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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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"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

Credits: Scott Kelly

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 CO_2, NO_2, 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 CO_2 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).

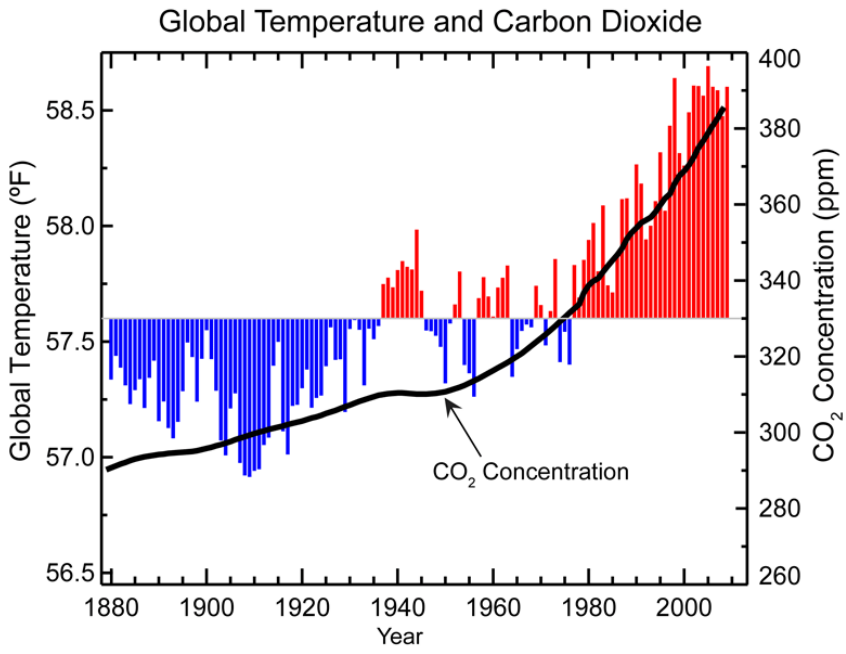


Figure 1. Global temperature and carbon dioxide from 1880 to 2012. *Global annual average temperature (as measured over both land and oceans) has increased by more than 1.5°F (0.8°C) since 1880. Red bars show temperatures above the long-term average, and blue bars indicate temperatures below the long-term average. The black line shows atmospheric carbon dioxide (CO_2) 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°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)	- 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	- Increased poleward heat transport in western boundary currents of the subtropical gyres
Warming of adjacent land masses	- 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	- Permanent land inundation, coastal erosion, loss of some coral atolls and islands
Negative feedback on the ocean carbon sink	- Higher sea surface temperature reduces pCO_2 uptake from the atmosphere - Higher global sea surface temperature and land surface temperature enhance the rate of increase in atmospheric CO_2
Deoxygenation	- Reduced oxygen solubility in warmer water - Reduced penetration of oxygen into deeper water due to enhanced stratification
Ocean Acidification	- 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 O_2 (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).

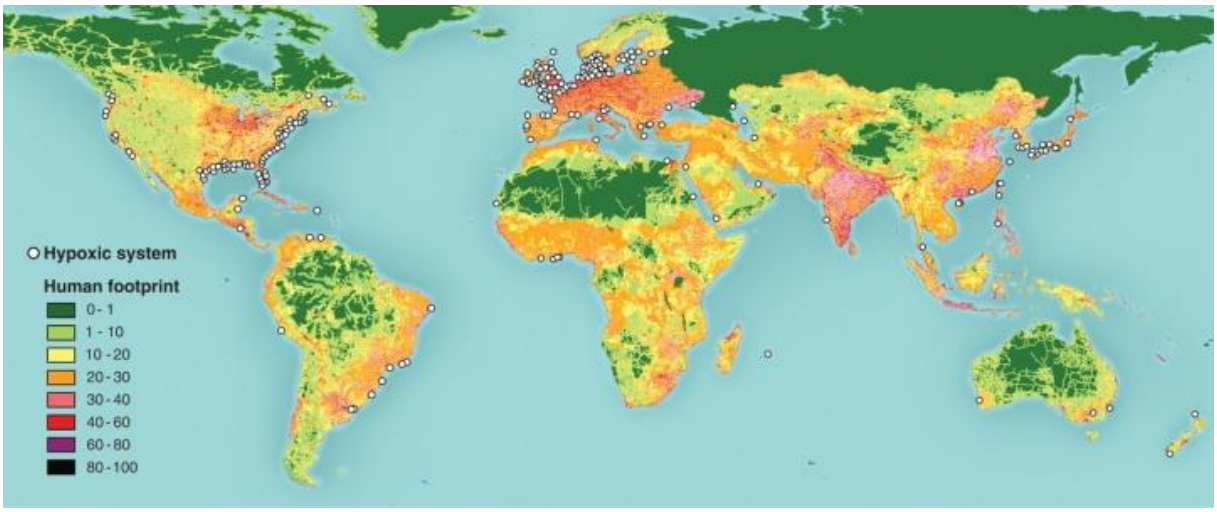


***Figure 2. Global distribution of hypoxic systems that have been associated to eutrophic areas**. Their distribution matches the global human footprint (the normalized human influence is expressed as a percent) (Diaz and Rosenberg, 2008).*

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 O_2 content. This is made possible since fish has developed a complex array of O_2 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 O_2 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 synthetized by two types of cellular respiration. Aerobic respiration is based on the utilization of O_2 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 O_2 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), O_2 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 O_2 availability which can potentially limits 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 O_2 demand and energy (nutrient) intake (Schulte, 2015). Therefore, combination of low O_2 and high temperature is exceptionally challenging since warming temperature increased metabolic demand for O_2 when hypoxia limits the availability of environmental O_2 (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 O_2 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 O_2 uptake from water and O_2 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 O_2 from the external fluid media (water and air) and the discharge of CO_2 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 O_2 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 O_2 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 O_2 to all body tissues by O_2-transport proteins such as hemoglobins (Hb), hemerythrins and hemocyanins (Mairbäurl and Weber, 2012; Terwilliger, 1998). Under hypoxic constraint, both O_2 uptake and transport have been shown to be regulated. Even if several marine fish species exhibit bimodal respiration (O_2 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 O_2 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 O_2 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).

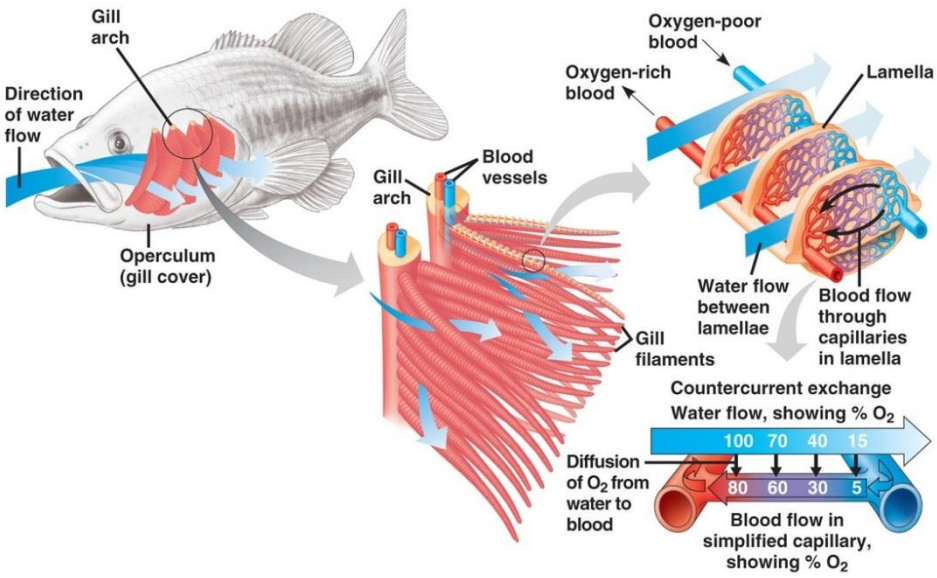


Figure 3. A simplified schematic of the oxygen extraction in fish. *Fish extract dissolved oxygen molecules from the water through the gills. Gill filaments have lamellae which increase the surface area available for diffusion. As water passes over the gill filaments, blood inside the capillaries picks 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 O_2 environment (Perry et al., 2009; Steffensen et al., 1982) (Figure 4). Such responses occur once O_2 chemoreceptors located in the neuroepithelial cells of the gill filament detect lower O_2 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 O_2 uptake, particularly by increasing gills surface area (Nilsson, 2007; Sollid and Nilsson, 2006). Indeed, the surface area available for O_2 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).

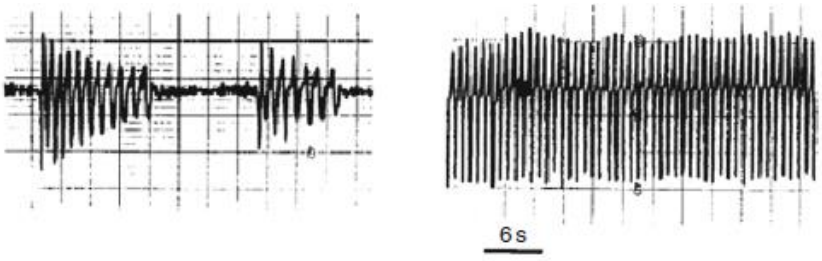


Figure 4. Ventilation under normoxic and hypoxic conditions in the sturgeon (Acipenser baeri). *Ventilation is episodic under normoxic conditions (left-hand panel) but becomes continuous upon exposure to hypoxic water (right-hand panel; PO2 = 60 Torr). Note the increase in ventilation amplitude that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

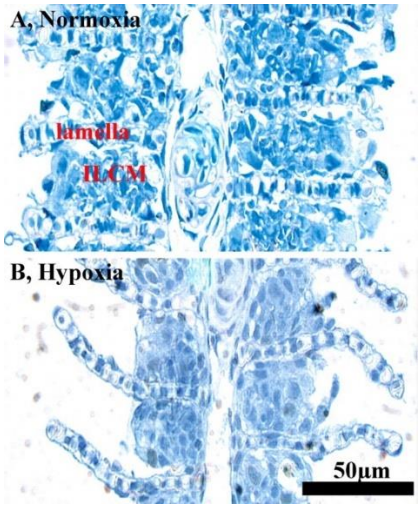


Figure 5 (A,B) Light micrographs of gills from crucian carp kept in normoxic (A) or hypoxic (B) water at 8°C. *Note that the lamellae are present in both conditions but that a regression of the interlamellar cell mass (ILCM) during hypoxia makes the lamellae protrude, thereby greatly increasing the respiratory 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 Hbα and Hbβ 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).

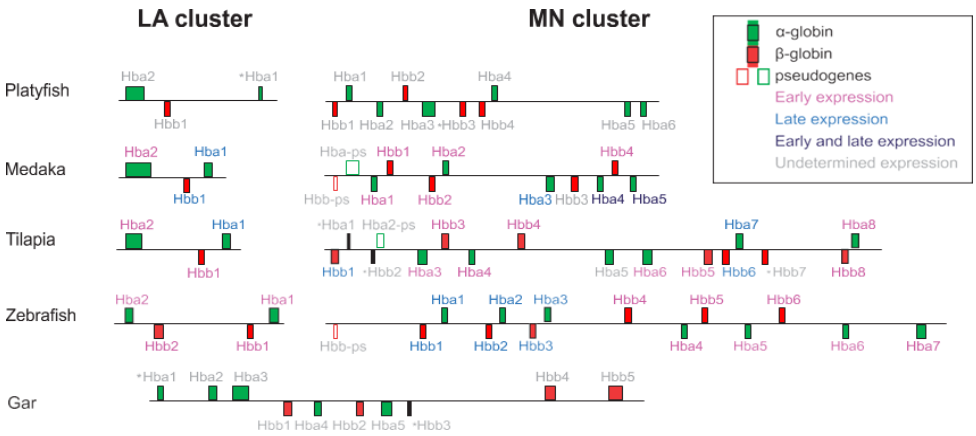


Figure 6. Genomic structure of the MN and LA globin gene clusters of some teleost fish*. Genes in the forward orientation are shown on top of the chromosome, whereas genes in the reverse orientation are shown below. Gene labels are color coded based on the timing of their expression (adapted from Opazo 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 O_2 binding properties of Hb are depicted by the O_2 equilibrium curve (OEC), which describes the relationship between partial pressure of O_2 (PO_2) and the degree of Hb saturation with O_2 (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 PO_2 is often used to determine Hb-O_2 affinity, characterized by one point, the PO_2 at which 50% of Hb is oxygenated (P50). A low P50 indicates a higher Hb-O_2 affinity, which favours O_2 loading in the gills. A high P50 indicates a lower Hb-O_2 affinity, which favours unloading of O_2 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 O_2 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 O_2 affinity of Hb, shifting the OEC to the right (Bohr et al., 1904). The Bohr effect facilitates O_2 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 O_2 loading at the gills where CO_2 excretion increases pH (Souza and Bonilla-Rodriguez, 2007).

The pH also affects O_2 affinity via the Root effect, which is only observed in fishes (Brittain, 1987), where low pH reduce the maximum carrying capacity of Hb for O_2 (Figure 7) (Fago et al. 1993, 1997). This allows for rapid O_2 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-O_2 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-O_2 affinity (Val, 2000).

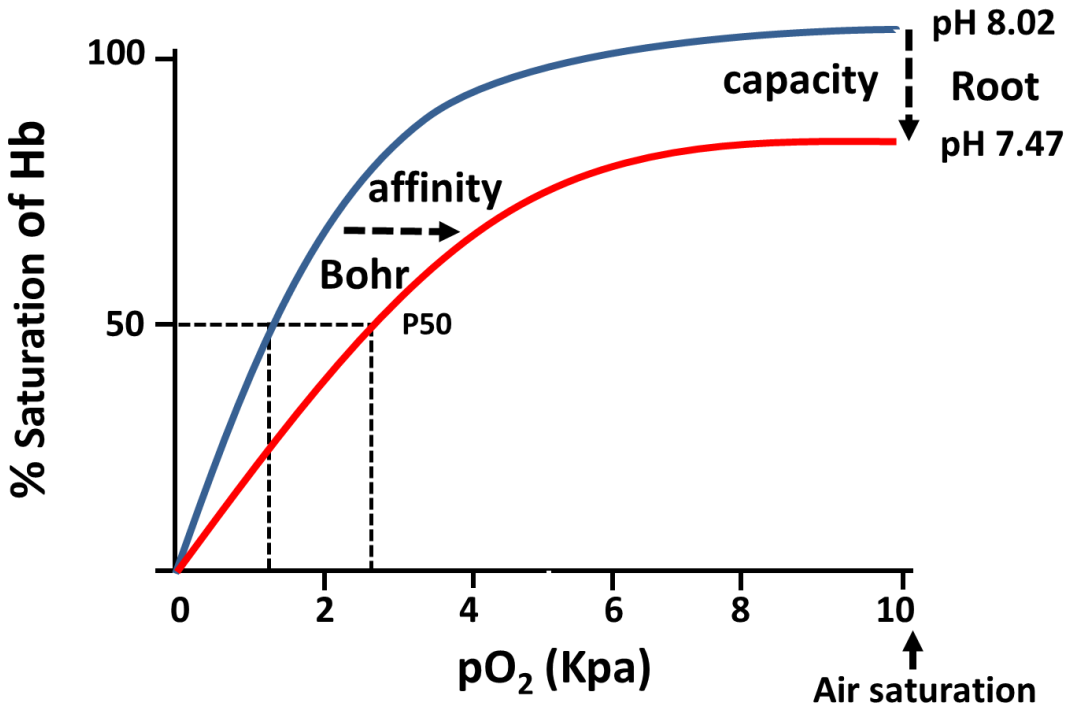


Figure 7. A theoretical oxygen equilibrium curve (OEC) depicting the relationship between hemoglobin-oxygen saturation (%Hb-O_2) and blood partial pressure for O_2 (PO_2; Kpa). *A reduced pH results in a Bohr effect (low affinity) rightward shift and Root effect (low carry-capacity) rightward and downward shift. Thin black drop lines depict P50 values, the PO_2 at which 50% of Hb is saturated with O_2, for each of the two OECs.*

Under hypoxic conditions, changes occur at the level of the erythrocyte to increase blood O_2 carrying capacity and Hb-O_2 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-O_2 (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 O_2 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 O_2 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 O_2 carrying capacity (e.g. by increasing Hb-O_2 affinity; Pichavant et al., 2003).

Hypoxia is known to regulate in fish the amount of Hb proteins and/or Hb transcripts levels produced by erytropoietic 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 O_2 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 O_2 deprivation.

	Fish species	***Stage***	***Hypoxia duration***	***Hypoxia intensity***	***Protein/mRNA level quantity***	***Reference***
Hb protein	*Poecilia latipinna*	*Adult*	*6 weeks*	*PO_2 = ~2.7 kPa*	*Increase*	*Timmerman and Chapman 2004*
	Carassius auratus	*Adult*	*- 48 h* *- 8 h*	*- PO_2 = ~6.7 kPa* *- PO_2 = ~1.7 kPa*	*Unchanged*	*Roesner et al. 2008*
	Oncorhynchus mykiss	*Larval*	*47 days*	*30% air saturation*	*Decrease*	*Bianchini and Wright, 2013*
Hb transcript	*Danio rerio*	*Embryonic*	*24h*	*5% air saturation*	*Decrease*	*Ton et al. 2003*
	Danio rerio	*Adult*	*48h*	*- PO2 = ~8.6 kPa)* *- PO2 = ~4.1 kPa*	*Decrease*	*Roesner et al. 2006*
	Carassius auratus	*Adult*	*- 48 h* *- 8 h*	*- PO_2 = ~6.7 kPa* *- PO_2 = ~1.7 kPa*	*Unchanged*	*Roesner et al. 2008*
	Poecilia latipinna	*Adult*	*6 weeks*	*PO_2 = ~2.7 kPa*	*Increase*	*Timmerman and Chapman 2004*
	Oryzias latipes	*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 O_2-limited hypoxic environments, duration of survival requires a well-coordinated response to defend against the metabolic consequences of low O_2 supply to the mitochondria, which limits aerobic ATP production. Under these O_2 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 O_2 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 CO_2, the final oxidation product of organic fuels (Campbell and Reece, 2005). The hydrogen atoms generated by the Krebs cycle are transferred via NADH and $FADH_2$ 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. O_2 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 O_2 decreases, the mitochondria are limited in their capacity to generate ATP due to the lack of O_2 available to act as the terminal electron acceptor in the electron transport chain. Under this threshold, vertebrates including fish can activate O_2-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).

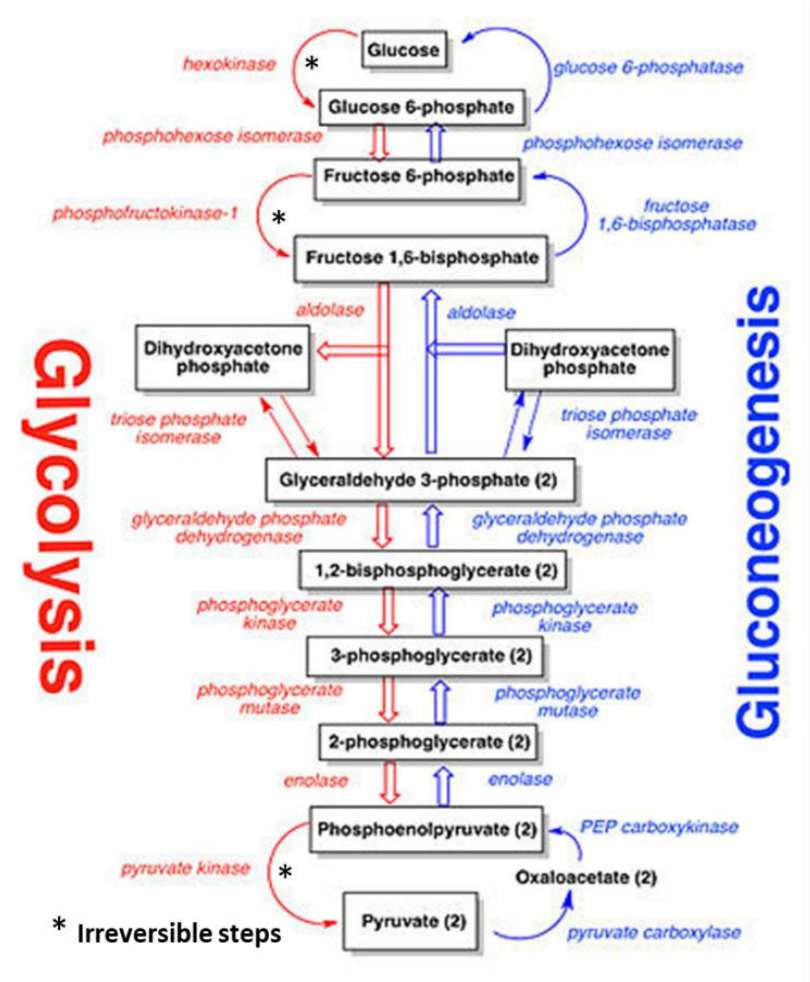
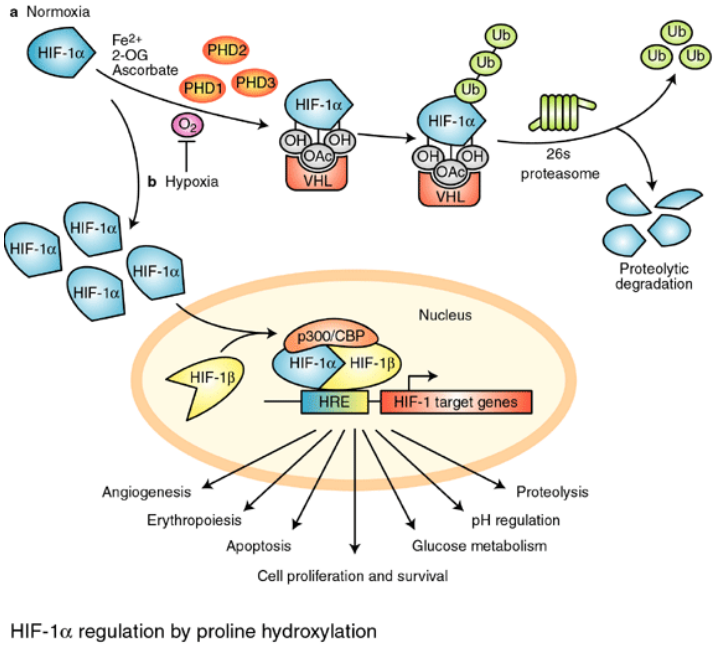


Figure 8. Representation of the pathways of glucose phosphorylation (anaerobic glycolysis) and glucose production (gluconeogenesis). *Irreversible steps were marked with an asterisk.*

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-1α, HIF-2α or HIF-3α) and the β-subunit (HIF-1β) (Kaelin and Ratcliffe, 2008). HIF-1β 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, HIFα 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 O_2 to catalize HIF hydroxylation, this reaction does not efficiently occur under low O_2 tension. Therefore, under hypoxia, HIFα 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.



HIF-1α regulation by proline hydroxylation

Figure 9. HIF-1a regulation by proline hydroxylation. *(a) In normoxia, hypoxia-inducible factor (HIF)-1a is hydroxylated by proline hydroxylases (PHD1, 2 and 3) in the presence of O_2, Fe^{2+}, 2-oxoglutarate (2-OG) and ascorbate. (b) In response to hypoxia, proline hydroxylation is inhibited, which leads to HIF-1a accumulation and translocation to the nucleus. There, HIF-1a dimerises with HIF-1b, 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 Kreb'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 O_2 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 O_2 also depends on the duration and the intensity of hypoxia event.

The effects of low O_2 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 O_2 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 CO_2 (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 that dominant individuals in coping with acute hypoxia, experiencing higher ventilation but failing to defend blood O_2 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 O_2 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 O_2 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, O_2 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 O_2 likely represent one of the major stress for organisms in development since they have to sustain high O_2 requirements for energy-linked functions related to developmental processes as cell proliferation, differentiation, migration and apoptosis, while facing an overall decrease in ambient O_2 (Nilsson and Östlund-Nilsson 2008; Wu et al. 2009). Warming waters may exacerbate the hypoxic constraint since it increases metabolism and, therefore, the O_2 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 O_2 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 O_2 and the O_2 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 (*Pseudocredilabrus mutlicolor 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 O_2 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 approximatively 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).

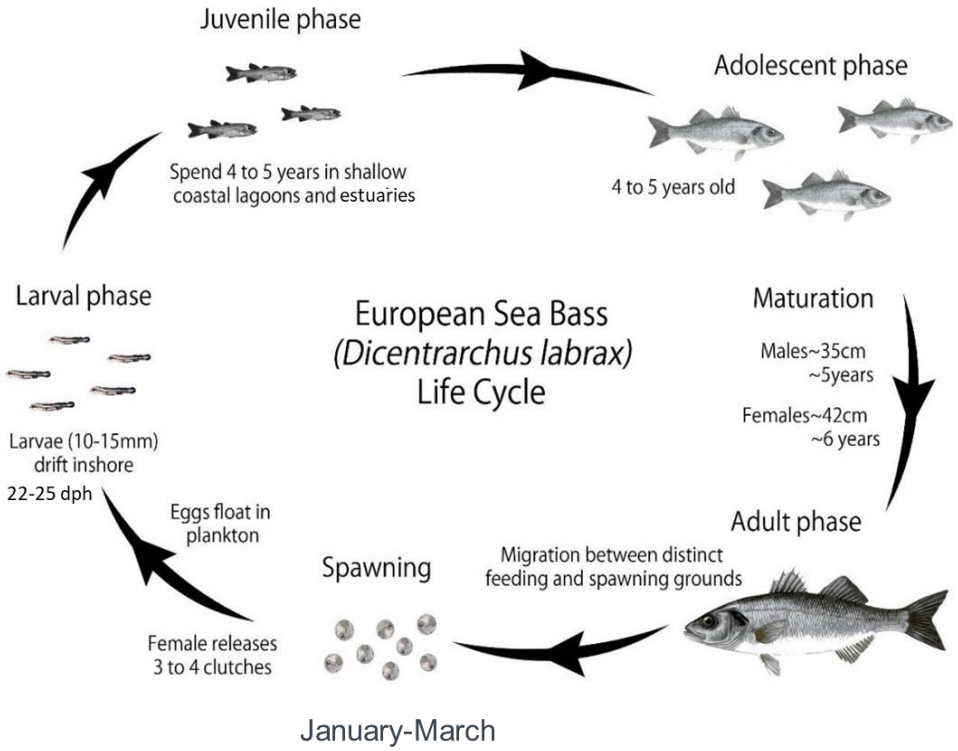


Figure 10. **Life cycle of European sea bass (*Dicentrarchus labrax*)** (from Dando and Demir 1985; Pawson et al. 1987; Pawson, et al. 2007).

5. Thesis goals and hypotheses

As previously mentioned, high temperature and low O_2 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 asses 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 O_2 extraction and transport, cardiovascular functions and energy metabolism. The overall appearance of operculum, which contributes to water flow across the gills lamellae and allows O_2 extraction, was also examined.

In parallel, our second question was approached in other inmature 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 O_2 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 O_2 transport, understanding changes in Hb gene expression could give new insights related to the tolerance to the lack of O_2. 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 O_2 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.

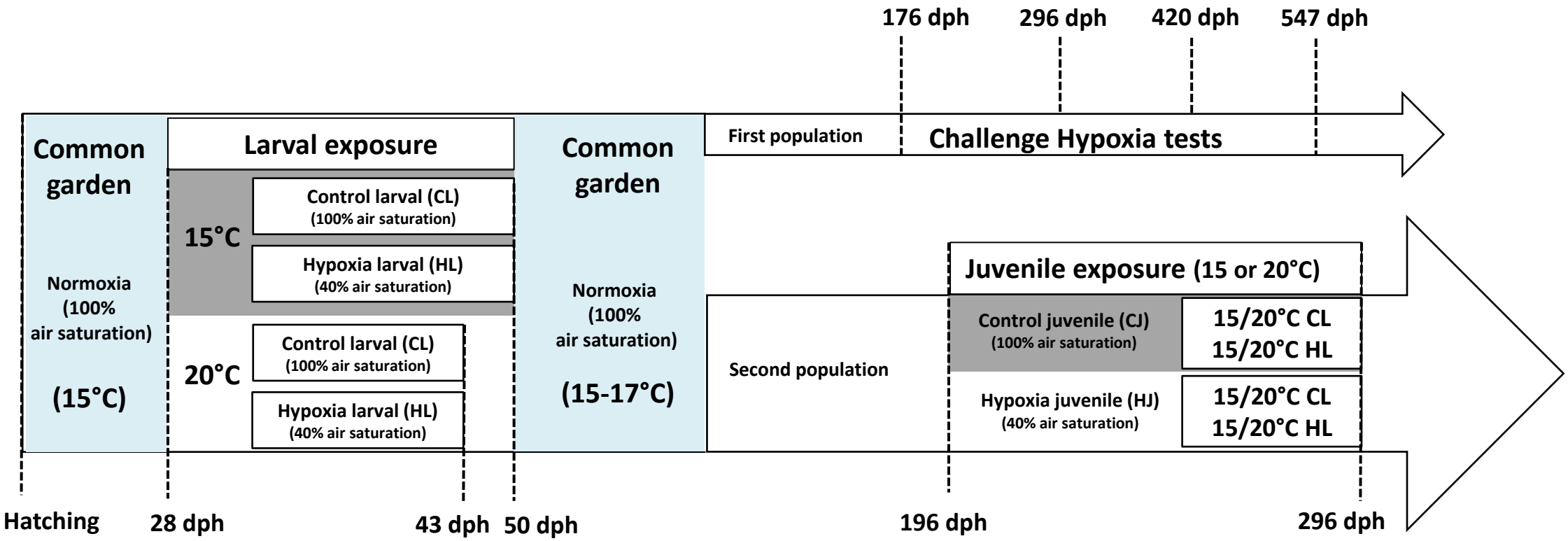


Figure 11. Schema of the experimental protocol. *Sea bass larvae were exposed to each combination of oxygenation level [40% air saturation: hypoxia larval group (HL); and 100% saturation: control larval group (CL)] and temperature (15°C and 20°C) from 28 to 43dph (20°C) or 50 dph (15°C). Following the 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

Credits: Frédéric Lancien

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 O_2 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 O_2 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 O_2 extraction together with operculum.

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

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Abstract

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

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

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

1. Introduction

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

Coastal marine areas are particularly prone to spatial and temporal fluctuations in terms of environmental conditions. These fluctuations are intensifying as a result of the multifaceted on-going global change, which is reflected by several episodes of variable magnitude combining warming and hypoxia (Díaz and Rosenberg, 2011; Levin and Breitburg, 2015). Marine coastal and estuarine ecosystems serve as nursery for a large number of fish species which are thus likely to be exposed to periods of low dissolved oxygen (O_2) and high temperature at a particularly sensitive life stages (Korwin-Kossakowski, 2008).

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

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

European sea bass (*Dicentrarchus labrax*) larvae generally enter shallow coastal areas after the flexion stage and could therefore be exposed to environmental fluctuations, while their larval development is not totally completed (Dufour et al., 2009). Previous studies on European sea bass revealed that exposure to moderate hypoxia at the larval stage could induce long lasting effects on metabolic parameters and on digestive and hemoglobin systems (Cadiz et al., 2017b; Cadiz et al., 2017c; Vanderplancke et al., 2015a; Zambonino-Infante et al., 2017). However, the global impact of early exposure to realistic environmental fluctuations, such as combined temperature and hypoxia, on the overall individual fitness of sea bass remains poorly understood. The present study aims to acquire data related to hypoxia tolerance in European sea bass juveniles exposed to the combination of different environmentally realistic oxygen (40% and 100% air saturation) and temperature (15°C and 20°C) conditions at larval stage. Following the larval period, fish experienced a five-month period of common garden (15-17°C, 100% air saturation).Then, fish tolerance to hypoxic challenge test (HCT) was investigated four times at regular time intervals over one year to evaluate the global physiological status of juveniles. HCT was used since the performance of fish to resist to HCT depends on the ability of individuals to regulate physiological processes related to oxygen extraction and transport, cardiovascular functions and energy metabolism (Roze et al., 2013). The operculum, which contributes to water flow across the gills lamellae and allows O_2 extraction, was also examined. The individuals' responses to the challenge tests were examined in relation to their early-life environment and to the presence of opercular deformities.

2. Materials 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 mg O_2 L^{-1}): hypoxia larval group; and 100% saturation (7.35 mg O_2 L^{-1}): control larval group] and temperature (15°C and 20°C). Hypoxic conditions were created 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 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 (745°C·day), for larvae exposed at 20°C, and at 50 dph (750°C·day), for larvae exposed to 15°C, to ensure that larval treatments were applied at the same stage of development. Replicate tanks were then pooled into one 1 m^3-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 152 dph, 100 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-m^3 tank.

2.2 Hypoxia challenge test (HCT)

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

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

2.3 Identification of opercular deformities

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

2.4 Data analysis and statistics

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

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

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

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

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

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

3. Results

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

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

Table 1. Results of two-way ANOVA for effects of larval treatments (Temperature (Temp.) and Hypoxia (Hyp.)) on larval (43/50 dph) and juvenile (549 dph) body mass. Df means degrees of freedom. N for each group= 100 juveniles. Significant differences between groups are identified with 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 $p< 1*10^{-6}$	F=75. 46 $p< 1*10^{-5}$	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

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

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

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

	Chisq	Df	p- value
Oxygen	18.7	1	$1.5*10^{-5}$
Temperature	7.5	1	0.07

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

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

Table 3. Results of the Weibull regression model on the effect of date and opercular deformity on 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	$<1*10^{-4}$

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

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

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

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

4. Discussion

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

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

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

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

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

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

In conclusion, we assume that exposure to moderate hypoxia and to a lesser extent to warm temperature in European sea bass larvae impact the fitness of part of the future juveniles. Indeed, it has to be pointed out that, in addition to the adverse effect on hypoxia tolerance, 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). Impact on opercular formation associated to decrease in hypoxia tolerance confirms that developmental, morphological and physiological responses to the early-life environment do 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. Nevertheless, we cannot exclude other potential effects of early oxygen and temperature conditions on key physiological function (e.g. reproduction) not investigated in the present study. Physiological trade off associated with compensatory growth could particularly be addressed. Future research will help to better characterize the long term impact of early hypoxic and thermal conditions on components of fitness which could influence population recruitment success.

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References

Abdel, I., Abellan, E., Lopez-Albors, O., Valdes, P., Nortes, M. J. and Garcia-Alcazar, A. (2004). Abnormalities in the juvenile stage of sea bass (Dicentrarchus labrax L.) reared at different temperatures: types, prevalence and effect on growth. *Aquaculture International* **12**, 523–538.

Ali, M., Nicieza, A. and Wootton, R. J. (2003). Compensatory growth in fishes: A response to growth depression. *Fish and Fisheries* **4**, 147–190.

Bagatto, B. (2005). Ontogeny of cardiovascular control in zebrafish (Danio rerio): effects of developmental environment. *Comparative biochemistry and physiology. Part A, Molecular & integrative physiology* **141**, 391–400.

Baroiller, J. F., D'Cotta, H. and Saillant, E. (2009). Environmental effects on fish sex determination and differentiation. *Sexual Development* **3**, 118–135.

Bateson, P., Gluckman, P. and Hanson, M. (2014). The biology of developmental plasticity and the Predictive Adaptive Response hypothesis. *The Journal of physiology* **592**, 2357–2368.

Beraldo, P., Pinosa, M., Tibaldi, E. and Canavese, B. (2003). Abnormalities of the operculum in gilthead sea bream (Sparus aurata): Morphological description. *Aquaculture* **220**, 89–99.

Cadiz, L., Servili, A., Quazuguel, P., Madec, L., Zambonino-Infante, J.-L. and Mazurais, D. (2017b). Early exposure to chronic hypoxia induces short- and long-term regulation of hemoglobin gene expression in European sea bass (*Dicentrarchus labrax*). *The Journal of Experimental Biology* **220**, 3119–3126.

Cadiz, L., Zambonino-Infante, J.-L., Quazuguel, P., Madec, L., Le Delliou, H. and Mazurais, D. (2017c). Metabolic response to hypoxia in European sea bass (Dicentrarchus labrax) displays developmental plasticity. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **215**, 1–9.

Darias, M. J., Lan Chow Wing, O., Cahu, C., Zambonino-Infante, J. L. and Mazurais, D. (2010). Double staining protocol for developing European sea bass (Dicentrarchus labrax) larvae. *Journal of Applied Ichthyology* **26**, 280–285.

Díaz, R. J. and Rosenberg, R. (2011). Introduction to Environmental and Economic Consequences of Hypoxia. *International Journal of Water Resources Development* **27**, 71–82.

Dufour, V., Cantou, M. and Lecomte, F. (2009). Identification of sea bass (Dicentrarchus labrax) nursery areas in the north-western Mediterranean Sea. *Journal of the Marine Biological Association of the United Kingdom* **89**, 1367–1374.

Fox, J. and Weisberg, S. (2011). Multivariate Linear Models in R. *An R Companion to Applied Regression* 1–31.

Hassell, K. L., Coutin, P. C. and Nugegoda, D. (2008a). Hypoxia impairs embryo development and survival in black bream (Acanthopagrus butcheri). *Marine Pollution Bulletin* **57**, 302–306.

Hassell, K. L., Coutin, P. C. and Nugegoda, D. (2008b). Hypoxia, low salinity and lowered temperature reduce embryo survival and hatch rates in black bream Acanthopagrus butcheri (Munro, 1949). *Journal of Fish Biology* **72**, 1623–1636.

Hubeaux, S. and Rufibach, K. (2015). SurvRegCensCov: Weibull Regression for a Right-Censored Endpoint with Interval-Censored Covariate. R package version 1.4. https://CRAN.R-project.org/package=SurvRegCensCov

Hothorn, T., Bretz, F. and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical Journal* **50**, 346–363.

Jonsson, B. and Jonsson, N. (2014). Early environment influences later performance in fishes. *Journal of Fish Biology* **85**, 151–188.

Kaplan and Meier (1958). Nonparametric estimation from incomplete observations. Journal of the American Statistical Association. *Journal of the American Statistical Association* **53**, 457–81.

Killen, S., Adriaenssens, B., Marras, S., Claireaux, G. and Cooke, S. J. (2016). Context dependency of trait repeatability and its relevance for management and conservation of fish populations Themed Issue Article : Conservation Physiology of Marine Fishes Context dependency of trait repeatability and its relevance for management and. *Conservation Physiology* **4**, 1–19.

Korwin-Kossakowski, M. (2008). the Influence of Temperature During the Embryonic Period on Larval Growth and Development in Carp, Cyprinus Carpio L., and Grass Carp, Ctenopharyngodon Idella (Val.): Theoretical and Practical Aspects. *Fisheries (Bethesda)* **16**, 231–314.

Koumoundouros, G., Oran, G., Divanach, P., Stefanakis, S. and Kentouri, M. (1997). The opercular complex deformity in intensive gilthead sea bream (Sparus aurata L.) larviculture. *Aquaculture* **156: 165-1**, 165–177.

Levin, L. A. and Breitburg, D. L. (2015). Linking coasts and seas to address ocean deoxygenation. *Nature Climate Change* **5**, 401–403.

Lindström, J. (1999). Early development and fitness in birds and mammals. *Trends in Ecology and Evolution* **14**, 343–348.

Lupien, S. J., McEwen, B. S., Gunnar, M. R. and Heim, C. (2009). Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nature Reviews Neuroscience* **10**, 434–445.

Mandic, M., Todgham, A. E. and Richards, J. G. (2009). Mechanisms and evolution of hypoxia tolerance in fish. *Proceedings. Biological sciences / The Royal Society* **276**, 735–44.

Marks, C., West, T. N., Bagatto, B. and Moore, F. B.-G. (2005). Developmental Environment Alters Conditional Aggression in Zebrafish. *Copeia* **2005**, 901–908.

Mejri, S., Tremblay, R., Lambert, Y. and Audet, C. (2012). Influence of different levels of dissolved oxygen on the success of Greenland halibut (Reinhardtius hippoglossoides) egg hatching and embryonic development. *Marine Biology* **159**, 1693–1701.

Monaghan, P. (2008). Early growth conditions, phenotypic development and environmental change. *Philosophical Transactions of the Royal Society B: Biological Sciences* **363**, 1635–1645.

Nicholson, G., Jenkins, G. P., Sherwood, J. and Longmore, A. (2008). Physical environmental conditions, spawning and early-life stages of an estuarine fish: Climate change implications for recruitment in intermittently open estuaries. *Marine and Freshwater Research* **59**, 735–749.

O'Connor, M. I., Bruno, J. F., Gaines, S. D., Halpern, B. S., Lester, S. E., Kinlan, B. P. and Weiss, J. M. (2007). Temperature control of larval dispersal and the implications for marine ecology, evolution, and conservation. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 1266–71.

Peto and Peto (1972). Statistics in Town Planning Author (s): B . Benjamin Source : Journal of the Royal Statistical Society . Series A (General), Vol . 132 , No . 1 (1969), Published by : Wiley for the Royal Statistical Society Stable URL : http://www.jstor.org/stable/2. *Journal of the Royal Statistical Society* **132**, 185–207.

Richards, J. G., Farell, A. P. and Brauner, C. J. (2009). Hypoxia in fish. **27**, 528.

Robertson, C. E., Wright, P. A., Köblitz, L., Bernier, N. J., B, P. R. S. and Ko, L. (2014). Hypoxia-

inducible factor-1 mediates adaptive developmental plasticity of hypoxia tolerance in zebrafish , Danio rerio. *Proceeding of the royal society B* **281**,.

Rödel, H. G. and Monclús, R. (2011). Long-term consequences of early development on personality traits: A study in European rabbits. *Behavioral Ecology* **22**, 1123–1130.

Rombough, P.J. (1997)**.** The effects of temperature on embryonic and larval development. In Global Warming: Implications for Freshwater and Marine Fish , 177–223. Cambridge, UK: Cambridge Univ. Press

Roze, T., Christen, F., Amerand, A. and Claireaux, G. (2013). Trade-off between thermal sensitivity, hypoxia tolerance and growth in fish. *Journal of Thermal Biology* **38**, 98–106.

Sheriff, M. J. and Love, O. P. (2013). Determining the adaptive potential of maternal stress. *Ecology Letters* **16**, 271–280.

Therneau,T.M. and Grambsch, P.M (2000). Modeling Survival Data: Extending the Cox Model. Springer, New York. ISBN 0-387-98784-3.

Therneau, T.M. (2015). A Package for Survival Analysis in S. version 2.38, https://CRAN.R-project.org/package=survival.

Vanderplancke, G., Claireaux, G., Quazuguel, P., Madec, L., Ferraresso, S., Sévère, A., Luis, J., Infante, Z. and Mazurais, D. (2015a). Hypoxic episode during the larval period has long‐term effects on European sea bass juveniles (*Dicentrarchus labrax*). **162**, 367–376.

Vanderplancke, G., Claireaux, G., Quazuguel, P., Huelvan, C., Corporeau, C., Mazurais, D. and Zambonino-Infante, J.-L. (2015b). Exposure to chronic moderate hypoxia impacts physiological and developmental traits of European sea bass (*Dicentrarchus labrax*) larvae. *Fish physiology and biochemistry* **41**, 233–242.

Venables, W. N. and Ripley, B. D. (2002). *Statistics Complements to Modern applied statistics with S*.

Widmer, S., Moore, F. B. G. and Bagatto, B. (2006). The effects of chronic developmental hypoxia on swimming performance in zebrafish. *Journal of Fish Biology* **69**, 1885–1891.

Wood, A. T., Clark, T. D., Andrewartha, S. J., Elliott, N. G. and Frappell, P. B. (2017). Developmental Hypoxia Has Negligible Effects on Long-Term Hypoxia Tolerance and Aerobic Metabolism of Atlantic Salmon (*Salmo salar*). *Physiological and Biochemical Zoology* **90**, 494–501.

Wu, R. S. S. (2009). *Effects of Hypoxia on Fish Reproduction and Development*. 1st ed. Elsevier Inc.

Zambonino, J. L., Calm, C. L., Peres, A., Quazuguel, P. and Gall, M. M. Le (1996). Sea bass (Dicentrarchus lab-ax) larvae fed different Artemia rations : growth , pancreas enzymatic response and development of digestive functions. *Aquaculture* **139**, 129–138.

Zambonino-Infante, J. L., Mazurais, D., Servili, A., Cahu, C., Vanderplancke, G., Le Bayon, N., Huelvan, C. and Claireaux, G. (2017). An early-life hypoxia event has a long-term impact on protein digestion and growth in European sea bass juvenile. *The Journal of Experimental Biology* **220**, 1846–1851.

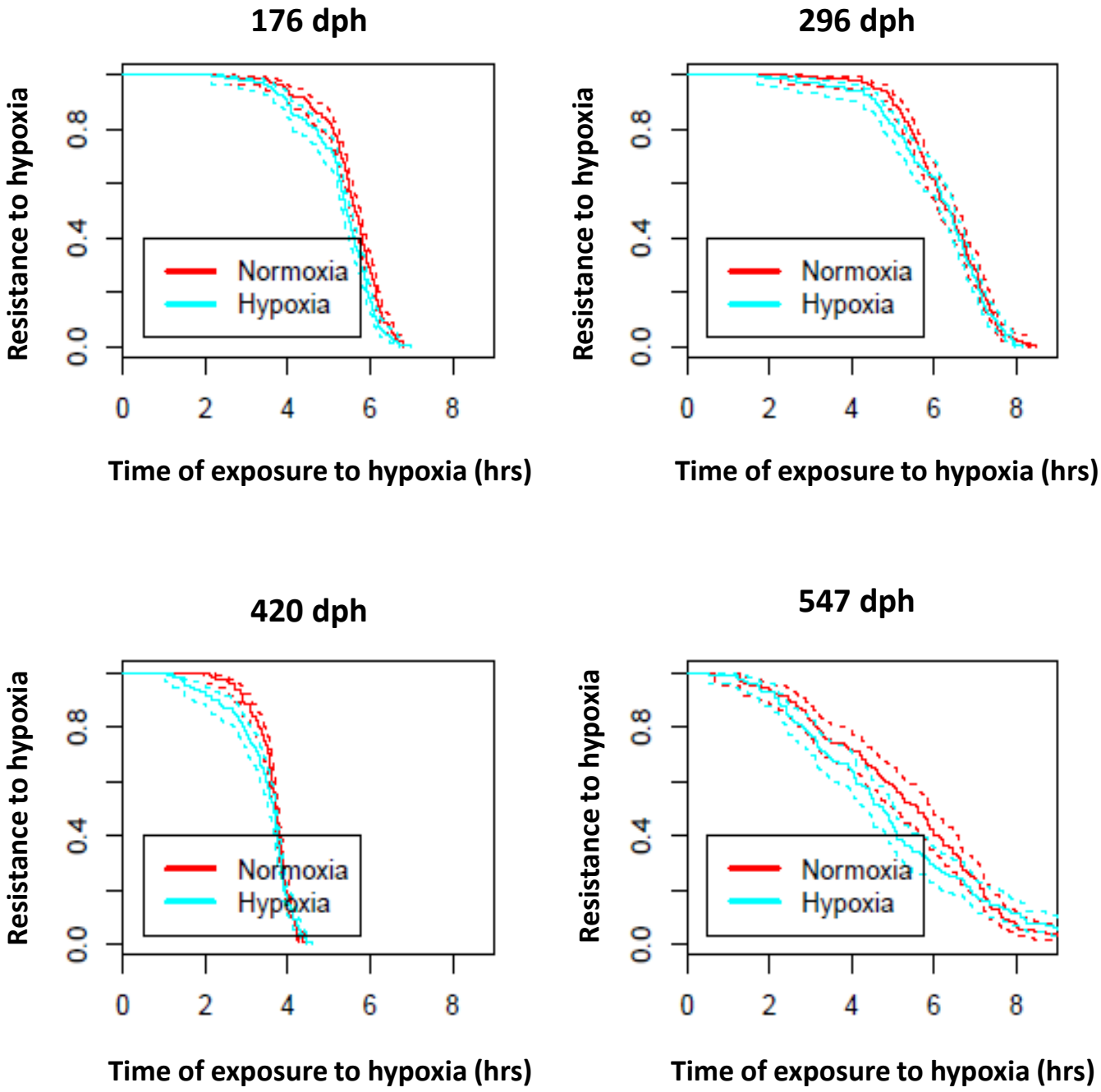


Figure 1. Kaplan-Meier probability plot of tolerance time during a Hypoxia Challenge in juveniles early exposed during larval stage to normoxia (red lines) or hypoxia (blue lines). Continuous lines represent the percentage of individuals that resisted hypoxia up to the time point considered (also called 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).

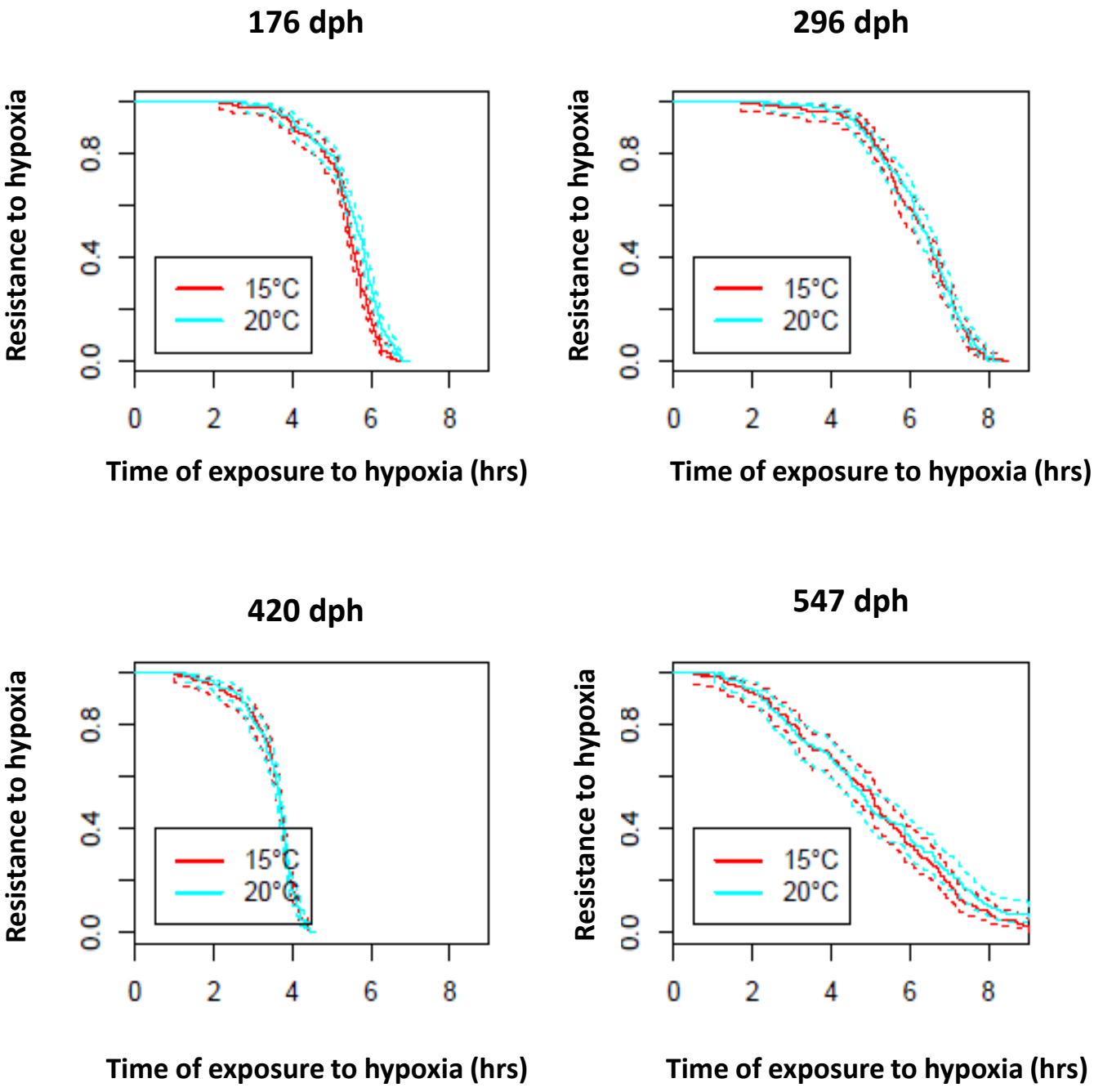


Figure 2. Kaplan-Meier probability plot of tolerance time during a Hypoxia Challenge in juveniles early exposed during larval stage to 15°C (red lines) or 20°C (blue lines). Continuous lines represent the percentage of individuals that resisted hypoxia up to the time point considered (also called the 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).

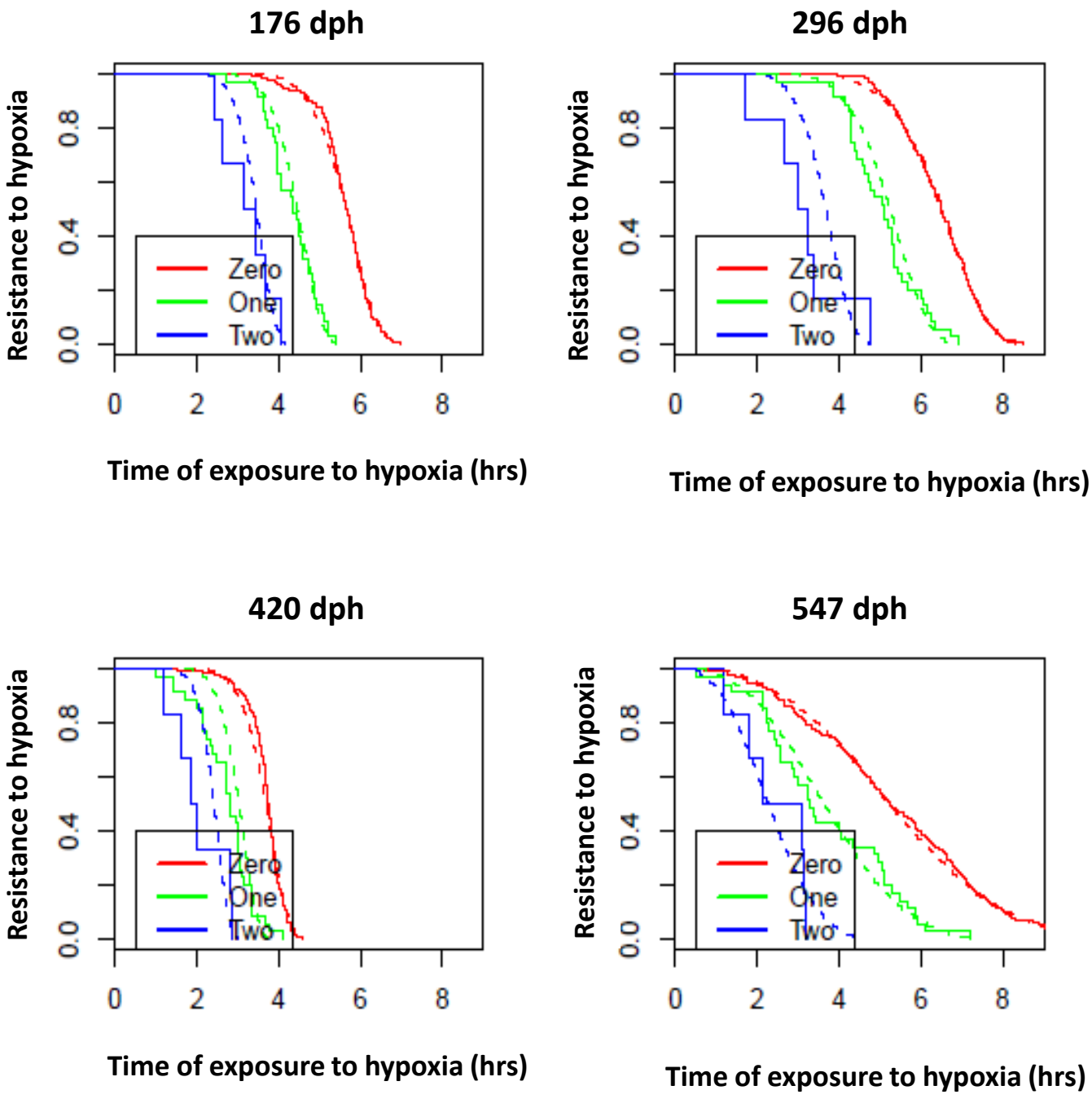
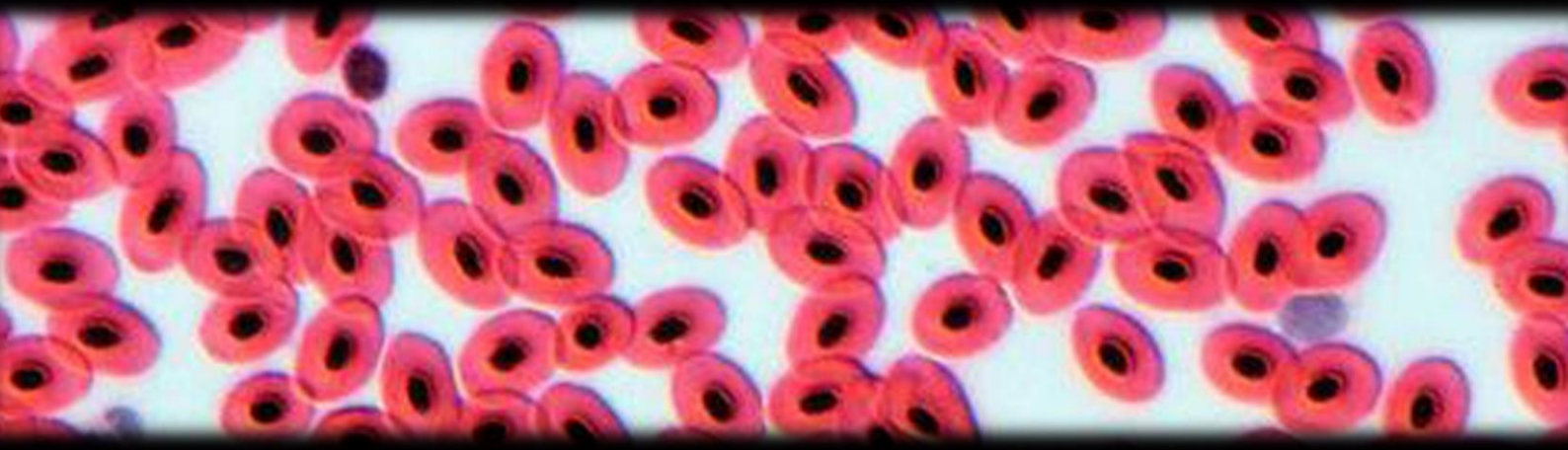


Figure 3. Kaplan-Meier probability plot of tolerance time during a Hypoxia Challenge on the basis of the incidence of opercular deformities in sea bass juveniles: zero (red line), one-unilateral (green line) and two-bilateral (blue line). Continuous lines represent the percentage of individuals that 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.

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ORIGINAL PAPER

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 Hbα genes named MN-*Hbα1-5* and six Hbβ genes named MN-*Hbβ1–6*) and the "LA" cluster consisting of three genes (two Hbα genes named LA-*Hbα1-2* and one Hbβ gene named LA-*Hbβ1*). 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-*Hbβ3*. Six genes were mainly expressed during early life (MN-*Hbα3-5*, MN-*Hbβ4–6*), 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.

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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

mammals are clustered on one chromosome, teleost α and β globin genes (Hbα and Hbβ 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 (sometime 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.mpipz.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

Hbα and Hbβ 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 Hbα and Hbβ 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 ± 0.4 °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× 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-*Hbα1*, LA-*Hbα2*, LA-*Hbβ1*, MN-*Hbα1*, MN-*Hbα2*, MN-*Hbβ1*, MN-*Hbβ2*, MN-*Hbβ3*, MN-*Hbβ4*, and reference genes; 62 °C for MN-*Hbβ5* and MN-*Hbβ6*; and 70 °C for MN-*Hbα3*, MN-*Hbα4*, and MN-*Hbα5*). 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 EF1α) genes

Gene	Accession numbers	Forward primer sequences (5′–3′)	Reverse primer sequences (5′–3′)
LA-*Hbα1*	KX196178	CAGTGGGACAGGATCTTGAAGT	GGTGATGGGTGGAATCAATC
LA-*Hbα2*	KX196180	TTTCCCATGAGAGAGCAGGT	TCAGATGCGCTTCTTAGGATGT
MN-*Hbα1*	KX196190	GGCCAGGATGCTGACTGTA	CCAGCAAGGTCATCCATCTT
MN-*Hbα2*	KX196188	CCTGCCAACTTCAAGATTCTG	TTTCTCAGACAAGGCACGAG
MN-*Hbα3*	KX196184	ACAGACAAGATGACCAGTCTCACT	GCCAATGTCCTCTGCCTTC
MN-*Hbα4*	KX196183	ACAGACAAGATGACCAGTCTCACT	GCCAATGTCCTCTGCCTTT
MN-*Hbα5*	KX196181	ACAGACAAGATGACCAGTCTCACA	GCCAATGTCCTCTGCCTTC
LA-*Hbβ1*	KX196179	CCCGACAACTTCAAACTGCT	CCTGCGTCTCTGGTGTGAAG
MN-*Hbβ1*	KX196191	TGATTTGAGCAAAGATCCTGAA	CATGGACGACATCAAGAACG
MN-*Hbβ2*	KX196189	GTCAGCCAGCAGCCTGAAAT	GCAGCTCTTTCCAGGTGTCT
MN-*Hbβ3*	KX196187	CAGAAGCTTTGGCAAGAGTG	GCTGCTACTTTGGCGTTACC
MN-*Hbβ4*	KX196186	GTCGTTTACCCCTGGACTCA	GTTTTGCGACCATCGGATTT
MN-*Hbβ5*	KX196185	ACCATCCAGGACATCTTCTCT	GTTTTGCGACCAACGGATTC
MN-*Hbβ6*	KX196182	ACCATCCAGGACATTTTCTCC	GTTTTGCGACCAACGGATTC
18 S	AY831388.1	TGGTTCCTTTGATCGCTCTT	AGCAGCTCGTTGGCATGTAT
Ef1α	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 $\Delta\Delta Ct$ 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 (*ef1α*) 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 uncentered) 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 Hbα and seven Hbβ genes (Fig. 1). As shown in stickleback (*Gasterosteus aculeatus*), the MN cluster included five Hbα genes annotated MN-*Hbα1* to MN-*Hbα5* and six Hbβ genes annotated MN-*Hbβ1* to MN-*Hbβ6* following the suggested nomenclature (Opazo et al. 2013). The cluster consisted of about 42 kb from the first codon of MN-*Hbα5* to the stop codon of MN-*Hbβ1*. Whereas stickleback MN-Hbα 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-*Hbβ4* and MN-*Hbβ5* .

The LA cluster included two Hbα and one Hbβ genes, annotated LA-*Hbα1*, LA-*Hbα2,* and LA-*Hbβ1* (Fig. 1). These three genes spanned a sequence length of about 14 kb from the first codon of LA-*Hbα2* to the stop codon of LA-*Hbα1*. The Hbα genes of the LA cluster were encoded by one strand, whereas *Hbβ1* was encoded by the reverse strand. LA-*Hbα1* and LA-*Hbβ1* were organized head to head, whereas LA-*Hbα2* and LA-*Hbβ1* were arranged tail to tail. The organization of the LA cluster was conserved between the European sea bass and tilapia (*Oreochromis niloticus*).

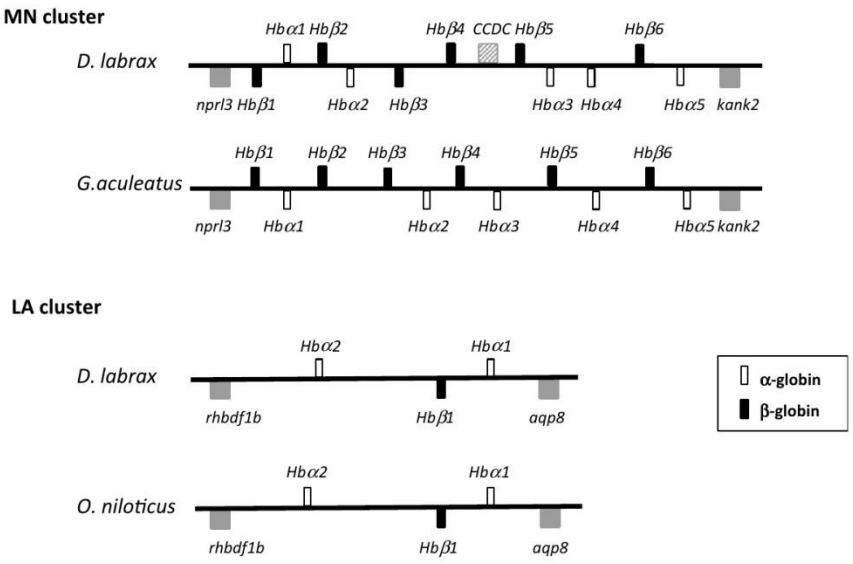

Fig. 1 Genomic organization of the European sea bass MN and LA clusters. Genomic structures of the D. labrax MN and LA clusters are compared with *G. aculeatus* and *O. niloticus*, respectively. The genes in the forward and the reverse orientations are shown boxed above and below the line, respectively. Hbα and Hbβ 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 Hbα and Hbβ genes typically consist of three exons separated by two introns (Table 2). While all the Hbβ genes coded for proteins of 147 aa, the size of the proteins encoded by Hbα 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 Hbα and Hbβ globins (Figs. 2, 3). The His residue at position 122 of Hbα globins seems to be involved in the Bohr effect (Bellelli and Brunori 2011) and was found in all Hbα sequences of European sea bass. Similarly, the C-terminal His residue of Hbβ 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 Hbα and Hbβ genes

Genes	Length (bp)						
	Exon 1	Intron 1	Exon 2	Intron 2	Exon 3	CDS	AA
LA-*Hbα1*	95	629	208	355	129	432	143
LA-*Hbα2*	92	1103	205	1010	129	426	141
MN-*Hbα1*	95	107	208	176	129	432	143
MN-*Hbα2*	95	105	208	506	129	432	143
MN-*Hbα3*	98	422	208	180	129	435	144
MN-*Hbα4*	98	110	208	184	129	435	144
MN-*Hbα5*	98	348	208	180	129	435	144
LA-*Hbβ1*	92	91	223	246	129	444	147
MN-*Hbβ1*	92	142	223	94	129	444	147
MN-*Hbβ2*	92	110	223	97	129	444	147
MN-*Hbβ3*	92	737	223	106	129	444	147
MN-*Hbβ4*	92	143	223	99	129	444	147
MN-*Hbβ5*	92	146	223	99	129	444	147
MN-*Hbβ6*	92	145	223	99	129	444	147

Fig. 2 Amino acid sequence alignment of European sea bass Hbα. 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 root effect are boxed in *grey*. The residues responsible for the Bohr effect are indicated in *bold*

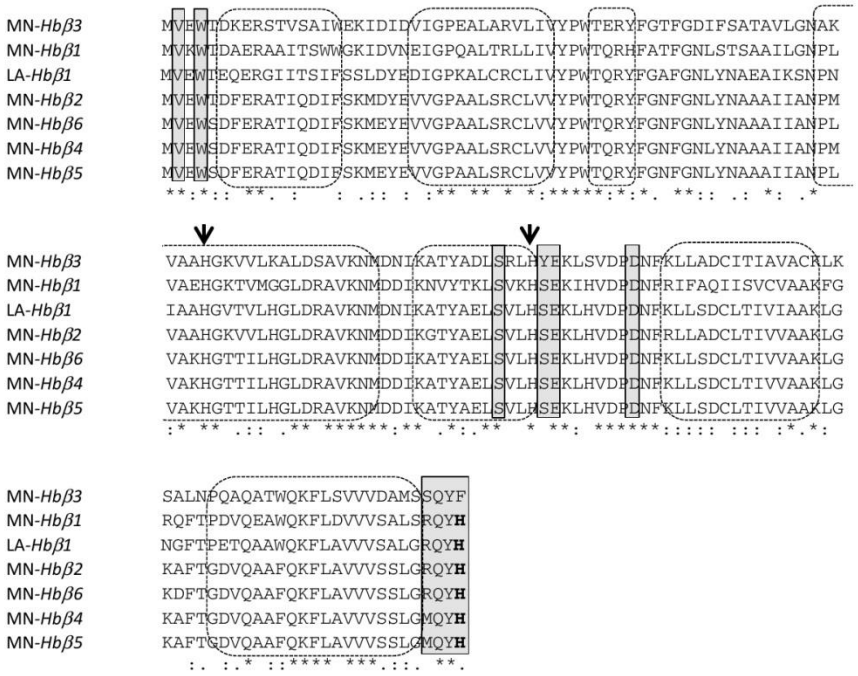

Fig. 3 Amino acid sequence alignment of European sea bass Hbβ. The amino acid similarities between all proteins are given as follows: *asterisks* indicate identical amino acids, *colons* or *dots* indicate similar amino acids, and *empty spaces* represent absence or low degree of similarity. Predicted alpha-helices are shown in *dashed boxes*. Histidine 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 Hbβ sequences, except for MN-*Hbβ3*, where it is substituted by a Phe residue. European sea bass Hbα and Hbβ genes possessed all the putative residues responsible for the Root effect, such as Val2β, Ser2α, Trp4β, Ser90β, Ser94β, Glu95β, Asp95α, Asp101β, Glu140α, Arg144β, Gln145β, Tyr146β, and His147β (Bonaventura et al. 2004), except MN-Hbβ3 which was devoid of Ser94β and Arg144β.

Phylogenetic analysis of European sea bass Hb

Phylogenetic analysis was performed based on the deduced Hbα and Hbβ 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 Hbα in many clades, two of them composed with genes from the LA cluster. Hbβ 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-Hbα3–5 on one hand and MN-Hbβ2 and MN-Hbβ4–6 on the other clustered together in the MN-Hbα and MN-Hbβ 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 Hbα and Hbβ genes were very low or not detected (Cq > 30) (Supplementary file 6). Several genes (notably LA-*Hbα2*, MN-*Hbα2*, MN-*Hbβ2*, MN-*Hbα3*, MN-*Hbβ5*, MN-*Hbβ4*, and MN-*Hbα5)* exhibited a substantial increase in expression between 60 hpf and 5 day post hatching

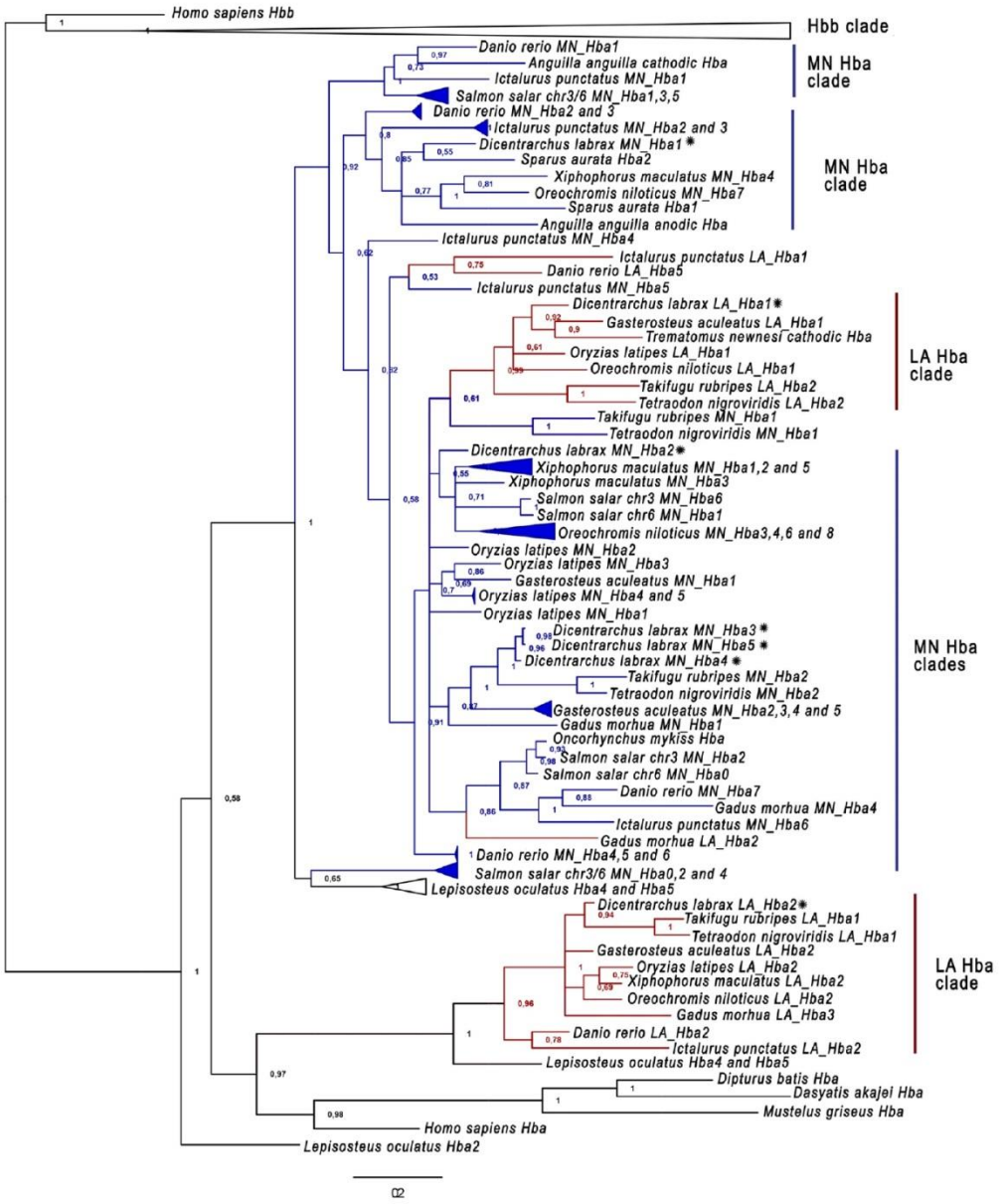

Fig. 4 Bayesian phylogenetic tree of Hbα sequences. Phylogenetic analysis was performed on both Hbα and Hbβ following the method detailed in "Materials and methods", but only Hbα sequences are presented here. European sea bass Hbα genes are marked with asterisks. Hbα amino acid sequences from other species 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 Hbβ genes of European sea bass and other species have been collapsed in an Hbβ 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-*Hbα2*, LA-*Hbβ1*, MN-*Hbβ1*, MN-*Hbα2*, MN-*Hbβ4*, MN-*Hbα5*, MN-*Hbα4*, and MN-*Hbβ6*) 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-*Hbβ1*, MN-*Hbα2*, LA-*Hbα1*, MN-*Hbα1*, MN-*Hbβ2*, and MN-*Hbα2)* during the juvenile stage, while it dropped significantly for others (MN-*Hbβ4*, MN-*Hbα5*,

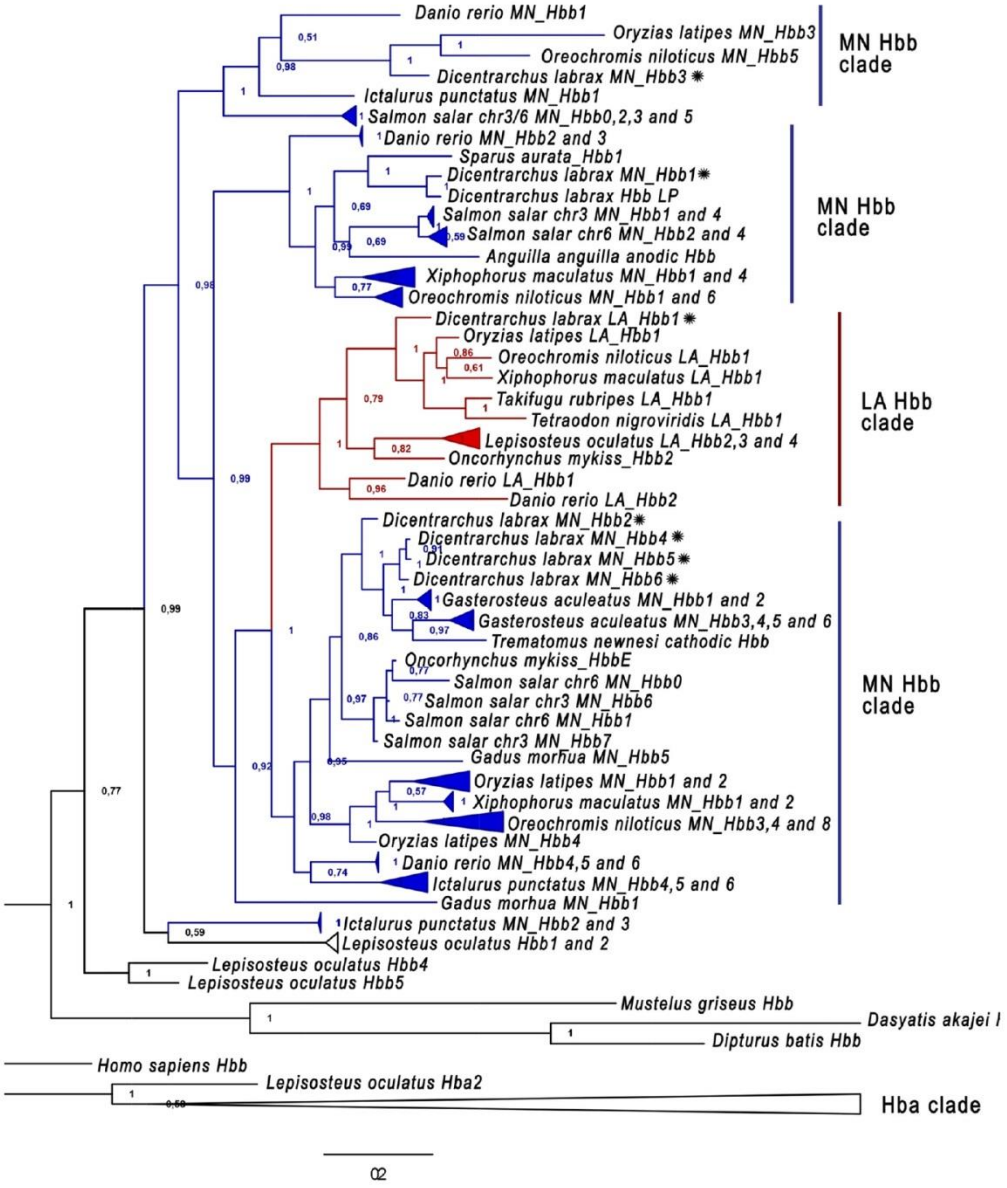

Fig. 5 Bayesian phylogenetic tree of Hbβ sequences. Phylogenetic analysis was performed on both Hbα and Hbβ following the method detailed in "Materials and methods", but only Hbβ sequences are presented here. European sea bass Hbβ genes are marked with asterisks. Hbβ amino acid sequences from other species 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 Hbα genes of European sea bass and other species have been collapsed in an Hbα 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-*Hbα4*, and MN-*Hbβ6* genes). MN-*Hbβ3* 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-*Hbα1* and MN-*Hbβ1* exhibited the highest expression levels in the adult fish, irrespective of the tissue considered

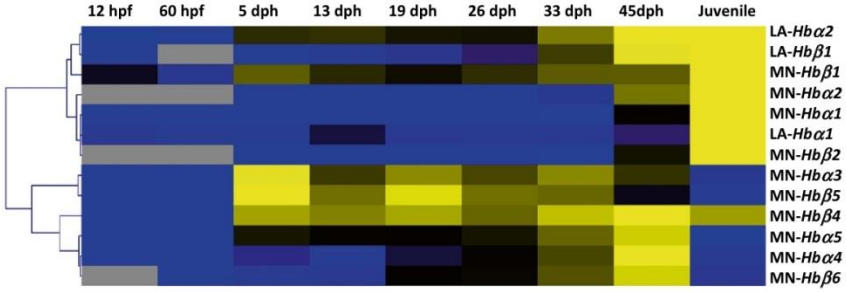

Fig. 6 Relative expression profiles of Hb α and β genes during development of European sea bass. The relative expression profiles of Hbα and β genes during development of European sea bass were determined using qPCR. MN-Hbβ3 expression profile is not shown since Cq values were up to 35 from 12 hpf until 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-*Hbα3*, MN-*Hbα4*, and MN-*Hbα5*, as well as MN-*Hbβ4*, MN-*Hbβ5*, and MN-*Hbβ6*, were expressed at very low levels in the different adult tissues investigated. MN-*Hbα1*, MN-*Hbβ1*, and MN-*Hbβ3* showed the highest expression levels in the head kidney and lesser levels in the spleen (Fig. 7; Supplementary file 7). MN-*Hbα2* and MN-*Hbβ2* 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 Hbα and 1 Hbβ) 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 Hbα genes were separated by the Hbβ 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-*Hbβ1* seems to have been lost in stickleback, which yet exhibits high overall orthologous

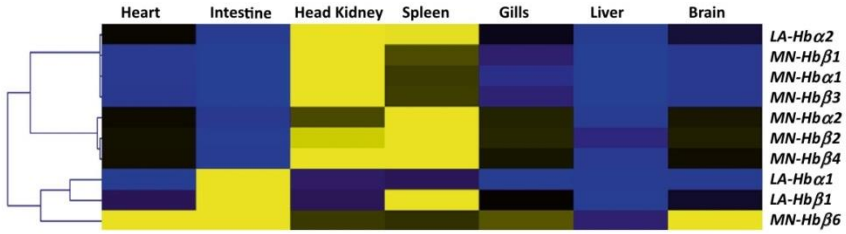

Fig. 7 Expression pattern of Hbα and β genes in different tissues of adult European sea bass. The relative expression levels of Hbα and β genes were determined in different tissues of European sea bass using qPCR. MN-Hbα3, MN-Hbα4, MN-Hbα5, and MN-Hbβ5 expression profiles are not shown, since Cq values were up to 33 among the tissues. Following normalization with 18 S and Ef1a levels, relative 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 Hbα and six Hbβ) were flanked by *nprl3* and *kank2* genes similar to the MN cluster in teleosts. Intriguingly, a *CCDC106-like* gene was found inserted between MN-*Hbβ4* and MN-*Hbβ5*, 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-*Hbα3*, MN-*Hbα4*, MN-*Hbα5* and MN-*Hbβ2*, MN-*Hbβ4*, MN-*Hbβ5*, MN-*Hbβ6*) each consist of close paralogous copies and are probably the results of recent duplication events that occurred in the Moronidae lineage. In comparison, MN-*Hbα4* and MN-*Hbβ2* 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-*Hbα3−5* genes and three MN-*Hbβ4−6* genes exhibited high larval expression compared to juvenile and adult levels. Intriguingly, the expression patterns throughout development were similar in the closely linked MN-*Hbα4*, MN-*Hbα5* , and MN-*Hbβ6* on one hand and in the neighbour genes MN-Hbα3 and MN-Hbβ5 on the other hand, suggesting that these Hbα and Hbβ 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-*Hbα1*, LA-*Hbα2*, MN-*Hbα1*, MN-*Hbα2*, LA-*Hbβ1*, MN-*Hbβ1*, MN-*Hbβ2* and MN-*Hbβ3*) 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-*Hbα1*, MN-*Hbβ1* and to a lesser extent LA-*Hbα2*, MN-*Hbβ3*, MN-*Hbα2*, and MN-*Hbβ2* were the most predominantly expressed genes at the adult stage. The expression of three different Hbα and three different Hbβ 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 Hbβ 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 Hbβ-like (highly similar to the present MN-*Hbβ1* 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, Asp95α and Asp101β, 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, Ser94β, Arg144β, and His147β 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 Hbβ genes except for MN-*Hbβ3*. The substitution of key functional residues is partially found in *Hbβ1, Hbβ4*, and *Hbβ5* 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.

References

Abascal F, Zardoya R, Posada D (2005) ProtTest: selection of best-fit models of protein evolution. Bioinformatics 21:2104–2105

Bellelli A, Brunori M (2011) Hemoglobin allostery: variations on the theme. Biochim Biophys Acta (BBA) Bioenergetics 1807:1262–1272

Biagioli M, Pinto M, Cesselli D, Zaninello M, Lazarevic D, Roncaglia P, Simone R, Vlachouli C, Plessy C, Bertin N, Beltrami A, Kobayashi K, Gallo V, Santoro C, Ferrer I, Rivella S, Beltrami CA, Carninci P, Raviola E, Gustincich S (2009) Unexpected expression of alpha- and beta-globin in mesencephalic dopaminergic neurons and glial cells. Proc Natl Acad Sci USA 106:15454–15459

Bonaventura C, Crumbliss AL, Weber RE (2004) New insights into the proton-dependent oxygen affinity of root effect haemoglobins. Acta Physiol Scand 182:245–258

Brownlie A, Zon L (1999) The Zebrafish as a model system for the study of hematopoiesis: Zebrafish mutants point the way to novel genes involved in the generation of vertebrate blood cells. Bioscience 49:382–392

Burge C, Karlin S (1997) Prediction of complete gene structures in human genomic DNA. J Mol Biol 268:78–94

Campo S, Nastasi G, D'Ascola A, Campo GM, Avenoso A, Traina P, Calatroni A, Burrascano E, Ferlazzo A, Lupidi G, Gabbianelli R, Falcioni G (2008) Hemoglobin system of Sparus aurata: changes in fishes farmed under extreme conditions. Sci Total Environ 403:148–153

Claireaux G, Lagardère JP (1999) Influence of temperature, oxygen and salinity on the metabolism of the European sea bass. J Sea Res 42:157–168

Darias MJ, Zambonino-Infante JL, Hugot K, Cahu CL, Mazurais D (2008) Gene expression patterns during the larval development of European sea bass (dicentrarchus labrax) by microarray analysis. Mar Biotechnol (NY) 10: 416–428

Esteban MA, Meseguer J, Garcia Ayala A, Agulleiro B (1989) Erythropoiesis and thrombopoiesis in the head-kidney of the sea bass (*Dicentrarchus labrax* L.): an ultrastructural study. Arch Histol Cytol 52:407–419

Feng J, Liu S, Wang X, Wang R, Zhang J, Jiang Y, Li C, Kaltenboeck L, Li J, Liu Z (2014) Channel catfish hemoglobin genes: identification, phylogenetic and syntenic analysis, and specific induction in response to heat stress. Comp Biochem Physiol Part D Genomics Proteomics 9:11–22

Gabbianelli R, Zolese G, Bertoli E, Falcioni G (2004) Correlation between functional and structural changes of reduced and oxidized trout hemoglobins I and IV at different pHs. A circular dichroism study. Eur J Biochem 271:1971–1979

Hardison RC (2008) Globin genes on the move. J Biol 7:35

Helfman G, Collette B, Facey D (1997) The Diversity of Fishes Malden, Mass

Ishibashi Y, Kotaki T, Yamada Y, Ohta H (2007) Ontogenic changes in tolerance to hypoxia and energy metabolism of larval and juvenile Japanese flounder *Paralichthys olivaceus*. J Exp Mar Biol Ecol 352:42–49

Kulkeaw K, Sugiyama D (2012) Zebrafish erythropoiesis and the utility of fish as models of anemia. Stem Cell Res Ther 3:55

Le SQ, Gascuel O (2008) An improved general amino acid replacement matrix. Mol Biol Evol 25:1307–1320

Lu W, Mayolle A, Cui G, Luo L, Balment RJ (2011) Molecular characterization and expression of alpha-Globin and beta-Globin Genes in the Euryhaline Flounder (*Platichthys flesus*). Evid Based Complement Alternat Med 965153

Marinakis P, Tamburrini M, Carratore V, di Prisco G (2003) Unique features of the hemoglobin system of the Antarctic notothenioid fish Gobionotothen gibberifrons. Eur J Biochem 270:3981–3987

Maruyama K, Yasumasu S, Naruse K, Mitani H, Shima A, Iuchi I (2004) Genomic organization and developmental expression of globin genes in the teleost *Oryzias latipes*. Gene 335:89–100

Mazzarella L, Bonomi G, Lubrano MC, Merlino A, Riccio A, Vergara A, Vitagliano L, Verde C, di Prisco G (2006) Minimal structural requirements for root effect: crystal structure of the cathodic hemoglobin isolated from the antarctic fish *Trematomus newnesi*. Proteins 62:316–321

McKenzie DJ, Lund I, Pedersen PB (2008) Essential fatty acids influence metabolic rate and tolerance of hypoxia in Dover sole (Solea solea) larvae and juveniles. Marine Biology 154:1041–1051

Mylvaganam SE, Bonaventura C, Bonaventura J, Getzoff ED (1996) Structural basis for the root effect in haemoglobin. Nat Struct Mol Biol 3:275–283

Near TJ, Parker SK, Detrich HW 3rd (2006) A genomic fossil reveals key steps in hemoglobin loss by the antarctic icefishes. Mol Biol Evol 23:2008–2016

Nishi H, Inagi R, Kato H, Tanemoto M, Kojima I, Son D, Fujita T, Nangaku M (2008) Hemoglobin is expressed by mesangial cells and reduces oxidant stress. J Am Soc Nephrol 19:1500–1508

Opazo JC, Butts GT, Nery MF, Storz JF, Hoffmann FG (2013) Whole-genome duplication and the functional diversification of teleost fish hemoglobins. Mol Biol Evol 30:140–153

Perez JE, Maclean N (1976) Multiple globins and haemoglobins in the bass, *Dicentrarchus labrax* (L.) (Serranidae: Teleostei). J Fish Biol 8:413–417

Perez-Ruzafa A, Marcos C (2014) Ecology and distribution of *Dicentrarchus labrax* (Linnaeus 1758). In: Sanchez Vazquey J, Munoz-Cueto J (eds) Biology of European Sea Bass. CRC Press, Boca Raton, FL, pp 3–33

Perutz MF (1983) Species adaptation in a protein molecule. Mol Biol Evol 1:1–28

Quesada J, Villena MI, Navarro V (1994) Ontogeny of the sea bass spleen (Dicentrarchus labrax): a light and electron microscopic study. J Morphol 221:161–176

Quinn NL, Boroevich KA, Lubieniecki KP, Chow W, Davidson EA, Phillips RB, Koop BF, Davidson WS (2010) Genomic organization and evolution of the Atlantic salmon hemoglobin repertoire. BMC Genomics 11:539

Rinaldi L, Basso P, Tettamanti G, Grimaldi A, Terova G, Saroglia M, de Eguileor M (2005) Oxygen availability causes morphological changes and a different VEGF/Flk-1/HIF-2 expression pattern in sea bass gills. Ital J Zool 72:103–111

Rutjes HA, Nieveen MC, Weber RE, Witte F, Van den Thillart GE (2007) Multiple strategies of Lake Victoria cichlids to cope with lifelong hypoxia include hemoglobin switching. Am J Physiol Regul Integr Comp Physiol 293:R1376–R1383

Saha D, Patgaonkar M, Shroff A, Ayyar K, Bashir T, Reddy KV (2014) Hemoglobin expression in nonerythroid cells: novel or ubiquitous? Int J Inflam 803237

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729

Terova G, Cattaneo AG, Preziosa E, Bernardini G, Saroglia M (2011) Impact of acute stress on antimicrobial polypeptides mRNA copy number in several tissues of marine sea bass (*Dicentrarchus labrax*). BMC Immunol 12:69

Tezel G, Yang X, Luo C, Cai J, Kain AD, Powell DW, Kuehn MH, Pierce WM (2010) Hemoglobin expression and regulation in glaucoma: insights into retinal ganglion cell oxygenation. Invest Ophthalmol Vis Sci 51:907–919

Tiedke J, Gerlach F, Mitz SA, Hankeln T, Burmester T (2011) Ontogeny of globin expression in zebrafish (Danio rerio). J Comp Physiol B 181:1011–1021

Tine M, Kuhl H, Gagnaire PA, Louro B, Desmarais E, Martins RS, Hecht J, Knaust F, Belkhir K, Klages S, Dieterich R, Stueber K, Piferrer F, Guinand B, Bierne N, Volckaert FA, Bargelloni L, Power DM, Bonhomme F, Canario AV, Reinhardt R (2014) European sea bass genome and its variation provide insights into adaptation to euryhalinity and speciation. Nat Commun 5:5770

Tortonese E (1986) Moronidae. In: Whitehead PJP, Bauchot ML, Hureau JC, Nielsen J ET (eds) Fishes of the North-eastern Atlantic and the Mediterranean, Paris, pp 793–796

Ullal AJ, Wayne Litaker R, Noga EJ (2008) Antimicrobial peptides derived from hemoglobin are expressed in epithelium of channel catfish (Ictalurus punctatus, Rafinesque). Developmental and Comparative. Immunology 32:1301–1312

Verde C, Carratore V, Riccio A, Tamburrini M, Parisi E, Di Prisco G (2002) The functionally distinct hemoglobins of the Arctic spotted wolffish Anarhichas minor. J Biol Chem 277:36312–36320

Verde C, Balestrieri M, de Pascale D, Pagnozzi D, Lecointre G, di Prisco G (2006) The oxygen transport system in three species of the boreal fish family Gadidae. Molecular phylogeny of hemoglobin. J Biol Chem 281:22073–22084

Verde C, Vergara A, Mazzarella L, di Prisco G (2008) The hemoglobins of fishes living at polar latitudes—current knowledge on structural adaptations in a changing environment. Curr Protein Pept Sci 9:578–590

von der Heyden S, Toms JA, Teske PR, Lamberth SJ, Holleman W (2015) Contrasting signals of genetic diversity and historical demography between two recently diverged marine and estuarine fish species. Mar Ecol Prog Ser 526:157–167

Weber RE (1990) Functional significance and structural basis of multiple hemoglobins with special reference to ectothermic vertebrates. In: Animal Nutrition and Transport Processes. 2. Transport, Respiration and Excretion: Comparative and Environmental Aspects. Comparative Physiology. Karger, Basel, pp 58–75

Weber RE (2000) Adaptations for oxygen transport: lessons from Fish Hemoglobins hemoglobin function in vertebrates: molecular adaptation in extreme and temperate environments. Springer Milan, Milano, pp 23–37

Wetten OF, Nederbragt AJ, Wilson RC, Jakobsen KS, Edvardsen RB, Andersen O (2010) Genomic organization and gene expression of the multiple globins in Atlantic cod: conservation of globin-flanking genes in chordates infers the origin of the vertebrate globin clusters. BMC Evol Biol 10:315

Zambonino-Infante JL, Cahu CL, Peres A, Quazuguel P, Le Gall MM (1996) Sea bass (*Dicentrarchus labrax*) larvae fed different Artemia rations: growth, pancreas enzymatic response and development of digestive functions. Aquaculture 139:129–138

Zhou J, Qiao X, Xiao L, Sun W, Wang L, Li H, Wu Y, Ding X, Hu X, Zhou C, Zhang J (2010) Identification and characterization of the novel protein CCDC106 that interacts with p53 and promotes its degradation. FEBS Lett 584:1085–1090

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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-*Hbα3*, MN-*Hbβ4* and MN-*Hbβ5* and increase of MN-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* 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-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* 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.

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List of abbreviations used:

Aqp8	Aquaporin-8
CJ	Control juvenile group
CL	Control larval group
Cq	Quantification cycle
Dph	Days Post Hatching
Ef1α	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
PHD-3	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 (O_2) 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 O_2 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 O_2 extraction and transport (Nilsson, 2007), as well as metabolic regulation in order to limit O_2 demands (Hopkins and Powell, 2001). In fish, as in other vertebrates, hemoglobins (Hb) are involved in O_2 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 O_2. Teleost fish species have the characteristic of expressing several Hbα and Hbβ genes which are grouped into two unliked 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 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 O_2 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-*Hbα3-5* and MN-*Hbβ4-6*), while others (LA-*Hbα1-2,* LA-*Hbβ1,* MN-*Hbα1-2* and MN-*Hbβ1*-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

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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 ± 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. 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 mg O_2 L^{-1}), while in the other three tanks (control larval group (CL)) it was maintained at 100% saturation (7.35 mg O_2 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 ± 0.4°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)).

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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): $\frac{(final\ weight - initial\ weight)}{initial\ weight}$.

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).

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Hematological parameters

Hematocrit was determined from blood samples after centrifugation at 13,000 × 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).

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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-*Hbα1*, LA-*Hbβ1*, MN-*Hbα2*, MN-*Hbα3*, MN-*Hbβ4* and MN-*Hbβ5*) and genes highly expressed in the head kidney and spleen at the juvenile stage, i.e. MN-*Hbα1* and MN-*Hbβ1* (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 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, 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 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-*Hbα1*, LA-*Hbα2*, LA-*Hbβ1*, MN-*Hbα1*, MN-*Hbα2*, MN-*Hbβ1*, MN-*Hbβ2*, MN-*Hbβ3*, MN-*Hbβ4* and reference genes; 62°C for MN-*Hbβ5* and MN-*Hbβ6*; and 70°C for MN-*Hbα3*, MN-*Hbα4* and MN-*Hbα5*). 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 ΔΔCt 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* (*ef1α*)

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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 O_2 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 O_2 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 *Hbα* genes and 7 *Hbβ* 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-*Hbα1*, LA-*Hbβ1* and MN-*Hbα2* genes were significantly higher (7-fold, 6-fold and 14-fold, respectively) in larvae exposed to hypoxia ($p = 2\times10^{-5}$, 1×10^{-5} and 1×10^{-6}, respectively). Conversely, the MN-*Hbα3*, MN-*Hbβ4* and MN-*Hbβ5* transcript levels were reduced (3-fold, 5-fold and 4-fold, respectively) in

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larvae under hypoxia ($p = 4\times10^{-4}$, 1×10^{-4} and 1×10^{-4}, respectively). Other Hb genes (LA-*Hbα2*, MN-*Hbα4*, MN-*Hbα5* and MN-*Hbβ6*) did not exhibit any differential expression or were not sufficiently expressed (Cq>33 for MN-*Hbα1,* MN-*Hbβ1*, MN-*Hbβ2* and MN-*Hbβ3*) 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 ($p = 2\times10^{-5}$).

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-*Hbα1*, LA-*Hbβ1* and MN-*Hbα2* 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-*Hbα1* and MN-*Hbβ1* remained unchanged under moderate hypoxia in the head kidney and spleen tissues. The transcript levels of MN-*Hbα3*, MN-*Hbβ4* and MN-*Hbβ5* 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_{O2}, 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

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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-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* transcripts, respectively, while MN-*Hbα3*, MN-*Hbβ4* and MN-*Hbβ5* 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-*Hbα3*, MN-*Hbβ4* and MN-*Hbβ5* transcripts are more abundant than MN-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* 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-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* gene expressions on the composition of the Hb tetramers and on the O_2 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-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* 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

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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 O_2 transport (e.g. blood oxygen affinity), our results are in agreement with previous data obtained in *D. labrax* juveniles, showing that the O_2 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 O_2 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

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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-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* 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-*Hbα1* and MN-*Hbβ1* (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-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* could have specific functional

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characteristics (e.g. binding capacities, Root and Bohr effects), we cannot rule out the hypothesis that their regulation may be associated with enhanced O_2 transport capacity, allowing fish to cope better with mild hypoxia, as previously suggested by (Wawrowski et al., 2011). Moreover, regulation of LA-*Hbα1* and LA-*Hbβ1* expression may impact other cellular/physiological functions than those strictly related to O_2 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 O_2 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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References

Bateson, P., Gluckman, P. and Hanson, M. (2014). The biology of developmental plasticity and the Predictive Adaptive Response hypothesis. *The Journal of physiology* **592**, 2357–2368.

Bianchini, K. and Wright, P. a (2013). Hypoxia delays hematopoiesis: retention of embryonic hemoglobin and erythrocytes in larval rainbow trout, Oncorhynchus mykiss, during chronic hypoxia exposure. *The Journal of experimental biology* **216**, 4415–4425.

Breitburg, D. L., Hondorp, D. W., Davias, L. a and Diaz, R. J. (2009). Hypoxia, nitrogen, and fisheries: integrating effects across local and global landscapes. *Annual review of marine science* **1**, 329–349.

Cadiz, L., Desmarais, E., Servili, A., Quazuguel, P., Madec, L., Huelvan, C., Andersen, O., Zambonino-Infante, J. and Mazurais, D. (2017). Genomic organization and spatio-temporal expression of the hemoglobin genes in European sea bass (Dicentrarchus labrax). *Marine Biology* **164**, 82–95.

Dufour, V., Cantou, M. and Lecomte, F. (2009). Identification of sea bass (Dicentrarchus labrax) nursery areas in the north-western Mediterranean Sea. *Journal of the Marine Biological Association of the United Kingdom* **89**, 1367–1374.

Dupont-Prinet, a, Chatain, B., Grima, L., Vandeputte, M., Claireaux, G. and McKenzie, D. J. (2010). Physiological mechanisms underlying a trade-off between growth rate and tolerance of feed deprivation in the European sea bass (Dicentrarchus labrax). *The Journal of experimental biology* **213**, 1143–52.

Ekau, W., Auel, H., Pörtner, H.-O. and Gilbert, D. (2010). Impacts of hypoxia on the structure and processes in pelagic communities (zooplankton, macro-invertebrates and fish). *Biogeosciences* **7**, 1669–1699.

Gollock, M. J., Currie, S., Petersen, L. H. and Gamperl, a K. (2006). Cardiovascular and haematological responses of Atlantic cod (Gadus morhua) to acute temperature increase. *The Journal of experimental biology* **209**, 2961–2970.

Gracey, A. Y., Troll, J. V and Somero, G. N. (2001). Hypoxia-induced gene expression profiling in the euryoxic fish Gillichthys mirabilis. *Proceedings of the National Academy*

Journal of Experimental Biology • Advance article

of Sciences **98**, 1993–1998.

Ho, D. H. and Burggren, W. W. (2012). Parental hypoxic exposure confers offspring hypoxia resistance in zebrafish (Danio rerio). *The Journal of experimental biology* **215**, 4208–4216.

Hopkins, S. . and Powell, F. . (2001). Common themes of adaptation to hypoxia: insights from comparative physiology. *Advances in Experimental Medicine and Biology* **502**, 153–167.

Ishibashi, Y., Kotaki, T., Yamada, Y. and Ohta, H. (2007). Ontogenic changes in tolerance to hypoxia and energy metabolism of larval and juvenile Japanese flounder Paralichthys olivaceus. *Journal of Experimental Marine Biology and Ecology* **352**, 42–49.

Jennings, S. and Pawson, M. G. (1992). The origin and recruitment of bass, Dicentrarchus labrax, larvae to nursery areas. *Journal of the Marine Biological Association of the United Kingdom* **72**, 199–212.

Kong, Y., Zhou, S., Kihm, a J., Katein, a M., Yu, X., Gell, D. a, Mackay, J. P., Adachi, K., Foster-Brown, L., Louden, C. S., et al. (2004). Loss of alpha hemoglobin stabilizing protein (AHSP) impairs erythropoiesis and exacerbates b thalassemia. *J. Clin. Invest.* **114**, 1457–1466.

Lai, J. C. C., Kakuta, I., Mok, H. O. L., Rummer, J. L. and Randall, D. (2006). Effects of moderate and substantial hypoxia on erythropoietin levels in rainbow trout kidney and spleen. 2734–2738.

Levin, L. a., Ekau, W., Gooday, a. J., Jorissen, F., Middelburg, J. J., Naqvi, S. W. a., Neira, C., Rabalais, N. N. and Zhang, J. (2009). Effects of natural and human-induced hypoxia on coastal benthos. *Biogeosciences* **6**, 2063–2098.

McDonald, F. B., Dempsey, E. M. and O'Halloran, K. D. (2016). Early Life Exposure to Chronic Intermittent Hypoxia Primes Increased Susceptibility to Hypoxia-Induced Weakness in Rat Sternohyoid Muscle during Adulthood. *Frontiers in physiology* **7**, 69.

Nikinmaa, M. (2001). Haemoglobin function in vertebrates : evolutionary changes in cellular regulation in hypoxia. *Respiration physiology* **128**, 317–329.

Nikinmaa, M. and Rees, B. B. (2005). Oxygen-dependent gene expression in fishes.

Journal of Experimental Biology • Advance article

American journal of physiology. Regulatory, integrative and comparative physiology **288**, 1079–1090.

Nilsson, G. E. (2007). Gill remodeling in fish a new fashion or an ancient secret? *The Journal of experimental biology* **210**, 2403–2409.

Opazo, J. C., Butts, G. T., Nery, M. F., Storz, J. F. and Hoffmann, F. G. (2012). Whole-Genome Duplication and the Functional Diversification of Teleost Fish Hemoglobins. 1–14.

Pichavant, K., Bayon, N. Le, Severe, A., Roux, A. Le and Boeuf, G. (2001). Comparative effects of hypoxia on growth and metabolism of turbot and European sea bass. *Journal of Fish Biology* **59**, 875–883.

Pichavant, K., Maxime, V., Soulier, P., Boeuf, G. and Nonnotte, G. (2003). A comparative study of blood oxygen transport in turbot and sea bass: effect of chronic hypoxia. *Journal of Fish Biology* **62**, 928–937.

Pihl, L., Baden, S. P., Diaz, R. J. and Schaffner, L. C. (1992). Hypoxia-induced structural changes in the diet of bottom-feeding fish and crustacea. *Marine Biology* **361**, 349–361.

Richards, J. G. (2011). Physiological, behavioral and biochemical adaptations of intertidal fishes to hypoxia. *The Journal of experimental biology* **214**, 191–199.

Robertson, C. E., Wright, P. A., Ko, L. and Bernier, N. J. (2014). Hypoxia-inducible factor-1 mediates adaptive developmental plasticity of hypoxia tolerance in zebrafish , Danio rerio. *Proceedings of the royal society B* **281**, pii: 20140637.

Roesner, A., Hankeln, T. and Burmester, T. (2006). Hypoxia induces a complex response of globin expression in zebrafish (Danio rerio). 2129–2137.

Roesner, A., Mitz, S. A., Hankeln, T. and Burmester, T. (2008). Globins and hypoxia adaptation in the goldfish, Carassius auratus. *The FEBS journal* **275**, 3633–3643.

Roessig, J. M., Woodley, C. M., Cech, J. J. and Hansen, L. J. (2004). Effects of global climate change on marine and estuarine fishes and fisheries. *Reviews in Fish Biology and Fisheries* **14**, 251–275.

Somero, G. N. (2012). The physiology of global change: linking patterns to mechanisms. *Annual review of marine science* **4**, 39–61.

Journal of Experimental Biology • Advance article

Val, A. L., Gomes, K. R. M. and de Almeida-Val, V. M. F. (2015). Rapid regulation of blood parameters under acute hypoxia in the Amazonian fish Prochilodus nigricans. *Comparative Biochemistry and Physiology -Part A : Molecular and Integrative Physiology* **184**, 125–131.

van der Meer, D. L. M., van den Thillart, G. E. E. J. M., Witte, F., de Bakker, M. a G., Besser, J., Richardson, M. K., Spaink, H. P., Leito, J. T. D. and Bagowski, C. P. (2005). Gene expression profiling of the long-term adaptive response to hypoxia in the gills of adult zebrafish. *American journal of physiology. Regulatory, integrative and comparative physiology* **289**, 1512–1519.

Vanderplancke, G., Claireaux, G., Quazuguel, P., Huelvan, C., Corporeau, C., Mazurais, D. and Zambonino-Infante, J.-L. (2015). Exposure to chronic moderate hypoxia impacts physiological and developmental traits of European sea bass (Dicentrarchus labrax) larvae. *Fish physiology and biochemistry* **41**, 233–242.

Wawrowski, A., Gerlach, F., Hankeln, T. and Burmester, T. (2011). Changes of globin expression in the Japanese medaka (Oryzias latipes) in response to acute and chronic hypoxia. *Journal of comparative physiology. B, Biochemical, systemic, and environmental physiology* **181**, 199–208.

Weber, R. E. and Jensen, F. B. (1988). Functional adaptations in hemoglobins from ectothermic vertebrates. *Annual Review of Physiology* **50**, 161–179.

Zambonino, J. L., Calm, C. L., Peres, A., Quazuguel, P. and Gall, M. M. Le (1996). Sea bass (Dicentrarchus lab-ax) larvae fed different Artemia rations : growth , pancreas enzymatic response and development of digestive functions. *Aquaculture* **139**, 129–138.

Zhu, C.-D., Wang, Z.-H. and Yan, B. (2013). Strategies for hypoxia adaptation in fish species: a review. *Journal of comparative physiology. B, Biochemical, systemic, and environmental physiology* **183**, 1005–1013.

Zi-sheng, W., Zhi-tao, Q. I., Jing-yun, T., Ming, Q. I. U., Wei-hong, Z., Ai-min, W., Jin-tian, H. and Xi-jie, G. U. O. (2011). Cloning of hemoglobin- α 1 from half-smooth tongue sole (Cynoglossus semilaevis) and its expression under short-term hy poxia. *Zoological research* **32**, 641–646.

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Tables

Table 1. Specific primers used for real-time amplification of Hb, PHD-3 and reference (28S and EF1α) genes.

Gene	Accession number on NCBI (*) or unipd (#)	Forward primer sequences (5'-3')	Reverse primer sequences (5'-3')
LA-*Hbα1*	KX196178 (*)	CAGTGGGACAGGATCTTGAAGT	GGTGATGGGTGGAATCAATC
LA-*Hbα2*	KX196180 (*)	TTTCCCATGAGAGAGCAGGT	TCAGATGCGCTTCTTAGGATGT
MN-*Hbα1*	KX196190 (*)	GGCCAGGATGCTGACTGTA	CCAGCAAGGTCATCCATCTT
MN-*Hbα2*	KX196188 (*)	CCTGCCAACTTCAAGATTCTG	TTTCTCAGACAAGGCACGAG
MN-*Hbα3*	KX196184 (*)	ACAGACAAGATGACCAGTCTCACT	GCCAATGTCCTCTGCCTTC
MN-*Hbα4*	KX196183 (*)	ACAGACAAGATGACCAGTCTCACT	GCCAATGTCCTCTGCCTTT
MN-*Hbα5*	KX196181 (*)	ACAGACAAGATGACCAGTCTCACA	GCCAATGTCCTCTGCCTTC
LA-*Hbβ1*	KX196179 (*)	CCCGACAACTTCAAACTGCT	CCTGCGTCTCTGGTGTGAAG
MN-*Hbβ1*	KX196191 (*)	TGATTTGAGCAAAGATCCTGAA	CATGGACGACATCAAGAACG
MN-*Hbβ2*	KX196189 (*)	GTCAGCCAGCAGCCTGAAAT	GCAGCTCTTTCCAGGTGTCT
MN-*Hbβ3*	KX196187 (*)	CAGAAGCTTTGGCAAGAGTG	GCTGCTACTTTGGCGTTACC
MN-*Hbβ4*	KX196186 (*)	GTCGTTTACCCCTGGACTCA	GTTTTGCGACCATCGGATTT
MN-*Hbβ5*	KX196185 (*)	ACCATCCAGGACATCTTCTCT	GTTTTGCGACCAACGGATTC
MN-*Hbβ6*	KX196182 (*)	ACCATCCAGGACATTTTCTCC	GTTTTGCGACCAACGGATTC
PHD-3	DLPD06823 (#)	TCCTACTCCACCAGGTACGC	GCAGTCATGTTTGCTCTCCA
28S	AH011863.2 (*)	CAAGAACATCCAGCTGCTGAC	GGTGATATGTCGGCCATAAA
Ef1α	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						
				CJ		HJ		p-value		
	CL	HL	p-value	CL	HL	CL	HL	larval oxygenation	juvenile oxygenation	larval oxygenation x juvenile oxygenation
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.

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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).

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Figures

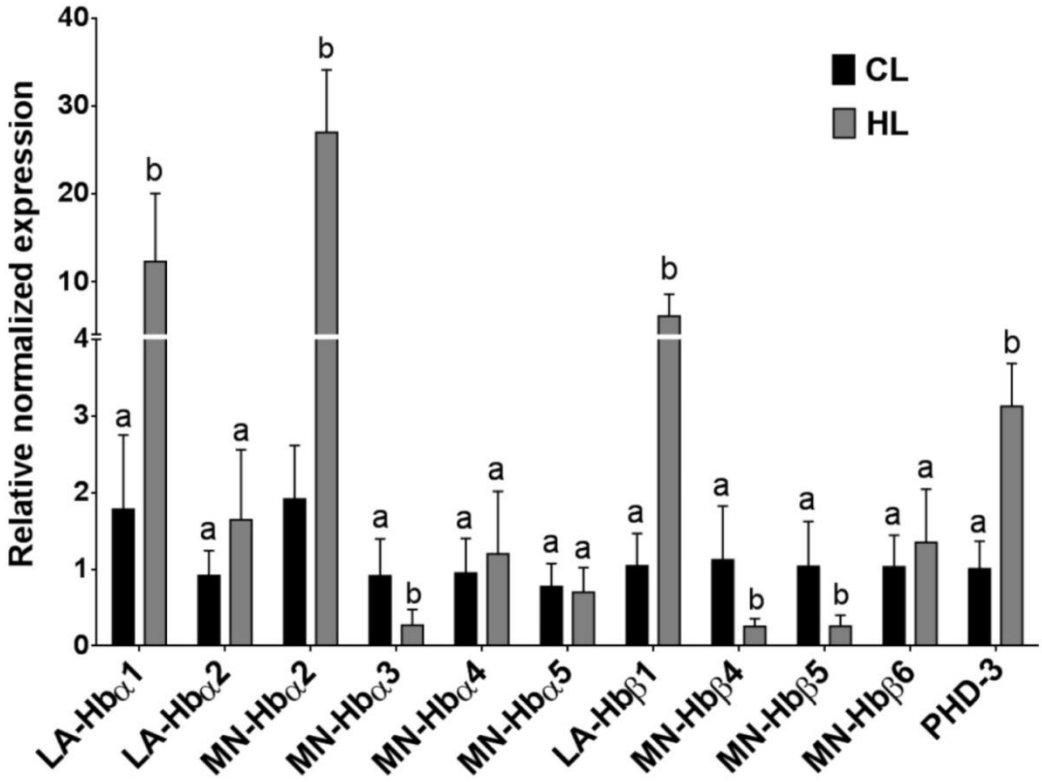

Fig.1. Expression pattern of Hb genes (LA-Hbα1-2; LA-Hbβ1; MN-Hbα2-5 and MN-Hbβ4-6) and PHD-3 gene under normoxia (CL) and moderate hypoxia (HL, 40 % air saturation) in the whole larvae (50dph). The Y-axis represents the relative expression normalized by expression levels of Ef1-α. Relative expression levels are expressed relative to normoxia group (×1). Significant differences for each gene between groups are identified with different letters (one-way ANOVA followed by Tukey's test).

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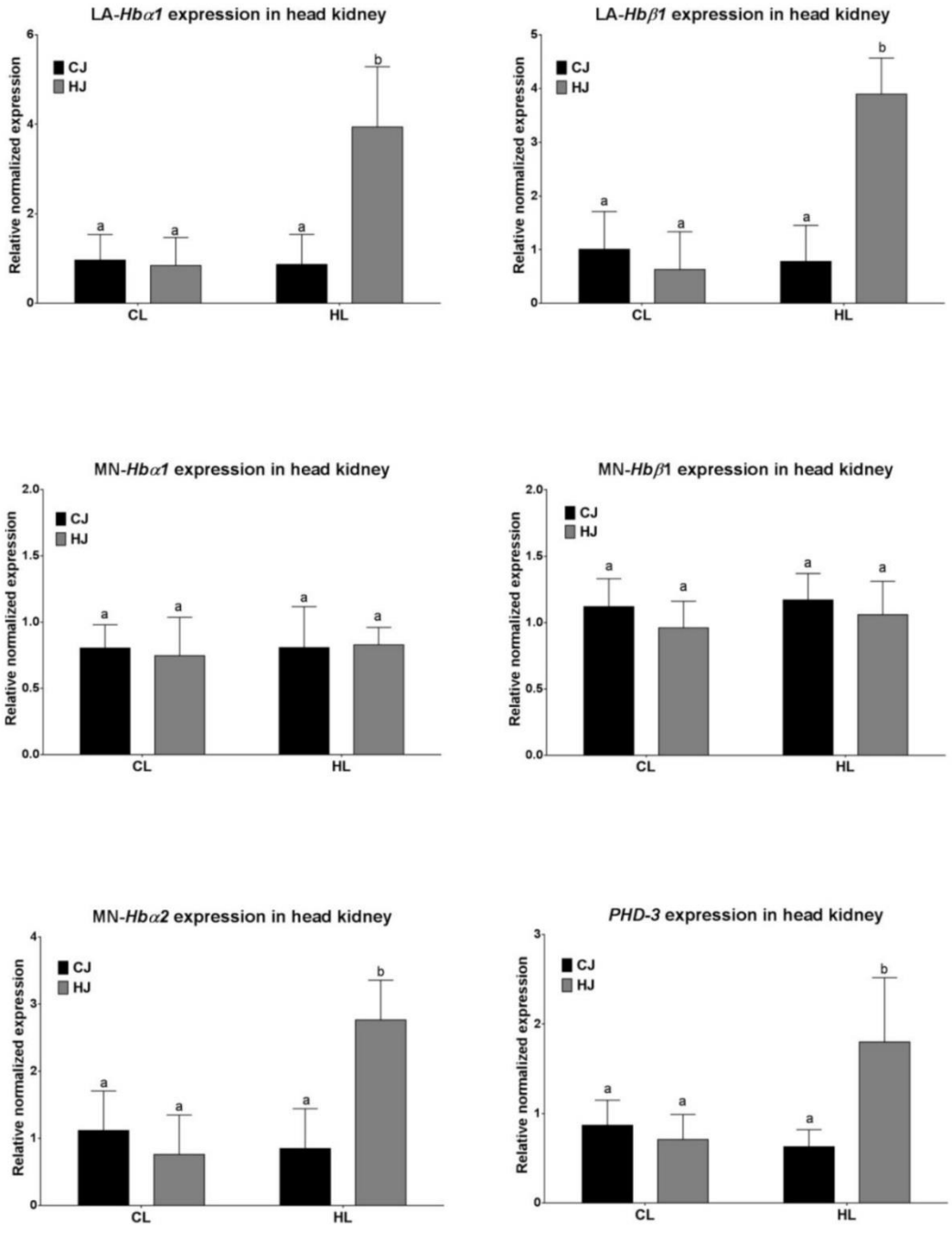

Fig.2. Expression pattern of Hb genes (LA-Hbα1, LA-Hbβ1, MN-Hbα1, MN-Hbα2 and MN-Hbβ1) and the PHD-3 gene under normoxia (CJ) and moderate hypoxia (HJ, 40 % air saturation) in the head kidney of juveniles previously exposed (HL) or not (CL) to larval hypoxia. *The Y-axis represents the relative expression normalized by expression levels of 28S.*

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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).

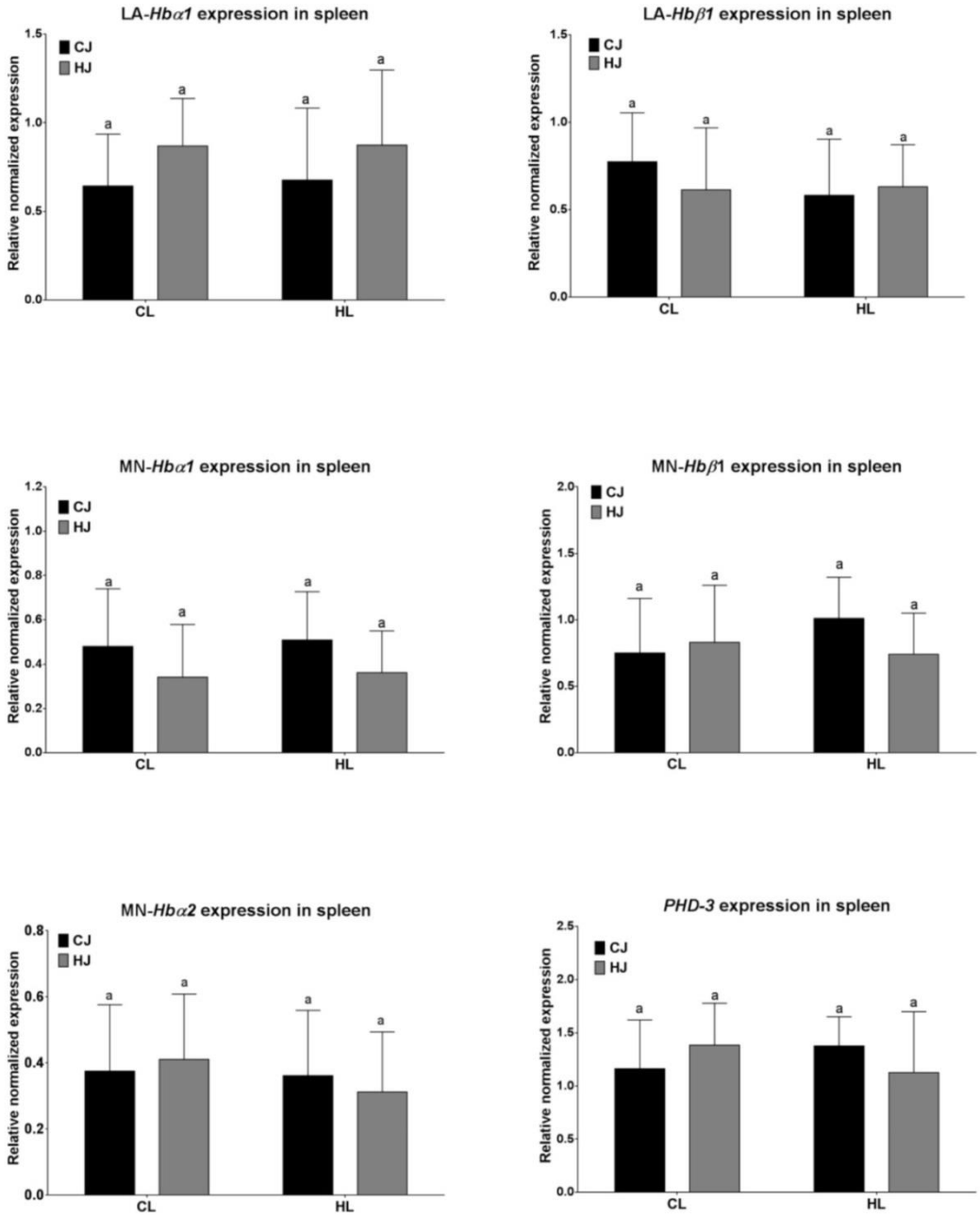

Fig.3. Expression patterns of Hb genes (LA-Hbα1, LA-Hbβ1, MN-Hbα1, MN-Hbα2 and MN-Hbβ1) and the PHD-3 gene under normoxia (CJ) and moderate hypoxia (HJ, 40 % air saturation) in the spleen of juveniles previously exposed (HL) or not (CL) to larval hypoxia. *The Y-axis represents relative expression normalized by expression levels of 28S. Relative expression levels are expressed relative to the juvenile control group in normoxia (×1).*

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

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

		CL		HL			
	Genes	**Relative expression**	**Cq**	**Relative expression**	**Cq**	**p-value**	**Fold**
Hb	**LA-*Hbα1***	1.79	30	12.34	28	**$2x10^{-5}$**	**+7**
	LA-*Hbα2*	0.92	27	1.65	27	0.07	+2
	MN-*Hbα1*	-	33	-	33	-	-
	MN-*Hbα2*	1.92	28	27.04	24	**$1x10^{-6}$**	**+14**
	MN-*Hbα3*	0.92	21	0.27	24	**$4x10^{-4}$**	**-3**
	MN-*Hbα4*	0.95	20	1.20	20	0.44	+1
	MN-*Hbα5*	0.78	21	0.70	22	0.61	+1
	LA-*Hbβ1*	1.05	23	6.12	21	**$1x10^{-5}$**	**+6**
	MN-*Hbβ1*	-	33	-	33	-	-
	MN-*Hbβ2*	-	33	-	32	-	-
	MN-*Hbβ3*	-	35	-	35	-	-
	MN-*Hbβ4*	1.13	22	0.25	24	**$1x10^{-4}$**	**-5**
	MN-*Hbβ5*	1.04	21	0.26	23	**$1x10^{-4}$**	**-4**
	MN-*Hbβ6*	1.03	19	1.36	19	0.24	+1
HIF pathway	***PHD-3***	1.01	31	3.13	30	**$2x10^{-5}$**	**+3**

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.

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Table S2. Relative expression levels of selected Hb genes (LA-Hbα1, LA-Hbβ1, MN-Hbα1, MN-Hbβ1 and MN-Hbα2) 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.

		CJ head kidney				HJ head kidney				p-value		
	Genes	CL Relative expression	Cq	HL Relative expression	Cq	CL Relative expression	Cq	HL Relative expression	Cq	larval ox.	juvenile ox.	larval ox. x juvenile ox.
Hb	LA-*Hbα1*	0.97	22	0.87	22	0.84	22	3.94	20	**0.02**	**0.02**	**$1x10^{-3}$**
	LA-*Hbβ1*	1.01	20	0.78	20	0.63	20	3.90	18	**0 .01**	**0.02**	**$5x10^{-4}$**
	MN-*Hbα1*	0.80	11	0.81	11	0.75	11	0.83	11	0.58	0.84	0.43
	MN-*Hbβ1*	1.11	11	1.17	11	0.96	11	1.06	11	0.37	0.12	0.76
	MN-*Hbα2*	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.

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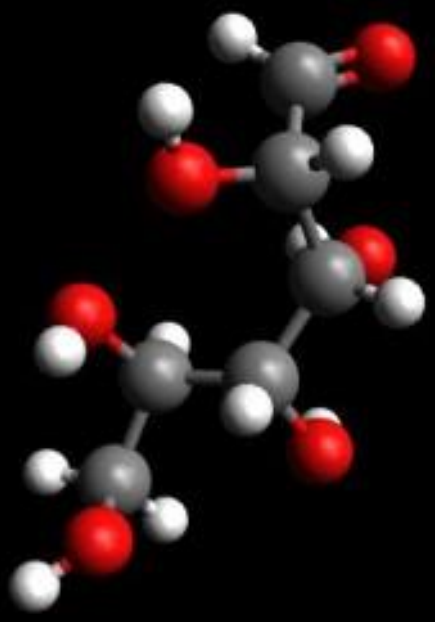
Table S3. Relative expression levels of selected Hb genes (LA-Hbα1, LA-Hbβ1, MN-Hbα1, MN-Hbβ1 and MN-Hbα2) and the PHD-3 gene under normoxia (CJ) and moderate hypoxia (HJ, 40 % air saturation) in the spleen of juveniles previously exposed (HL) or not (CL) to larval hypoxia.

		CJ spleen				HJ spleen				p-value		
		CL		HL		CL		HL				
	Genes	Relative expression	Cq	Relative expression	Cq	Relative expression	Cq	Relative expression	Cq	larval ox.	juvenile ox.	larval ox. x juvenile ox.
Hb	LA-*Hbα1*	0.64	19	0.68	19	0.87	18	0.87	18	0.93	0.78	0.95
	LA-*Hbβ1*	0.77	17	0.58	18	0.61	18	0.63	18	0.66	0.75	0.92
	MN-*Hbα1*	0.48	13	0.51	12	0.34	13	0.36	13	0.64	0.40	0.43
	MN-*Hbβ1*	0.75	13	1.01	12	0.83	13	0.75	13	0.60	0.56	0.32
	MN-*Hbα2*	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 O_2 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 O_2 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 O_2 demands, while hypoxia limits O_2 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; *Ef1α*, 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 mg O_2 L^{-1}): hypoxia larval group (HL); and 100% saturation (7.35 mg O_2 L^{-1}): control larval group (CL)] and temperature (15 °C and 20 °C). Hypoxic conditions were created 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 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 m^3-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-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 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 ($RGR = \frac{(final\ weight - initial\ weight)}{initial\ weight}$) between 196 and 296 dph.

2.3. Sampling

Sampling of immature juveniles was performed at 296 dph, after

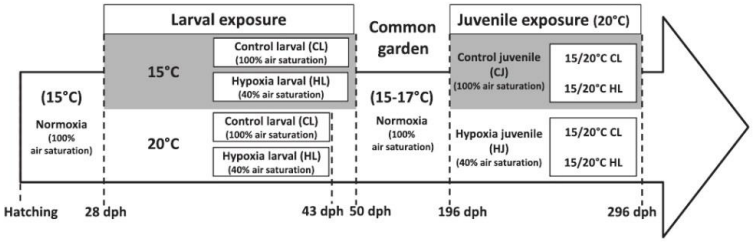

Fig. 1. Schema of the experimental protocol. Sea bass larvae were exposed to each combination of oxygenation level [40% air saturation: hypoxia larval group (HL); and 100% saturation: control larval group (CL)] and temperature (15 °C and 20 °C) from 28 to 43 dph (20 °C) or 50 dph (15 °C). Following the 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 $HSI = \frac{liver\ weight}{fish\ weight}$. 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 K_2CO_3 (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 10000*g*, 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 10000*g* 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; 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 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 ef1α*) available from the NCBI (https://www.ncbi.nlm.nih.gov/) and the Sigenae website (http://www.sigenae.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 $R^2 > 0.99$. 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. Ef1α was used as housekeeping gene. Accession numbers of cDNA sequences (*trypsin*, *glut2*, *pk*, *atgl*, *phd3* and *ef1α*) 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′)
trypsin	AJ006882.1 (*)	CTCCACTGCTGACAGGAACA	CATGCCAGGGTAGGAGTTGT
glut2	EF014277.2 (*)	CAGCTGATCGTTGCACTCAT	TGTGGCATAGACTGGCTGAG
gp	CBXY010002083.1 (#)	TTCCCAGACAAAGTCGCTGT	GTCTTGTGAGGTCCCATGC
gs	CBXY010017321.1 (#)	GACAAGGAGGCAGGTGAGAG	GAAGACGTGAGCACAGTGGA
pk	KF857578.1 (*)	CAAGGTGGAAAGCCGGCAAGGC	GGTCACCCCTGGCAACCATCA
pepck	CBXY010006528.1 (#)	TCAGGTCGGACCATGTATGT	GTCACCTGGACGCCATATTT
atgl	KF857294.1 (*)	GCTCCTTTCCTGGTGCATAA	ATTCGCACCAGTCTCTCCAA
slc27	CBXY010013343.1 (#)	GCTTCCCAATTCTGGAATGA	TGTCTCTCTTTGGTGCGTTG
dgat1	CBXY010017042.1 (#)	CCACAGAGGGGCATGAGTAT	CCATGACCCCAGGAGTCTAC
phd3	DLPD06823 (**)	TCCTACTCCACCAGGTACGC	GCAGTCATGTTTGCTCTCCA
ef1α	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, $MgCl_2$, 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 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* (*ef1α*) 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°				
	CL	HL	CL	HL	CL	HL	CL	HL	L. Ox	L. T.	J. Ox.
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	$\mathbf{1 \times 10^{-6}}$	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	$\mathbf{1 \times 10^{-6}}$

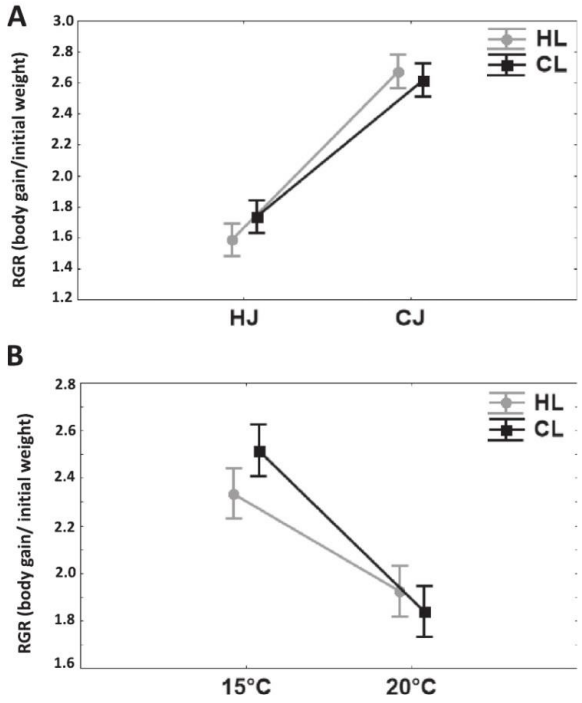

Fig. 2. A–B. Impact of juvenile and larval conditions on juvenile relative growth rates. A. Graph representing significant interaction (see Table 3) between hypoxia exposures at juvenile and larval stages on juvenile relative growth rates. Juveniles reared under moderate hypoxia (HJ, $n = 120$) or normoxia (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 ($p = 2 \times 10^{-3}$) 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

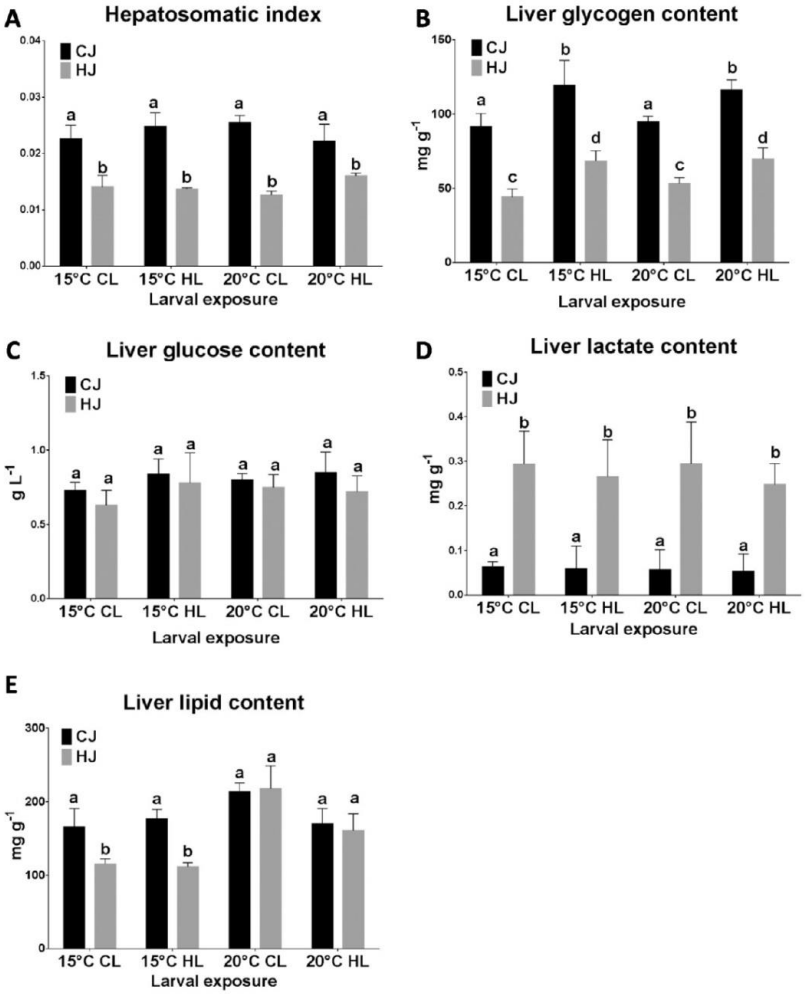

Fig. 3. Hepatosomatic index (HSI) (A) and hepatic metabolites: glycogen (B), glucose (C), lactate (D) and lipids (E) in European sea bass juveniles. Juveniles were either exposed (HJ) or not (CJ) to hypoxia and were previously exposed (HL) or not (CL) to hypoxia at 15 °C or 20 °C during the larval stage (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 (Mommsen 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).

			CJ				HJ				p-Value		
			15°		20°		15°		20°				
			CL	HL	CL	HL	CL	HL	CL	HL	L. Ox	L.T.	J. Ox.
Specific activity U mg^{-1}		*GP*	0.097 ± 0.009	0.092 ± 0.007	0.087 ± 0.005	0.092 ± 0.002	0.154 ± 0.01	0.153 ± 0.003	0.152 ± 0.02	0.151 ± 0.01	NS	NS	1×10^{-6}
		Pancreatic lipase	0.021 ± 0.004	0.021 ± 0.002	0.022 ± 0.004	0.022 ± 0.004	0.023 ± 0.005	0.024 ± 0.003	0.023 ± 0.006	0.023 ± 0.006	NS	NS	NS
Relative expression	Digestion	*Trypsin*	2.3 ± 0.4	1.56 ± 0.7	1.6 ± 0.6	1.03 ± 0.4	1.26 ± 0.3	0.62 ± 0.6	0.15 ± 0.1	0.46 ± 0.5	NS	8×10^{-3}	7×10^{-6}
	Glucose metabolism	*glut2*	1.81 ± 0.9	1.41 ± 0.3	1.64 ± 0.5	1.58 ± 0.6	1.73 ± 1	1.78 ± 1.1	1.64 ± 0.7	1.36 ± 0.5	NS	NS	NS
		gp	1.62 ± 0.3	1.49 ± 0.3	1.62 ± 0.2	1.26 ± 0.3	1.10 ± 0.5	1.34 ± 0.6	1.29 ± 0.3	1.57 ± 0.5	NS	NS	NS
		gs	0.80 ± 0.1	0.70 ± 0.1	0.65 ± 0.1	0.54 ± 0.1	1.17 ± 0.1	1.17 ± 0.4	1.12 ± 0.3	1.08 ± 0.2	NS	NS	1×10^{-6}
		pk	1.94 ± 0.6	1.58 ± 0.3	1.91 ± 0.4	1.59 ± 0.3	1.72 ± 0.7	1.77 ± 0.8	1.62 ± 0.1	1.72 ± 0.8	NS	NS	NS
		pepck	0.57 ± 0.3	0.56 ± 0.1	0.58 ± 0.2	0.57 ± 0.1	1.22 ± 0.2	1.34 ± 0.3	1.32 ± 0.2	0.86 ± 0.3	NS	NS	1×10^{-6}
		atgl	1.42 ± 0.6	1.23 ± 0.2	1.31 ± 0.3	1.48 ± 0.4	1.27 ± 0.5	1.71 ± 0.5	1.53 ± 0.1	1.52 ± 0.4	NS	NS	NS
		slc27	0.77 ± 0.3	1.42 ± 0.6	1.39 ± 0.4	1.25 ± 0.8	1.81 ± 0.7	1.72 ± 0.3	2.18 ± 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	1.26 ± 0.3	1.47 ± 0.8	1.56 ± 0.2	1.39 ± 0.2	NS	NS	NS
	HIF pathway	*phd3*	0.54 ± 0.2	0.48 ± 0.2	0.57 ± 0.3	0.61 ± 0.2	0.81 ± 0.2	1.34 ± 0.3	0.95 ± 0.2	1.38 ± 0.3	NS	NS	2×10^{-3}

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 O_2 deprivation episodes during the juvenile period.

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References

Ali, M., Nicieza, A., Wootton, R.J., 2003. Compensatory growth in fishes: a response to growth depression. Fish Fish. 4, 147–190. http://dx.doi.org/10.1046/j.1467-2979.2003.00120.x.

Anttila, K., Lewis, M., Prokkola, J.M., Kanerva, M., Seppänen, E., Kolari, I., Nikinmaa, M., 2015. Warm acclimation and oxygen depletion induce species-specific responses in salmonids. J. Exp. Biol. 218, 1471–1477. http://dx.doi.org/10.1242/jeb.119115.

Bateson, P., Gluckman, P., Hanson, M., 2014. The biology of developmental plasticity and the predictive adaptive response hypothesis. J. Physiol. 592, 2357–2368. http://dx.doi.org/10.1113/jphysiol.2014.271460.

Bickler, P.E., Buck, L.T., 2007. Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. Annu. Rev. Physiol. 69, 145–170. http://dx.doi.org/10.1146/annurev.physiol.69.031905.162529.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. http://dx.doi.org/10.1016/0003-2697(76)90527-3.

Carr, R.S., Neff, J.M., 1984. Quantitative semi-automated enzymatic assay for tissue glycogen. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 77, 447–449. http://dx.doi.org/10.1016/0305-0491(84)90258-X.

Chippari-Gomes, A.R., Gomes, L.C., Lopes, N.P., Val, A.L., Almeida-Val, V.M.F., 2005. Metabolic adjustments in two Amazonian cichlids exposed to hypoxia and anoxia. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 141, 347–355. http://dx.doi.org/10.1016/j.cbpc.2005.04.006.

Diaz, R.J., 2001. Overview of hypoxia around the world. J. Environ. Qual. 30, 275–281. http://dx.doi.org/10.2134/jeq2001.302275x.

Diaz, R.J., Rosenberg, R., 2008. Spreading dead zones and consequences for marine ecosystems. Science 321, 926–929. http://dx.doi.org/10.1126/science.1156401.

Díaz, R.J., Rosenberg, R., 2011. Introduction to environmental and economic consequences of hypoxia. Int. J. Water Resour. Dev. 27, 71–82. http://dx.doi.org/10.1080/07900627.2010.531379.

Dufour, V., Cantou, M., Lecomte, F., 2009. Identification of sea bass (*Dicentrarchus labrax*) nursery areas in the north-western Mediterranean Sea. J. Mar. Biol. Assoc. U. K. 89, 1367–1374. http://dx.doi.org/10.1017/S0025315409000368.

Fernandez-Twinn, D.S., Ozanne, S.E., 2010. Early life nutrition and metabolic programming. Ann. N. Y. Acad. Sci. 1212, 78–96. http://dx.doi.org/10.1111/j.1749-6632.2010.05798.x.

Ficke, A.D., Myrick, C.A., Hansen, L.J., 2007. Potential impacts of global climate change on freshwater fisheries. Rev. Fish Biol. Fish. 17, 581–613. http://dx.doi.org/10.1007/s11160-007-9059-5.

Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509.

Fuiman, L.A., Perez, K.O., 2015. Metabolic programming mediated by an essential fatty acid alters body composition and survival skills of a marine fish. Proc. R. Soc. B Biol. Sci. 282. http://dx.doi.org/10.1098/rspb.2015.1414.

Geurden, I., Aramendi, M., Zambonino-Infante, J., Panserat, S., 2007. Early feeding of carnivorous rainbow trout (*Oncorhynchus mykiss*) with a hyperglucidic diet during a short period: effect on dietary glucose utilization in juveniles. Am. J. Phys. Regul. Integr. Comp. Phys. 292, 2275–2283. http://dx.doi.org/10.1152/ajpregu.00444.2006.

Gillanders, B.M., Elsdon, T.S., Halliday, I.A., Jenkins, G.P., Robins, J.B., Valesini, F.J., 2011. Potential effects of climate change on Australian estuaries and fish utilising estuaries: a review. Mar. Freshw. Res. 62, 1115–1131. http://dx.doi.org/10.1071/MF11047.

Gracey, A.Y., Troll, J.V., Somero, G.N., 2001. Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. Proc. Natl. Acad. Sci. 98, 1993–1998. http://dx.doi.org/10.1073/pnas.98.4.1993.

Gracey, A.Y., Lee, T.-H., Higashi, R.M., Fan, T., 2011. Hypoxia-induced mobilization of stored triglycerides in the euryoxic goby *Gillichthys mirabilis*. J. Exp. Biol. 214, 3005–3012. http://dx.doi.org/10.1242/jeb.059907.

Green, B.W., Rawles, S.D., Fuller, S.A., Beck, B.H., Mcentire, M.E., 2015. Hypoxia affects performance traits and body composition of juvenile hybrid striped bass (*Morone chrysops* x *M. saxatilis*). Aquac. Res. 47, 2266–2275. http://dx.doi.org/10.1111/are.12678.

Hanley, B., Dijane, J., Fewtrell, M., Grynberg, A., Hummel, S., Junien, C., Koletzko, B., Lewis, S., Renz, H., Symonds, M., Gros, M., Harthoorn, L., Mace, K., Samuels, F., van Der Beek, E., 2010. A review of metabolic programming, imprinting and epigenetics. Br. J. Nutr. 104, S1–25. http://dx.doi.org/10.1017/S0007114510003338.

Iijima, N., Tanaka, S., Ota, Y., 1998. Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish Physiol. Biochem. 18, 59–69. http://dx.doi.org/10.1023/A:1007725513389.

Jennings, S., Pawson, M.G., 1992. The origin and recruitment of bass, *Dicentrarchus labrax*, larvae to nursery areas. J. Mar. Biol. Assoc. U. K. 72, 199–212. http://dx.doi.org/10.1017/S0025315400048888.

Laiz-carrión, R., Martín Del Río, M.P., Miguez, J.M., Mancera, J.M., Soengas, J.L., 2003. Influence of cortisol on osmoregulation and energy metabolism in gilthead seabream *Sparus aurata*. J. Exp. Zool. A Comp. Exp. Biol. 298A, 105–118. http://dx.doi.org/10.1002/jez.a.10256.

Levin, L.a., Ekau, W., Gooday, a.J., Jorissen, F., Middelburg, J.J., Naqvi, S.W.a., Neira, C., Rabalais, N.N., Zhang, J., 2009. Effects of natural and human-induced hypoxia on coastal benthos. Biogeosciences 6, 2063–2098. http://dx.doi.org/10.5194/bg-6-2063-2009.

Lewis, J.M., Costa, I., Val, A.L., Almeida-Val, V.M.F., Gamperl, A.K., Driedzic, W.R., 2007. Responses to hypoxia and recovery: repayment of oxygen debt is not associated with compensatory protein synthesis in the Amazonian cichlid, *Astronotus ocellatus*. J. Exp. Biol. 210, 1935–1943. http://dx.doi.org/10.1242/jeb.005371.

Liu, J., Plagnes-Juan, E., Geurden, I., Panserat, S., Marandel, L., 2017. Exposure to an acute hypoxic stimulus during early life affects the expression of glucose metabolism-related genes at first-feeding in trout. Sci Rep 7, 363. http://dx.doi.org/10.1038/s41598-017-00458-4.

Mandic, M., Speers-Roesch, B., Richards, J.G., 2013. Hypoxia tolerance in sculpins is associated with high anaerobic enzyme activity in brain but not in liver or muscle. Physiol. Biochem. Zool. 86, 92–105. http://dx.doi.org/10.1086/667938.

Martínez, M.L., Landry, C., Boehm, R., Manning, S., Cheek, A.O., Rees, B.B., 2006. Effects of long-term hypoxia on enzymes of carbohydrate metabolism in the Gulf killifish, *Fundulus grandis*. J. Exp. Biol. 209, 3851–3861. http://dx.doi.org/10.1242/jeb.02437.

McBryan, T.L., Anttila, K., Healy, T.M., Schulte, P.M., 2013. Responses to temperature and hypoxia as interacting stressors in fish: implications for adaptation to environmental change. Integr. Comp. Biol. 53, 648–659. http://dx.doi.org/10.1093/icb/ict066.

McBryan, T.L., Healy, T.M., Haakons, K.L., Schulte, P.M., 2016. Warm acclimation improves hypoxia tolerance in *Fundulus heteroclitus*. J. Exp. Biol. 219, 474–484. http://dx.doi.org/10.1242/jeb.133413.

Mommsen, T.P., Danulat, E., Gavioli, M.E., Foster, G.D., Moon, T.W., 1991. Separation of enzymatically distinct populations of trout hepatocytes. Can. J. Zool. J. Canadien de Zoologie 69, 420–426. http://dx.doi.org/10.1139/z91-066.

Moyson, S., Jung, H., Diricx, M., Kumar, A., Blust, R., Boeck, G. De, 2015. The combined effect of hypoxia and nutritional status on metabolic and ionoregulatory responses of common carp (*Cyprinus carpio*). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 179, 133–143. http://dx.doi.org/10.1016/j.cbpa.2014.09.017.

Mustafa, S.A., Karieb, S.S., Davies, S.J., Jha, A.N., 2015. Assessment of oxidative damage to DNA, transcriptional expression of key genes, lipid peroxidation and histopathological changes in carp *Cyprinus carpio* L. following exposure to chronic hypoxic and subsequent recovery in normoxic conditions. Mutagenesis 30, 107–116. http://dx.doi.org/10.1093/mutage/geu048.

Nagelkerken, I., Munday, P.L., 2016. Animal behaviour shapes the ecological effects of ocean acidification and warming: moving from individual to community-level responses. Glob. Chang. Biol. 22, 974–989. http://dx.doi.org/10.1111/gcb.13167.

Patel, M.S., Srinivasan, M., 2010. Metabolic programming due to alterations in nutrition in the immediate postnatal period. J. Nutr. 140, 658–661. http://dx.doi.org/10.3945/jn.109.110155.

Pedersen, B.H., Ugelstad, I., Hjelmeland, K., 1990. Effects of a transitory, low food supply in the early life of larval herring (*Clupea harengus*) on mortality, growth and digestive capacity. Mar. Biol. 107, 61–66. http://dx.doi.org/10.1007/BF01313242.

Pichavant, K., Bayon, N. Le, Severe, A., Roux, A. Le, Boeuf, G., 2001. Comparative effects of hypoxia on growth and metabolism of turbot and European sea bass. J. Fish Biol. 59, 875–883. http://dx.doi.org/10.1111/j.1095-8649.2001.tb00158.x.

Polakof, S., Arjona, F.J., Sangiao-Alvarellos, S., Martin Del Rio, M.P., Mancera, J.M., Soengas, J.L., 2006. Food deprivation alters osmoregulatory and metabolic responses to salinity acclimation in gilthead sea bream *Sparus auratus*. J. Comp. Physiol. Biochem. Syst. Environ. Physiol. 176, 441–452. http://dx.doi.org/10.1007/s00360-006-0065-z.

Portner, H.O., Farrell, A.P., 2008. Ecology: physiology and climate change. Science 322, 690–692. http://dx.doi.org/10.1126/science.1163156.

Richards, J.G., 2009. Metabolic and molecular responses of fish to hypoxia. In: Jeffrey, G., APF, Richards, Colin, J.B. (Eds.), Fish Physiology, Vol 27. Academic Press, pp. 443–485 (Chapter 10).

Richards, J.G., 2011. Physiological, behavioral and biochemical adaptations of intertidal fishes to hypoxia. J. Exp. Biol. 214, 191–199. http://dx.doi.org/10.1242/jeb.047951.

Robertson, C.E., Wright, P.A., Köblitz, L., Bernier, N.J., B, P.R.S., Ko, L., 2014. Hypoxia-inducible factor-1 mediates adaptive developmental plasticity of hypoxia tolerance in zebrafish, *Danio rerio*. Proc. R. Soc. B Biol. Sci. 281 (1786), 20140637. http://dx.doi.org/10.1098/rspb.2014.0637.

Rungruangsak-Torrissen, K., Moss, R., Andresen, L.H., Berg, A., Waagbø, R., 2006. Different expressions of trypsin and chymotrypsin in relation to growth in Atlantic salmon (*Salmo salar L.*). Fish Physiol. Biochem. 32, 7–23. http://dx.doi.org/10.1007/s10695-005-0630-5.

Schaefer, J., Ryan, A., 2006. Developmental plasticity in the thermal tolerance of zebrafish *Danio rerio*. J. Fish Biol. 69, 722–734. http://dx.doi.org/10.1111/j.1095-8649.2006.01145.x.

Scott, G.R., Johnston, I.A., 2012. Temperature during embryonic development has persistent effects on thermal acclimation capacity in zebrafish. Proc. Natl. Acad. Sci. 109,

14247–14252. http://dx.doi.org/10.1073/pnas.1205012109.
Terblanche, J.S., Chown, S.L., 2006. The relative contributions of developmental plasticity and adult acclimation to physiological variation in the tsetse fly, *Glossina pallidipes* (Diptera, Glossinidae). J. Exp. Biol. 209, 1064–1073. http://dx.doi.org/10.1242/jeb.02129.
Terova, G., Rimoldi, S., Brambilla, F., Gornati, R., Bernardini, G., Saroglia, M., 2009. In vivo regulation of GLUT2 mRNA in sea bass (*Dicentrarchus labrax*) in response to acute and chronic hypoxia. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 152, 306–316. http://dx.doi.org/10.1016/j.cbpb.2008.12.011.
Thetmeyer, H., Waller, U., Black, K.D., 1999. Growth of European sea bass (*Dicentrarchus labrax L.*) under hypoxic and oscillating oxygen conditions. Aquaculture 174, 355–367. http://dx.doi.org/10.1016/S0044-8486(99)00028-9.
van den Thillart, G., Vianen, G., Zaagsma, J., 2002. Adrenergic regulation of lipid mobilization in fishes; a possible role in hypoxia survival. Fish Physiol. Biochem. 27, 189–204. http://dx.doi.org/10.1023/B:FISH.0000032754.42056.cb.
Vanderplancke, G., Claireaux, G., Quazuguel, P., Madec, L., Ferraresso, S., Sévère, A., Luis, J., Infante, Z., Mazurais, D., 2015. Hypoxic Episode During the Larval Period has Long-term Effects on European Sea Bass Juveniles (*Dicentrarchus labrax*). 162. pp. 367–376. http://dx.doi.org/10.1007/s00227-014-2601-9.
Wu, R.S., 2002. Hypoxia: from molecular responses to ecosystem responses. Mar. Pollut. Bull. 45, 35–45. http://dx.doi.org/10.1016/S0025-326X(02)00061-9.
Yang, Y., Cao, Z.D., Fu, S.J., 2015. Variations in temperature acclimation effects on glycogen storage, hypoxia tolerance and swimming performance with seasonal acclimatization in juvenile Chinese crucian carp. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 185, 16–23. http://dx.doi.org/10.1016/j.cbpa.2015.03.009.
Zambonino, J.L., Calm, C.L., Peres, A., Quazuguel, P., Gall, M.M. Le, 1996. Sea bass (*Dicentrarchus labrax*) larvae fed different Artemia rations: growth, pancreas enzymatic response and development of digestive functions. Aquaculture 139, 129–138. http://dx.doi.org/10.1016/0044-8486(95)01149-8.

Chapter 5

General Discussion

Credits: Patrick Quazuguel

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 O_2 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 O_2 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 O_2 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 O_2 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 O_2 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 O_2 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.

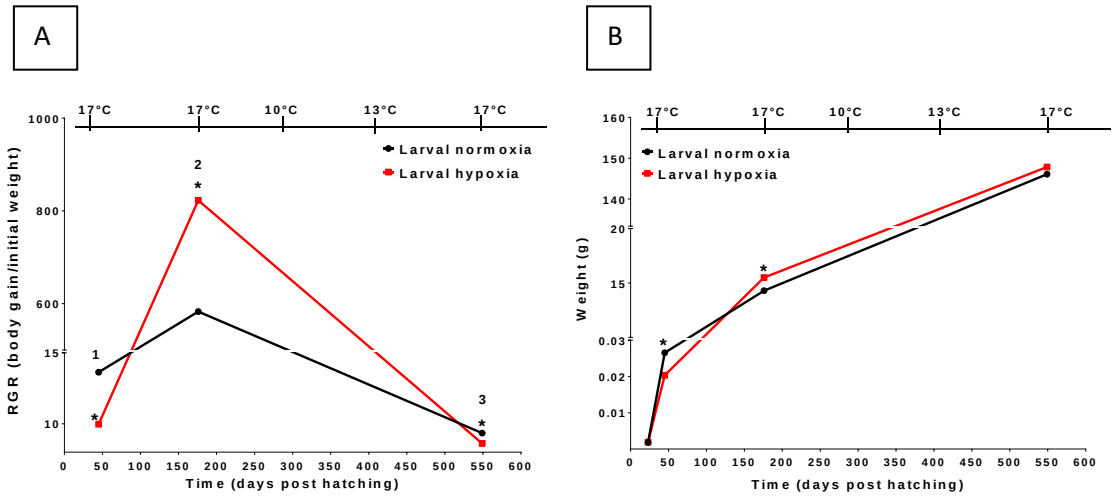

Figure 1. Relative growth rate (body gain/initial weight) (A) and weight (B) of juveniles early exposed or not to hypoxia at larval stage *(including fish early exposed to 15°C and 20°C at larval stage) (n for each group= 200 juveniles). Water temperature followed the natural thermal cycle (10 - 17°C). 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.

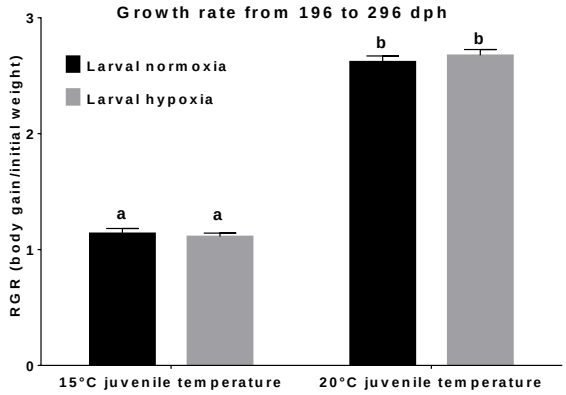

Figure 2. Relative growth rate (body gain/initial weight) from 196 to 296 dph of juveniles under normoxic conditions at 15°C or 20°C*. Juveniles were early exposed or not to hypoxia at larval stage (including fish early exposed to 15°C and 20°C at larval stage) (n for each group= 60 juveniles). Significant 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).

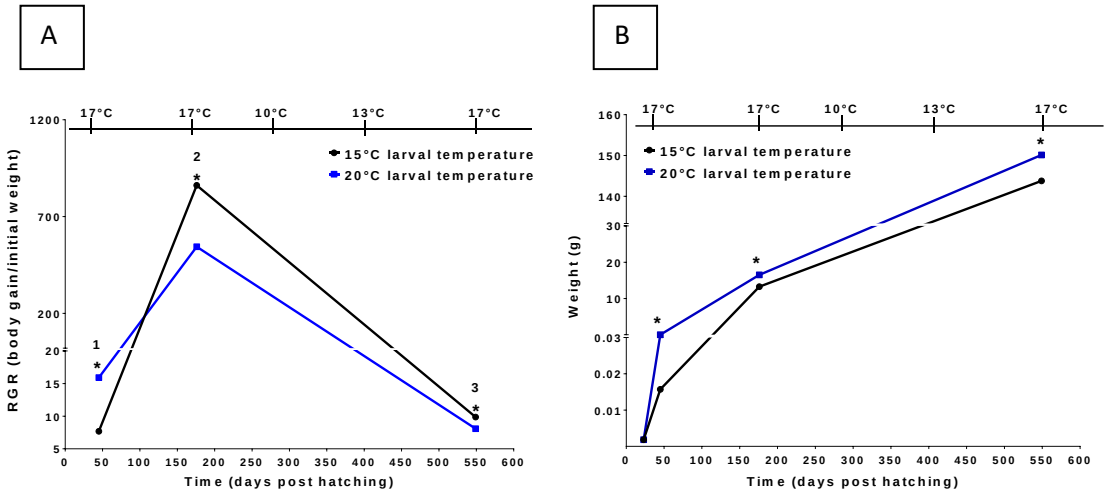

Figure 3. Relative growth rate (body gain/initial weight) (A) and weight (B) of juveniles early exposed to 15°C or 20°C at larval stage (including fish early exposed or not to hypoxia at larval stage) *(n for each group= 200 juveniles)*. Water temperature followed the natural thermal cycle (10 -17°C). *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 Haussmann 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 (Metcalfe 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 O_2 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.

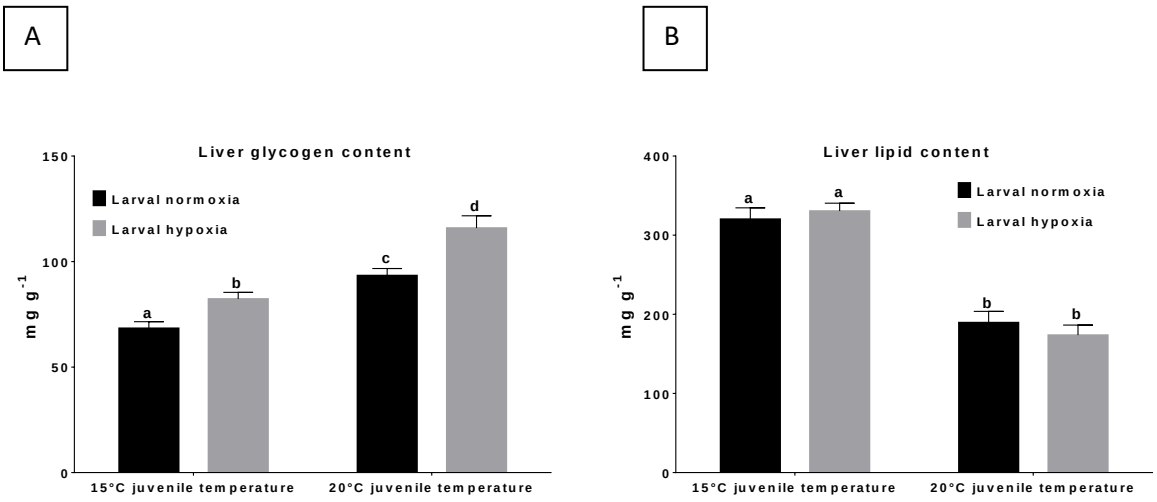

Figure 4. Hepatic metabolites (glycogen (A) and lipid (B)) at 296 dph of juveniles under normoxic conditions at 15°C or 20°C. *Juveniles were early exposed or not to hypoxia at larval stage (including fish early exposed to 15°C and 20°C at larval stage) (n for each group= 8 pools of 2 juveniles). Significant 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.

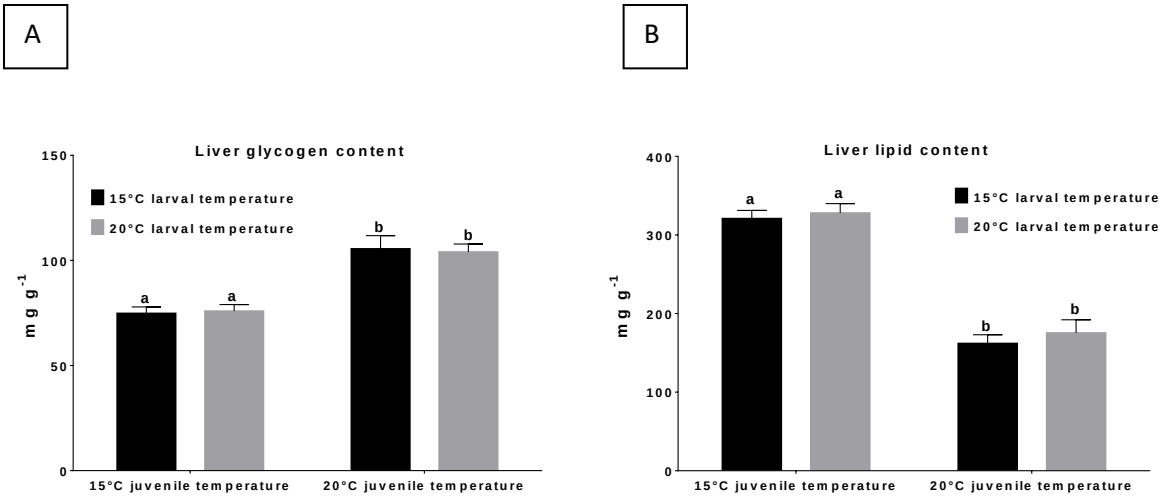

Figure 5. Hepatic metabolites (glycogen (A) and lipid (B)) at 296 dph of juveniles exposed under normoxic conditions at 15°C or 20°C. *Juveniles were early exposed to 15°C or 20°C at larval stage (including fish early exposed or not to hypoxia at larval stage) (n for each group= 8 pools of 2 juveniles). 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 O_2 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 O_2 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 O_2 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 O_2 demands, while hypoxia limits the availability of environmental O_2 (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 O_2 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 O_2 (induced by warming) and a limited O_2 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).

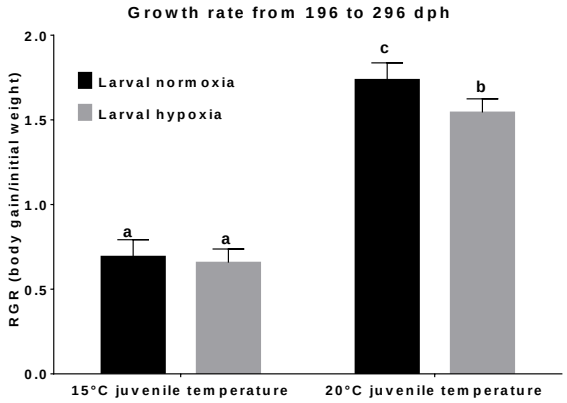

Figure 6. Relative growth rate (body gain/initial weight) from 196 to 296 dph of juveniles under moderate hypoxia (40% air saturation) at 15°C or 20°C. *Juveniles were early exposed or not to hypoxia at larval stage (including fish early exposed to 15°C and 20°C at larval stage) (n for each group= 60 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.

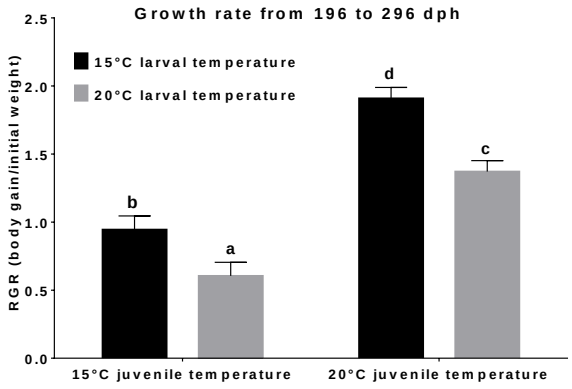

Figure 7. Relative growth rate (body gain/initial weight) from 196 to 296 dph of juveniles under moderate hypoxia (40% air saturation) at 15°C or 20°C. *Juveniles were early exposed to 15°C or 20°C at larval stage (including fish early exposed or not to hypoxia at larval stage) (n for each*

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.

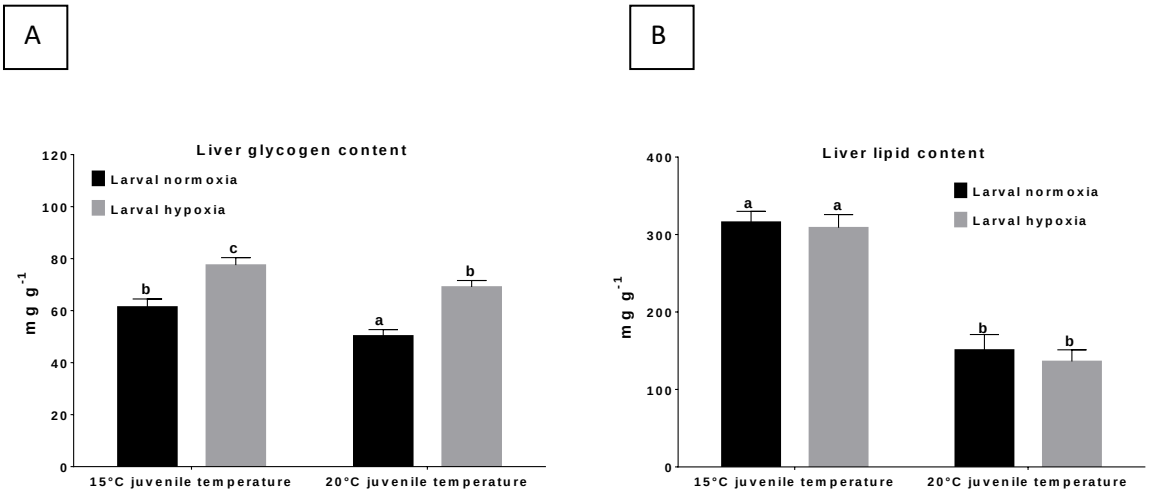

Figure 8. Hepatic metabolites (glycogen (A) and lipid (B)) at 296 dph of juveniles exposed to moderate hypoxia at 15°C or 20°C. *Juveniles were early exposed or not to hypoxia at larval stage (including fish early exposed to 15°C and 20°C at larval stage) (n for each group= 8 pools of 2 juveniles). Significant 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.

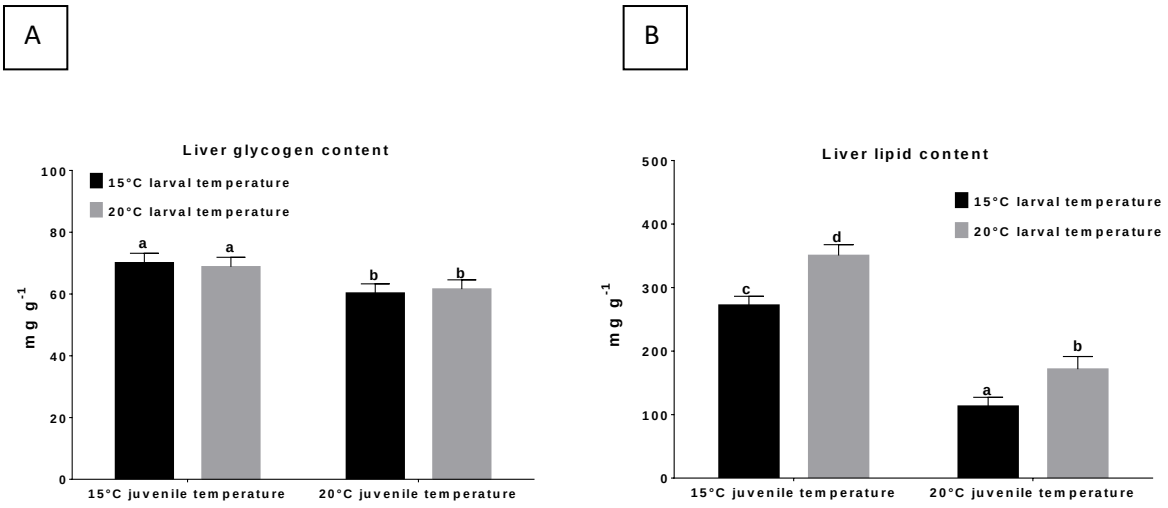

Figure 9. Hepatic metabolites (glycogen (A) and lipid (B)) at 296 dph of juveniles exposed to moderate hypoxia at 15°C or 20°C. *Juveniles were early exposed to 15°C or 20°C at larval stage (including fish early exposed or not to hypoxia at larval stage) (n for each group= 8 pools of 2 juveniles). Significant 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 O_2. 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 O_2 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 O_2 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

Credits: ORG save our sea bass

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 Haussmann 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 O_2 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 O_2 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.

References

-A-

Abdel, I., Abellan, E., Lopez-Albors, O., Valdes, P., Nortes, M. J. and Garcia-Alcazar, A. (2004). Abnormalities in the juvenile stage of sea bass (*Dicentrarchus labrax* L.) reared at different temperatures: types, prevalence and effect on growth. *Aquaculture International* 12, 523–538.

Able, K. W. (2005). A re-examination of fish estuarine dependence: Evidence for connectivity between estuarine and ocean habitats. *Estuarine, Coastal and Shelf Science* 64, 5–17.

Akram, M. (2014). Citric acid cycle and role of its intermediates in metabolism. *Cell biochemistry and biophysics* 68*,* 75-478.

Ali, M., Nicieza, A. and Wootton, R. J. (2003). Compensatory growth in fishes: A response to growth depression. *Fish and Fisheries* 4, 147–190.

Almeida-Val, V. M. F., Val, A. L., Duncan, W. P., Souza, F. C. A., Paula-Silva, M. N., and Land, S. (2000). Scaling effects on hypoxia tolerance in the Amazon fish Astronotus ocellatus (Perciformes: Cichlidae): contribution of tissue enzyme levels. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 125, 219-226.

Aprelikova, O., Chandramouli, G. V. R., Wood, M., Vasselli, J. R., Riss, J., Maranchie, J. K., Linehan, W. M. and Barrett, J. C. (2004). Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. *Journal of Cellular Biochemistry* 92, 491–501.

Arendt, J. D. (1997). Adaptive intrinsic growth rates: an integration across taxa. Quarterly Review of Biology 72, 149-177.

Axelsson M, Farrell AP, Nilsson S. (1990). Effects of hypoxia and drugs on the cardiovascular dynamics of the Atlantic hagfish *Myxine glutinosa*. *Journal of Experimental Biology* 151, 297–316.

-B-

Bagatto, B. (2005). Ontogeny of cardiovascular control in zebrafish (*Danio rerio*): effects of developmental environment. *Comparative biochemistry and physiology. Part A, Molecular and integrative physiology* 141, 391–400.

Bagherzadeh Lakani, F., Sattari, M., and Falahatkar, B. (2013). Effect of different oxygen levels on growth performance, stress response and oxygen consumption in two weight groups of great sturgeon Huso huso. Iranian Journal of Fisheries Sciences 12, 533-549.

Bailey, J. R., Val, A. L., Almeida-Val, V. M. F., Driedzic, W. R. (1999). Anoxic cardiac performance in Amazonian and north-temperate-zone teleosts. *Canadian Journal of Zoology* 77, 683–89.

Balfour, D. L. (1999). Ontogenetic Changes and Environmental Hypoxia: Responses of Two Fish Species to Low Oxygen Concentrations at Early Life Stages (Doctoral dissertation).

Barnes, R. K., King, H., and Carter, C. G. (2011). Hypoxia tolerance and oxygen regulation in Atlantic salmon, Salmo salar from a Tasmanian population. *Aquaculture* 318, 397-401.

Barrionuevo, W. R., Fernandes, M. N. and Rocha, O. (2010). Aerobic and anaerobic metabolism for the zebrafish, *Danio rerio,* reared under normoxia and hypoxic conditions and exposed to acute hypoxia during development. *Brazilian Journal of Biology* 70, 425-434

Bateson, P., Gluckman, P. and Hanson, M. (2014). The biology of developmental plasticity and the Predictive Adaptive Response hypothesis. *The Journal of physiology* 592, 2357–2368.

Beck, M. W., Heck, K. L., Able, K. W., Childers, D. L., Eggleston, D. B., Gillan, B. M., Ha, B., Hays, C. G., Hoshino, K., Minello, T. J., et al. (2001). The Identification, Conservation, and Management of Estuarine and Marine Nurseries for Fish and Invertebrates. *BioScience* 51, 633.

Beldade, P., Mateus, A. R. A., and Keller, R. A. (2011). Evolution and molecular mechanisms of adaptive developmental plasticity. *Molecular Ecology* 20, 1347-1363.

Benson, B. B. and Krause, D. (1984). The concentration and isotopic fractionation of oxygen dissolved in freshwater and seawater in equilibrium with the atmosphere1. *Limnology and Oceanography* 29, 620–632.

Bento, E. G., Grilo, T. F., Nyitrai, D., Dolbeth, M., Pardal, M. Â. and Martinho, F. (2016). Climate influence on juvenile European sea bass (*Dicentrarchus labrax*) populations in an estuarine nursery: A decadal overview. *Marine environmental research* 122, 93-104.

Beraldo, P., Pinosa, M., Tibaldi, E. and Canavese, B. (2003). Abnormalities of the operculum in gilthead sea bream (Sparus aurata): Morphological description. *Aquaculture* 220, 89–99.

Bianchini, K., and Wright, P. A. (2013). Hypoxia delays hematopoiesis: retention of embryonic hemoglobin and erythrocytes in larval rainbow trout, *Oncorhynchus mykiss*, during chronic hypoxia exposure. *Journal of Experimental Biology* 216, 4415-4425.

Bickler, P. E. and Buck, L. T. (2007). Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. *Annual Review of Physiology* 69, 145–170.

Blomstrand, E., Rådegran, G., and Saltin, B. (1997). Maximum rate of oxygen uptake by human skeletal muscle in relation to maximal activities of enzymes in the Krebs cycle. *The Journal of Physiology* 501, 455-460.

Bohr, C., Hasselbalch, K. and Krogh, A. (1904). Ueber einen in biologischer beziehungwichtigen einfluss, den die Kohlensauerspannung des blutes auf dessen sauerstoffbindung uebt. *Skandinavisches Archiv fur Physiologie* 16, 402-412.

Braasch, I., Volff, J. N. and Schartl, M. (2009). The endothelin system: Evolution of vertebrate-specific ligand-receptor interactions by three rounds of genome duplication. *Molecular Biology and Evolution* 26, 783–799.

Braasch, I., Brunet, F., Volff, J. N. and Schartl, M. (2010). Pigmentation Pathway Evolution after Whole-Genome Duplication in Fish. *Genome Biology and Evolution* 1, 479–493.

Bracken, C. P., Whitelaw, M. L., and Peet, D. J. (2003). The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. *Cellular and Molecular Life Sciences CMLS* 60, 1376-1393.

Breitburg, D. L., Steinberg, N., DuBeau, S., Cooksey, C., and Houde, E. D. (1994). Effects of low dissolved oxygen on predation on estuarine fish larvae. *Marine Ecology Progress Series*, 235-246.

Brett, J. R. (1979). Environmental factors and growth. In 'Fish Physiology. Vol. VIII: Bioenergetics and Growth'. Eds WS Hoar, DJ Randall and JR Brett, pp. 599–675.

Bricker, S. B., Longstaff, B., Dennison, W., Jones, A., Boicourt, K., Wicks, C. and Woerner, J. (2008). Effects of nutrient enrichment in the nation's estuaries: A decade of change. *Harmful Algae* 8, 21–32.

Brittain, T. (1987). The Root effect. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 86, 473-481.

Buentello, J. a, Gatlin III, D. M. and Neill, W. H. (2000). Effects of water temperature and dissolved oxygen\ron daily feed consumption, feed utilization and growth of channel catfish (Ictalurus punctatus). *Aquaculture* 182, 339–352.

Bushnell PG, Brill RW, Bourke RE. (1990). Cardiorespiratory responses of skipjack tuna (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus albacares*), and bigeye tuna (*Thunnus obesus*) to acute reductions of ambient oxygen. *Canadian Journal of Zoology* 68:1857–1865.

-C-

Cannon, W. B. (1932). Homeostasis. *The wisdom of the body. Norton, New York*.

Carroll, V. A., and Ashcroft, M. (2005). HIF-1a regulation by proline hydroxylation. *Expert Reviews in Molecular Medicine*, 7.

Champagne, F. A., and Meaney, M. J. (2007). Transgenerational effects of social environment on variations in maternal care and behavioral response to novelty. *Behavioral neuroscience* 121, 1353-1363.

Campbell, N., and Reece, J. (2005). Membrane structure and function. *Biology*, *7*, 124-140.

Chapman, L. J. and Hulen, K. G. (2001). Implications of hypoxia for the brain size and gill morphometry of mormyrid fishes. *Journal of Zoology (London)* 254, 461–472.

Chapman L, Galis F and Shinn J, 2000. Phenotypic plasticity and the possible role of genetic assimilation- Hypoxia-induced trade-offs on the morphological traits of an African cichlid. *Ecological Letters* 3: 387-393.

Chown, S. L. and Gaston, K. J. (2008). Macrophysiology for a changing world. *Proceedings of the Royal Society B: Biological Sciences* 275, 1469–1478.

Ciftci, Y., Castilho, R., and McAndrew, B. J. (2002). More polymorphic microsatellite markers in the European sea bass (*Dicentrarchus labrax*). *Molecular Ecology Resources* 2, 575-576.

Cioffi, C. L., Liu, X. Q., Kosinski, P. A., Garay, M., and Bowen, B. R. (2003). Differential regulation of HIF-1α prolyl-4-hydroxylase genes by hypoxia in human cardiovascular cells. *Biochemical and biophysical research communications* 303, 947-953.

Crowley, T. J. and Hyde, W. T. (2008). Transient nature of late Pleistocene climate variability. *Nature* 456, 226–230.

-D-

Dando, P. R., and Demir, N. (1985). On the spawning and nursery grounds of bass, Dicentrarchus labrax, in the Plymouth area. *Journal of the Marine Biological Association of the United Kingdom*, 65 (1), 159-168.

Darias, M. J., Lan Chow Wing, O., Cahu, C., Zambonino-Infante, J. L. and Mazurais, D. (2010). Double staining protocol for developing European sea bass (*Dicentrarchus labrax*) larvae. *Journal of Applied Ichthyology* 26, 280–285.

Dehal, P. and Boore, J. L. (2005). Two rounds of whole genome duplication in the ancestral vertebrate. *PLoS Biology* 10, 314.

Dejours, P. (1981). Principles of comparative respiratory physiology. sole distributors for the USA and Canada, Elsevier North-Holland.

Dias, J., Alvarez, M. J., Diez, A., Arzel, J., Corraze, G., Bautista, J. M. and Kaushik, S. J. (1998). Regulation of hepatic lipogenesis by dietary protein/energy in juvenile European seabass (Dicentrarchus labrax). *Aquaculture* 161, 169–186.

Diaz, R. J. (2001). Overview of Hypoxia around the World. *Journal of Environment Quality* 30, 275–281.

Diaz, R. J. and Rosenberg, R. (2008). Spreading Dead Zones and Consequences for Marine Ecosystems. *Science* 321, 926–929.

Diez, J. M., and Davenport, J. (1990). Energy exchange between the yolk and embryo of dogfish (Scyliorhinus canicula L.) eggs held under normoxic, hypoxic and transient anoxic conditions. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* 96, 825-830.

Diffenbaugh, N. S. and Field, C. B. (2013). Changes in Ecologically Critical Terrestrial Climate Conditions. *Science* 341, 486–492.

Dixson, D. L., Jones, G. P., Munday, P. L., Planes, S., Pratchett, M. S., and Thorrold, S. R. (2014). Experimental evaluation of imprinting and the role innate preference plays in habitat selection in a coral reef fish. *Oecologia*, 174 (1), 99-107.

Douard, V., Brunet, F., Boussau, B., Ahrens-Fath, I., Vlaeminck-Guillem, V., Haendler, B., Laudet, V. and Guiguen, Y. (2008). The fate of the duplicated androgen receptor in fishes: a late neofunctionalization event? *BMC evolutionary biology* 8, 336.

Dufour, V., Cantou, M. and Lecomte, F. (2009). Identification of sea bass (Dicentrarchus labrax) nursery areas in the north-western Mediterranean Sea. *Journal of the Marine Biological Association of the United Kingdom* 89, 1367–1374.

-E-

Eckle, T., Köhler, D., Lehmann, R., El Kasmi, K. C., and Eltzschig, H. K. (2008). Hypoxia-inducible factor-1 is central to cardioprotection. *Circulation*, *118*(2), 166-175.

Edeline, E., Lacroix, G., Delire, C., Poulet, N., and Legendre, S. (2013). Ecological emergence of thermal clines in body size. *Global change biology*, *19*(10), 3062-3068.

Elliot, M. and Dewailly, F. (1995). 114719.pdf. *Journal of aquatic ecology* 29, 397–417.

Eroldoğan, O. T., Kumlu, M., and Aktaş, M. (2004). Optimum feeding rates for European sea bass Dicentrarchus labrax L. reared in seawater and freshwater. *Aquaculture* 231, 501-515.

Esteban, M., Muñoz, J., and Meseguer, J. (2000). Blood cells of sea bass (*Dicentrarchus labrax*). Flow cytometric and microscopic studies. *The Anatomical Record*, 258, 80-89.

Evans, D. H., Piermarini, P. M., and Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiological reviews*, 85, 97-177.

-F-

Fago, A., Romano, M., Tamburrini, M., Coletta, M., D'avinno, R., Prisco, G. (1993). A polymerising Root-effect fish hemoglobin with high subunit heterogeneity. *The FEBS Journal* 218, 829-835.

Fago, A., Bendixen, E., Malte, H. and Weber, R. E. (1997). The anodic hemoglobin of Anguilla anguilla. *Journal of Biological Chemistrry* 272, 15628–15635.

Faust HA, Gamperl AK, Rodnick KJ. 2004. All rainbow trout (*Oncorhynchus mykiss*) are not created equal: intraspecific variation in cardiac hypoxia tolerance. *Journal of Experimental Biology* 207, 1005-1015.

Fedele AO, Whitlaw ML and Peet DJ, 2002. Regulation of gene expression by the hypoxia-inducible factors. *Molecular Interventions* 2, 229-243.

Feng, J., Liu, S., Wang, X., Wang, R., Zhang, J., Jiang, Y., and Liu, Z. (2014). Channel catfish hemoglobin genes: identification, phylogenetic and syntenic analysis, and specific induction in response to heat stress. Comparative Biochemistry and Physiology Part D: *Genomics and Proteomics* 9, 11-22.

Fischer, S., Bessert-Nettelbeck, M., Kotrschal, A., and Taborsky, B. (2015). Rearing-group size determines social competence and brain structure in a cooperatively breeding cichlid. *The American Naturalist*, 186, 123-140.

Fitzgibbon, Q. P., Seymour, R. S., Ellis, D. and Buchanan, J. (2007). The energetic consequence of specific dynamic action in southern bluefin tuna *Thunnus maccoyii*. *Journal of Experimental Biology* 210, 290–298.

Fonds, M., Cronie, R., Vethaak, A. D., and Van der Puyl, P. (1992). Metabolism, food consumption and growth of plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*) in relation to fish size and temperature. *Netherlands Journal of Sea Research* 29, 127-143.

Foss, A. and Imsland, A. K. (2002). Compensatory growth in the spooted wolffish Anarhichas minor(Olafsen) after a period of limited oxygen supply. *Aquaculture Research* 33, 1097–1101.

Franco, A., Elliott, M., Franzoi, P. and Torricelli, P. (2008). Life strategies of fishes in European estuaries: The functional guild approach. *Marine Ecology Progress Series* 354, 219–228.

Franz-Guess, S. and Starck, J. M. (2016). Histological and ultrastructural analysis of the respiratory tracheae of Galeodes granti (Chelicerata: Solifugae). *Arthropod Structure and Development* 45, 452–461.

Freyhof, J. and Kottelat, M. (2008). Dicentrarchus labrax. The IUCN Red List of Threatened Species 2008.

Fuller, A., Dawson, T., Helmuth, B., Hetem, R. S., Mitchell, D. and Maloney, S. K. (2010). Physiological Mechanisms in Coping with Climate Change. *Physiological and Biochemical Zoology* 83, 713–720.

-G-

Gamperl, A. K., Todgham, A. E., Parkhouse, W. S., Dill, R., and Farrell, A. P. (2001). Recovery of trout myocardial function following anoxia: preconditioning in a non-mammalian model. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 281, 1755-1763.

Garland, T. and Kelly, S. A. (2006). Phenotypic plasticity and experimental evolution. *Journal of Experimental Biology* 209, 2344–2361.

Garrett, R.H., Grisham, C.M. (1995). The synthesis and degradation of nucleotides. In: Garrett, R.H., Grisham, C.M. (Eds.), Biochemistry. Saunders College Publishing, NewYork, pp. 882.

Gibson, R. N. (1994). Impact of habitat quality and quantity on the recruitment of juvenile flatfishes. *Netherlands Journal of Sea Research* 32, 191–206.

Gillanders, B. M., Able, K. W., Brown, J. A., Eggleston, D. B., and Sheridan, P. F. (2003). Evidence of connectivity between juvenile and adult habitats for mobile marine fauna: an important component of nurseries. *Marine Ecology Progress Series*, 247, 281-295.

Gracey, A. Y., Troll, J. V and Somero, G. N. (2001). Hypoxia-induced gene expression profiling in the euryoxic fish Gillichthys mirabilis. *Proceedings of the National Academy of Sciences* 98, 1993–1998.

Gracey, A. Y., Lee, T.-H., Higashi, R. M. and Fan, T. (2011). Hypoxia-induced mobilization of stored triglycerides in the euryoxic goby Gillichthys mirabilis. *The Journal of experimental biology* 214, 3005–3012.

Graham, J. B. (1990). Ecological, evolutionary, and physical factors influencing aquatic animal respiration. *American Zoologist*. 30, 137-146.

Graham, J. B. (1994). An evolutionary perspective for bimodal respiration: A biological synthesis of fish air breathing. *Integrative and Comparative Biology* 34, 229–237.

Gorr, T. A., Gassmann, M., and Wappner, P. (2006). Sensing and responding to hypoxia via HIF in model invertebrates. *Journal of insect physiology*, 52 (4), 349-364.

Gurney, W. S. C. and Nisbet, R. M. (2004). Resource allocation, hyperphagia and compensatory growth. *Bulletin of Mathematical Biology* 66, 1731–1753.

-H-

Hallgrímsson, B., Willmore, K. and Hall, B. K. (2002). Canalization, developmental stability, and morphological integration in primate limbs. *Yearbook of Physical Anthropology* 45, 131–158.

Handelsman, C., Claireaux, G., and Nelson, J. A. (2010). Swimming ability and ecological performance of cultured and wild European sea bass (*Dicentrarchus labrax*) in coastal tidal ponds. *Physiological and Biochemical Zoology* 83, 435-445.

Hardison, R. C. (2008). Globin genes on the move. *Journal of Biology* 7, 35.1-35.5.

Haselmair, A., Stachowitsch, M., Zuschin, M. and Riedel, B. (2010). Behaviour and mortality of benthic crustaceans in response to experimentally induced hypoxia and anoxia in situ. *Marine Ecology Progress Series* 414, 195–208.

Hassell, K. L., Coutin, P. C. and Nugegoda, D. (2008). Hypoxia impairs embryo development and survival in black bream (*Acanthopagrus butcheri*). *Marine Pollution Bulletin* 57, 302–306.

Herbing, I. (2002). Effects of temperature on larval fish swimming performance: the importance of physics to physiology. *Journal of Fish Biology*, 61, 865-876.

Hidalgo, F., and Alliot, E. (1988). Influence of water temperature on protein requirement and protein utilization in juvenile sea bass, *Dicentrarchus labrax*. *Aquaculture* 72, 115-129.

Ho, D. H. and Burggren, W. W. (2012). Parental hypoxic exposure confers offspring hypoxia resistance in zebrafish (Danio rerio). *The Journal of experimental biology* 215, 4208–4216.

Hoegh-Guldberg O., Bruno J.F. (2010). The impact of climate change on the world's marine ecosystems. *Science* 328, 1523–28.

Holk, K., and Lykkeboe, G. (1995). Catecholamine-induced changes in oxygen affinity of carp and trout blood. *Respiration physiology* 100, 55-62.

Hollowed, A. B., Barange, M., Beamish, R. J., Brander, K., Cochrane, K., Drinkwater, K., Foreman, M. G. G., Hare, J. A., Holt, J., Ito, S., et al. (2013). *Marine Science*. 70, 1023–1037.

Holmgren, K. 2003. Omitted spawning in compensatory-growing perch. *Journal of Fish Biology* 62, 918-927.

Homechaudhuri, S., and Jha, A. (2001). A technique to evaluate the erythropoietic efficiency in fish. Asian Fisheries *Science* 14, 453-455.

Hsia, C. C., Schmitz, A., Lambertz, M., Perry, S. F., and Maina, J. N. (2013). Evolution of air breathing: oxygen homeostasis and the transitions from water to land and sky. *Comprehensive Physiology* 3, 849-915.

Hundahl, C., Fago, A., Malte, H., and Weber, R. E. (2003). Allosteric effect of water in fish and human hemoglobins. *Journal of Biological Chemistry*, *278*, 42769-42773.

Hylland, P., Milton, S., Pek, M., Nilsson, G. E. and Lutz, P. L. (1997). Brain Na+/K+-ATPase activity in two anoxia tolerant vertebrates: crucian carp and freshwater turtle. *Neuroscience Letters* 235, 89–92.

-I-

Ishibashi, Y., Inoue, K., Nakatsukasa, H., Ishitani, Y., Miyashita, S. and Murata, O. (2005). Ontogeny of tolerance to hypoxia and oxygen consumption of larval and juvenile red sea bream, Pagrus major. *Aquaculture* 244, 331–340.

Ishibashi, Y., Kotaki, T., Yamada, Y. and Ohta, H. (2007). Ontogenic changes in tolerance to hypoxia and energy metabolism of larval and juvenile Japanese flounder Paralichthys olivaceus. *Journal of Experimental Marine Biology and Ecology* 352, 42–49.

-J-

Jennings, S. and Pawson, M. G. (1992). The origin and recruitment of bass, *Dicentrarchus labrax*, larvae to nursery areas. *Journal of the Marine Biological Association of the United Kingdom* 72, 199–212.

Jennings, B. J., Ozanne, S. E.and C. N. Hales. 2000. Nutrition, oxidative damage, telomere shortening, and cellular senescence: individual or connected agents of aging? *Molecular Genetics and Metabolism* 71, 32-42.

Jensen, F. B. (2004). Red blood cell pH, the Bohr effect, and other oxygenation-linked phenomena in blood O2 and CO2 transport. *Acta Physiologica* 182, 215-227.

Jiang, L., Zhang, J., Wang, J. J., Wang, L., Zhang, L., Li, G., Yang, X., Ma, X., Sun, X., Cai, J., et al. (2013). Sperm, but not oocyte, DNA methylome is inherited by zebrafish early embryos. *Cell* 153, 773–784.

Jibb, L. A., and Richards, J. G. (2008). AMP-activated protein kinase activity during metabolic rate depression in the hypoxic goldfish, *Carassius auratus*. *Journal of Experimental Biology* 211, 3111-3122.

Johnston, I. A. 2003. Muscle metabolism and growth in Antarctic fishes (suborder Notothenioidei): evolution in a cold environment. *Comparative Biochemistry and Physiology B-Biochemistry and Molecular Biology* 136, 701-713.

Jonsson, B. and Jonsson, N. (2014). Early environment influences later performance in fishes. *Journal of Fish Biology* 85, 151–188.

Justić, D., Rabalais, N. N., Eugene Turner, R. and Wiseman, W. J. (1993). Seasonal coupling between riverborne nutrients, net productivity and hypoxia. *Marine Pollution Bulletin* 26, 184–189.

Joyce, W., Ozolina, K., Mauduit, F., Ollivier, H., Claireaux, G. and Shiels, H. A. (2016). Individual variation in whole-animal hypoxia tolerance is associated with cardiac hypoxia tolerance in a marine teleost. *Biology Letters* 12, 1-6.

-K-

Kaelin, W. G., and Ratcliffe, P. J. (2008). Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Molecular cell* 30, 393-402.

Kajimura S, Aida K and Duan C, 2006. Understanding hypoxia-induced gene expression in early development: In vitro and in vivo analysis of hypoxia-inducible factor 1- regulated zebra fish insulin-like growth factor binding protein 1 gene expression. *Molecular and Cellular Biology* 26, 1142-1155.

Kaluz, S., Kaluzová, M., and Stanbridge, E. J. (2008). Regulation of gene expression by hypoxia: integration of the HIF-transduced hypoxic signal at the hypoxia-responsive element. *Clinica Chimica Acta* 395, 6-13.

Kamaci, H. O., Çoban, D., Suzer, C., Aksu, B., Saka, Ş., and Firat, K. (2010). Exocrine pancreas development and trypsin expression in cultured European sea bass (*Dicentrarchus labrax*) larvae. *Turkish Journal of Fisheries and Aquatic Sciences*, 10.

Karasov, W. H. (1986). Energetics, physiology and vertebrate ecology. *Trends in Ecology and Evolution 1*, 101-104.

Karl, T. R., J. T. Melillo, and T. C. Peterson, 2009: *Global Climate Change Impacts in the United States*. Eds. Cambridge University Press.

Keckeis, H., Kamler, E., Bauer-Nemeschkal, E., and Schneeweiss, K. (2001). Survival, development and food energy partitioning of nase larvae and early juveniles at different temperatures. *Journal of Fish Biology* 59, 45-61.

Keeling, R. F. and Garcia, H. E. (2002). The change in oceanic O2 inventory associated with recent global warming. *Proceedings of the National Academy of Sciences* 99, 7848–7853.

Kennedy, M., and Fitzmaurice, P. (1972). The biology of the bass, *Dicentrarchus labrax*, in irish waters. *Journal of the Marine Biological Association of the United Kingdom* 52, 557-597.

Khakh, B. S. and Burnstock, G. (2010). NIH Public Access. *Scientific American* 119, 5124–5136.

Kieffer, J. D. (2000). Limits to exhaustive exercise in fish. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* 126, 161-179.

Kojima, T., Ishii, M., Kobayashi, M., and Shimizu, M. (2004). Blood parameters and electrocardiogram in squeezed fish simulating the effect of net damage and recovery. *Fisheries science* 70, 860-866.

Kousoulaki, K., Sæther, B. S., Albrektsen, S., and Noble, C. (2015). Review on European sea bass (*Dicentrarchus labrax*, Linnaeus, 1758) nutrition and feed management: a practical guide for optimizing feed formulation and farming protocols. *Aquaculture Nutrition* 21, 129-151.

-L-

Labbé, C., Robles, V. and Herraez, M. P. (2016). Epigenetics in fish gametes and early embryo. *Aquaculture* 472, 93–106.

Lai, J. C., Kakuta, I., Mok, H. O., Rummer, J. L., and Randall, D. (2006). Effects of moderate and

substantial hypoxia on erythropoietin levels in rainbow trout kidney and spleen. *Journal of Experimental Biology* 209, 2734-2738.

Landsman, S. J., Gingerich, A. J., Philipp, D. P., and Suski, C. D. (2011). The effects of temperature change on the hatching success and larval survival of largemouth bass *Micropterus salmoides* and smallmouth bass *Micropterus dolomieu*. *Journal of fish biology* 78, 1200-1212.

Lee, W.-S., Monaghan, P. and Metcalfe, N. B. (2010). The trade-off between growth rate and locomotor performance varies with perceived time until breeding. *Journal of Experimental Biology* 213, 3289–3298.

Lee, W. S., Monaghan, P. and Metcalfe, N. B. (2012). The pattern of early growth trajectories affects adult breeding performance. *Ecology* 93, 902–912.

Lefevre, S., Huong, D. T. T., Wang, T., Phuong, N. T. and Bayley, M. (2011). Hypoxia tolerance and partitioning of bimodal respiration in the striped catfish (*Pangasianodon hypophthalmus*). *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* 158, 207–214.

Lendahl, U., Lee, K. L., Yang, H., and Poellinger, L. (2009). Generating specificity and diversity in the transcriptional response to hypoxia. *Nature Reviews Genetics* 10, 821-832.

Levin, L. a., Ekau, W., Gooday, a. J., Jorissen, F., Middelburg, J. J., Naqvi, S. W. a., Neira, C., Rabalais, N. N. and Zhang, J. (2009). Effects of natural and human-induced hypoxia on coastal benthos. *Biogeosciences* 6, 2063–2098.

Lewis, J. M., Costa, I., Val, A. L., Almeida-Val, V. M. F., Gamperl, A. K. and Driedzic, W. R. (2007). Responses to hypoxia and recovery: repayment of oxygen debt is not associated with compensatory protein synthesis in the Amazonian cichlid, Astronotus ocellatus. *Journal of Experimental Biology* 210, 1935–1943.

Lieb, M. E., Menzies, K., Moschella, M. C., Ni, R., and Taubman, M. B. (2002). Mammalian EGLN genes have distinct patterns of mRNA expression and regulation. *Biochemistry and cell biology* 80, 421-426.

Lindström, J. (1999). Early development and fitness in birds and mammals. *Trends in Ecology and Evolution* 14, 343–348.

Liu, J., Plagnes-Juan, E., Geurden, I., Panserat, S. and Marandel, L. (2017). Exposure to an acute hypoxic stimulus during early life affects the expression of glucose metabolism-related genes at first-feeding in trout. *Scientific reports*, 7, 363.

Livingstone, D. R. (1991). Origins and evolution of pathways of anaerobic metabolism in the animal kingdom. *American zoologist* 31, 522-534.

Lu, W., Mayolle, A., Cui, G., Luo, L. and Balment, R. J. (2011). Molecular Characterization and Expression of α -Globin and β -Globin Genes in the Euryhaline Flounder (*Platichthys flesus*). *Evidence-Based Complementary and Alternative Medicine*, 2011-2021.

Ludsin, S. A. and D. R. DeVries. 1997. First-year recruitment of largemouth bass: the interdependency of early life stages. *Ecological Applications* 7, 1024-1038.

Lutz, P. L., and Nilsson, G. E. (2004). Vertebrate brains at the pilot light. *Respiratory physiology and neurobiology* 141, 285-296.

-M-

Maclean, A. and N. B. Metcalfe (2001). Social status, access to food, and compensatory growth in juvenile Atlantic salmon. *Journal of Fish Biology* 58, 1331-1346.

Maina, J. N. (2002). Structure, function and evolution of the gas exchangers: Comparative perspectives. *Journal of Anatomy* 201, 281–304.

Mairbäurl, H. and Weber, R. E. (2012). Oxygen transport by hemoglobin. *Comprehensive Physiology* 2, 1463–1489.

Mallin, M. A., Johnson, V. L., Ensign, S. H., and MacPherson, T. A. (2006). Factors contributing to hypoxia in rivers, lakes, and streams. *Limnology and Oceanography* 51, 690-701.

Mandic, M., Todgham, A. E. and Richards, J. G. (2009). Mechanisms and evolution of hypoxia tolerance in fish. *Proceedings. Biological sciences / The Royal Society* 276, 735–744.

Marks, C., West, T. N., Bagatto, B. and Moore, F. B.-G. (2005). Developmental Environment Alters Conditional Aggression in Zebrafish. *Copeia* 2005, 901–908.

Martínez, M. L., Landry, C., Boehm, R., Manning, S., Cheek, A. O. and Rees, B. B. (2006). Effects of long-term hypoxia on enzymes of carbohydrate metabolism in the Gulf killifish, Fundulus grandis. *The Journal of experimental biology* 209, 3851–3861.

Massa, F., Delorme, C., Bagliniere, J. L., Prunet, P., and Grimaldi, P. (1999). Early life development of brown trout (*Salmo trutta*) eggs under temporary or continuous hypoxial stress: effects on the gills, yolk sac resorption and morphometric parameters. *Bulletin Francais de la Peche et de la Pisciculture (France)*.

Matear, R. J., Hirst, A. C. and Mcneil, B. I. (2000). Changes in dissolved oxygen in the Southern Ocean with climate change.

Matey, V., Richards, J. G., Wang, Y., Wood, C. M., Rogers, J., Davies, R., Murray, B. W., Chen, X.-Q., Du, J. and Brauner, C. J. (2008). The effect of hypoxia on gill morphology and ionoregulatory status in the Lake Qinghai scaleless carp, Gymnocypris przewalskii. *Journal of Experimental Biology* 211, 1063–1074.

McBryan, T. L., Anttila, K., Healy, T. M. and Schulte, P. M. (2013). Responses to temperature and hypoxia as interacting stressors in fish: implications for adaptation to environmental change. *Integrative and comparative biology* 53, 648–59.

McBryan, T. L., Healy, T. M., Haakons, K. L. and Schulte, P. M. (2016). Warm acclimation improves hypoxia tolerance in Fundulus heteroclitus. *Journal of Experimental Biology* 219, 474–484.

McCarthy, I. D. (2001). Competitive ability is related to metabolic asymmetry in juvenile rainbow trout. *Journal of Fish Biology* 59, 1002-1014.

McDonald, F. B., Dempsey, E. M. and O'Halloran, K. D. (2016). Early Life Exposure to Chronic Intermittent Hypoxia Primes Increased Susceptibility to Hypoxia-Induced Weakness in Rat Sternohyoid Muscle during Adulthood. *Frontiers in physiology* 7, 69.

McKenzie, D. J., Piraccini, G., Steffensen, J. F., Bolis, C. L., Bronzi, P. and Taylor, E.W. (1995). Effects of diet on spontaneous locomotor activity and oxygen consumption in Adriatic sturgeon (*Acipenser naccarii*). *Fish Physiology and Biochemistry* 14, 341–355.

Meire, L., Soetaert, K. E. R. and Meysman, F. J. R. (2013). Impact of global change on coastal oxygen dynamics and risk of hypoxia. *Biogeosciences* 10, 2633–2653.

Mejri, S., Tremblay, R., Lambert, Y. and Audet, C. (2012). Influence of different levels of dissolved oxygen on the success of Greenland halibut (Reinhardtius hippoglossoides) egg hatching and embryonic development. *Marine Biology* 159, 1693–1701.

Meseguer, J., Esteban, M. A., Ayala, A. G., Ruiz, A. L., and Agulleiro, B. (1990). Granulopoiesis in the head-kidney of the sea bass (Dicentrarchus labrax L.): an ultrastructural study. *Archives of histology and cytology* 53, 287-296.

Metcalfe, N. B. and C. Alonso-Alvarez. 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. *Functional Ecology* 24, 984-996.

Monaghan, P. (2008). Early growth conditions, phenotypic development and environmental change. *Philosophical Transactions of the Royal Society B: Biological Sciences* 363, 1635–1645.

Monaghan, P., N. B. Metcalfe, and R. Torres. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecology Letters* 12, 75-92.

Moreno, P., Garcia-Rosado, E., Borrego, J. J., and Alonso, M. C. (2016). Genetic characterization and transcription analyses of the European sea bass (*Dicentrarchus labrax*) isg15 gene. *Fish and shellfish immunology* 55, 642-646.

Muller, M., Mentel, M., van Hellemond, J. J., Henze, K., Woehle, C., Gould, S. B., Yu, R.-Y., van der Giezen, M., Tielens, A. G. M. and Martin, W. F. (2012). Biochemistry and Evolution of Anaerobic Energy Metabolism in Eukaryotes. *Microbiology and Molecular Biology Reviews* 76, 444–495.

Myllyharju, J. (2009). HIF prolyl 4-hydroxylases and their potential as drug targets. *Current pharmaceutical design* 15, 3878-3885.

-N-

Naguib, M., Nemitz, A., and Gil, D. (2006). Maternal developmental stress reduces reproductive success of female offspring in zebra finches. *Proceedings of the Royal Society of London B: Biological Sciences* 273, 1901-1905.

Naqvi, S. W. A., Jayakumar, D. A., Narvekar, P. V., Naik, H., Sarma, V. V. S. S., D'souza, W., and George, M. D. (2000). Increased marine production of N2O due to intensifying anoxia on the Indian continental shelf. *Nature* 408, 346-349.

Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Di Croce, L. and Piferrer, F. (2011). DNA methylation of the gonadal aromatase (cyp19a) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. *PLoS Genetics* 7, 1-11.

Nettle, D., and Bateson, M. (2015). Adaptive developmental plasticity: what is it, how can we recognize it and when can it evolve?. *Proceeding of the Royal Society* 282, 1812-1834.

Nicholson, G., Jenkins, G. P., Sherwood, J. and Longmore, A. (2008). Physical environmental conditions, spawning and early-life stages of an estuarine fish: Climate change implications for recruitment in intermittently open estuaries. *Marine and Freshwater Research* 59, 735–749.

Nikinmaa, M. (1983). Adrenergic regulation of haemoglobin oxygen affinity in rainbow trout red cells. Journal of comparative physiology 152: 67-72.

Nikinmaa, M., A. Salama, A. Bogdanova and L. V. Virkki (2001). beta-Adrenergic Stimulation of Volume-Sensitive Chloride Transport in Lamprey Erythrocytes. *Physiological and Biochemical Zoology* 74(1): 45-51.

Nikinmaa M and Rees BB, 2005. Oxygen-dependent gene expression in fishes. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* 288, 1079-1090

Nilsson, G. E., Rosen, P. R. and Johansson, D. (1993). Anoxic depression of spontaneous locomotor activity in crucian carp quantified by a computerized imaging technique. *Journal of Experimental Biology* 180, 153-162.

Nilsson, G. E., and Renshaw, G. M. (2004). Hypoxic survival strategies in two fishes: extreme anoxia tolerance in the North European crucian carp and natural hypoxic preconditioning in a coral-reef shark. *Journal of Experimental Biology* 207, 3131-3139.

Nilsson, G. E. (2007). Gill remodeling in fish a new fashion or an ancient secret? *The Journal of experimental biology* 210, 2403–2409.

Nilsson, G. E. and Ostlund-Nilsson, S. (2008). Does size matter for hypoxia tolerance in fish? *Biological reviews of the Cambridge Philosophical Society* 83, 173–89.

Ninness, M. M., Stevens, E. D., and Wright, P. A. (2006). Energy expenditure during hatching in rainbow trout (*Oncorhynchus mykiss*) embryos. *Canadian Journal of Fisheries and Aquatic Sciences* 63, 1405-1413.

Nonnotte, G., Maxime, V., Truchot, J. P., Williot, P., and Peyraud, C. (1993). Respiratory responses to progressive ambient hypoxia in the sturgeon, *Acipenser baeri*. Respiration physiology 91, 71-82.

-O-

Opazo, J. C., Butts, G. T., Nery, M. F., Storz, J. F. and Hoffmann, F. G. (2012). Whole-Genome Duplication and the Functional Diversification of Teleost Fish Hemoglobins. *Molecular biology and evolution* 30, 140-153.

-P-

Padilla, P. A., and Roth, M. B. (2001). Oxygen deprivation causes suspended animation in the zebrafish embryo. *Proceedings of the National Academy of Sciences* 98, 7331-7335.

Parpoura, A. C., and Alexis, M. N. (2001). Effects of different dietary oils in sea bass (*Dicentrarchus labrax*) nutrition. *Aquaculture international* 9, 463-476.

Pawson, M. G., Pickett, G. D., Leballeur, J., Brown, M., and Fritsch, M. (2007). Migrations, fishery interactions, and management units of sea bass (Dicentrarchus labrax) in Northwest Europe. *ICES Journal of Marine Science* 64, 332-345.

Peck, L. S. and Uglow, R. F. (1990). Two methods for the assessment of the oxygen content of small volumes of seawater. *Journal of Experimental Marine Biology and Ecology* 141, 53–62.

Pelster, B., and Randall, D. J. (1998). The physiology of the Root effect. *Fish Respiration 17*, 113-139.

Person-Le Ruyet, J., Lacut, A., Le Bayon, N., Le Roux, A., Pichavant, K. and Quéméner, L. (2003). Effects of repeated hypoxic shocks on growth and metabolism of turbot juveniles. *Aquatic Living Resources* 16, 25–34.

Perry, S. F. and K. M. Gilmour (1996). Consequences of catecholamine release on ventilation and blood oxygen transport during hypoxia and hypercapnia in an elasmobranch (*Squalus acanthias*) and a teleost (*Oncorhynchus mykiss*). *Journal of Experimental Biology* 199, 2105-2118.

Perry, S. F., Jonz, M. G. and Gilmour, K. M. (2009). Oxygen sensing and the hypoxic ventilatory response. *Fish Physiology: Hypoxia* 27, 193–253.

Perutz, M. F., Rossman, M. G., Cullis, A. F., Muirhead, H., Will, G. and North, A.C. (1960). Structure of haemoglobin: a three-dimensional Fourier synthesis at 5.5-A. resolution, obtained by X-ray analysis. *Nature* 185, 416-422.

Pescador, N., Cuevas, Y., Naranjo, S., Alcaide, M., Villar, D., Landázuri, M. O. and del Peso, L. (2005). Identification of a functional hypoxia-responsive element that regulates the expression of the egl nine homologue 3 (egln3/phd3) gene. *Biochemical Journal* 390, 189-197.

Pescador, N., Villar, D., Cifuentes, D., Garcia-Rocha, M., Ortiz-Barahona, A., Vazquez, S., Ordoñez, A., Cuevas, Y., Saez-Morales, D., Garcia-Bermejo, M. L., et al. (2010). Hypoxia promotes glycogen accumulation through hypoxia inducible factor (HIF)-mediated induction of glycogen synthase 1. *PLoS ONE* 5.

Pichavant, K., Bayon, N. Le, Severe, A., Roux, A. Le and Boeuf, G. (2001). Comparative effects of hypoxia on growth and metabolism of turbot and European sea bass. *Journal of Fish Biology* 59, 875–883.

Pichavant, K., Maxime, V., Soulier, P., Boeuf, G. and Nonnotte, G. (2003). A comparative study of blood oxygen transport in turbot and sea bass: effect of chronic hypoxia. *Journal of Fish Biology* 62, 928–937.

Piersma, T., and Drent, J. (2003). Phenotypic flexibility and the evolution of organismal design. *Trends in Ecology and Evolution*, 18, 228-233.

Pittman, R. N. (2011). Oxygen transport in normal and pathological situations: defects and compensations. In: Pittman RN ed. Regulation of Tissue Oxygenation. San Rafael, CA: Morgan and Claypool Life Sciences; 2011: 47–50.

Plattner, G.-K., Joos, F., Stocker, T. F. and Marchal, O. (2001). Feedback mechanisms and sensitivities of ocean carbon uptake under global warming. *Tellus B* 53, 564–592.

Podrabsky, J. E., Lopez, J. P., Fan, T. W., Higashi, R., and Somero, G. N. (2007). Extreme anoxia tolerance in embryos of the annual killifish Austrofundulus limnaeus: insights from a metabolomics analysis. *Journal of Experimental Biology* 210, 2253-2266.

Pope, E. C., Ellis, R. P., Scolamacchia, M., Scolding, J. W. S., Keay, A., Chingombe, P., and Lewis, C. (2014). European sea bass, *Dicentrarchus labrax*, in a changing ocean. *Biogeosciences* 11, 2519-2527.

Pörtner, H. (2001). Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. Naturwissenschaften, 88, 137-146.

Pörtner, H. O. (2002). Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, 132, 739-761.

Portner, H. O. and Farrell, A. P. (2008). ECOLOGY: Physiology and Climate Change. *Science* 322, 690-692.

Pörtner, H. O., and Lannig, G. (2009). Oxygen and capacity limited thermal tolerance. *Fish physiology*, 27, 143-191.

Pörtner, H. O. (2010). Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. *Journal of Experimental Biology* 213, 881-893.

-R-

Rabalais, N. N., Turner, R. E., Díaz, R. J., and Justić, D. (2009). Global change and eutrophication of coastal waters. ICES *Journal of Marine Science*, 66, 1528-1537.

Ramler, D., Mitteroecker, P., Shama, L. N. S., Wegner, K. M. and Ahnelt, H. (2014). Nonlinear effects of temperature on body form and developmental canalization in the threespine stickleback. *Journal of Evolutionary Biology* 27, 497-507.

Randall, D. (1982). The control of respiration and circulation in fish during exercise and hypoxia. *Journal of Experimental Biology* 100, 275–288.

Randall, D. J. and Perry, S. F. (1992). Catecholamines. In *Fish Physiology*, vol. 12B eds. W. S. Hoar, D. J. Randall, and A. P. Farrell), pp. 255-300. New York: Academic Press.

Remen, M., Oppedal, F., Torgersen, T., Imsland, A. K., and Olsen, R. E. (2012). Effects of cyclic environmental hypoxia on physiology and feed intake of post-smolt Atlantic salmon: initial responses and acclimation. *Aquaculture*, *326*, 148-155.

Remen, M., Aas, T. S., Vågseth, T., Torgersen, T., Olsen, R. E., Imsland, A. and Oppedal, F. (2014). Production performance of Atlantic salmon (Salmo salar L.) postsmolts in cyclic hypoxia, and following compensatory growth. *Aquaculture Research* 45, 1355-1366.

Richards, J. G. (2009). Metabolic and molecular responses of fish to hypoxia. Fish physiology, 27, 443-485.

Rijnsdorp, A. D., Peck, M. A., Engelhard, G. H., Möllmann, C. and Pinnegar, J. K. (2009). Resolving the effect of climate change on fish populations. *ICES Journal of Marine Science* 66, 1570–1583.

Roesner, A., Hankeln, T., and Burmester, T. (2006). Hypoxia induces a complex response of globin expression in zebrafish (*Danio rerio*). *Journal of experimental biology* 209, 2129-2137.

Roesner, A., Mitz, S. A., Hankeln, T., and Burmester, T. (2008). Globins and hypoxia adaptation in the goldfish, Carassius auratus. *The FEBS journal* 275, 3633-3643.

Rombout, J. H. W. M., Huttenhuis, H. B. T., Picchietti, S., and Scapigliati, G. (2005). Phylogeny and ontogeny of fish leucocytes. *Fish and shellfish immunology* 19, 441-455.

Roussel, J. M. (2007). Carry-over effects in brown trout (Salmo trutta): hypoxia on embryos impairs predator avoidance by alevins in experimental channels. *Canadian Journal of Fisheries and Aquatic Sciences* 64, 786-792.

Royer, W. E., Zhu, H., Gorr, T. A., Flores, J. F. and Knapp, J. E. (2005). Allosteric hemoglobin assembly: Diversity and similarity. *Journal of Biological Chemistry* 280, 27477–27480.

Roze, T., Christen, F., Amerand, A. and Claireaux, G. (2013). Trade-off between thermal sensitivity, hypoxia tolerance and growth in fish. *Journal of Thermal Biology* 38, 98–106.

Rytkönen, K. T., Renshaw, G. M., Vainio, P. P., Ashton, K. J., Williams-Pritchard, G., Leder, E. H., and Nikinmaa, M. (2012). Transcriptional responses to hypoxia are enhanced by recurrent hypoxia (hypoxic preconditioning) in the epaulette shark. *Physiological genomics 44*, 1090-1097.

-S-

Sandersfeld, T., Davison, W., Lamare, M. D., Knust, R. and Richter, C. (2015). Elevated temperature causes metabolic trade-offs at the whole-organism level in the Antarctic fish Trematomus bernacchii. *Journal of Experimental Biology* 218, 2373–2381.

Sato, Y., Hashiguchi, Y. and Nishida, M. (2009). Temporal pattern of loss/persistence of duplicate genes involved in signal transduction and metabolic pathways after teleost-specific genome duplication. *BMC Evolutionary Biology* 9, 127.

Schofield, C. J., and Ratcliffe, P. J. (2004). Oxygen sensing by HIF hydroxylases. *Nature reviews Molecular cell biology 5*, 343-354.

Schulte, P. M. (2015). The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *Journal of Experimental Biology* 218, 1856–1866.

Schurmann, H. and Steffensen, J. F. (1992). Lethal oxygen levels at different temperatures and the preferred temperature during hypoxia of the Atlantic cod, Gadus morhua L. *Journal of Fish Biology* 41, 927–934.

Scott, G. R. and Johnston, I. A. (2012). Temperature during embryonic development has persistent effects on thermal acclimation capacity in zebrafish. *Proceedings of the National Academy of Sciences* 109, 14247–14252.

Seebacher, F. (2009). Responses to temperature variation: integration of thermoregulation and metabolism in vertebrates. *Journal of Experimental Biology 212*, 2885-2891.

Semenza GL and Wang GL, 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gen enhancer at a site required for transcriptional activation. *Molecular and Cellular Biology* 12, 5447-544

Semenza, G. L. (1999). Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. *Annual review of cell and developmental biology* 15, 551-578.

Semenza, G. L. (2000). HIF-1 and human disease: one highly involved factor. *Genes and development* 14, 1983-1991.

Shang, E. H. H. and Wu, R. S. S. (2004). Aquatic hypoxia is a teratogen and affects fish embryonic development. *Environmental Science and Technology* 38, 4763–4767.

Sheridan, J. A., and Bickford, D. (2011). Shrinking body size as an ecological response to climate change. *Nature climate change* 1, 401-406.

Shoji, J., Masuda, R., Yamashita, Y., and Tanaka, M. (2005). Effect of low dissolved oxygen concentrations on behavior and predation rates on red sea bream Pagrus major larvae by the jellyfish *Aurelia aurita* and by juvenile Spanish mackerel *Scomberomorus niphonius*. *Marine Biology* 147, 863-868

Soitamo, A. J., Råbergh, C. M., Gassmann, M., Sistonen, L., and Nikinmaa, M. (2001). Characterization of a Hypoxia-inducible Factor (HIF-1α) from Rainbow Trout accumulation of protein occurs at normal venous oxygen tension. *Journal of Biological Chemistry* 276, 19699-19705.

Sokolova, I. M. and Lannig, G. (2008). Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: Implications of global climate change. *Climate Research* 37, 181-201.

Sokolova, I. M., Frederich, M., Bagwe, R., Lannig, G. and Sukhotin, A. A. (2012). Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Marine Environmental Research* 79, 1–15.

Sollid, J., Angelis, P., Gundersen, K. and Nilsson, G. E. (2003). Hypoxia induces adaptive and reversible gross morphological changes in crucian carp gills. *Journal of Experimental Biology* 206, 3667–3673.

Sollid, J. and Nilsson, G. E. (2006). Plasticity of respiratory structures--adaptive remodeling of fish gills induced by ambient oxygen and temperature. *Respiratory physiology and neurobiology* 154, 241–51.

De Souza, P. C. and Bonilla-Rodriguez, G. O. (2007). Fish hemoglobins. Brazilian Journal of Medical and Biological Research, 40, 769-778.

Smith, R. W., Houlihan, D. F., Nilsson, G. E., and Alexandre, J. (1999). Tissue-specific changes in RNA synthesis in vivo during anoxia in crucian carp. American Journal of Physiology 277, 690-697.

Spees, J. L., Olson, S. D., Whitney, M. J. and Prockop, D. J. (2006). Mitochondrial transfer between cells can rescue aerobic respiration. *Proceedings of the National Academy of Sciences* 103, 1283–1288.

Stecyk, J. A. W., Overgaard, J., Farrell, A. P., and Wang, T. (2004). α-Adrenergic regulation of systemic peripheral resistance and blood flow distribution in the turtle Trachemys scripta during anoxic submergence at 5 C and 21 C. Journal of Experimental Biology 207, 269-283.

Steffensen, J. F., Lomholt, J. P. and Johansen, K. (1982). Gill ventilation and O2 extraction during graded hypoxia in two ecologically distinct species of flatfish, the flounder (Platichthys flesus) and the plaice (Pleuronectes platessa). *Environmental Biology of Fishes* 7, 157–163.

Stierhoff, K. L., Targett, T. E., and Miller, K. (2006). Ecophysiological responses of juvenile summer and winter flounder to hypoxia: experimental and modeling analyses of effects on estuarine nursery quality. Marine Ecology Progress Series 325, 255-266.

Storey, K. B., and Storey, J. M. (1990). Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. The Quarterly Review of Biology 65, 145-174.

-T-

Terova, G., Cattaneo, A. G., Preziosa, E., Bernardini, G. and Saroglia, M. (2011). Impact of acute stress on antimicrobial polypeptides mRNA copy number in several tissues of marine sea bass (*Dicentrarchus labrax*). *BMC Immunology* 12, 69.

Terwilliger, N. B. (1998). Functional adaptations of oxygen-transport proteins. *The Journal of experimental biology* 201, 1085–1098.

Thomas, C. D., Thomas, C. D., Cameron, A., Cameron, A., Green, R. E., Green, R. E., Bakkenes, M., Bakkenes, M., Beaumont, L. J., Beaumont, L. J., et al. (2004). Extinction risk from climate change. *Nature* 427, 145–8.

Thomas, J. B., and Gilmour, K. M. (2012). Low social status impairs hypoxia tolerance in rainbow trout (*Oncorhynchus mykiss*). *Journal of Comparative Physiology B* 182, 651-662.

Timmerman, C. M., and Chapman, L. J. (2004). Behavioral and physiological compensation for chronic hypoxia in the sailfin molly (Poecilia latipinna). Physiological and Biochemical Zoology 77, 601-610.

Tine, M., Kuhl, H., Gagnaire, P.-A., Louro, B., Desmarais, E., Martins, R. S. T., Hecht, J., Knaust, F., Belkhir, K., Klages, S., et al. (2014). European sea bass genome and its variation provide insights into adaptation to euryhalinity and speciation. *Nature Communications* 5, 5770.

Ton, C., Stamatiou, D., and Liew, C. C. (2003). Gene expression profile of zebrafish exposed to hypoxia during development. *Physiological Genomics* 13, 97-106.

Tsuneshige, A., Park, S., and Yonetani, T. (2002). Heterotropic effectors control the hemoglobin function by interacting with its T and R states a new view on the principle of allostery. *Biophysical chemistry* 98, 49-63.

-U-

Ullal, A. J., Litaker, R. W., and Noga, E. J. (2008). Antimicrobial peptides derived from hemoglobin are expressed in epithelium of channel catfish (*Ictalurus punctatus*, Rafinesque). *Developmental and Comparative Immunology* 32, 1301-1312.

-V-

Val, A. L. (2000). Organic phosphates in the red blood cells of fish. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* 125, 417-435.

van Buskirk, J., and Steiner, U. K. (2009). The fitness costs of developmental canalization and plasticity. *Journal of evolutionary biology* 22, 852-860.

van de Pol, I., Flik, G. and Gorissen, M. (2017). Comparative physiology of energy metabolism: Fishing for endocrine signals in the early vertebrate pool. *Frontiers in Endocrinology* 8, 1–18.

van den Thillart, G. and Van Raaij, M. (1995). Endogenous fuels; non invasive *versus* invasive approaches. In: Hochachka, P.W. and Mommsen, P. (eds.). Biochemistry and Molecular Biology of Fishes, Vol. 4, Metabolic Biochemistry. Elsevier Science, Amsterdam, pp. 33-63.

van der Meer, D. L., van den Thillart, G. E., Witte, F., de Bakker, M. A., Besser, J., Richardson, M. K., ... and Bagowski, C. P. (2005). Gene expression profiling of the long-term adaptive response to hypoxia in the gills of adult zebrafish. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *289*(5), R1512-R1519.

Vanderplancke, G., Claireaux, G., Quazuguel, P., Huelvan, C., Corporeau, C., Mazurais, D., & Zambonino-Infante, J. L. (2015a). Exposure to chronic moderate hypoxia impacts physiological and developmental traits of European sea bass (Dicentrarchus labrax) larvae. *Fish physiology and biochemistry* 41, 233-242.

Vanderplancke, G., Claireaux, G., Quazuguel, P., Madec, L., Ferraresso, S., Sévère, A., Luis, J., Infante, Z. and Mazurais, D. (2015b). Hypoxic episode during the larval period has long - term effects on European sea bass juveniles (Dicentrarchus labrax). 162, 367–376.

van Tuyl, M. and Post, M. (2000). From fruitflies to mammals: mechanisms of signalling via the Sonic hedgehog pathway in lung development. *Respiratory research* 1, 30–5.

Varsamos, S., Diaz, J., Charmantier, G., Blasco, C., Connes, R. and Flik, G. (2002). Location and morphology of chloride cells during the post-embryonic development of the european sea bass, *Dicentrarchus labrax*. Anatomy and embryology, 205, 203-213.

Vazquez, A., Liu, J., Zhou, Y., and Oltvai, Z. N. (2010). Catabolic efficiency of aerobic glycolysis: the Warburg effect revisited. *BMC systems biology* 4, 58.

Vornanen, M., Stecyk, J. A. W. and Nilsson, G. E. (2009). The Anoxia-Tolerant Crucian Carp (*Carassius Carassius* L.). In *Fish Physiology Volume 27* (Richards, J. G., Farrell, a. P. and Brauner, C. J., Eds.) 397–441. Elsevier.

-W-

Wang, T., Lefevre, S., Thanh Huong, D. T., Cong, N. V. and Bayley, M. (2009). The Effects of Hypoxia On Growth and Digestion. In *Fish Physiology Volume 27* (Richards, J. G., Farrell, a. P. and Brauner, C. J., Eds.) 361–396. Elsevier.

Wang, Y., and Richards, J. G. (2011). HYPOXIA.Anaerobic Metabolism in Fish. Elsevier 1757-1763

Waser, W., and Heisler, N. (2005). Oxygen delivery to the fish eye: Root effect as crucial factor for elevated retinal PO2. *Journal of experimental biology* 208, 4035-4047.

Wawrowski, A., Gerlach, F., Hankeln, T., and Burmester, T. (2011). Changes of globin expression in the Japanese medaka (Oryzias latipes) in response to acute and chronic hypoxia. *Journal of Comparative Physiology B* 181, 199-208.

Weber, R. E. and Jensen, F. B. (1988). Functional adaptations in hemoglobins from ectothermic vertebrates. *Annual Review of Physiology* 50, 161–179.

Weber, R. E. and Fago, A. (2004). Functional adaptation and its molecular basis in vertebrate hemoglobins, neuroglobins and cytoglobins. *Respiratory Physiology and Neurobiology* 144, 141–159.

Weber, J. M., Choi, K., Gonzalez, A. and Omlin, T. (2016). Metabolic fuel kinetics in fish: swimming, hypoxia and muscle membranes. *Journal of Experimental Biology* 219, 250–258.

Wells, 2009. Blood-gas transport and hemoglobin function: adaptations for functional and environmental hypoxia. J.G. Richards, A.P. Farrell, C.J. Brauner (Eds.), Fish Physiology, Academic Press (2009), pp. 255-299

Weltzien, F., Doving, K. B., and Carr, W. E. (1999). Avoidance reaction of yolk-sac larvae of the inland silverside *Menidia beryllina* (Atherinidae) to hypoxia. *Journal of experimental biology* 202, 2869-2876.

Wenger, R. H. (2000). Mammalian oxygen sensing, signalling and gene regulation. *Journal of Experimental Biology* 203, 1253-1263.

Wheeler, T. and von Braun, J. (2013). Climate Change Impacts on Global Food Security. *Science* 341, 508–513.

Widmer, S., Moore, F. B. G. and Bagatto, B. (2006). The effects of chronic developmental hypoxia on swimming performance in zebrafish. *Journal of Fish Biology* 69, 1885–1891.

Wieser, W. (1995). Energetics of fish larvae, the smallest vertebrates. *Acta Physiologica* 154, 279-290.

Wikelski, M. and Cooke, S. J. (2006). Conservation physiology. *Trends in Ecology and Evolution* 21, 38–46.

Wikelski, M., Tarlow, E. M., Raim, A., Diehl, R. H., Larkin, R. P., and Visser, G. H. (2003). Avian metabolism: costs of migration in free-flying songbirds. Nature 423, 704-704.

Willett, C. E., Cortes, A., Zuasti, A., and Zapata, A. G. (1999). Early hematopoiesis and developing lymphoid organs in the zebrafish. *Developmental Dynamics* 214, 323-336.

Witeska, M. (2013). Erythrocytes in teleost fishes: a review. *Zoology and Ecology* 23, 275-281.

Wood, A. T., Clark, T. D., Andrewartha, S. J., Elliott, N. G. and Frappell, P. B. (2017). Developmental Hypoxia Has Negligible Effects on Long-Term Hypoxia Tolerance and Aerobic Metabolism of Atlantic Salmon (*Salmo salar*). *Physiological and Biochemical Zoology* 90, 494–501.

Wu, R. S. . (2002). Hypoxia: from molecular responses to ecosystem responses. *Marine Pollution Bulletin* 45, 35–45.

Wu, R. S. S. (2009). Effects of Hypoxia on Fish Reproduction and Development. In *Fish Physiology Volume 27* (Richards, J. G., Farrell, a. P. and Brauner, C. J., Eds.) 79–141. Elsevier.

-Z-

Zachos, J. (2001). Trends, Rhythms, and Aberrations in Global Climate 65 Ma to Present. *Science* 292, 686–693.

Zambonino-Infante, J. L., Mazurais, D., Servili, A., Cahu, C., Vanderplancke, G., Le Bayon, N., Huelvan, C. and Claireaux, G. (2017). An early-life hypoxia event has a long-term impact on protein digestion and growth in European sea bass juvenile. *The Journal of Experimental Biology* 220, 1846–1851.

Zhang, Y., Shao, Z., Zhai, Z., Shen, C., and Powell-Coffman, J. A. (2009). The HIF-1 hypoxia-inducible factor modulates lifespan in C. elegans. *PloS one* 4.

Zhang, J., Gilbert, D., Gooday, A. J., Levin, L., Naqvi, S. W. A., Middelburg, J. J., Scranton, M., Ekau, W., Peña, A., Dewitte, B., et al. (2010). Natural and human-induced hypoxia and consequences for coastal areas: Synthesis and future development. *Biogeosciences* 7, 1443–1467.

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