *n* = 120) to moderate hypoxia (combining 15 °C and 20 °C) at larval stage. B. Graph representing significant interaction (see Table 3) between oxygen (HL, *n* = 120 and CL, *n* = 120) and thermic conditions (15 °C, *n* = 120 and 20 °C, *n* = 120) at larval stage on juvenile relative growth rates (combining HJ and CJ groups). The Y-axis represents the relative growth rate (body gain/initial weight). Error bars indicate standard deviation.

the liver of juveniles exposed to hypoxia, while *glut2*, *gp* and *pk* mRNA levels remained unchanged. In addition, conditions experienced at the larval stage did not influence subsequent expression of the genes involved in glucose metabolism at the juvenile stage (*p* > 0.06). Transcripts related to genes implicated in triglyceride metabolism (*atgl*, *slc27*, *dgat1*) did not exhibit any significantly different levels among the experimental groups, although there was a tendency for higher *slc27* mRNA levels (*p* = 0.06) in hypoxia-exposed juveniles. Finally, hypoxia induced a stimulation of *phd3* gene expression (*p* = 2 × 10⁻³) in hypoxia-exposed juveniles while larval conditions had no significant effect.

4. Discussion

Exposure to environmental hypoxia lowers metabolic activity in fish, which have to cope with limited aerobic ATP production while maintaining vital physiological functions. This effect requires a fine-tuned regulation of the metabolic pathways especially in the liver, which has a pivotal role in energy metabolism. As hypoxia can often be associated with warming episodes, we postulate that oxygen and temperature conditions at early life stages may influence metabolic programming, which could have a long-term influence on hypoxia-induced metabolic responses.

Our data revealed that exposure of juvenile European sea bass to moderate but chronic hypoxia induced significantly higher levels of hepatic *phd3* gene expression. This result indicates a stimulation of the HIF pathway and then hypoxia sensitivity after almost three months of exposure to 40% dissolved oxygen. Since it is acknowledged that the HIF pathway underlies cellular/tissular hypoxia response, its activation in the liver likely results in the regulation of different biological processes, including energy metabolism, and possibly impacts fish fitness components (Richards, 2009).

In European sea bass it has been shown that hypoxia induces a decrease in feed intake concurrent to a metabolic depression (Pichavant et al., 2001; Thetmeyer et al., 1999). It was therefore not surprising to observe a marked decrease in growth of juveniles exposed to hypoxia in the present study. Accordingly, in the pancreatic tissue of fish under hypoxia, we observed a significantly lower expression of *trypsin*, which is an indicator of food consumption and digestion (Rungruangsak-

Table 3

Statistical analysis of relative growth rate, hepatosomatic index (HSI) and hepatic metabolites (glycogen, glucose, lactate and lipids) in European sea bass exposed to different thermic and oxygen conditions at larval and juvenile stages. The *p*-values were calculated from a three-way ANOVA analysis. L.Ox.: larval oxygenation; L.T.: larval temperature; J. Ox.: juvenile oxygenation. NS means non-significant.

	L. Ox	L. T.	J. Ox.	L. Ox. x L. T.	L. Ox. x J. Ox.	L. T. x J. Ox	L. Ox.x L. T. x J. Ox.
Relative growth rate	NS	1 × 10 ⁻⁶	1 × 10 ⁻⁶	5 × 10 ⁻³	0.02	NS	NS
Hepatosomatic index (HSI)	NS	NS	1 × 10 ⁻⁶	NS	NS	NS	NS
Glycogen	1 × 10 ⁻⁶	NS	1 × 10 ⁻⁶	NS	NS	NS	NS
Glucose	NS	NS	NS	NS	NS	NS	NS
Lactate	NS	NS	1 × 10 ⁻⁶	NS	NS	NS	NS
Lipids	NS	1 × 10 ⁻⁵	1 × 10 ⁻⁵	NS	NS	1 × 10 ⁻³	NS

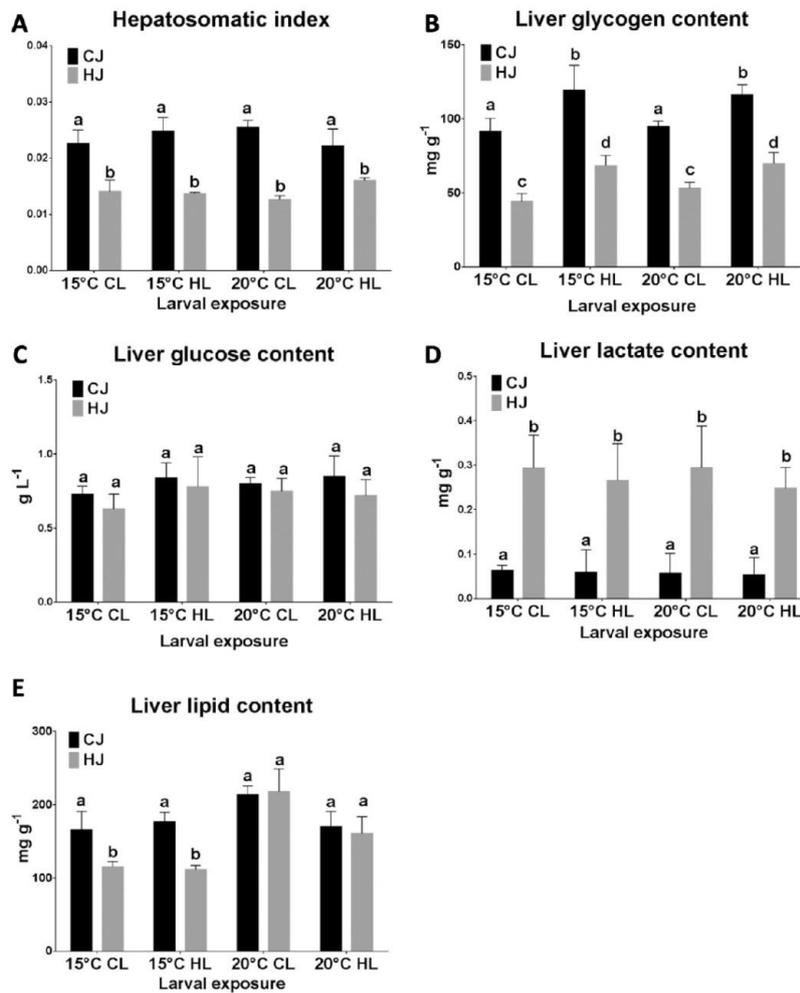


Fig. 3. Hepatosomatic index (HSI) (A) and hepatic metabolites: glycogen (B), glucose (C), lactate (D) and lipids (E) in European sea bass juveniles. Juveniles were either exposed (HJ) or not (CJ) to hypoxia and were previously exposed (HL) or not (CL) to hypoxia at 15 °C or 20 °C during the larval stage ($n = 4$ pools of 2 juveniles for each combination of larval and juvenile conditions). Error bars indicate standard deviation. Significant differences between groups are identified with different letters (three-way ANOVA followed by Tukey's test).

Torrissen et al., 2006). In addition, the lower hepatosomatic index (HSI) observed in juveniles exposed to chronic hypoxia, regardless of the larval conditions they experienced, confirmed the diminution of feed intake together with the mobilization and loss of energetic reserves, as reported in other teleosts, e.g., hybrid striped bass (Green et al., 2015) or common carp (Moyson et al., 2015).

The present data showed that a hypoxia event experienced during early-life history did not confer any benefit to sea bass in terms of growth when they were subsequently exposed to chronic hypoxia. On the contrary, juveniles that had experienced early hypoxia exposure showed a significantly lower relative growth rate under hypoxia conditions at the juvenile stage. Previous data obtained in European sea bass already indicated a negative impact of mild hypoxia exposure during the larval period on subsequent juvenile growth (Vanderplancke et al., 2015). Although we cannot rule out a potential effect of early hypoxia exposure on other components of fish fitness, our data does not support the idea that early exposure to low oxygen conditions improves the subsequent ability of European sea bass to cope with hypoxia, as observed in zebrafish (Robertson et al., 2014). Interestingly, we observed the highest growth rates in juveniles that had been reared at 15 °C as larvae compared to those reared at 20 °C. These higher growth rates likely resulted from compensatory growth that occurred after the reduced food consumption observed in larvae reared at low

temperature. Such compensatory growth is suggested by the higher *trypsin* expression found in juveniles previously exposed to 15 °C during their larval stage; *trypsin* is a recognized indicator of feed ingestion (Pedersen et al., 1990) and it is accepted that hyperphagia is the most common mechanism of growth compensation in fish (Ali et al., 2003).

Hepatic glycogen is an important energy resource during hypoxia episodes, able to provide sufficient glucose substrate for a heightened ATP demand under anaerobic metabolism. Accordingly, our data revealed lower glycogen stores in the livers of juveniles exposed to chronic hypoxia. Because the level of glycogen stores in a tissue depends on the balance between the rates of glycogenogenesis and glycogenolysis, our data suggest a higher rate of glycogen breakdown in the liver of fish exposed to chronic hypoxia. Both actors involved in hepatic glycogenogenesis (GS gene expression) and glycogenolysis (GP activity) appeared stimulated in juveniles under hypoxia. This result is in agreement with previous data obtained by Martínez et al. (2006) in the liver of Gulf killifish (*Fundulus grandis*) exposed to hypoxia. These authors attributed the stimulation of both anabolic and catabolic glycogen pathways in the liver to the existence of distinct hepatic cell populations that differ from one another in their glycolytic and gluconeogenic capacities (Mommensen et al., 1991). In *D. labrax* exposed to mild chronic hypoxia, higher GP activity revealed the stimulation of glycogenolysis in the liver, which could be explained by the enhanced

Table 4 Specific activity of GP and pancreatic lipase and relative expression levels of genes involved in the HIF pathway, digestion, glucose and triglyceride metabolism in European sea bass juveniles. Juveniles were exposed (HJ) or not (CJ) to hypoxia and previously subjected to larval hypoxia (HL) or not (CL) at two different temperatures (15°C and 20°C). N = 4 pools of 2 juveniles for each combination of larval and juvenile conditions. Standard deviation was indicated for each value. The p-values were calculated from a three-way ANOVA analysis. L.Ox.: larval oxygenation; L.T.: larval temperature; J. Ox.: juvenile oxygenation. NS means non-significant. The p-values of the interaction between treatments were omitted (p-value = NS).

Relative expression	P-Value																
	CJ				HJ				L. Ox				L.T.		J. Ox.		
	15°		20°		15°		20°		20°		20°		L. Ox	L.T.			
	CL	HL	HL	HL	NS	NS											
Specific activity U mg ⁻¹																	
GP	0.097 ± 0.009	0.092 ± 0.007	0.087 ± 0.005	0.092 ± 0.002	0.022 ± 0.004	0.022 ± 0.004	0.023 ± 0.005	0.154 ± 0.01	0.153 ± 0.003	0.023 ± 0.006	0.023 ± 0.006	0.152 ± 0.02	0.151 ± 0.01	0.023 ± 0.006	NS	NS	1 × 10 ⁻⁶
Pancreatic lipase	0.021 ± 0.004	0.021 ± 0.002	0.022 ± 0.004	0.022 ± 0.004	0.022 ± 0.004	0.022 ± 0.004	0.023 ± 0.005	0.023 ± 0.005	0.024 ± 0.003	0.023 ± 0.006	0.023 ± 0.006	0.023 ± 0.006	0.023 ± 0.006	0.023 ± 0.006	NS	NS	8 × 10 ⁻³
Trypsin	2.3 ± 0.4	1.56 ± 0.7	1.6 ± 0.6	1.03 ± 0.4	1.03 ± 0.4	1.03 ± 0.4	1.26 ± 0.3	1.26 ± 0.3	1.26 ± 0.3	1.26 ± 0.3	1.26 ± 0.3	1.26 ± 0.3	1.26 ± 0.3	1.26 ± 0.3	NS	NS	7 × 10 ⁻⁶
glut2	1.81 ± 0.9	1.41 ± 0.3	1.64 ± 0.5	1.58 ± 0.6	1.58 ± 0.6	1.58 ± 0.6	1.73 ± 1	1.78 ± 1.1	1.78 ± 1.1	1.78 ± 1.1	1.78 ± 1.1	1.78 ± 1.1	1.78 ± 1.1	1.78 ± 1.1	NS	NS	NS
gp	1.62 ± 0.3	1.49 ± 0.3	1.62 ± 0.2	1.26 ± 0.3	1.26 ± 0.3	1.26 ± 0.3	1.10 ± 0.5	1.34 ± 0.6	1.34 ± 0.6	1.34 ± 0.6	1.34 ± 0.6	1.34 ± 0.6	1.34 ± 0.6	1.34 ± 0.6	NS	NS	NS
gs	0.80 ± 0.1	0.70 ± 0.1	0.65 ± 0.1	0.54 ± 0.1	0.54 ± 0.1	0.54 ± 0.1	1.17 ± 0.1	1.17 ± 0.4	1.17 ± 0.4	1.17 ± 0.4	1.17 ± 0.4	1.17 ± 0.4	1.17 ± 0.4	1.17 ± 0.4	NS	NS	1 × 10 ⁻⁶
pk	1.94 ± 0.6	1.58 ± 0.3	1.91 ± 0.4	1.59 ± 0.3	1.59 ± 0.3	1.59 ± 0.3	1.72 ± 0.7	1.77 ± 0.8	1.77 ± 0.8	1.77 ± 0.8	1.77 ± 0.8	1.77 ± 0.8	1.77 ± 0.8	1.77 ± 0.8	NS	NS	NS
pepck	0.57 ± 0.3	0.56 ± 0.1	0.58 ± 0.2	0.57 ± 0.1	0.57 ± 0.1	0.57 ± 0.1	1.22 ± 0.2	1.34 ± 0.3	1.34 ± 0.3	1.34 ± 0.3	1.34 ± 0.3	1.32 ± 0.2	1.32 ± 0.2	1.32 ± 0.2	NS	NS	1 × 10 ⁻⁶
atgl	1.42 ± 0.6	1.23 ± 0.2	1.31 ± 0.3	1.48 ± 0.4	1.48 ± 0.4	1.48 ± 0.4	1.27 ± 0.5	1.71 ± 0.5	1.71 ± 0.5	1.71 ± 0.5	1.71 ± 0.5	1.71 ± 0.5	1.71 ± 0.5	1.71 ± 0.5	NS	NS	NS
slc27	0.77 ± 0.3	1.42 ± 0.6	1.39 ± 0.4	1.25 ± 0.8	1.25 ± 0.8	1.25 ± 0.8	1.81 ± 0.7	1.72 ± 0.3	1.72 ± 0.3	1.72 ± 0.3	1.72 ± 0.3	2.18 ± 0.6	1.62 ± 0.6	1.62 ± 0.6	NS	NS	NS
dgat1	1.43 ± 0.6	1.41 ± 0.4	1.55 ± 0.4	1.67 ± 0.5	1.67 ± 0.5	1.67 ± 0.5	1.26 ± 0.3	1.47 ± 0.8	1.47 ± 0.8	1.47 ± 0.8	1.47 ± 0.8	1.56 ± 0.2	1.39 ± 0.2	1.39 ± 0.2	NS	NS	NS
phd3	0.54 ± 0.2	0.48 ± 0.2	0.57 ± 0.3	0.61 ± 0.2	0.61 ± 0.2	0.61 ± 0.2	0.81 ± 0.2	1.34 ± 0.3	1.34 ± 0.3	1.34 ± 0.3	1.34 ± 0.3	0.95 ± 0.2	1.38 ± 0.3	1.38 ± 0.3	NS	NS	2 × 10 ⁻³
HIF pathway																	

glucose needs of glycolysis during anaerobic metabolism (Chippari-Gomes et al., 2005; Lewis et al., 2007; Richards, 2009). The necessity for fish to produce glucose as anaerobic fuel was also confirmed by the stimulation of the *pepck* gene, which is a key actor involved in gluconeogenesis. However, this stimulation of glycogenolysis and gluconeogenesis did not result in a higher hepatic glucose accumulation, likely due to an immediate utilization through glycolysis or outflow. Even though we could not detect any stimulation in the expression of genes involved in glycolysis (e.g., *pk*), higher concentrations of hepatic lactate observed in juveniles under hypoxia suggested a stimulation of the anaerobic glycolytic pathway in the liver. Previous studies on *D. labrax* revealed that mild chronic hypoxia induced a stimulation of glucose transporter *glut2* gene expression in liver and suggested that glucose produced from glycogen could leave the liver to serve as fuel in other tissues (Terova et al., 2009). In the present study we could not find any regulation of *glut2* gene expression in juveniles exposed to hypoxia, which does not rule out a potential regulation of glucose transport at the post-transcriptional level. Although we found higher glycogen stores in the livers of juvenile fish that had been exposed to hypoxia during their larval stage, we did not find any changes in several molecular indicators of glycogen catabolic and anabolic pathways that could help unravel the potential mechanisms involved. Particularly, while GP activity increased during hypoxic exposure of juvenile bass, GP transcript levels did not, which suggest post-transcriptional (e.g. phosphorylation) regulation of this enzyme. Considering that liver glycogen content is positively associated with chronic hypoxia tolerance in vertebrates, including fish (Bickler and Buck, 2007; Yang et al., 2015), we cannot rule out such a possible tolerance in the European sea bass juveniles that had experienced an early-life hypoxic event in the present study. Since early exposure to hypoxia did not enhance juvenile growth potential (as mentioned above), the potential advantage in terms of hypoxia tolerance will require further and additional investigation of physiological parameters such as those related to metabolic rates and key functions (e.g., reproduction).

In most vertebrates, including fish, glycogen is considered the primary source of stored energy for organisms exposed to environmental hypoxia. However, recent findings in burrow-dwelling goby, *Gillichthys mirabilis* and common carp *Cyprinus carpio* revealed triglyceride mobilization and lipid peroxidation in the liver during exposure to prolonged environmental hypoxia (Gracey et al., 2011; Mustafa et al., 2015). Accordingly, our data revealed a significant decrease of lipid content under hypoxia in the liver of juveniles that had experienced 15 °C at the larval stage; this difference in lipid content could be the consequence of a higher energy demand due to the higher growth observed in these fish. Measures of metabolic rates under mild chronic hypoxia would have made it possible to test this hypothesis. While Gracey et al. (2011) showed in goby that triglyceride mobilization under hypoxia involved the stimulation of key genes related to triglyceride hydrolysis (e.g., *atgl*) and fatty acid transport (*slc27*), as well as the downregulation of genes associated with triglyceride synthesis (*dgat1*), our data only revealed a tendency for higher *slc27* expression in juveniles exposed to hypoxia. In addition, we did not observe any regulation in the activity of pancreatic lipase, which plays a role during periods of lipid mobilization (van den Thillart et al., 2002). Further investigation will be necessary to elucidate the different mechanisms involved in the control of lipid storage (e.g., lipid peroxidation) as well as the functional significance of lipid depletion under hypoxic conditions in European sea bass.

5. Conclusion

In conclusion, the present study reveals that oxygen and temperature conditions experienced by European sea bass at early life stages have long term effects on features that could affect the metabolic response to hypoxia at the juvenile stage. These effects are reflected through long lasting regulation of glycogen and lipid stores by early oxygen and temperature condition, respectively. These effects can be

attributed to developmental plasticity, although the specific underlying mechanisms remain to be determined. Further investigations are now required to clarify whether early-life exposure to hypoxia conditions (low oxygen and warm temperature) actually results in a higher tolerance to moderate O₂ deprivation episodes during the juvenile period.

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Chapter 5

General Discussion

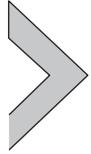




Climate change is altering oceanic conditions and marine fish could be faced to important modifications of their environment all along their life. One puzzling issue relates to the fact that environmental encountered during young stages could shape future juveniles and one could expect that this process allows them to better face similar environmental conditions (adaptive developmental plasticity). There are many studies that have investigated the consequences of this early stressing exposure conditioning in fish (Robertson et al. 2014; Vanderplancke et al. 2015b; Zambonino-Infante et al., 2013), but most of them have mainly focused on the effect of only one stressor. Although these studies reported valuable information, they didn't represent a realistic picture of what actually happens in nature, where marine organisms encounter several stressors simultaneously. Multiple stressor experiments are therefore crucial to gain a better understanding of future vulnerability of species, populations and ecosystems. This thesis specifically addressed this question by considering the effect of water deoxygenation in different temperature conditions acting during the larval period on subsequent life-history traits of resulting juveniles. Further, this dissertation highlights the attempting to understand how these specific developmental environments may influence fish future ability to cope with low dissolved O₂ levels.

The experiments performed during my PhD led to the collection of original data, most of which having been published. In chapter 2, our results indicated that the ability of juveniles to tolerate acute hypoxia stress was negatively impacted by hypoxia but not by the thermal conditions experienced at larval stage. We also showed that this impact was related to the prevalence of unilateral and, in a lesser extent, bilateral opercular abnormalities that likely negatively influence the capacity for oxygen extraction. In chapter 3, we characterized for the first time the Hb system in European sea bass. We revealed stage- and tissue-specific Hb gene expression patterns, suggesting a broad range of roles of Hb proteins over sea bass life cycle. Above all, our data showed that the regulation of Hb gene expression is involved in the molecular response to hypoxia in European sea bass. Such regulation appears to be specific to early-life stages and involves different Hb genes, reinforcing the idea of distinct functional properties of Hb subtypes. At juvenile stage, moderate hypoxia did not seem to regulate Hb gene expression, except in fish that have experienced an early-life hypoxic episode. In those fish, we evidenced that Hb gene expression was still regulated by hypoxia in the head kidney, suggesting a likely long-term effect of the early-life exposure to moderate hypoxia. Finally, chapter 4 revealed that oxygen and temperature conditions experienced by sea bass larvae have long term effects on metabolic features. These effects are reflected through long lasting regulation of glycogen and lipid stores by early oxygen and temperature conditions, respectively. These long term effects are likely to influence the tolerance of fish to hypoxia at the juvenile stage.

Here after we briefly discuss some of the issues which we assume to be especially important to understand the long term implications of early exposure to non-lethal low dissolved O₂ waters and elevated temperature. In particular, we will focus on understanding whether early exposition to an environmental constraint makes fish able to better cope with the same stressor in later life stages.



1. Relevance of environmental constraints at larval stage

The aim of the present experimental protocol was to test the effect of moderate hypoxia under different temperature conditions that European sea bass larvae may experience in temperate coastal areas. Such environmental conditions were applied in a fine tune balance ensuring larval response but avoiding any significant larvae mortality that could induce a potential genetic selection. The hypoxia intensity (40% air saturation) was determined based on previous experimental exposures performed in European sea bass larvae (Vanderplancke et al., 2015a). The fact that any significant mortality was observed even when we combined hypoxia with elevated temperature (20°C) suggests that larvae were able to tolerate these experimental conditions and that any genetic selection was induced. Moreover, we also noted that sea bass larvae have implemented physiological responses to cope to the hypoxic and thermal conditions encountered, as illustrated by the regulation of physiological functions (see below) known to be linked to these two abiotic parameters. Globally, our results showed that the experimental conditions applied during the early-life period, totally felt in the buffering-capacity range of sea bass larvae.

Environmental constraints of higher intensity (e.g. dissolved oxygen <30% air saturation; temperature >20°C) and/or earlier developmental window of stimulus (e.g. embryonic stage) would likely have been more efficient in terms of “programming” effects for surviving fish (Lindström, 1999), and would also have induced more contrasting physiological responses. However, the objective of this study was to mimic a realistic field condition. An earlier developmental window for the experimental environmental constraints would not have ecological relevance since, after hatching in odd-shore waters, sea bass larvae drift into coastal waters at the flexion stage (i.e. from day 22 post-hatching). Similarly, higher temperatures would have been unrealistic considering the mean winter temperature in temperate Atlantic areas. Despite our willingness to give a realistic dimension to the experimental design, we are aware that larvae can simultaneously experience other environmental stressors in the wild (acidification, pollution, food availability...) that are out of our understanding in the present study.



2. Larval sensitivity to experimental conditions

We hypothesized in the present work that exposure to hypoxia at different temperatures during early life stage could have long lasting effects on physiological parameters related to O₂ uptake/transport and to energy metabolism influencing physiological performance in European sea bass. This hypothesis was based on the fact that abiotic conditions encountered by organisms during their early life developmental period can have lasting effects on future animal traits. Several studies related these long-lasting effects to developmental plasticity (Jonsson and Jonsson, 2014; Monaghan, 2008). Specially, it has been shown that exposure to severe hypoxia at embryonic stage in zebrafish enhanced hypoxia tolerance in subsequent developmental stages due to a modification of developmental trajectories (Robertson et al., 2014). In fact, this effect observed in zebrafish, resulted from biased sex ratio in favor of males, which exhibit higher tolerance to hypoxia than females. It was also shown that the early stimulation of HIF-1 pathway was involved in the mechanisms underlying this long-term impact of hypoxia in zebrafish.

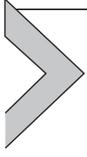
In the present work, we planned to investigate HIF-1 pathway to determine whether moderate hypoxia condition had a physiological impact on larvae. We first tried to assess the regulation of HIF-1 pathway through the detection and quantification of HIF-1 protein. Indeed, HIF-1 protein is stabilized by hypoxic condition, and functions as a master transcriptional regulator of the adaptive cellular response to hypoxia. Unfortunately, no commercial HIF-1 antibody allowed us to detect HIF-1 protein in European sea bass and our attempts to develop specific HIF-1 antibodies failed. Consequently, we have relied on the mRNA level of *phd-3* gene which is considered as an oxygen sensor whose expression is stimulated via HIF-1 pathway (Aprelikova et al., 2004). The higher quantity of *phd-3* mRNA observed in larvae exposed to moderate hypoxia (chapter 3) suggested the stimulation of HIF-1 pathway and the implementation of physiological responses.

Sensitivity of European sea bass larvae to our experimental hypoxic conditions was also clearly revealed by the huge regulation of Hb genes (chapter 3). Hb gene expression response depended on subtypes considered, which reveals the fine tune regulation of O₂ transport. Strong hypoxia-induced regulation of some Hb gene expression (until 14 fold induction) during larval development is consistent with the necessity for larvae to sustain the high O₂ requirements associated with tissue and organ development (Nilsson and Ostlund-Nilsson, 2008).

Finally, also the analysis of growth reflected the sensitivity of European sea bass larvae to the tested environmental conditions (chapter 2; see below section 3.1). Higher growth rates observed for larvae reared at 20°C compared to those reared at 15°C is consistent with the physiological

response expected in ectotherm organisms. Even if feed intake was not monitored during larval exposure, we assume that larvae reared at 20°C ingested more food in order to provide enough energy and sustain increasing metabolism (Fonds et al., 1992; Keckeis et al. 2001). By contrast, a marked decrease in growth rate was observed in larvae exposed to our hypoxia condition compared to those reared in normoxic condition. Stunting induced by hypoxia is known to be due to reduced feed intake and metabolic depression [e.g. European sea bass (*Dicentrarchus labrax*) (Pichavant et al., 2001); summer flounder (*Paralichthys dentatus*) and winter flounder (*Pseudopleuronectes americanus*) (Stierhoff et al. 2006) and great sturgeon (*Huso huso*) (Bagherzadeh-Lakani et al. 2013)]. This finding is consistent with previous one obtained in European sea bass suggesting metabolic depression in larvae exposed to similar moderate hypoxia Vanderplancke et al. (2015b). It is admitted in several organisms, including fish, that environmentally-induced regulation of growth rate at early life stages may influence subsequent growth trajectories associated to physiological trade-offs (Lee et al., 2012). The role of the thermal condition during development on growth has been especially investigated. The growth compensation (i.e. accelerating growth trajectory) resulting from atypical temperatures during early life stage was associated in sticklebacks with alteration of swimming activity, modification of sex maturation and decline of life span (Lee et al., 2010; 2012). Mechanisms behind such long term impacts are not well known, but energy allocation, oxidative stress and carry over effects on muscular development appear to underlie these effects (Johnston et al., 2003; Metcalfe and Alonso-Alvarez 2010; Monaghan et al. 2009). In the present work, we assumed that the regulation of growth by oxygen and thermal conditions observed at larval stage could also be associated with subsequent modification of growth trajectories and related physiological trade-offs (discussed in paragraph 3).

Altogether, our findings suggest that sea bass larvae do perceive the early environmental stressors and react to them. In fact, larvae likely use a suite of physiological regulations to cope with thermic and hypoxic conditions, which may be interpreted as larval sensitivity to these constraints. Such physiological regulations implemented by sea bass larvae could also result in long-lasting effects on animal performance and fitness at the juvenile stage.



3. An early environmental constraint at larval stage has long-term effects in juveniles

3.1 Long-lasting phenotypic effects at juvenile stage under normoxic conditions

The potential long-term effects of early exposure to thermic and hypoxic conditions at larval stage were first investigated in juveniles reared under normoxic conditions. The overall strategy was to apply different water temperatures in order to better reveal physiological trade-offs in juveniles. In fact, some trade-offs may become evident and detectable only when fish has to cope with specific temperature. Over one year, the overall physiological status of juveniles was then evaluated through hypoxia challenge tests and growth rate. In one group, water temperature was intentionally not controlled and it therefore followed the natural thermal cycle (10 -17°C). Another group of sea bass juveniles was exposed to two different constant temperatures (15°C and 20°C) for three months (from 196 to 296 dph). It is admitted that metabolic rate increases exponentially with rising temperature as a result of thermodynamic effects on biochemical reactions (Schulte, 2015). Thus, an increase in water temperature (20°C) in the present study have likely forced the metabolic machinery, and in turn allowed the detection of long-lasting effects more easily than lower temperatures. Characterization of physiological traits related to metabolism and O₂ transport (Hb system) was then performed in these juveniles reared at 15°C and 20°C.

➤ **Growth**

The regulation of growth rate at larval stage under hypoxic and thermic conditions on subsequent growth trajectories was investigated during the natural thermal cycle (10-17°C) and under controlled constant temperatures (15°C and 20°C).

Hypoxia conditioning

First, growth trajectories were investigated in juveniles following the natural thermal cycle (10-17°C). After the marked decrease in growth rate observed at the end of the early-life hypoxia exposition (50 dph, Fig. 1A), fish displayed an elevated growth rate (1.4 times higher than control from 50 to 172 dph; Fig.1A) when back to normoxic conditions. This is a well-known strategic adjustment documented in several organisms, including fish, which occurs after a period of food restriction or starvation and was generally termed as compensatory growth (Maclean and Metcalfe, 2001; Lee et al., 2010; 2012). The mechanism underlying such compensatory growth observed in our study may be related to an increase in food consumption (i.e. hyperphagia), as previously reported

by Remen et al. (2014) in juvenile Atlantic salmon (*Salmo salar*) which had experimented 69 days of hypoxia at 50% air saturation. These authors noted that those fish increased their feed intake when back to normoxia, and exhibited a more rapid growth after only 30 days. In the present study, sea bass juveniles early exposed to hypoxia at larval stage not only recovered the normal size but also showed higher body weight than control after the period of growth compensation, which is likely akin to an overcompensation (172 dph, Fig. 1B). Overcompensation has been documented in fish and other vertebrates when the severity of the growth depression increases the duration of the hyperphagic phase (Ali et al., 2003; Gurney and Nisbet, 2004). The literature indicated that such compensatory growth is highly variable depending on the species and on the nature of the hypoxic constraint (e.g. intensity, duration, developmental window) (Remen et al. 2012; Foss and Imsland, 2002; Person-Le Ruyet et al., 2003), and sometimes it could also lead to a partial growth recovery (Remen et al. 2012). Finally, from 172 to 540 dph growth rate regularly diminished in juveniles that had experienced hypoxia at larval stage, and ended at 1.1 times significantly lower than that of the control group (Figure 1A). As feed ingestion was not measured in our study, we are not able to indicate if this process corresponds to a return to a more normal feeding rate.

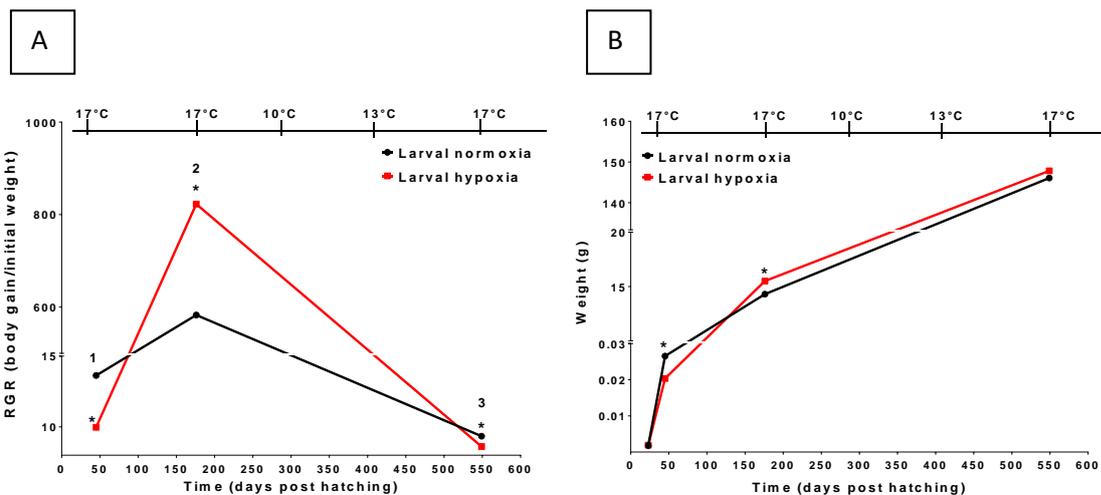


Figure 1. Relative growth rate (body gain/initial weight) (A) and weight (B) of juveniles early exposed or not to hypoxia at larval stage (including fish early exposed to 15°C and 20°C at larval stage) (n for each group= 200 juveniles). Water temperature followed the natural thermal cycle (10 - 17°C). Asterisks means significant difference for each point (one-way ANOVA). Growth rate (A) was calculated in the following periods: 1 (from 23 to 50 dph); 2 (from 50 to 172 dph) and 3 (from 172 to 540 dph).

At 196 dph, a subgroup of juveniles was selected from fish reared under the natural thermal cycle. Then, from 196 to 296 dph growth was monitored for these juveniles at two controlled

temperatures 15°C or 20°C (Fig. 2). As expected, the higher the temperature, the higher the growth rate (2.3 times higher); however, although 20°C has clearly pushed fish metabolism and growth, we did not reveal any long-lasting effect of early exposure to hypoxic conditions. This result is in contrast with previous data obtained in European sea bass showing a long-term negative impact on growth rate in juveniles that have experienced an early-life hypoxia event (Vanderplancke et al., 2015b). It is likely that a brief period of 3 months at 20°C used in the present study, compared to 7 months at 20°C in Vanderplancke et al., (2015b), was not sufficient to reveal any potential adverse effect on growth.

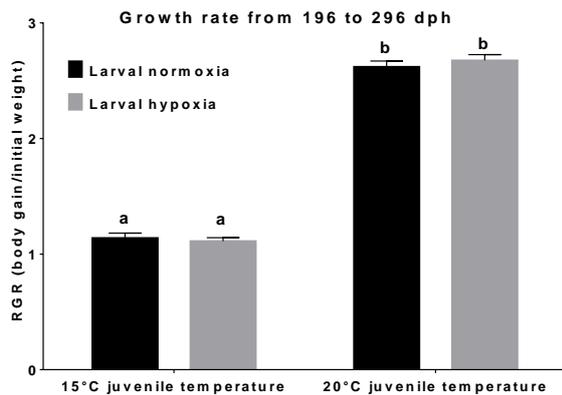


Figure 2. Relative growth rate (body gain/initial weight) from 196 to 296 dph of juveniles under normoxic conditions at 15°C or 20°C. Juveniles were early exposed or not to hypoxia at larval stage (including fish early exposed to 15°C and 20°C at larval stage) (n for each group= 60 juveniles). Significant differences between groups are identified with different letters (two-way ANOVA followed by Tukey's test).

Thermal conditioning

As mentioned before (section 2), our data showed that larvae exposition to 15°C led to 2,1 times lower growth rate at 50 dph than larvae exposed to 20°C (Fig. 3A). A compensatory growth (1.7 times higher) was observed in fish that have been reared at 15°C compared to 20°C, when the natural thermal cycle (starting at 17°C) was applied (from 50 dph to 172 dph, Fig. 3A). The influence of the thermal condition history on the compensatory growth has been extensively investigated in fish (Maclean and Metcalfe, 2001; Lee et al., 2010; 2012) since temperature acts as a dominant abiotic factor controlling feeding and growth in ectotherms (Brett, 1979). However, this compensatory growth did not allow to catch up the delay in growth at 540 dph (chapter 2, Fig. 3B) in natural thermal cycle; an additional yearly cycle would have been necessary. Previous studies in teleost fish showed that the time required to reach normal size after a period of cooler temperature

is highly variable, depending on species, range of temperatures used before and after growth compensation and developmental window of exposure. For instance, juvenile Atlantic salmon (*Salmo salar*) subjected for three weeks of cooler temperatures (8.4 °C) caught up in size 20 weeks later at 16.4 °C (Maclean and Metcalfe, 2001). In the case of three-spined stickleback juvenile (*Gasterosteus aculeatus*), 12 days at 10°C were needed to reach the normal adult size after 4 weeks of at 6°C (Lee et al., 2010).

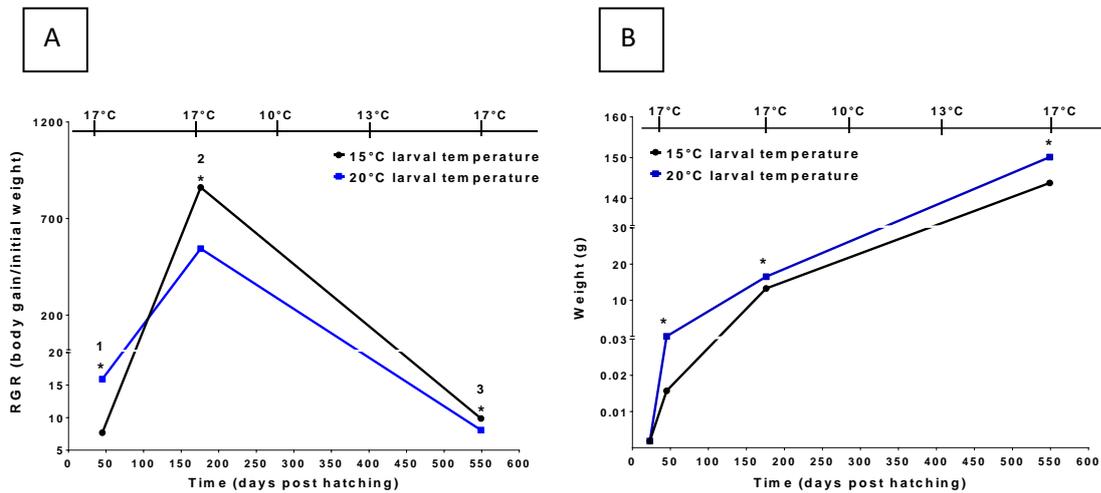


Figure 3. Relative growth rate (body gain/initial weight) (A) and weight (B) of juveniles early exposed to 15°C or 20°C at larval stage (including fish early exposed or not to hypoxia at larval stage) (n for each group= 200 juveniles). Water temperature followed the natural thermal cycle (10 -17°C). Asterisks means significant difference for each point (one-way ANOVA). Growth rate (A) was calculated in the following periods: 1 (from 23 to 50 dph); 2 (from 50 to 172 dph) and 3 (from 172 to 540 dph).

General considerations

It has to be pointed out that, the benefits of compensatory growth to reach large body size (e.g. mating, reproduction and survival), could be counterbalanced by the oxidative stress linked to fast growth; oxidative stress generally results in increased rates of cellular damage (Jennings et al. 2000; Monaghan and Hausmann 2009). Such damage has also been shown to modify muscle structure leading to reduced locomotor performance in Antarctic fishes (Johnston et al., 2003) and decreased reproductive capacity in females of perch (*Perca fluviatilis*) (Holmgren 2003). The recovery from tissue damages often needs activation of protein turnover or repair (Johnston 1999), leading to increase metabolic rates (Metcalf and Alonso-Alvarez 2011). However, we did not find any influence of the early exposure to thermic and hypoxic conditions on standard metabolic rate (SMR), routine

metabolic rate (RMR), and maximal active metabolic rate (MMR) (collaboration with Pr. Guy Claireaux and Dr. Helene Ollivier; data not shown): the potential impact of compensatory growth requires then further and additional investigation of physiological parameters such as those related to oxidative stress, swimming performance and reproductive output.

➤ ***Metabolism related to growth compensation***

Allocation of energy reserves (i.e. glycogen and lipid) related to growth compensation was investigated in juveniles exposed at 15°C and 20°C under 100% air saturation. Juvenile metabolism was evaluated in hepatic tissue, which has a pivotal role in energy production.

Hypoxia conditioning

Glycogen stores were higher at 20°C than at 15°C, which was not really surprising since warmer temperatures had likely induced higher food consumption (Buentello et al., 2000; Richards, 2009). We also noted that juveniles early exposed to hypoxia at larval stage exhibited at 296 dph higher hepatic glycogen stores at the two different temperatures [20°C (chapter 4, Fig. 4A) and 15°C (Fig. 4 A)], which could mean that hypoxia conditioning would have oriented the energy metabolism towards glycogen production as a carry-over strategy in order to cope with future hypoxia; indeed, this metabolite is mainly mobilized during anaerobia. However, we did not find any changes in several molecular indicators of glycogen catabolic and anabolic pathways (e.g. glycogen synthase and glycogen phosphorylase) that could confirm our hypothesis or help to unravel the potential mechanisms behind such glycogen storage. In human cells, prolonged hypoxia promotes glycogen accumulation that might serve to replenish glycogen stores and improve cell survival following subsequent hypoxic episodes (Pescador et al., 2010). Considering that the capacity to support ATP turnover during long-term hypoxia exposure relies on glycogen reserves in fish species (Nilsson and Renshaw, 2004; Stecyk et. al, 2004), we cannot rule out a potential effect of early hypoxia exposure on subsequent tolerance to low O₂ levels in sea bass juveniles.

When reared at 20°C, juvenile fish exhibited lower lipid reserves than at 15°C; it very likely that this could be a consequence of the rapid growth and increased metabolism at 20°C, and the need of energy to sustain such physiological demands (Fig. 4B, Keckeis et al. 2001). We did not observe any long-term effect of early-life exposure to hypoxia or any possible remaining “metabolic trace” of growth overcompensation (Fig. 5B). We should consider that lipids are mainly deposited in visceral tissue in European sea bass (Dias et al., 1998). Since energy allocation results from a complex interplay between several tissues, further and additional investigation in other storage tissues (i.e.

muscle, visceral...) would represent useful information for a better understanding of energy metabolism related to compensatory growth in European sea bass.

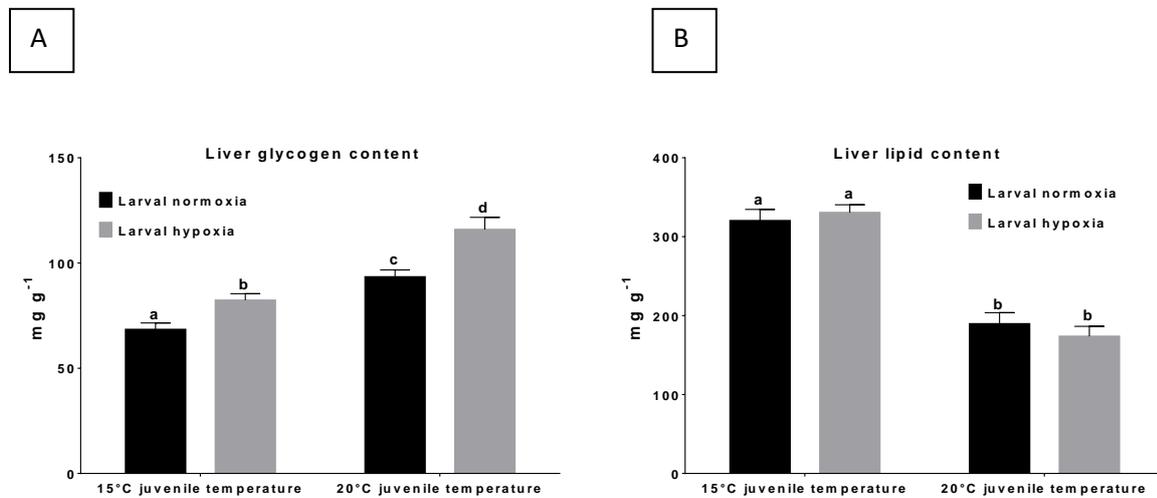


Figure 4. Hepatic metabolites (glycogen (A) and lipid (B)) at 296 dph of juveniles under normoxic conditions at 15°C or 20°C. Juveniles were early exposed or not to hypoxia at larval stage (including fish early exposed to 15°C and 20°C at larval stage) (n for each group= 8 pools of 2 juveniles). Significant differences between groups are identified with different letters (two-way ANOVA followed by Tukey's test).

Thermal conditioning

We did not find any long-term effects of early-life thermal conditions on juvenile hepatic stores (glycogen and lipids; Figure 5A and B), whatever the juvenile rearing temperature. In addition, gene expression and activity of proteins involved in carbohydrate and lipid metabolism were not influenced by thermal conditions experienced at larval stage.

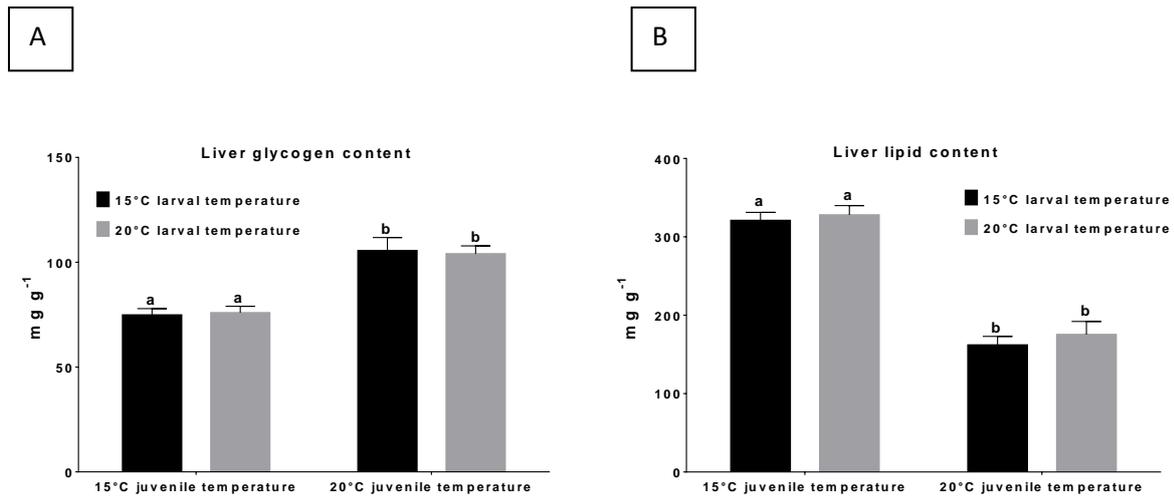


Figure 5. Hepatic metabolites (glycogen (A) and lipid (B)) at 296 dph of juveniles exposed under normoxic conditions at 15°C or 20°C. Juveniles were early exposed to 15°C or 20°C at larval stage (including fish early exposed or not to hypoxia at larval stage) (n for each group= 8 pools of 2 juveniles). Significant differences between groups are identified with different letters (two-way ANOVA followed by Tukey's test).

➤ Oxygen extraction and transport

Since O_2 is an essential component in producing energy resources for an organism and serves as substrate in several enzymatic reactions, we focused our attention on the capacity of juvenile fish to acquire O_2 from its environment (opercular structure) and to transport it by O_2 -carry proteins (hemoglobins) to all body tissues. Analysis of branchial parameters (e.g. gill surface, cellular composition and diffusion distance) are in progress (collaboration with Isabelle Leguen, INRA) since the gills acts as the main structure involved in O_2 extraction together with operculum.

Hypoxia conditioning

We showed that an early-life moderate hypoxic episode caused an incidence of unilateral and bilateral opercular deformities in sea bass juveniles. It is well known that hypoxia exposure can disrupt morphogenesis and organogenesis during embryonic and early larval stage development resulting in hatching anomalies and skeletal deformities in ocular, spinal and craniofacial areas (Hassell et al., 2008; Mejri et al., 2012; Nicholson et al., 2008; Wu, 2009). The present work reveals that some deformities may be induced by hypoxia during the late stages of larval development in European sea bass. The detection of larval deformities is not easy because it requires an accurate histological analysis at early-life stages, and in some cases such malformations are only evidenced in

later life stages. In the present study, only a 10% of the juveniles early-life exposed to moderate hypoxia developed opercular deformities, which were difficult to detect in a tank with 400 fish. Such opercular deformity was fortuitously detected after an acute hypoxia challenge test when we noted that less tolerant fish had this deformity. Previous studies reported that the ability of an organism to acquire O₂ from its environment is an important determinant of hypoxia tolerance (Mandic et al., 2009). According to that, we found that the difference in hypoxia tolerance related to early-life exposure to acute hypoxia is totally explained by the existence of opercular abnormalities that likely impact the capacity for oxygen extraction.

The mechanisms underlying these hypoxia-induced opercular abnormalities still need to be deciphered. In a previous study performed in the gilthead sea bream (*Sparus aurata*), Beraldo et al., (2003) suggested that an increase in ventilation frequency at the beginning of operculum ossification (i.e. 400 °C-days) resulted in opercular malformations. It is very likely that such hypothesis could also be valid in the present study, because larvae were exposed to hypoxia during the developmental window corresponding to opercular ossification in sea bass (around 500°C-days; Darias et al., 2010). However, only 10% of juveniles early exposed to hypoxia exhibited opercular deformities that revealed a significant inter-individual variation in such malformations. Many factors (e.g. genetic; physiological sensitivity) could be related to this variability and future research would help to better characterize the intra-specific variation in the incidence of opercular deformities in sea bass larvae. The early-life hypoxia exposure period may also be refined to determine more precisely the critical window of larval development.

As mentioned above, only a very low proportion of sea bass juveniles were affected by these deformities. In the wild, larvae with such deformities are likely vulnerable to environmental constraints (e.g. predation, infection, hypoxia...) and we assume that they have short lifetime. For these reasons, we have made the choice to not use fish with opercular deformities for the investigation of long term effect on other physiological parameters (metabolism and capacity of oxygen transport).

The capacity of oxygen transport in sea bass juveniles at 15°C (chapter 3) and 20°C (data not shown) that experienced an early life hypoxia-event was evaluated through the analysis of (i) Hb gene expression in hematopoietic tissues (head kidney and spleen) and (ii) haematological parameters (i.e. Hb protein levels and erythrocyte volume, chapter 3) in arterial blood. The huge regulation of Hb genes observed in larvae exposed to hypoxia did not lead to any long-term disturbance at juvenile stage in Hb gene expressions under normoxic conditions. Even when the temperature increased up to 20°C at juvenile stage, which may result in an increase of oxygen

demand (Schulte, 2015) and therefore in a regulation of O₂ transport capacity (Richards, 2009), we did not find any long-lasting effect on the Hb gene expression in the head kidney and spleen. In line with Hb expression, the Hb protein levels and erythrocyte volume (hematocrit) in the blood of juveniles were not impacted by the early-life exposure to hypoxia. However, additional functional analysis would be required to evaluate the potential effects of early-life hypoxia on Hb properties (e.g. blood oxygen affinity). Considering that the Hb expression has been found in many non-hematopoietic organs (e.g. intestine, gills, brain, heart...) (chapter 3; Feng et al., 2014; Terova et al., 2011; Ullal et al., 2008) of fish, it could also be worth evaluating potential long-lasting effect in other tissues.

Thermal conditioning

Our results also showed an impact of the warmer temperature at larval stage on the incidence of opercular deformities in sea bass juveniles. The mechanisms underlying these effects may be also related to an increase in the ventilation frequency at larval stage due to the higher metabolism resulting from warmer temperatures. The analyses of the capacity of O₂ transport (e.g. Hb gene expression, Hb protein levels) in juveniles exposed to thermic conditions at early-life stage did not reveal any potential long-lasting effect (data not shown).

➤ ***Long-term interaction between hypoxia and thermal conditioning on juvenile physiology***

In the range of temperature and oxygen conditions applied at the larval stage, we did not observe any long-term interaction of these two environmental factors in sea bass juveniles reared under normoxic conditions, even when the temperature increased up to 20°C. These results contrasts with both empirical and theoretical data which indicates that warm temperature and hypoxia are likely to interact synergistically in fishes, because both of these stressors affect aerobic metabolism (McBryan et al., 2013; McBryan et al., 2016; Pörtner and Farrell, 2008); indeed, raised temperatures increase metabolism, resulting in higher O₂ demands, while hypoxia limits the availability of environmental O₂ (McBryan et al., 2013; McBryan et al., 2016). The interaction of temperatures and hypoxia levels applied at the larval stage, in the present study, probably felt in the buffering capacities of sea bass larvae. It is also likely that higher environmental constraints (e.g. dissolved oxygen <30% air saturation; temperature >20°C) would have induced stronger effects at the larval stages, and would allow us to reveal long-lasting synergistic effects at the juveniles stage.

3.2 Long-lasting phenotypic effects at juvenile stage under moderate hypoxia

As often mentioned, one of the main objective of this thesis was to test whether the early-life conditioning to specific environmental conditions better prepare juveniles to cope with similar environments at later life stages. In order to answer this question, juvenile fish were exposed for three months to the same environmental constraints that they have experienced at larval stage [(40% air saturation at two different temperatures (15°C and 20°C)]. Long-term effects of the early exposure were then evaluated in terms of growth rate, metabolic parameters in the liver and O₂ transport (Hb system).

➤ Growth

As a fitness component, growth rate was used to evaluate the overall physiological status of sea bass juveniles under hypoxia.

Hypoxia conditioning

We showed that a hypoxia event experienced during sea bass larval development did not confer any benefit in terms of growth when they were subsequently exposed to chronic hypoxia at juvenile stage. On the contrary, we revealed a physiological limitation since, when hypoxia was combined with elevated temperature (20°C) at juvenile stage, the growth rate was even more depressed (chapter 4; Fig. 6). This limitation likely resulted from a lower capacity of those fish early-life exposed to hypoxia to find a balanced trade-off between increased metabolic demand for O₂ (induced by warming) and a limited O₂ supply (induced by hypoxia) (Mc Bryan et al., 2016, 2013). Vanderplancke et al. (2015b) also reported such a long-term negative effect on growth after an early-life hypoxia exposure, and this effect was associated with lower protein digestive capacity in the intestine (Zambonino et al. 2017) and long-lasting down-regulation of some genes involved in energy consuming metabolic pathways in the liver (Vanderplancke et al. 2015b). In the present study, we wanted to go further in the understanding by analyzing carbohydrate and lipid metabolism functioning under low oxygen availability, in those juveniles early exposed to hypoxia at larval stage (see below).

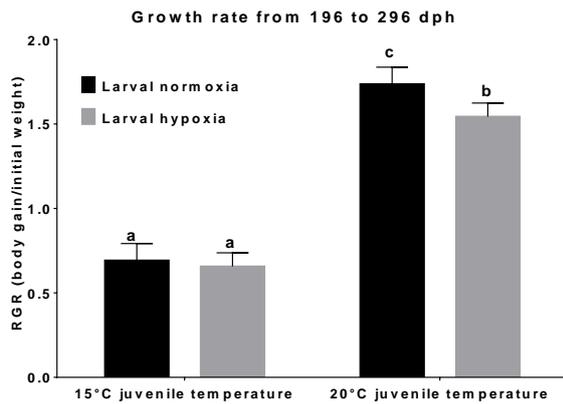


Figure 6. Relative growth rate (body gain/initial weight) from 196 to 296 dph of juveniles under moderate hypoxia (40% air saturation) at 15°C or 20°C. Juveniles were early exposed or not to hypoxia at larval stage (including fish early exposed to 15°C and 20°C at larval stage) (n for each group= 60 juveniles). Significant differences between groups are identified with different letters (two-way ANOVA followed by Tukey's test).

Thermal conditioning

Juveniles reared at 15°C during the larval stage showed higher growth rates than those reared at 20°C. As already mentioned and discussed for fish reared under normal oxygenation (in section 3.1), this result also reinforces the hypothesis that a compensatory growth occurs even under chronic hypoxia, after an early-life cold thermal exposure (chapter 4, Figure 7). The compensating growth resulting from early exposure to lower temperature is observed at whatever temperature the fish is exposed as juvenile.

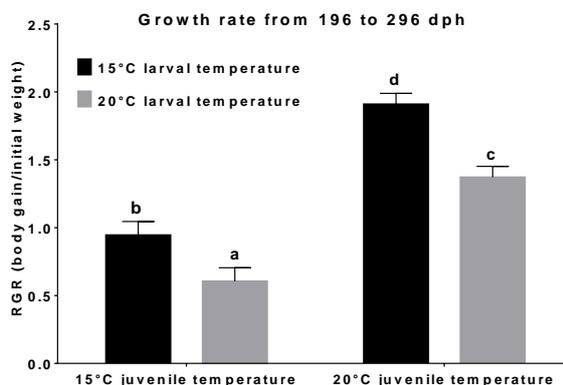


Figure 7. Relative growth rate (body gain/initial weight) from 196 to 296 dph of juveniles under moderate hypoxia (40% air saturation) at 15°C or 20°C. Juveniles were early exposed to 15°C or 20°C at larval stage (including fish early exposed or not to hypoxia at larval stage) (n for each

group= 60 juveniles). Significant differences between groups are identified with different letters (two-way ANOVA followed by Tukey's test).

➤ Metabolism

The coping metabolic capacity of sea bass juveniles to hypoxia was analyzed by assessing different metabolites (e.g. glycogen and lipid) content and by investigating activity or gene expression of different proteins involved in carbohydrate and lipid metabolism.

Hypoxia conditioning

After chronic hypoxia, the stores of hepatic glycogen were more rapidly depleted in juveniles reared at 20°C than at 15°C, likely due to a higher metabolic demand at elevated temperature, leading to a higher use of glucose for anaerobic glycolysis [chapter 4; Figure 8A (Richards, 2009)]. We also observed at the end of hypoxia exposure (296 dph) that glycogen depletion was similar for juveniles whatever oxygen condition encountered at larval stage. These data suggest that metabolic pathway involved in the mobilization of glycogen under hypoxic condition has not been affected by the early oxygen conditions. Therefore, as observed under normoxic condition, juveniles early exposed to hypoxia at larval stage continued to exhibit higher hepatic glycogen stores at the two different temperatures after 100 days of chronic hypoxia [20°C (chapter 4, Fig. 8A) and 15°C (Fig. 8A)]. Previous studies reported that species with larger glycogen stores (e.g., goldfish, carp, tilapia) can fuel glycolysis for longer time periods than species with smaller glycogen stores (e.g., rainbow trout), and this prolongs their hypoxic/anoxic survival time (Richards, 2009; Martinez et al., 2006). However, we were unable to reveal any advantage of the early-life exposure to hypoxia in term of juvenile growth potential under hypoxia (as mentioned above); nonetheless, we do not rule out the possibility that the potential advantage in terms of hypoxia tolerance may be revealed under more severe hypoxia regimes (higher intensity and duration) and/or by the analysis of other physiological parameters related to key functions (e.g. reproduction).

As shown in normoxic condition, liver lipid contents were lower in juveniles reared at 20°C compared to those reared at 15°C due to higher energy requested at warm temperature (Fig. 8B; chapter 4). Interestingly, we found that lipid contents were lower under hypoxic condition compared to normoxic condition only when juveniles were reared at 20°C. These data indicate that mobilization of lipid reserves to provide energy in hypoxia conditions occurs only at higher temperature, again likely due to higher energy requested at warmer temperature. Lipid could provide energy through oxidation cascade (aerobic metabolism) that generates acetyl CoA which enters the citric acid cycle.

Lipid can also provide energy through anaerobic metabolism since glycerol generated by breakdown of triacylglycerols can be converted to glucose (substrate for anaerobic glycolysis) by gluconeogenesis. Such metabolic strategy is indeed displayed in some hypoxia-tolerant species, such as burrow-dwelling goby (*Gillichthys mirabilis*), when they are exposed to hypoxia (Gracey et al., 2011). We did not find any long-lasting effect of early exposure to hypoxia in the hepatic lipid reserves and/or gene expression and activity of proteins involved in lipid metabolism.

Overall, under warm chronic hypoxia, we were unable to show any link between the growth depression and energetic metabolism in liver of juveniles early-life exposed to hypoxia. Further analysis in other relevant tissues (i.e. intestine) or/and an increase in the sample size used in our analysis will be necessary to better understand the mechanisms involved in such growth depression.

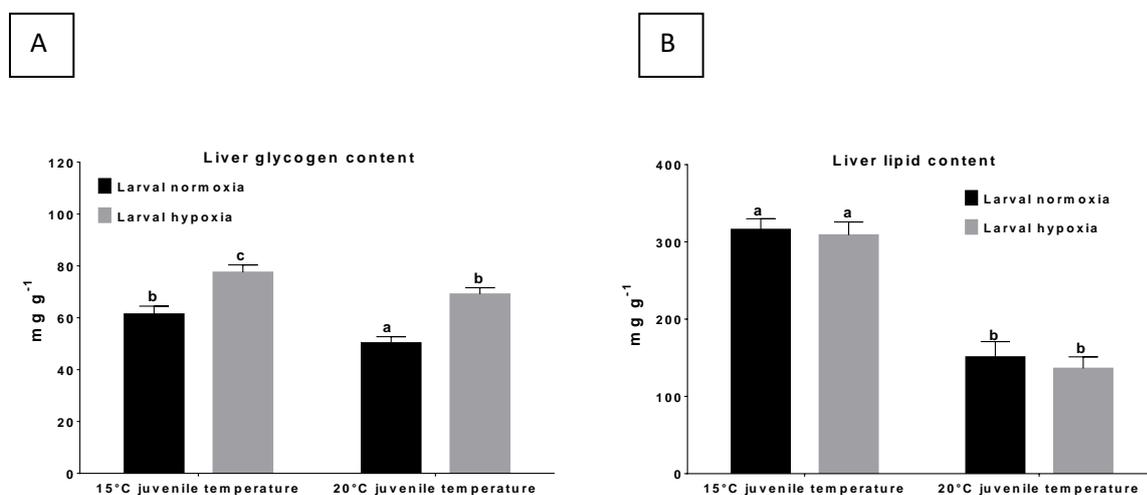


Figure 8. Hepatic metabolites (glycogen (A) and lipid (B)) at 296 dph of juveniles exposed to moderate hypoxia at 15°C or 20°C. Juveniles were early exposed or not to hypoxia at larval stage (including fish early exposed to 15°C and 20°C at larval stage) (n for each group= 8 pools of 2 juveniles). Significant differences between groups are identified with different letters (two-way ANOVA followed by Tukey's test).

Thermal conditioning

Early life thermal conditions did not induce any long-term effects in the hepatic glycogen and/or regulation of carbohydrate metabolism at juvenile stage under cold or warm chronic hypoxia (Figure 9A). We only observed that lipid contents were significantly lower under chronic hypoxia in the liver of juveniles that had experienced 15°C at the larval stage (compared to 20°C) whatever temperature considered at juvenile stage. One could hypothesize that this difference resulted from an increase in energy demand due to the higher compensatory growth observed in these fish [(20°C

(chapter 4, Fig. 9B) and 15°C (Fig. 9B)]. However, we did not observed any effects of larval temperature conditioning on lipid metabolism (several indicators of triglyceride hydrolysis, triglyceride synthesis and/or lipid mobilization). Additional investigation of the different mechanisms involved in the control of lipid storage (e.g., lipid peroxidation) will be necessary to elucidate the functional significance of lipid depletion under hypoxic conditions in European sea bass.

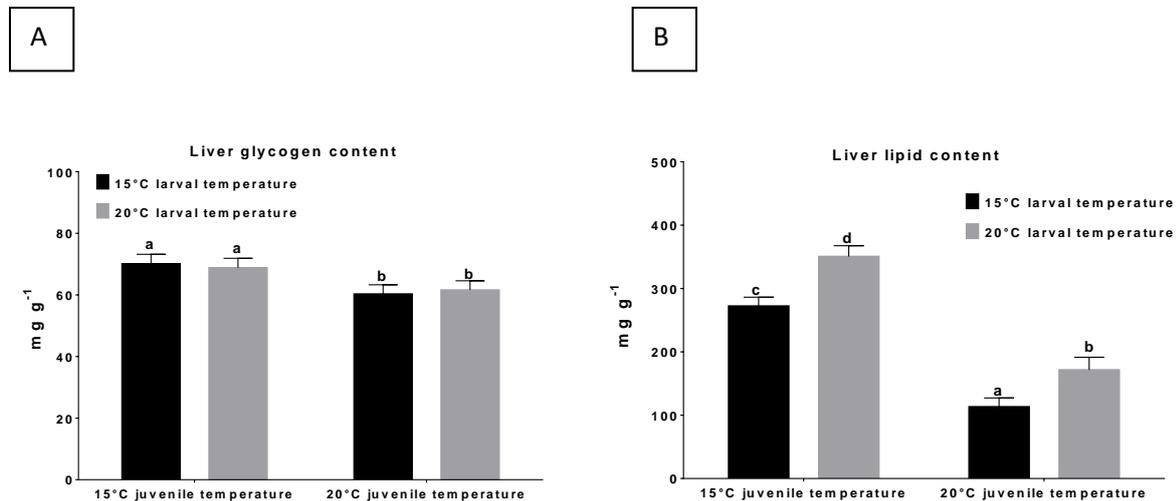


Figure 9. Hepatic metabolites (glycogen (A) and lipid (B)) at 296 dph of juveniles exposed to moderate hypoxia at 15°C or 20°C. Juveniles were early exposed to 15°C or 20°C at larval stage (including fish early exposed or not to hypoxia at larval stage) (n for each group= 8 pools of 2 juveniles). Significant differences between groups are identified with different letters (two-way ANOVA followed by Tukey's test).

➤ Oxygen transport

Considering that Hbs play a key role in mediating an adaptive response to hypoxia in vertebrates including fish, the Hb system (Hb gene expression and Hb protein levels) was closely investigated in the present study in juveniles reared under hypoxia.

Hypoxia conditioning

Early exposure to hypoxia at larval stage induced long-term up-regulation of certain Hb genes in the head kidney of juveniles exposed to cold (15°C) hypoxia (chapter 3). This long-lasting effect was not observed in juveniles reared under warm (20°C) hypoxia (data not shown). The fact that such fish are not able to regulate Hb mRNA could represent a trade-off due to fish under warm hypoxia exacerbated the metabolic need in energy, switched from aerobic to anaerobic metabolism (chapter

4). An alternative explanation could be that under warm deoxygenation, the available amount of Hb proteins was in excess of that of dissolved O₂. This would limit the expression of Hb mRNA to avoid an excess of Hb synthesis (Roesner et al., 2006).

The physiological consequence of the long-term regulation of Hb genes under cold hypoxia is still uncertain (chapter 3). Even if additional functional characterization of Hb genes in European sea bass (e.g. binding capacities, Root and Bohr effects) will be necessary, we assume that their stimulation under hypoxia could increase the O₂ transport capacity of the blood (Wawrowski et al., 2011). This result indicates that only juveniles exposed to hypoxia at the larval stage need to implement such a response when they are again exposed to hypoxia at 15°C, suggesting higher sensitivity to the oxygen constraint. According to that, it is interesting to note that the up-regulation of Hb genes in juveniles exposed to hypoxia at larval stage was associated with higher mRNA levels of oxygen sensor *phd-3* gene in the head kidney. Expression of *phd-3* gene is known to be stimulated in hypoxia condition notably via HIF-mediated pathway (Aprelikova et al., 2004). Thus, higher expression level of *phd-3* gene suggests an activation of the HIF pathway and then higher hypoxia sensitivity in juveniles that experienced hypoxia at larval stage. Previous studies in humans had already suggested that intermittent hypoxia during postnatal development increased susceptibility to hypoxia in adult muscle (McDonald et al., 2016). Since we could not detect other physiological consequences associated with this potential higher sensitivity to hypoxia (lower growth rates being observed only in juveniles under hypoxia at 20°C), further experiments should be considered. For example, higher hypoxic constraint at juvenile stage could reveal higher sensitivity of fish previously exposed to hypoxia whatever temperature considered.

These data contrast with the adaptive developmental plasticity revealed in zebrafish adults that benefited from early-life exposition to hypoxia at embryonic stage (Robertson et al., 2014). However, it has to be noted that the early hypoxia was not applied at the same developmental stage in zebrafish, likely inducing distinct response with different long-term consequences.

It must be emphasized that the Hb genes showing the strongest stimulation at larval stage during hypoxia were the ones up-regulated in the subsequent group of juveniles when exposed to hypoxia. In fish, it has been reported that the two main windows in which environmental exposures may affect the epigenome of an individual are: (1) the parental window, if the changes occur during the production of the gametes (Labbé et al., 2016), and (2) the post-conception window during early embryogenesis (Jiang et al., 2013; Navarro-Martín et al., 2011). Although further investigations will be necessary to determine the molecular mechanisms underlying Hb gene regulation reported in the present study, we hypothesize that an epigenetic modulation could be responsible for such hypoxia-

induced transcriptional imprinting as previously reported in fish (Liu et al., 2017). Vanderplancke et al. (2015b) had already suggested that transcriptional imprinting could have occurred during hypoxia exposure at the last stages of sea bass larval development. Our results shed light on the transcriptional imprinting in marine fish species, suggesting that not only the early embryonic stages of fish development are susceptible to be genetic programmed (Dixson et al., 2014). However, we cannot rule out that earlier developmental window of stimulus could have been more efficient in terms of “programming” effects but it would not correspond to an ecological reality (see section 2).

Thermal conditioning

The analyses of the capacity of O₂ transport (e.g. Hb gene expression, Hb protein levels....) at juvenile stage during cold and warm hypoxia did not reveal any long-term effect of early exposure to thermal conditions (data not shown).

In summary, the data obtained in juveniles submitted to chronic moderate hypoxia allowed us to reveal long-lasting effects in juveniles that would not have been possible to detect under normal oxygenation and thermal conditions. This challenging environment confirmed that thermal and hypoxic conditions at larval stage did not induce any long-lasting synergistic effects on physiological parameters we investigated in sea bass juveniles. Overall, our results showed that the early-life larval environment did not always allow a beneficial adaptive tuning of physiological functions; this result clearly questions a widespread concept by which it is considered that the early-life environment should prepare individuals to cope better with similar environmental conditions at later life stages.

Chapter 6

Conclusions and perspectives



In summary, this thesis has shown that a combination of moderate hypoxia and warm temperature in a critical window of sea bass larval development has persistent long-term effects on juvenile physiology. However, this long-lasting modification on the phenotype did not allow sea bass juveniles to better tolerate similar conditions that they encountered at larval stage. It has to be noted that these effects have been revealed using moderate environmental constraints and we assume that the impacts would be more pronounced by using more severe oxygen and temperature levels (e.g. dissolved oxygen <30% air saturation; temperature >20°C) at both larval and juvenile stages. These findings contribute to make predictions of how the marine fish communities could be altered by current climate change. However, to better evaluate the long-lasting impact of this early-life environment on the dynamics of fish populations, in terms of recruitment and/or reproductive effort, further investigations would be necessary.

➤ **Growth**

The growth depression observed at larval stage by low temperature and hypoxic conditions induced subsequent partial or completed compensatory growth, respectively. This implies that physiological mechanisms were triggered off during development to compensate the perturbations due to early stressing environment which may allow restoring the normal life trajectory. In other words, “developmental canalization” occurred (Hallgrímsson et al., 2002; Ramler et al., 2014; van Buskirk and Steiner, 2009). The advantages of developmental canalization appear evident for sea bass juveniles that reached the normal adult size after growth depression (a large body size could reduce predation rate or increase fecundity (Arendt 1997), or increase prey choice (Ludsin and DeVries 1997)). However, accelerated growth may also lead to damage accumulation (Jennings et al. 2000; Monaghan and Hausmann 2009). To be able to address this issue further analysis of rates of oxidative stress will be required. Furthermore, the extension of the juvenile phase would allow us to determine whether compensatory growth could influence the reproductive effort of sea bass individuals (sexual maturation, number of viable offspring...). Finally, additional analysis of feed intake and food conversion will be necessary to determine the mechanism underlying compensatory growth.

We also observed that an early life exposure to hypoxia could have negative impact on growth rates when juveniles face a decrease in O₂ availability in warming waters. The consequences of this lower growth phenotype could have large implications for trophic interactions as a reduction in reproductive rates (Rijnsdorp et al., 2009), and/or alteration of the ecosystem function (Sheridan and Bickford, 2011; Edeline et al., 2013). Considering that a long-term negative effect on growth

after an early-life hypoxia exposure has been associated with a lower protein digestive capacity in the intestine of European sea bass (Zambonino et al (2017), further analysis of enzymes involved in digestive functions in this tissue (e.g. alkaline phosphatase, aminopeptidase N) could shed light on the physiological trade-offs associated to growth depression observed in the present study.

➤ ***Metabolism***

We demonstrated that juveniles that experienced low oxygen and temperature conditions at larval stage are likely to exhibit a metabolism with specific features that could affect their subsequent response to chronic hypoxia. For instance, a long-term effect of the early-life hypoxia resulted in a metabolic conditioning towards higher hepatic glycogen accumulation. Since early exposure to hypoxia did not enhance juvenile growth potential (as mentioned above), future exposure of sea bass juveniles to more severe hypoxia regimes (higher intensity and duration) and/or the analysis of their reproductive output could shed light on the potential advantage of such glycogen storage. Furthermore, hypoxia only induced lower lipid content in the liver of juveniles that had experienced 15°C at the larval stage which likely resulted from an increase in energy demand due to the higher compensatory growth observed in these fish. Additional biochemical analyses (e.g. lipid peroxidation) are planned to clarify the functional significance of lipid depletion under hypoxic conditions in European sea bass.

➤ ***Oxygen extraction***

We showed that exposure to moderate hypoxia and warm temperature during larval stage caused a low incidence of opercular deformities in sea bass juveniles. It is likely that such malformations negatively influence the capacity for oxygen extraction affecting routine fish activities as searching food and predator avoidance. Moreover, opercular malformations may also predispose gills to pathological infections, which could induce delay in growth and high mortality rates in fish juveniles as a consequence of parasite infestation (Abdel et al., 2004). As a result, those juveniles that exhibited opercular deformities in the present study would rarely reach the adult size in wild populations, which could compromise population recruitment success. We can therefore hypothesize that along with overfishing and pollution, hypoxia even moderate in warming coastal waters may have a negative impact on sea bass stocks that are fallen during the last decades (FAO, 2013).

➤ ***Oxygen transport***

The physiological consequence of the long-term regulation of Hb gene expression under cold hypoxia in juveniles that experienced an early life hypoxia event requires further investigation.

However, based on these data, we can hypothesize that these juveniles were more sensitive to moderate O₂ deprivation which could explain the up-regulation of Hb gene expression associated with stimulation of the *phd-3* gene involved in the hypoxia-inducible factor oxygen-sensing pathway. The involvement of the HIF pathway in the long-term regulation of Hb genes would be supported by the future determination of functional hypoxia responsive elements (i.e. the binding sites through HIF-1 regulates target genes) in Hb genes.

Finally, the study of epigenetic processes (e.g. modifications of histones, DNA methylation...) may shed light on the underlying mechanisms of developmental plasticity of Hb gene regulation. In addition, the extension of the juvenile phase would allow us to determine whether this transcriptional imprinting of Hb genes could be transmitted to the next generation.

➤ **Final remarks**

The present study surprisingly revealed that warming and hypoxia do not always interact in fishes, as it is extensively reported (McBryan et al., 2013; McBryan et al., 2016; Pörtner and Farrell, 2008). Thus, in the range of temperature and oxygen concentration experimentally tested here, we did not observe any long-lasting (additive or synergistic) effects in European sea bass.

Our study also suggests that the early-life larval environment did not always allow a beneficial adaptive tuning of physiological functions, which should produce animals best suited for the environment they are likely to find later as juveniles or adults. Instead, we reported that exposure to moderate hypoxia and warm temperature during larval stage has long-lasting negative impact on sea bass juvenile physiology, which could compromise fitness and population recruitment success. Nevertheless, we cannot exclude other potential effect of early oxygen and temperature conditions on other components of fitness not investigated in the present study.

Given that the frequency and intensity of hypoxic events in warming coastal waters are predicted to increase (Diaz and Rosenberg, 2011), the data obtained through this thesis gain further value since they may help to identify successful conservation strategies that guarantee future fish population dynamics. For instance, our results could be integrated into population dynamic models for a better understanding of the connections between environmental constraints and fish recruitment. This will allow the development of more predictive management actions that, based on an analysis of environmental events having occurred in the major sea bass nurseries, would restrict annual fishing quotas in order to ensure the stock's survival.

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Title

Environment and early life stages in fish: developmental plasticity responds to seawater changes in oxygen and temperature

Abstract

In the context of global change, decrease in oxygen availability (hypoxia) combined with rising water temperature are especially prevalent in coastal regions, towards which marine fish larvae may drift at the end of their development. It is well admitted that the physiological regulations implemented by organisms to cope with their environment during the early life stages of life can cause profound consequences in their subsequent life-history trajectory (developmental plasticity). Therefore, the main objective of this thesis was to investigate whether ecologically relevant conditions of oxygenation (40% and 100% air saturation) combined with thermic conditions (15 and 20 °C), occurring at the last stages of larval development of European sea bass (*Dicentrarchus labrax*) larvae, could have long-lasting impacts on juvenile physiology. Our data showed that growth depression resulting from low temperature and hypoxic conditions at larval stage induced a subsequent compensatory growth. Moreover, our analyses of hepatic glycogen and lipid stores revealed that metabolic features of juvenile could be affected by early exposure to oxygen and temperature conditions. Furthermore, our data revealed that oxygen extraction capacity was affected due to opercular deformities caused by early exposure to hypoxia. Finally, when seabass juveniles, that have been exposed to hypoxia at larval stage, were under chronic hypoxic condition they show a long-term up-regulation of hemoglobin genes. Overall, our findings contribute to make predictions of how the marine fish communities could be altered by current climate change.

Key words

Hypoxia, Ocean warming, Physiology, European sea bass, Developmental plasticity

Titre

Environnement et jeunes stades de vie chez le poisson: la plasticité développementale comme réponse aux contraintes hypoxiques et thermiques

Résumé

Dans le contexte du changement global, la diminution de la disponibilité en oxygène (hypoxie) combinée à la hausse de la température sont deux phénomènes particulièrement présents dans les eaux côtières vers lesquelles les larves de poissons peuvent dériver à la fin de leur développement. Il est admis que les régulations mises en œuvre par les organismes pour faire face à leur environnement au cours des premières étapes de leur vie peuvent avoir des conséquences sur leur trajectoire de vie ultérieure (plasticité développementale). Ainsi, l'objectif principal de cette thèse était d'évaluer si des conditions d'oxygénation (40% et 100% de saturation) combinées à des conditions thermiques (15 et 20 °C) au stade larvaire, pouvaient avoir des impacts durables sur la physiologie des futurs juvéniles de bar (*Dicentrarchus labrax*). Nos résultats ont révélé que les retards de croissance associés à la plus basse température et à l'hypoxie au stade larvaire induisent une croissance compensatrice. De plus, les analyses des réserves hépatiques en glycogène et en lipides ont révélé que le métabolisme des juvéniles pourrait être affecté par les conditions environnementales au stade larvaire. Par ailleurs, nos données indiquent que la capacité d'extraction de l'oxygène a été affectée chez des juvéniles en raison de déformations operculaires causées par l'exposition précoce à l'hypoxie. Enfin, l'exposition précoce à l'hypoxie induit une sur-expression à long terme de gènes de l'hémoglobine de juvéniles replacés en situation d'hypoxie. Globalement, nos résultats contribuent à faire avancer les connaissances sur la façon dont les poissons marins font face aux changements climatiques actuels.

Mots clés

Hypoxie, Réchauffement des océans, Physiologie, Bar Européen, Plasticité développementale