EFFECTS OF OCEAN ACIDIFICATION ON THE PHENOTYPIC PLASTICITY AND FUNCTIONAL PROPERTIES OF EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*) HAEMOGLOBIN

MASTER THESIS

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ADAPTATION, REPRODUCTION ET NUTRITION

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Content

Index of figures

Abbreviations

$\Delta Ct_{GOI(c)}$	mean ΔCt_{GOI} of treatment pH 8.0
ATP	adenosine triphosphate
Ct	cycle threshold
dph	days post hatching
E	efficiency
EF1	elongation factor 1-alpha
f	expression fold change
GOI	gene of interest
GTP	guanosine triphosphate

Hb	haemoglobin
HKG	housekeeping gene
n50	Hill coefficient
NTC	no template control
OEC	oxygen equilibrium curve
P50	P_{O2} at 50% protein saturation
P_{CO2}	carbon dioxide partial pressure
pH50	pH at P50 CO_2
P_{O2}	oxygen partial pressure
rpm	rounds per minute
RT-	negative reverse transcription product; without reverse transcriptase
RT+	positive reverse transcript product
Y	fractional saturation of Hb
λ	Wavelength
pHe	extracellular pH
pHi	intracellular pH

1 Abstract

Due to anthropogenic emissions, sea water pH has decreased about 0.1 units since preindustrial times, and will continue to decline up to 0.4 units by the end of this century. The acclimation capacity of marine life to ocean acidification is a subject of rising interest and importance, still studies on animals with well-developed ion-regulating capacities, such as fish, are comparatively scarce. In this study, acclimation capacities of European sea bass (*Dicentrarchus labrax*) haemoglobin (Hb) on a functional and molecular level have been researched, by means of oxygen equilibrium curves and qPCR. Fish were reared under three different environmental relevant CO_2 partial pressures, with a total incubation time of 33 months. Results show that neither oxygen tension at 50% protein saturation (P50) and cooperativity (n50), nor haematocrit and isoform expression levels change under environmental hypercapnia. The pH dependant O_2 affinity (pH50) was found to significantly decrease in both high CO_2 treatments. Since molecular analyses suggest that changes in pH sensitivity are not caused by a shift in Hb isoforms to more pH sensitive proteins, an over-compensatory blood alkalosis, followed by chronically elevated erythrocytic organic phosphate levels (GTP and ATP) are assumed. Data indicate that sea bass Hb is adapted to changes in sea water pH, which can be linked to its ecological niche in coastal and estuarine habitats, where fluctuations in abiotic parameters, such as pH, are frequent. Nevertheless, if ocean acidification causes a chronic elevation in organic phosphate levels, sea bass would be facing an energetically costly regulation to maintain respiratory homeostasis, and it would consequently reduce the overall fitness of the animal.

2 Introduction

Oceans cover over 70% of the earth's surface and play a major role in the regulation of the global climate. Because of their ability to absorb and transport heat from one place to another, and due to water evaporation and precipitation, oceans shape our climate. Furthermore, oceans mitigate atmospheric CO_2 concentrations, effectively acting as a carbon sink. This is particularly relevant since CO_2 concentrations have been increasing steadily since preindustrial times, due to emissions by human activities. Seawater absorbs about half of the anthropogenic CO_2 from the atmosphere (Sabine et al., 2004). In water, CO_2 forms carbonic acid (H_2CO_3), which mostly dissociates to bicarbonate ions (HCO_3^-), carbonate ions (CO_3^{2-}) and protons (H^+) (Figure 1). Due to this increase in seawater [H^+],

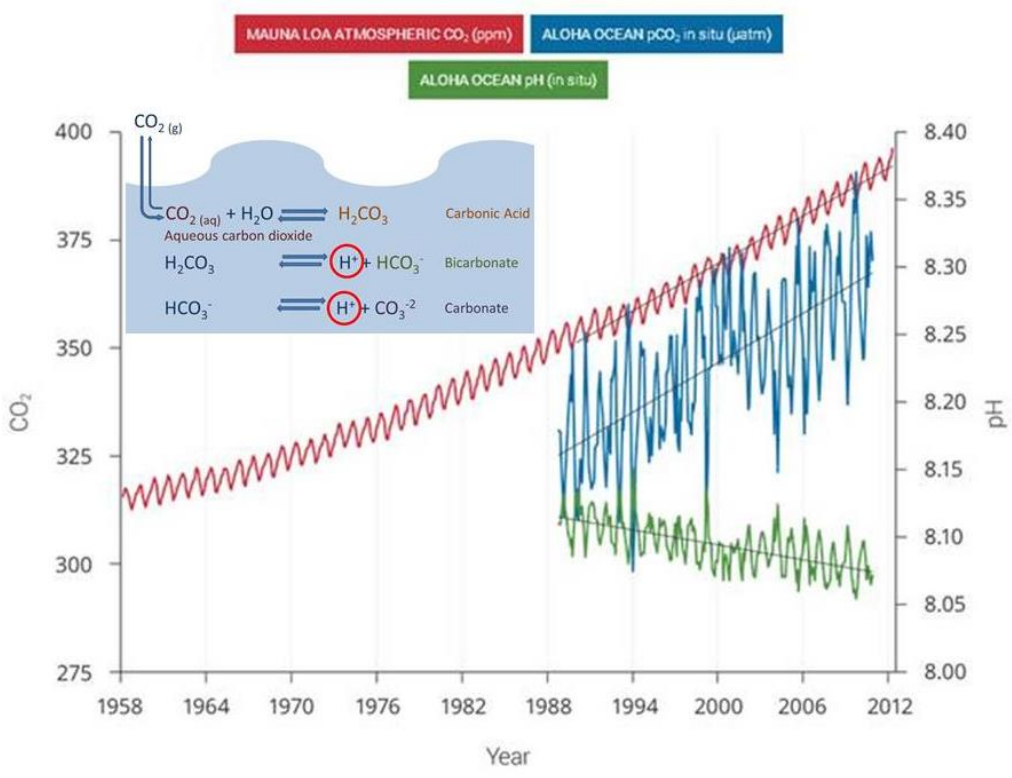

Figure 1: *Time series of atmospheric CO_2 at Mauna Loa (in ppmv) (red), surface ocean pH (green) and P_{CO2} (µatm) (*blue*) at Ocean Station ALOHA in the subtropical North Pacific Ocean, and schematic of the carbonate system species in seawater. Red cycled protons lead to a decrease in ocean pH (adapted after Doney et al. (2009) and http://snowcrablove.blogspot.de/2012/03/whats-ocean-acidification.html)*

the pH in ocean surface waters has already dropped by 0.1 during the last two decades (Chen & Millero, 1979; IPCC, 2014). If the current path of global CO_2 emissions is maintained, atmospheric CO_2 concentrations will reach between 800 (intermediate prediction “RCP6.0”) and 1200 ppm (high prediction “RCP8.5”) by the end of this century (IPCC, 2014). This will further reduce ocean pH levels by another 0.3-0.4 units compared to today (Caldeira & Wickett, 2005; Orr *et al.*, 2005). The process is called ocean acidification (OA) and together with ocean warming it poses an increasing threat to marine organisms, due to the change in physicochemical properties of the environment they inhabit. These alterations are projected to impact individual organisms, their population structure and size, community species composition, and therefore the structure and functioning of ecosystems (Pörtner & Knust, 2007). Growing scientific effort has been put into analysing the impact of OA on calcifying biota such as shellfish (Gazeau *et al.*, 2007), sea urchins (Havenhand *et al.*, 2008), and corals (Kleypas & Yates, 2009), whereas studies on the effect of elevated CO_2 concentrations on noncalifiers, including fish, are comparatively scarce. Since fish display well-developed ion regulation capacities, such as active proton transport (Deigweiher *et al.*, 2008; Melzner *et al.*, 2009), this group has been thought to be relatively unaffected by the predicated seawater pH change, caused by environmental hypercapnia. Thus, only a few studies focused on the long term effect of projected CO_2 concentrations on fish physiology (but see Deigweiher *et al.* (2008); Enzor *et al.* (2013); Frommel *et al.* (2012); Hoegh-Guldberg *et al.* (2007); Melzner *et al.* (2009); Munday *et al.* (2009)). However, early life stages of fish, including embryos (Dahlke *et al.*, 2016) and larvae (Frommel *et al.*, 2012; 2014; Stiasny *et al.*, 2016), might be a population bottleneck, since they still lack fully developed ion regulation capacities, and hence are particularly prone to OA related changes, such as environmental hypercapnia (Pörtner & Peck, 2010). But also adult fish exposed to OA scenarios experience strong physiological changes, such as transient blood acidosis, caused by increased body fluid CO_2 partial pressure (P_{CO2}) (Eddy *et al.*, 1977). In order to predict potential alterations in ecosystem structure it is essential to assess the acclimation capacity of fish of various life stages towards OA, as they account for over 50% of all vertebrates on earth and represent a highly diverse

taxon (Groombridge & Jenkins, 2002; Nelson *et al.*, 2016). Next to their ecological importance, the economic value of fish stocks is of major importance to our society. It is crucial to evaluate the ability of teleosts to respond to OA related changes, because the constantly growing fisheries and aquaculture sector provides income and livelihoods for more than 56 million people, which supply the world's population with 20 kg fish per capita per year. Globally, the trade of fish and fisheries products represents more than 9 percent of total agricultural exports, which translates into US$ 148 billion (FAO, 2016).

2.1 Study animals

Along the North Sea and the Atlantic coast, European sea bass (*Dicentrarchus labrax,* Figure 2) represents a temperate fish species with high economic and cultural value.

Figure 2: *Dicentrarchus labrax, European sea bass (Linnaeus, 1758)*

Sea bass is an euryhaline species, which is primarily ocean-going, but can also frequently be found in brackish and fresh waters along lagoons, estuaries, and rivers. Its biology, in particular its slow growth rate, temperature dependent recruitment and schooling behaviour inshore and offshore (Pickett & Pawson, 1994), makes it a particularly vulnerable species to overfishing. Together with an increased market demand, the natural and anthropogenic pressures on European sea bass have led to a 60 percent decline in stock during the last 6 years (ICES, 2016). The knowledge of underlying physiological processes contributing to potential acclimation to OA related

changes is crucial for any approach to sustainable fisheries management of this species.

Similar to thermal effects (Pörtner, 2002), influences of hypercapnia might show hierarchical differences from whole-animal to cellular and even molecular levels. The highest sensitivity might be seen in whole-organism-functioning, declining towards cellular and molecular levels (Pörtner, 2008). It is still important to identify and integrate responses of molecular mechanisms into whole-animal function in order to predict the effects of OA upon single species and eventually ecosystems.

2.2 Functional properties of haemoglobin (Hb)

Respiratory pigments, such as haemoglobin (Hb), embody unique complexes for studying acclimation capacities, as these oxygen-carrying proteins link ambient conditions to metabolic oxygen demands. In cells, oxygen acts as the final electron acceptor in oxidative catabolic reactions (Giardina *et al.*, 2004), and therefore Hb and its capacity to deliver oxygen to the tissues is elementary for survival of most vertebrates species. Hb is a tetrameric protein, with two α and two β globin chains, each bearing a haem group (de Souza & Bonilla-Rodriguez, 2007). This iron carrying porphyrin ring reversibly binds oxygen without a valence change of the iron atom, thus the bond is an oxygenation (not an oxidation), and enables the Hb to carry 4 O_2 molecules at a time (Heldmaier & Neuweiler, 2013). Haemes in all fish studied so far are identical. Globins on the other hand differ from species to species and between isoforms (de Souza & Bonilla-Rodriguez, 2007). By changing the spatial position of the haem group, the oxygenation can be regulated by the globin chains, and thus either strengthen or weaken the haem-oxygen bond. The four subunits of the Hb cooperate during gas exchange in order to facilitate oxygen discharge when oxygen partial pressures (P_{O2}) are low. With higher P_{O2}, at respiratory tissues for example, oxygen uptake is facilitated (Heldmaier & Neuweiler, 2013). The cooperativity is realized by a conformation change of Hb during oxygenation from the T ('tense') state, with low oxygen affinity, to the R ('relaxed') state, at which oxygen affinity is high (Monod *et al.*, 1965). The cooperative oxygen binding reaction can be described by sigmoidal O_2 equilibrium curves (OECs, Figure 3). The P50 (the P_{O2} at 50%

protein saturation) provides information about how readily the protein takes up or discharges the oxygen, and can be derived from the OECs. Furthermore the Hill coefficient n50 enables cooperativity comparisons between species and individuals, by defining the sigmoidal shape of the OEC (Heldmaier & Neuweiler, 2013), and thus enables the quantification of the degree of interaction between O_2 and its Hb binding sites.

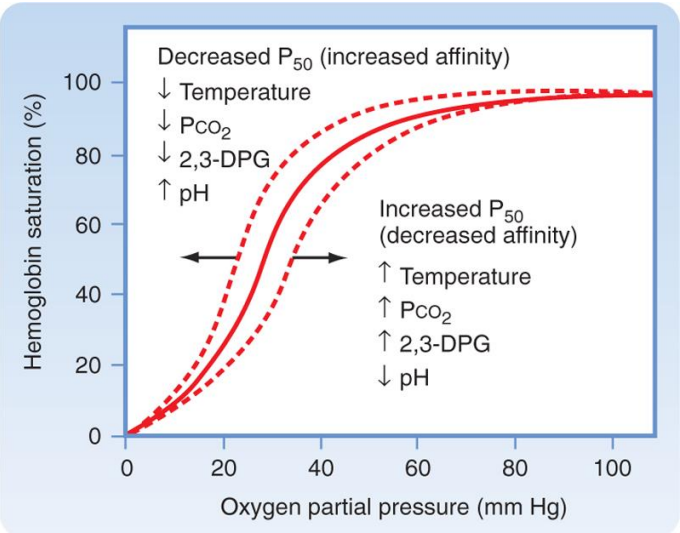

Figure 3: *Oxygen equilibrium curves (OECs), some allosteric effectors and the temperature effect on haemoglobin-oxygen affinity. Black arrows indicate the P50 shift towards the right or left (Koeppen & Stanton, 2009)*

Oxygenation of Hb is not only dependant on the available O_2, but is also closely linked to temperature. The oxygenation reaction is exothermic (Weber & Fago, 2004), thus when temperature rises, Hb-oxygen affinity decreases, reducing the oxygen uptake in the respiratory organs, and facilitating the oxygen discharge, and therefore changing the oxygen availability in the tissues (Weber & Campbell, 2011). Furthermore, the affinity to bind oxygen can be modulated by allosteric effectors, such as protons and CO_2. Both increasing P_{CO2} and decreasing pH shift the OEC and the P50 value towards higher blood P_{O2} levels and thus facilitating oxygen discharge (Bohr *et al.*, 1904). This so called Bohr effect is particularly important for the acute regulation of tissue oxygen supply. If the metabolic rate in tissues is elevated, the formation of CO_2 and therefore H^+ concentrations in tissues increases, which leads to a decrease in Hb-oxygen affinity and therefore a greater O_2 discharge to compensate

for the higher demand (Heldmaier & Neuweiler, 2013). Thus, the assessment of blood pH during OEC measurements provides information about the blood oxygen affinity and can yield the blood pH at half saturation (pH50). Transient environmental hypercapnic blood acidosis might lead to a reduced Hb-oxygen affinity and thus progressively reduces tissue-oxygen supply, which leads to a lower oxygen availability for mitochondria (Eddy *et al.*, 1977). Such acute responses are comparatively well studied (Cameron & Iwama (1987); Cameron & Randall (1972); Claiborne & Heisler (1986)). Teleostean fish generally exhibit a graded acidosis, partly or fully compensated for by an elevation of plasma bicarbonate levels (Brauner & Baker, 2009; Lloyd & White, 1967). This species-specific response varies in time and magnitude and depends on acid-base relevant ion-transfer mechanisms (Eddy *et al.*, 1977). Moreover, fish can fine-regulate the intracellular pH of red blood cells by the release of catecholamines, during early phases of metabolic hypercapnia, triggered by falling blood oxygen tensions due to elevated [H^+] (Perry *et al.*, 1989). In fish adenosine triphosphate (ATP) and guanosine triphosphate (GTP) (Johansen *et al.*, 1975), as well as in a few species also 2,3 bisphosphoglycerate (2,3 BPG) (Val, 1993) impart allosteric effects on Hb, reducing its O_2-affinity. Regulative mechanisms, such as the short term responses towards elevated CO_2, are energy consuming and maintaining them for longer periods of time would consequently reduce the fitness of the organism. Although high sensitivity to long term exposure is emphasized by some studies, most studies only analysed the effects of short- to medium-time exposure to hypercapnia (hours to a couple of weeks). Nevertheless, long-term studies are crucial to assess the patterns of sensitivity and the capacity for acclimation and adaptation of fish towards OA in order to support long-term management strategies.

2.3 Differential isoform expression

As a result of the Hb subunits showing the ability to interact with one another, new oxygen binding properties emerge (Brittain, 2005). In acclimating to OA, the high Hb polymorphism of fish could be an essential property. The isoforms can show marked differences in oxygen binding properties and sensitivity to allosteric factors, such as

protons, which ensure functional diversity and oxygen supply at various gas tensions (Binotti *et al.*, 1971; di Prisco & Tamburrini, 1992; Weber, 1990). Fish might ensure a better capacity of gas transportation during environmental variation, as they account for the highest number of Hb isoforms among vertebrates (Giardina *et al.*, 2004). Thus, Hb heterogeneity could be assumed to foster a selective advantage in changing environments. A very high genotypic plasticity has been observed for fish, which translates into modifications in their genetic material. These express in varying degrees in dependence of fluctuating environments (de Souza & Bonilla-Rodriguez, 2007). Due to the acclimation to different temperatures (Fago, Forest*, et al.*, 2001; Houston & Cyr, 1974) as well as hypoxia (Wells, 2009), a variation in Hb patterns of fish was found. However, neither the role of Hb isoforms in acclimation to different $P_{CO2,}$ nor the linkage of those expression levels to the functional properties of the respiratory pigments have been thoroughly explained .

The species *D. labrax* possesses 14 genes translating into different globins (Cadiz *et al.*, submitted May 2016), which could potentially foster acclimation capacity Assuming that shifts in Hb inventory are triggered by gene expression levels, the relative quantity of Hb subunit mRNA was analysed for this thesis.

In a second part, this study also looked at the effects of long term exposure to OA on functional properties of Hb from the economically important European sea bass, *D. labrax.* By trying to link the functional characterisation of Hb to the expression levels of its isoforms, an insight in the acclimation capacities of *D. labrax* in the light of ocean acidification was gained.

3 Materials and Methods

Fish were reared and kept at the PFOM-ARN facilities, Ifremer Campus, Plouzané, France. All experiments were conducted at the same location.

3.1 Experimental animals

European sea bass larvae (2- dph), originating from a domesticated population were obtained in October 2013 from a commercial supplier. 24000 larvae were randomly distributed to 12 rearing tanks (2000 larvae/tank), which were supplied with thermoregulated natural sea water (T = 15°C) with salinity = 34±1. Before entering the 12 rearing tanks, sea water flowed through 3 header tanks, each linked to 4 rearing tanks. There it was equilibrated with different amounts of CO_2, controlled by a pH-feedback system (iks Computer Systeme GmbH, Karlsbad, Germany).The pH-feedback system maintained the desired seawater pH by opening/closing a solenoid valve (Aqua Medic GmbH, Bissendorf, Germany) coupled to a bottle of pure CO_2. Three water P_{CO2} levels were adjusted, namely 400 µatm, 800 µatm, and 1200 µatm, representing average current ocean conditions, intermediate (RCP6.0) and high (RCP8.5) prediction for oceanic CO_2 partial pressures for the year 2100 (IPCC, 2014), respectively. The resulting water pH of approximately 8.0, 7.8, and 7.6 (±0.05 pH units, NIST scale), was checked daily with a WTW 340i pH meter and a WTW SenTix 81 electrode, which have been calibrated with NIST precision pH buffers (WTW, Weilheim, Germany) prior to each measurement. Seawater carbonate chemistry parameters were computed from the measured water pH and alkalinity values adopted from Clarke (Gaston *et al.*, 2009), using the CO2SYS macro for Microsoft Excel (Pierrot *et al.*, 2006) and the dissociation constant by Mehrbach (1973). Water pH was measured daily and alkalinity was measured weekly until sampling. Larvae were fed daily with live prey (Rotifera/*Artemia*) or a commercial diet (Le Gouessant, St Brieuc, France) by an automatic feeder. After metamorphosis to juvenile stage, fish were PIT tagged and moved to a room similar to the one mentioned afore, but equipped with 12 x 500 L tanks connected to the same pH controlling setup as during the larval phase. Here, seawater was not thermocontrolled, to ensure environmental fluctuation and a natural development of experimental animals.

Upon reaching an appropriate size, a subsample of fish was transferred to 3 larger tanks (1880 L, Figure 4) to ensure a proper fish density in the keeping facilities.

Figure 4: *Adult fish keeping facilities at the PFOM-ARN, Ifremer Campus, Plouzané. Each tank is equipped with an automatic feeder and is connected to a seawater flow through system. Sea water pH is held constant by equilibration with pure CO_2 (bottle on the left).*

The tanks were similarly equipped as the larval and juvenile tanks and water was again not thermocontrolled. The photoperiod was set to 12 h/day. Animals were fed daily with the commercial food mentioned before by means of an automatic feeder, but were starved 48 h before sampling. The final fish density at the day of sampling was 27.79 ± 1.29 kg/m^3. The total incubation period until sampling was 33 months.

3.2 Sampling & specimen preparation

Blood samples were taken in June 2016, at a water temperature of 16.4°C. Prior to sample collection, 8 PIT tag numbers per treatment were chosen at random in order to prevent selective sampling. Syringes were heparinised beforehand to prevent blood coagulation in the syringes and needles, by filling and ejecting heparin (5000 U/ml) three times. Syringes were kept on ice until sampling. Subsequently, all animals were anesthetised with tricaine mesylate (0.3 ppt; MS-222) to avoid excessive stress and resulting elevated haematocrit levels (Biron & Benfey, 1994). Fish with the respective tag numbers were selected, and total length, as well as weight were measured (mass 384.63 ± 90.94 g; total length 29.72 ± 2.6 cm). From each individual a blood sample of approximately 1 ml was taken quickly (under 2 min) through caudal vein punctuation and split for haematocrit determination, molecular

analysis, and for oxygen equilibrium curves (OECs). For haematocrit determination 25 µl of blood was drawn into a heparinised haematocrit capillary, and one end was sealed with Critoseal wax. Immediately after collection, the capillary was centrifuged for 5 min at 10500 rpm in a Sigma 201m haematocrit centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). Haematocrit reads were made with the aid of a haematocrit reader and are expressed as percent of total blood volume. For OEC analyses 300 µl of whole blood were frozen in liquid nitrogen and stored at -80°C for haemolysate preparation. Approximately 400 µl of blood was used to prepare plasma. Therefore, the fresh blood was centrifuged at 1000 g for 4 min in a Sigma 3-30k centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) and supernatant was collected and also frozen at -80°C (for dilution with haemolysate; see section 3.3). Moreover, 50 µl of blood was mixed with 250 µl of lysis buffer DL (from NucleoSpin RNA Blood kit; Macherey-Nagel GmbH & Co KG, Düren, Germany) and 200 µl of phosphate buffered saline. Due to unforeseeable difficulties during RNA extraction, blood sampling for RNA had to be repeated in August 2016 on the same individuals at a water temperature of 17.9°C

3.3 Functional properties of Hb

In order to detect possible changes in the oxygen carrying capacity of Hb from fish reared under OA scenarios, OECs of haemolysate were obtained through spectrophotometry. According to the model by Monod *et al.* (1965), Hb has two different conformations depending on the oxygen saturation: the ‘tense’ state (T) predominates in deoxygenated, whereas the ‘relaxed’ state (R) prevails in oxygenated Hb. This conformation change upon oxygen binding alters the light absorption spectra of the protein (Figure 5).

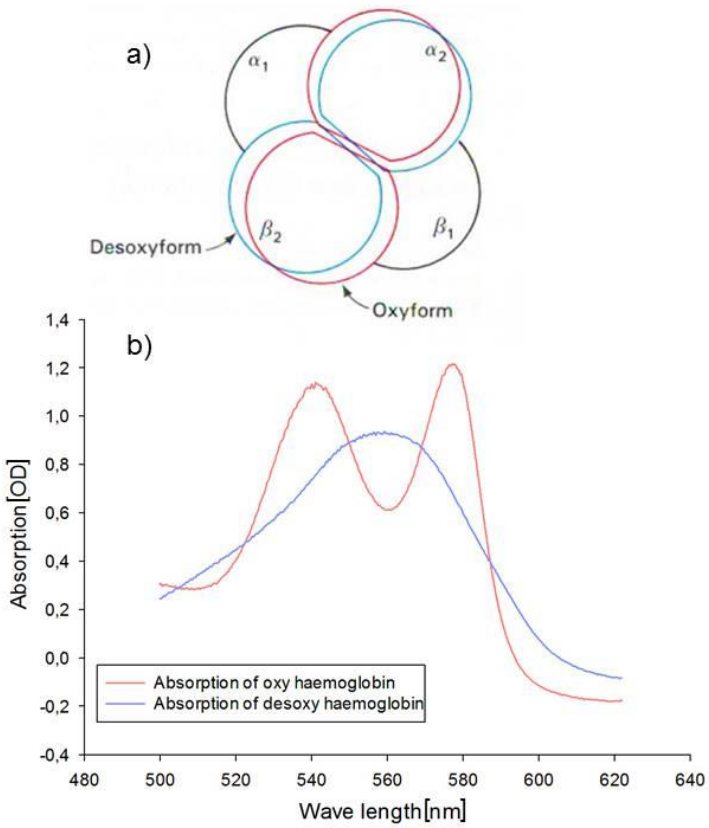

Figure 5: *a) schematic of quaternary structure change (a 15° rotation of one αβ- pair) upon oxygenation of haemoglobin (Hb) (after Stryer (1990); blue= 'tense' deoxyHb, red= 'relaxed' oxyHb b) Absorption spectra of European sea bass (Dicentrarchus labrax) haemolysate under pure oxygen (red) and pure nitrogen (blue) exposure*

Therefore the oxygenation status of the blood at different oxygen tension can be detected by spectrophotometry (Reeves, 1980), revealing changes in Hb oxygen affinity among the different treatments.

The present study made use of a method to measure pigment absorbance and pH simultaneously in a micro volume of blood under strictly controlled physicochemical condition, i.e. at two temperatures and stepwise changing nitrogen, oxygen and carbon dioxide partial pressures. OECs and blood parameters, including P50 and n_{50} were obtained by the stepwise increase in O_2 tension in equilibrating gas mixtures while maintaining P_{CO2} constant (experimental protocol 1) (Wells & Weber, 1989). Furthermore, OECs were recorded under constant P_{O2} and the stepwise increase of P_{CO2} (experimental protocol 2) (Pörtner, 1990) to obtain values for pH50, and gain a more physiological trajectory of O_2 binding that resembles pH changes *in vivo*. Since the absorbance of fresh blood would be too high to receive a clear spectrum, the sample needed to be treated as follows: 300µl of full blood and plasma were thawed

for each animal. In order to prevent amplitude perturbation due to cell debris originating from partial lysis during freezing, the thawed blood was centrifuged at 12000 g for 20 min (Weber *et al.*, 2010). Supernatant was collected and diluted with the same volume of corresponding plasma, in order to decrease light absorbance and therefore enhance signal strength during spectrophotometry. Furthermore, 5 µl of heparin (5000 u/ml) were added to prevent coagulation. Diluted haemolysate was aliquoted to 4 x 50-100 µl and refrozen at -80°C until measurements. No buffers were added (e.g. Tris, or HEPES (Brix *et al.*, 1995)), as these would inhibit pH changes during measurements and therefore prevent physiological changes relevant for the *in vivo* oxygen transport (Oellermann *et al.*, 2014).

3.3.1 Experimental set-up and protocol

In order to assess the oxygen carrying capacity of sea bass blood by means of OECs, a modified diffusion chamber (Eschweiler Co., Kiel, Germany) was used. For detailed description see Oellermann *et al.* (2014). During measurements the chamber was continuously perfused with defined gas mixtures (e.g. Weber *et al.* (2010)). An automatic gas mixing system (GMS, Loligo Systems, Aarhus, Denmark) containing four mass flow regulators, connected to one of the gas humidifiers of the chamber, ensured the desired gas concentrations. A LAUDA Ecoline Staredition RE104 thermostat (LAUDA Dr. R. Wobser GmbH & Co. KG, Lauda-Königshofen, Germany) was connected to the chamber and pumped thermostatted aqua bidest. through the water reservoir of the chamber during measurements. Temperature was constantly monitored with a checktemp 1 thermometer (Hanna Instruments, Limena, Italy) introduced in one of the gas humidifiers not used as part of the gas mixing procedure. As light source, a ^{2}H-halogen lamp (DT-Mini-2-GS, Ocean Optics, Winter Park, USA) was connected via fibre-optic cables to the chamber and directed the light beam through collimating lenses through the central cylinder containing the sample holder to a broad range fibre-optic spectrometer (λ = 200-1100 nm; USB 2000+, Ocean Optics, Winter Park, USA). The spectrophotometer was calibrated before each measurement run by acquiring a light (inserted sample holder without sample and switched on light) and a dark spectrum (light beam was blocked by an integrated shutter). To be able to measure sample pH simultaneously, a modified

plastic slide holder was used, housing a syringe containing a fibre optic micro-pH optode (NTH-HP5-L5-N5*25/0.8OIW, Regensburg, Germany, Figure 6; Oellermann *et al.* (2014)).

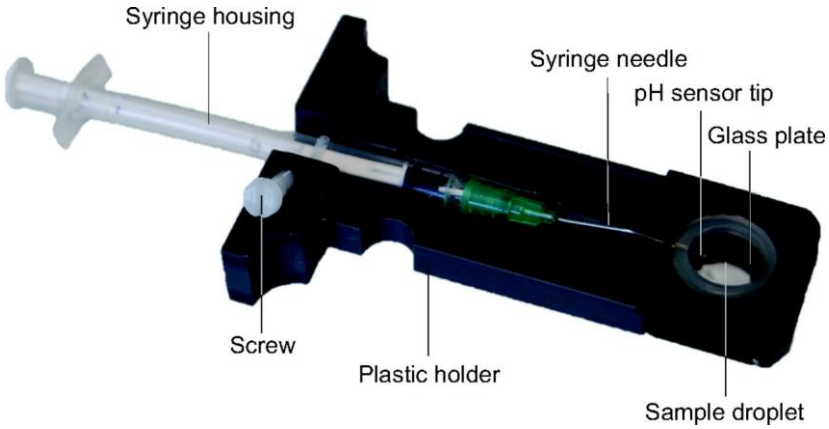

Figure 6: *Illustration of modified plastic holder. The syringe houses a pH micro optode, which can be inserted in a droplet of blood. The holder can be inserted in the gas tight compartment inside the diffusion chamber (Oellermann et al., 2014)*

The sensor tip is smaller than 150 µm, which makes pH measurements in minute sample volumes possible, and is covered with a combination of fluorescent indicator dyes. Protons react with the dyes on the sensor tip and thus change the emission spectra of the sensor, which can be detected by a phase detection device (µPDD 3470, PreSens, Regensburg, Germany). The pH optode accuracy is ±0.05 pH units (PreSens, 2004). To prevent gas leakage, the needle of the syringe was inserted through a silicone ring. Prior to each experimental run, the sensor had to be calibrated via a six-point-calibration in the relevant pH rage (6.8-8.4) and at the corresponding measurement temperature. The buffers consisted of 1:2 diluted filtered sea water (salinity 34) with aqua bidest., and were buffered with MOPS [40 mM, 3-(N-morpholino)-propanesulfonic acid]. Buffer pH was adjusted using 1 M NaOH, and was checked with a WTW SenTix 81 pH electrode connected to a WTW 340i pH meter (both WTW, Weilheim, Germany) before each experimental run, which was calibrated by a two point calibration in low strength (DIN/NIST) technical buffers (WTW, Weilheim, Germany). OEC data were recorded using the blood oxygen binding system software (Loligo Systems, Aarhus, Denmark).

The sample was slowly thawed on ice, vortexed and quickly spun down to ensure a homogeneous mixture during the measurement. A thin 10 µl droplet of Hb-solution

was applied on a glass slide without touching the sealing ring. The tip of the calibrated pH sensor was introduced at the edge of the droplet, in order to protect the sensor tip from photo bleaching. Then the sample slide holder including the pH sensor and the sample glass slide was pushed in the gas chamber. Integration time was set to 500 ms and the moving averages were adjusted to 20, as these settings were found most suitable for sea bass blood during preliminary experiments. OECs were obtained for each fish according to the two aforementioned experimental protocols and at two temperatures, i.e. 15 and 20 °C. In experimental protocol 1) the P_{CO2} was set to 1.3 kPa, resembling venous blood (Petochi *et al.*, 2011), which was chosen to gain an understanding of the ability of sea bass Hb to deliver O_2 to tissues. The P_{O2} on the other hand increased stepwise every 10 min to allow the sample to equilibrate to each O_2 tension (0, 1, 2, 4, 7, 10, 14, 21 kPa). For protocol 2) the P_{O2} was maintained at 3 kPa, as preliminary experiments revealed this to be the threshold oxygen tension, where sea bass Hb can still be fully oxygenised. P_{CO2} was increased stepwise from 0 to 6 kPa in 15 distinct steps (i.e. 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.75, 4.5, 5.25, and 6), leaving 10 min between each step for equilibration. Each experimental run was preceded and followed by a 100% (10 min) and a 0% (98% N_2, 2% CO_2 for 30 min, 100% N_2 for 10 min) oxygen calibration, in order to detect the drift of the maximum absorbance signal. A total of 4 OECs were obtained per fish which translates to a total of 96 curves for this study.

3.4 Differential isoform expression

In order to quantify globin isoform transcript levels, a real-time quantitative polymerase chain reaction (qPCR) on whole, frozen blood was performed. We chose blood for RNA extraction, because it contains a high amount of erythroid cells, which are one of the main Hb formation sites in fish (de Souza & Bonilla-Rodriguez, 2007). Furthermore taking blood represents a non-lethal sampling method, and experimental animals can still be used for other studies.

A qPCR is a biochemical method for nucleic acid proliferation, and can be used to quantify transcript levels of a target gene (defined by primers used). The underlying

assumption is that the amount of mRNA in a sample is proportional to the amount of protein generated (Figure 7).

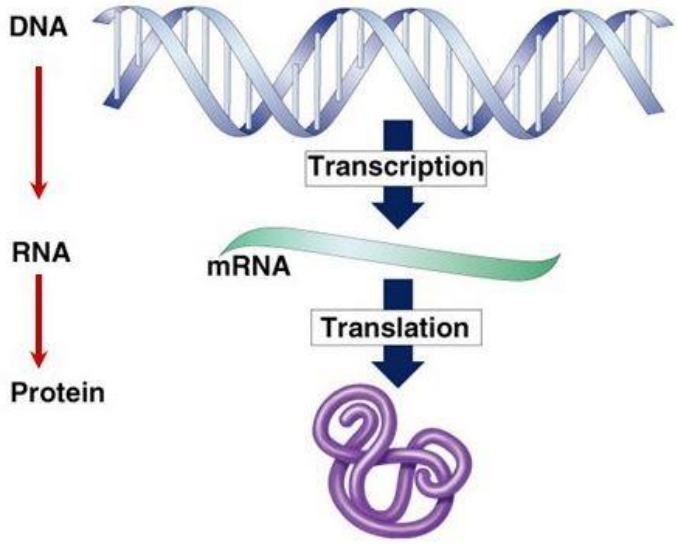

Figure 7: *Sketch summarising protein biosynthesis. The amounts of mRNA and generated protein are directly linked (Reece et al., 2011)*

To be able to quantify the transcript, mRNA needs to be translated back into cDNA by a reverse transcriptase, which can then be proliferated and relative quantities can be determined. Quantification is made by fluorescence measurements, which are recorded during PCR cycles in real time. To be able to detect the DNA proliferation, fluorescent DNA dyes (e.g. SYBR Green) are added. Under optimal conditions, cDNA quantity doubles during one thermal cycle (denaturation, annealing, and elongation). The dyes are deposited on the DNA during elongation, which enhances their fluorescence. The increase of stained target DNA is thus correlated to the increase in fluorescence from cycle to cycle. The quantity of cDNA is expressed relative to a stable, treatment unaffected expression of a reference gene (housekeeping gene, HKG). This method allows for conclusions on the initial mRNA quantity of the target gene in the examined tissue, which as mentioned before, is in turn related to the amount of specific protein.

3.4.1 RNA extraction, rDNase treatment, and RNA precipitation

RNA from sea bass was extracted using the NucleoSpin RNA Blood kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) with some modifications to the manufacturer's protocol. The kit was specifically designed for mammalian blood cells, which in contrast to fish erythrocytes do not contain nuclei. Fish erythrocytes thus called for adjustments in the extraction protocol. As preliminary experiments showed, the blood columns, provided in the kit, clogged when using a sample-lysis buffer DL ratio of 1:1. In order to prevent congestion due to only partial lysis of the erythrocyte nuclei, the samples were frozen as mentioned above (see section 3.2), to achieve a sample lysis buffer ratio of 1:5. All following steps were conducted at room temperature if not stated otherwise. The samples were thawed and 6.25 µl of ProteinaseK per sample was added (instead of 5 µl, as suggested by the manufacturer), to ensure a complete protein digestion in the samples, followed by 15 min of vigorous shaking on a thermomixer C (1400 rpm; Eppendorf, Hamburg, Germany). To clean the lid of the sample tubes, they were centrifuged briefly. Afterwards, 250 µl (instead of suggested 200 µl) of 70% ethanol was added and the mixture was vortexed, to ensure appropriate RNA binding conditions and to favour adsorption of RNA to the silica membrane of the column. Then, 610 µl of lysate was transferred into a column, which was placed in a collection tube and centrifuged for 1 min at 11000 g in a Sigma 3-30k centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). Flow-through was discarded and the column was placed in a new collection tube. In order to desalt the silica membrane, 350 µl of membrane desalting buffer (MDB) was added, and column was again centrifuged for 1 min at 11000 g. Subsequently 95 µl of rDNase was added and tubes were incubated for 15 min at room temperature to ensure a complete digestion of genomic DNA (gDNA), followed by an addition of 200 µl of buffer RB2 to inactivate the rDNase. The column was then centrifuged for 1 min at 11000 g, flow through was discarded and column was put in a new collection tube. Afterwards two washing steps with buffer RB3 followed. First 600 µl were added and again centrifuged at 11000 g for 1 min, flow-through was discarded, and the column was placed in a new collection tube, followed by adding 250 µl and centrifugation for 2 min at 11 000 g, and flow-through was again discarded. The column was placed in a 1.5 ml collection tube. Thereafter RNA was eluted by adding 60 µl of RNase-free H_2O onto the column

and centrifuging for 1 min at 11000 g. The column was discarded and the tube was stored on ice.

Preliminary experiments revealed an only partial gDNA digestion, therefore an additional DNA digestion followed by a RNA precipitation method had to be performed. The digestion was conducted according to the manufacturer's protocol: 3 µl of rDNase were added to the eluted RNA (0.5 µl/10 µl eluted RNA) and the tube was incubated for 20 min at 37°C. To remove the remaining rDNase, a RNA precipitation was performed. Therefore, the samples were frozen at -80°C over night, thawed and then kept on ice. The sample volume was adjusted to 150 µl by adding RNase free H_2O. Afterwards, 30 µl of 2 M potassium acetate and 450 µl of absolute ethanol was added, samples were vortexed, and frozen at -80°C over night. Then, samples were thawed on ice and centrifuged at 7500 g and 4°C for 5 min, afterwards the supernatant was removed. Later, the precipitate was rinsed with 500 µl of ice cold 75% ethanol, vortexed, again centrifuged at 7500 g and 4°C for 5 min, and supernatant was again removed. This procedure was repeated once. Tubes were then left open at room temperature for 15 min to ensure full evaporation of remnant ethanol. Finally, the pellet was re-suspended in 60 µl nuclease-free H_2O, vortexed, quickly spun down, to clean the lid of the tube, and kept on ice. RNA concentration and contamination with protein as well as ethanol, was checked in 1.5 µl of sample by means of a ND-1000 Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA), and all samples were diluted to the lowest RNA concentration detected (80 ng/µl). One sample possessed a very low RNA concentration (Fish 16; < 10 ng/µl) and therefore had to be excluded from the study. 2 µl were taken in order to analyse the sample for RNA integrity in an Agilent Bioanalyzer 2100 136 (Agilent Technologies Inc., Santa Clara, USA). Analysis was conducted according to the manufacturer's protocol. If the RIN (RNA integrity number) was ≥ 7, the samples were aliquoted to 2 x 10 µl and frozen at -80°C.

***Table 1**: Gene loci, Isoform name given for this study, annealing temperature, and primer sequences used in this study*

Gene locus	Isoform name	Annealing temperature [C°]	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
LA-Hbα1	a4	60	TTTCCCATGAGAGAGCAGGT	TCAGATGCGCTTCTTAGGATGT
LA-Hbα2	a3	60	CAGTGGGACAGGATCTTGAAGT	GGTGATGGGTGGAATCAATC
MN-Hbα1	a1	60	GGCCAGGATGCTGACTGTA	CCAGCAAGGTCATCCATCTT
MN-Hbα2	a2	60	CCTGCCAACTTCAAGATTCTG	TTTCTCAGACAAGGCACGAG
MN-Hbα3	-	70	ACAGACAAGATGACCAGTCTCACT	GCCAATGTCCTCTGCCTTC
MN-Hbα4	-	70	ACAGACAAGATGACCAGTCTCACT	GCCAATGTCCTCTGCCTTT
MN-Hbα5	-	70	ACAGACAAGATGACCAGTCTCACA	GCCAATGTCCTCTGCCTTC
LA-Hbβ1	ß2	60	CCCGACAACTTCAAACTGCT	CCTGCGTCTCTGGTGTGAAG
MN-Hbβ1	ß1	60	TGATTTGAGCAAAGATCCTGAA	CATGGACGACATCAAGAACG
MN-Hbβ2	ß3	60	GTCAGCCAGCAGCCTGAAAT	GCAGCTCTTTCCAGGTGTCT
MN-Hbβ3	ß4	60	CAGAAGCTTTGGCAAGAGTG	GCTGCTACTTTGGCGTTACC
MN-Hbβ4	-	60	GTCGTTTACCCCTGGACTCA	GTTTTGCGACCATCGGATTT
MN-Hbβ5	-	62	ACCATCCAGGACATCTTCTCT	GTTTTGCGACCAACGGATTC
MN-Hbβ6	-	62	ACCATCCAGGACATTTTCTCC	GTTTTGCGACCAACGGATTC
Ef1	-	60	GCTTCGAGGAAATCACCAAG	CAACCTTCCATCCCTTGAAC
28 S	-	60	CAAGAACATCCAGCTGCTGAC	GGTGATATGTCGGCCATAAA

Reverse transcription and qPCR

Reverse transcription (RT+) and negative reverse transcription (RT-) were performed for all samples. RT- is a RT without the enzyme reverse transcriptase, used to see possible contamination by genomic DNA later during amplification. An iScript 140 cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, USA) was used for conducting the RTs. 6.25 µl of sample were taken for RT+ (to reach a RNA quantity of 500 ng) and mixed with 4 µl 5x iScript reaction mix, 8.75 µl Nuclease-free H_2O and 1 µl of iScript reverse transcriptase. Another 6.25 µl of sample were taken to prepare the RT- by adding 4 µl 5x iScript reaction mix, and 9.75 µl nuclease-free H_2O. Both RTs were performed in a Thermo8 cycler TC-152 (Techne Barloworld Scientific, Staffordshire, UK) and the reaction protocol was set by the manufacturer (5

min at 25 °C (incubation), 30 min at 42 °C (reaction), 5 min at 85 °C (inactivation). The obtained cDNA was kept at -20°C until further procession.

Before the analysis of relative expression levels of Hb isoforms, a linear standard range was made on 14 Hb genes and 2 HKG (EF1 (elongation factor 1-alpha) and 28S), to test the functionality and efficiency of primer pairs, and to choose the appropriate sample concentration. Therefore, a 4-step dilution series of a sample pool for each gene was run in a qPCR system. For this procedure, 2 µl of each RT+ were pooled resulting in 48 µl of sample pool. Then, it was diluted with 432 µl of nuclease-free H_2O resulting in a dilution of 1:10. 160 µl of the 1:10 dilution was taken and further diluted with 320 µl nuclease-free H_2O to produce a dilution of 1:30. This step was repeated to obtain 1:90 and 1:270 dilutions. To prepare the qPCR reaction mix, a SsoAdvanced Universal SYBR Green Supermix kit (Bio-Rad Laboratories Inc, Hercules, USA) and primers [10µM] for 14 sea bass Hbs and 2 HKG designed by Cadiz *et al.* (submitted May 2016) and manufactured by AnaSpec (AnaSpec Inc., Seraing, Belgium) were used (Table 1). When possible, primer sequences were designed to lie in the inter-exon junction, to minimize gDNA amplification. Each dilution was run in triplicates in a final well volume of 15 µl, composed of 5 µl diluted sample, and 10 µl of reaction mix, itself containing 0.5 µl of respective forward and reverse primer, 1.5 µl nuclease-free H_2O, and 7.5 µl iQ SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, USA). Supermix contained antibody-mediated hot-started iTaq DNA polymerase, dNTPs, $MgCl_2$, SYBR Green I dye, enhancers, stabilisers, and fluorescein. A linear standard range was run for 14 genes, each in four dilutions on 96 well plates. No template controls (sample substituted by nuclease-free H_2O; NTCs), and a pool of RT+ (diluted 1:10) were systematically included for each gene to ensure absence of gDNA. Moreover, a positive control (*D. labrax* kidney from a former study) was included to reveal possible pipetting errors. After preparation, the plate was sealed and centrifuged several seconds in a PCR Plate Spinner Centrifuge (MIDSCI Inc, St. Louis, USA), placed in an iCycler MyiQ Single Colour Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, USA) and reaction was started. The qPCR profiles contain an activation step (95°C for 2 min), followed by 39 cycles, including 5 s at 95°C and 20 s at the gene specific annealing temperature listed in Table 1., to ensure the specific amplification of each cDNA Hb isoform. In the end, a melting curve was generated to ensure the amplification of a single product during each reaction. Sample cycle

threshold (Ct) and the resulting efficiency (the slope of log starting quantity versus ct; E) were determined automatically by the Bio-Rad iQ5 software (Bio-Rad Laboratories Inc., Hercules, USA). Only genes with an efficiency of 95-110% ($R^2 \geq 0.98$) were chosen for relative expression level analysis. Of the 14 genes tested, 8 had appropriate Es, the other six are categorized as larval/juvenile Hb genes (Cadiz *et al.*, submitted May 2016), and thus had a too low expression levels in adult blood to ensure accurate results, and were therefore excluded from further analyses.

Cts of 8 Hb genes in 24 samples (dilution 1:30) were determined in triplicates using a similar protocol as for the linear standard range. Instead of testing a sample pool, cDNA was proliferated individually per sample. NTC, RT-, and positive controls were again added systematically for each gene.

3.5 Data analysis

3.5.1 Oxygen binding parameters

All oxygen binding parameters were automatically determined by the Blood Oxygen Binding System Software (Loligo Systems, Aarhus, Denmark). The program interpolated P50 and pH50 from the respective OECs at half saturation and n50 was calculated by means of Hill plots. Here, $\log_{10} \frac{Y}{1-Y}$ is plotted against $\log_{10} p_{O2}$, with Y being the fractional saturation of the Hb molecule from P_{O2}-OEC. The slope (i.e. the derivation) of this curve represents n50. The target Wavelength (λ) 576,051 nm was chosen for analysis, as it was found to yield the most stable values. For each gas tension step a sample size of n = 10 was chosen and the mean was taken for calculation. A logarithmical model (LM) was fitted to the OECs. For pH50, a third degree polynomial function was chosen to optimise the fit. Linear drift was compensated and the target λ in nm was divided by λ = 586,975 nm for isosbestic compensation.

3.5.2 Differential isoform expression

The Ct for each sample was automatically determined by the Bio-Rad iQ5 software (Bio-Rad Laboratories Inc., Hercules, USA). Following calculations were made with MS Excel. The relative quantity of RNA was determined by the ΔΔCt method. Here, the difference in Cts (Δ) between two samples is converted into relative quantities by normalizing the Ct of the gene of interest (GOI) in the sample (i) by the HKG 28S (1) and an endogenous control (mean ΔCt_{GOI} from samples of control treatment pH 8; $\Delta Ct_{GOI(c)}$) (2) according to the formulae:

$$\Delta Ct_{GOI(i)} = Ct_{GOI(i)} - Ct_{HKG(i)} \quad (1)$$

$$\Delta\Delta Ct_{GOI(i)} = \Delta Ct_{GOI(i)} - \Delta Ct_{GOI(c)} \quad (2)$$

To express the results as an expression-fold-change compared to the control treatment (f), values are displayed as:

$$f = 2^{(-\Delta\Delta Ct_{GOI\,(i)})} \quad (3)$$

In order to get the percentage of the isoform in relation to respective globin subunit (α and β), formula (4) was applied.

$$\%_{GOI(i)} = \frac{2^{(\Delta Ct_{GOI\,(i)})} * 100}{\sum 2^{(\Delta Ct_{(i)})}} \quad (4)$$

With $\%_{GOI(i)}$ being the percentage of the respective GOI from the sample (i) and $\Delta Ct_{(i)}$ the other ΔCts of the respective globin subunit (α, or β) of the sample (i).

3.5.3 Statistical analyses

All results were tested for significance ($p < 0.05$) by conducting one-way analysis of variances (ANOVAs), accompanied by Tukey's Honestly Significant Difference post-hoc tests. In case of comparison between temperatures, ANOVAs of repeated

measurements were conducted. To warrant normal distribution and homogeneity of variances Shapiro-Wilk tests and a Levene's tests were applied, respectively. Statistics and plots were computed with RStudio (Version 1.0.136, R Development Core Team (2008)).

4 Results

4.1 Oxygen equilibrium curves & haematocrit

For each animal, OECs were recorded at 15 and 20°C each, in order to detect potential differences in temperature sensitivity among the three pH treatments. Haemoglobin saturation under changing P_{O2} (protocol 1) was measured to detect potential differences in Hb-oxygen affinity, expressed as the P50 and cooperativity among subunits (n50). Moreover, the pH50 was measured by means of stepwise increase in (ambient) P_{CO2} (protocol 2), to be able to reveal changing pH sensitives of Hb between groups. Haematocrit was taken from all blood samples in order to correlate the volume percentage of red blood cells with oxygen binding parameters and isoform expression levels. All mentioned values are shown in table 2.

Haematocrit varied between animals. The percentage of blood cells fluctuated between 31 ± 5.43% and 34.25 ± 3.07% (table 2), and was not significantly correlated with the pH treatment. The standard deviation was up to 5.43%, which is larger than the variation of means among the groups (maximal 3.25% between condition pH 7.8 and pH 7.6).

Table 2: *Mean blood parameters, expression level changes and percentage of all globin subunits of Dicentrarchus labrax haemoglobin (± standard deviation) reared at three different water pH treatments.*

		P50 [kPa]		pH50		n50		alpha 1		beta 1	
Treatment	Haematocrit [%]	15°C	20°C	15°C	20°C	15°C	20°C	fold change[1]	[%] of globin subunit	fold change[1]	[%] of globin subunit
pH 8	32.13 ± 4.54	2.4 ± 0.56	3.09 ± 1.12	7.09 ± 0.14	7.20 ± 0.08	1.75 ± 0.26	1.57 ± 0.38	1.04 ± 0.28	95.68 ± 3.18	1.03 ± 0.22	97.01 ± 2.84
pH 7.8	34.25 ± 3.07	3.06 ± 0.65	2.91 ± 0.41	7.3 ± 0.15	7.39 ± 0.19	1.87 ± 0.34	1.78 ± 0.26	1.41 ± 0.35	96.31 ± 1.27	1.52 ± 0.35	98.51 ± 0.51
pH 7.6	31.00 ± 5.43	2.72 ± 0.39	2.55 ± 0.47	7.35 ± 0.19	7.3 ± 0.14	1.82 ± 0.22	1.65 ± 0.27	1.33 ± 0.56	96.78 ± 1.47	1.43 ± 0.66	96.73 ± 1.46

[1] Relative to the mean of control condition pH 8

Fitting curves of the Hb saturation at different P_{O2} (OECs) obtained via protocol 1 are shown in Figure 8 a) for 15°C and b) for 20°C. Within the groups, a relatively high interindividual variability has been noticed for curve shape and P50, being highest at pH 8 and 20°C (Figure 8; table 2). At an assumed corresponding venous P_{CO2} of 1.3 kPa, most blood samples were almost fully saturated at the highest O_2 tension measured (21 kPa). The mean maximal saturation for condition pH 8 was 94.39 ± 3.67%, for pH 7.8 96.93 ± 3.84%, and for pH 7.6 96.91 ± 2.23% for 15°C. At 20°C the mean maximal saturation was 92.36 ± 8.28%, 96.1 ± 2.84%, and 95.1 ± 1.85%, for condition pH 8, 7.8, and 7.6 respectively.

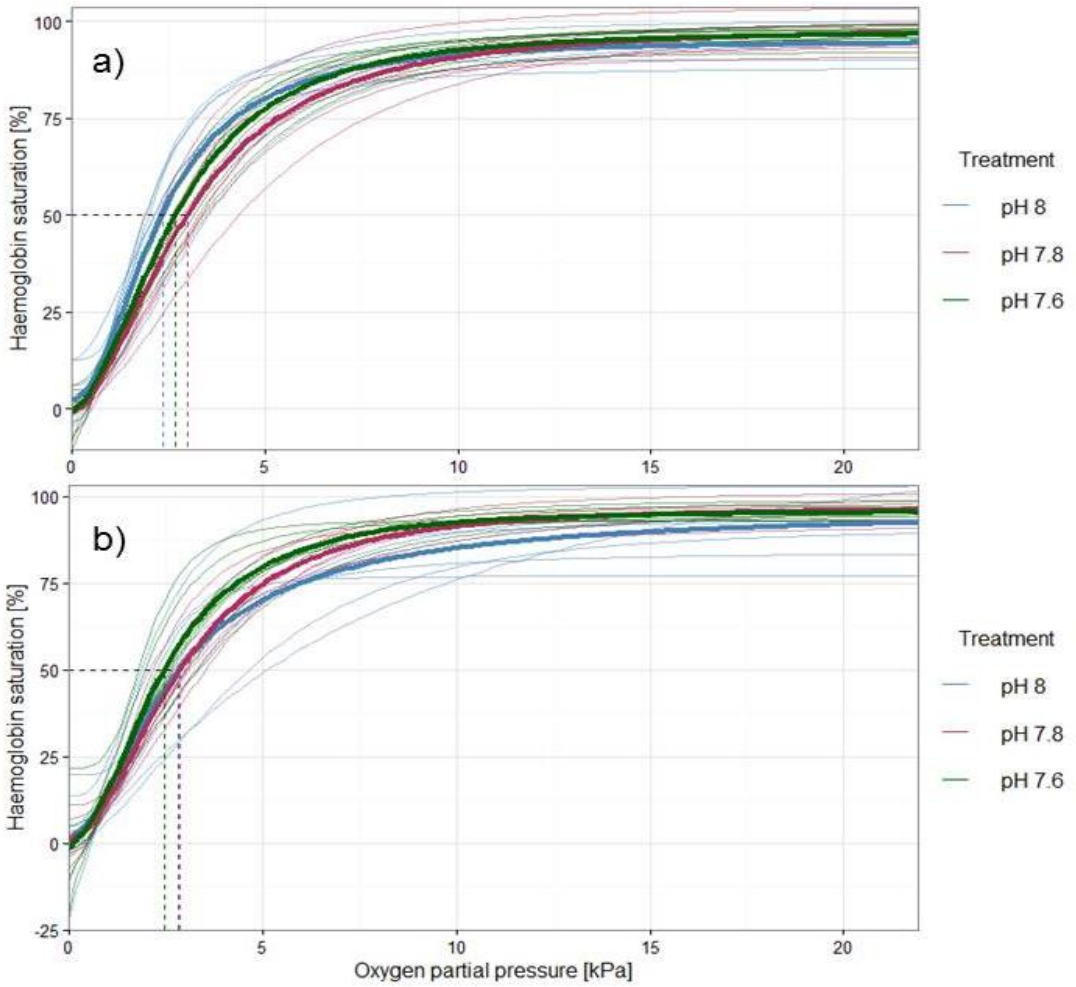

Figure 8: *Oxygen equilibrium curves at steady P_{CO2} (1.3 kPa) for sea bass (Dicentrarchus labrax) haemolysate measured at* ***T=15°C (a)),*** *and* ***T=20°C (b)),*** *experimental temperature. Blood originated from fish reared under different CO_2 partial pressures resulting in three pH treatments. In bold the mean of the respective pH group is displayed. Dashed lines indicate the pH at Hb half saturation (pH50)*

Values for P50 were similar between the two temperature treatments, lying between 2.4±0.56 and 3.06±0.65 kPa at 15°C and 2.55 ± 0.47 and 3.09 ± 1.12 kPa for 20°C, respectively (table 2).

In Figure 9 the oxygen tension at P50 for all treatments at both temperatures is displayed. Although the P50 increased slightly in the pH 7.8 treatment at both temperatures, no significant differences could be found.

Moreover, no temperature effect on oxygen affinity was detectable, since the P50 between temperatures were not found to be significantly different. A positive cooperativity was measured between the subunits among the treatments, with n50 varying from 1.75 ± 0.26 to 1.87 ± 0.34 for 15°C, and from 1.57 ± 0.38 to 1.78 ± 0.26 for 20°C (table 2). No significant differences were detectable among the treatments and in between temperatures.

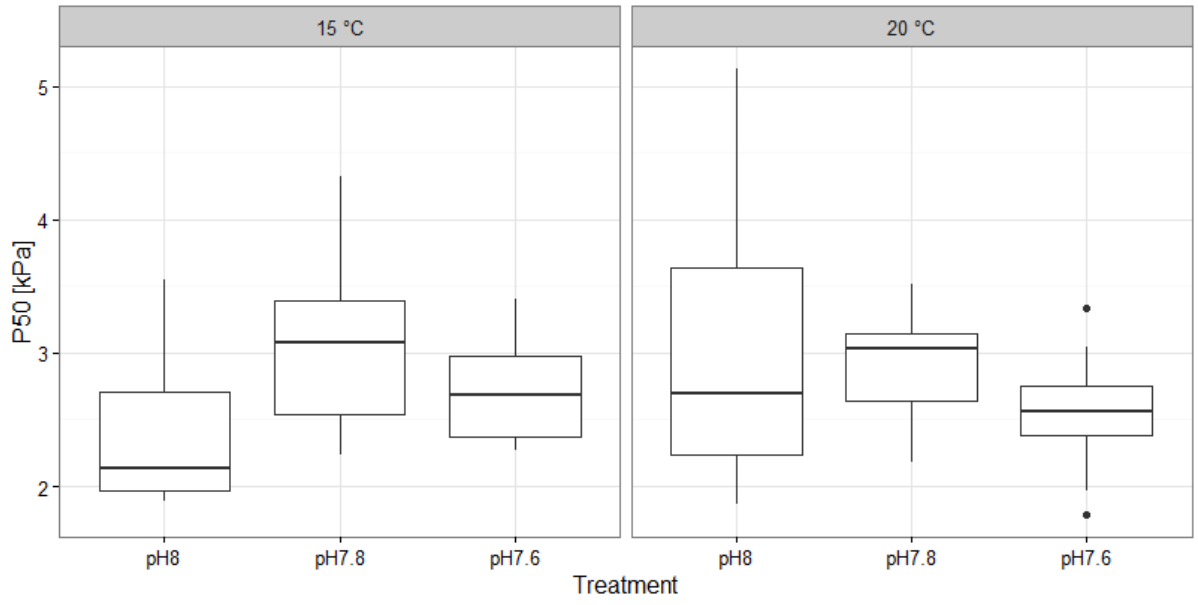

Figure 9: *P50s (P_{O2} at 50% haemoglobin oxygen saturation; steady P_{CO2} of 1.3 kPa) of sea bass (Dicentrarchus labrax) haemolysate measured at 15 and 20°C experimental temperature. Blood originated from fish reared under different CO_2 partial pressures resulting in three water pH treatments.*

The influence of pH on Hb saturation is shown in Figure 10 a) for 15°C and b) for 20°C. Here the fitting curves of data collected after protocol 2 (stable P_{O2}, variable P_{CO2}) are displayed. During measurements at 15°C, saturation at the lowest P_{CO2} (0.25 kPa) were found to be 90.06 ± 6.40% (treatment pH 8), decreasing to 85.93 ±

11.65% (pH 7.8), and 83.93 ± 11.66%. At 20°C maximum saturation decreased to 85.32 ± 10.01% (pH 8), 77.96 ± 10.75% (pH 7.8), and 81.57 ± 7.75%. Values for pH50 vary between pH 7.09±0.14 and pH 7.35±0.19 for 15°C and between pH 7.2 ±0.08 and pH 7.39±0.19. The interindividual variability of the pH50 was relatively high in the treatments (similar to P50 values), as seen in Figure 11, and in table 2.

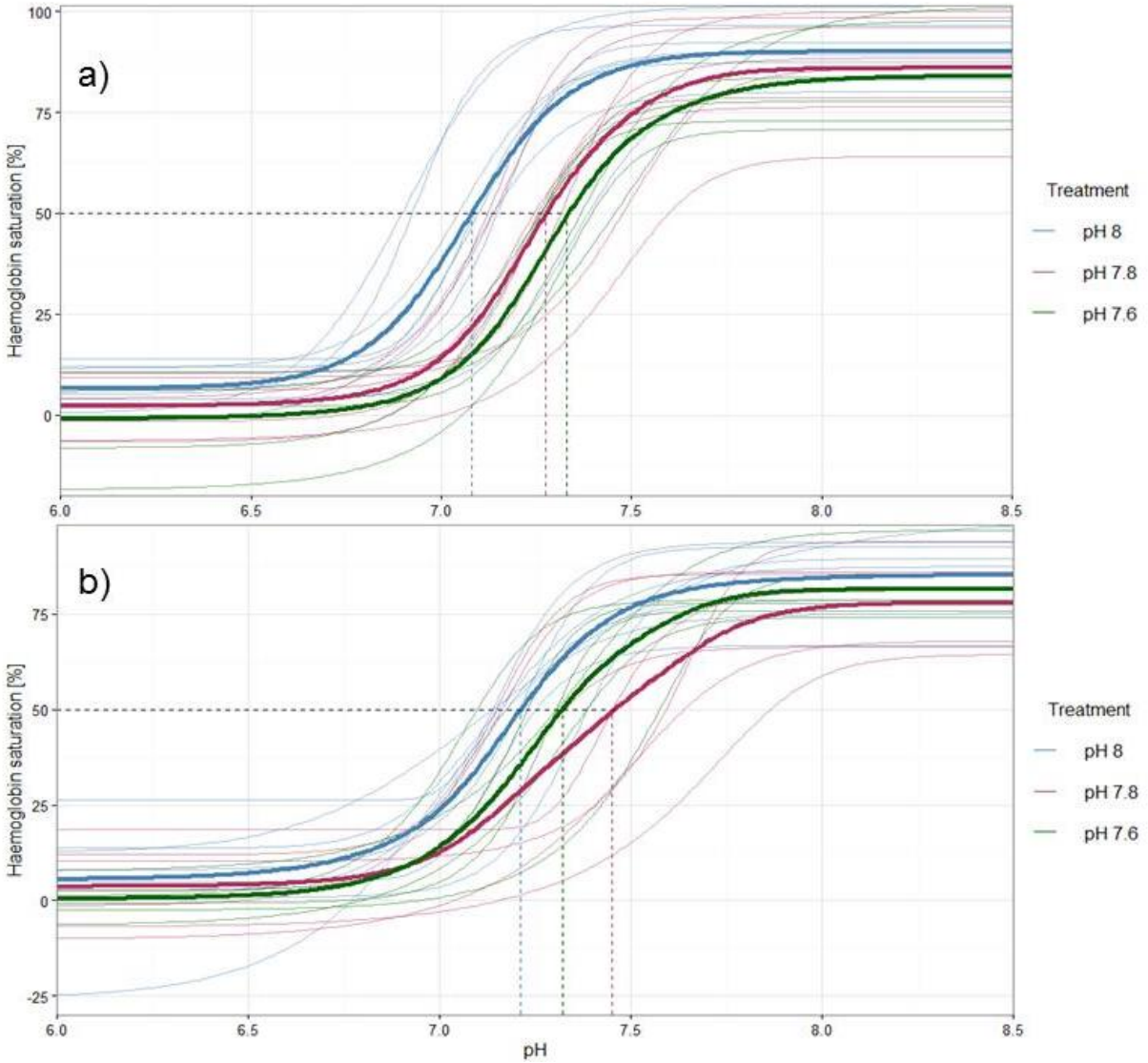

Figure 10: *Oxygen equilibrium curves at steady* P_{O2} *(3 kPa) for sea bass (Dicentrarchus labrax) haemolysate measured at* ***T=15°C (a),*** *and* ***T=20°C (b)*** *experimental temperature. Blood originated from fish reared under different* CO_2 *partial pressures resulting in three pH treatments. The mean of the respective pH group is displayed in bold lines. Dashed lines indicate the pH at Hb half saturation (pH50).*

In Figure 11 the pH at Hb half saturation is displayed for both temperatures and all treatments. The pH50s of the treatments pH 7.8 and pH 7.6 were found to be significantly higher than the control group pH 8 at 15°C (Pr (>F) = 0.028, and Pr (>F)

= 0.04, respectively), resulting in a clear shift of the OECs from the low pH treatments to the right (Figure 10). Also at 20°C, a shift towards a higher pH is noticeable, but in contrast, the measurements at 20°C show no significant differences, albeit a similar pattern between the treatments is observed. No differences of pH50 between the temperatures were detectable, although pH50 seems to be slightly elevated at 20°C.

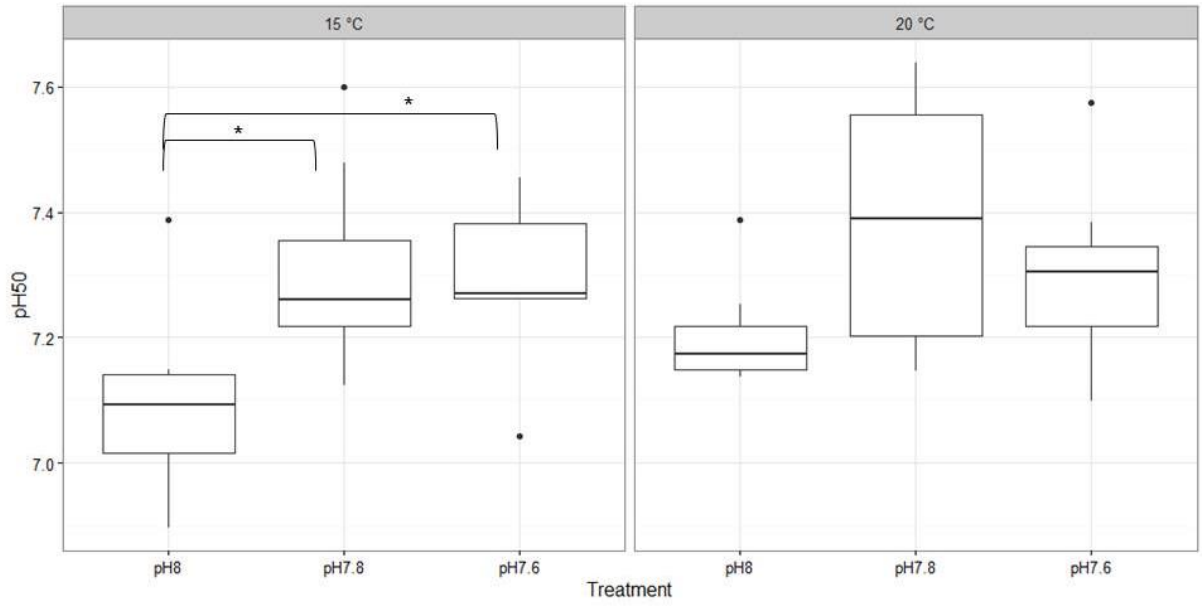

Figure 11: *pH50s (pH at 50% haemoglobin oxygen saturation) of sea bass (Dicentrarchus labrax) haemolysate measured at 15 and 20°C experimental temperature. Blood originated from fish reared under different CO_2 partial pressures resulting in three water pH treatments. Significant changes are marked by asterisks.*

4.2 Phenotypic plasticity

The mean percentage of alpha 1, beta 1 Hb subunits and the sum of the mean percentage of alpha 2,3 and 4, as well as beta 2,3, and 4 is depicted in Figure 12. As revealed by qPCR, the majority of the expressed alpha globin subunits are alpha 1 (between 95.68 ± 3.18% and 96.78 ± 1.47%), while in the beta globin subunit group, it is beta 1 (ranging from 96.73 ± 1.46% to 98.51 ± 0.51%; Figure 12; table 2). The other 3 isoforms from each group combined only account for 3.22 ± 1.47% to 4.32 ± 3.18% for alpha subunits, and 1.49 ± 0.51% and 4.32±3.18% for beta subunits (see Appendix I for detailed list of percentage).

The proportion of isoforms expressed in the respective globin group seems unaffected by the water pH, as there were no significant differences found among the different treatments.

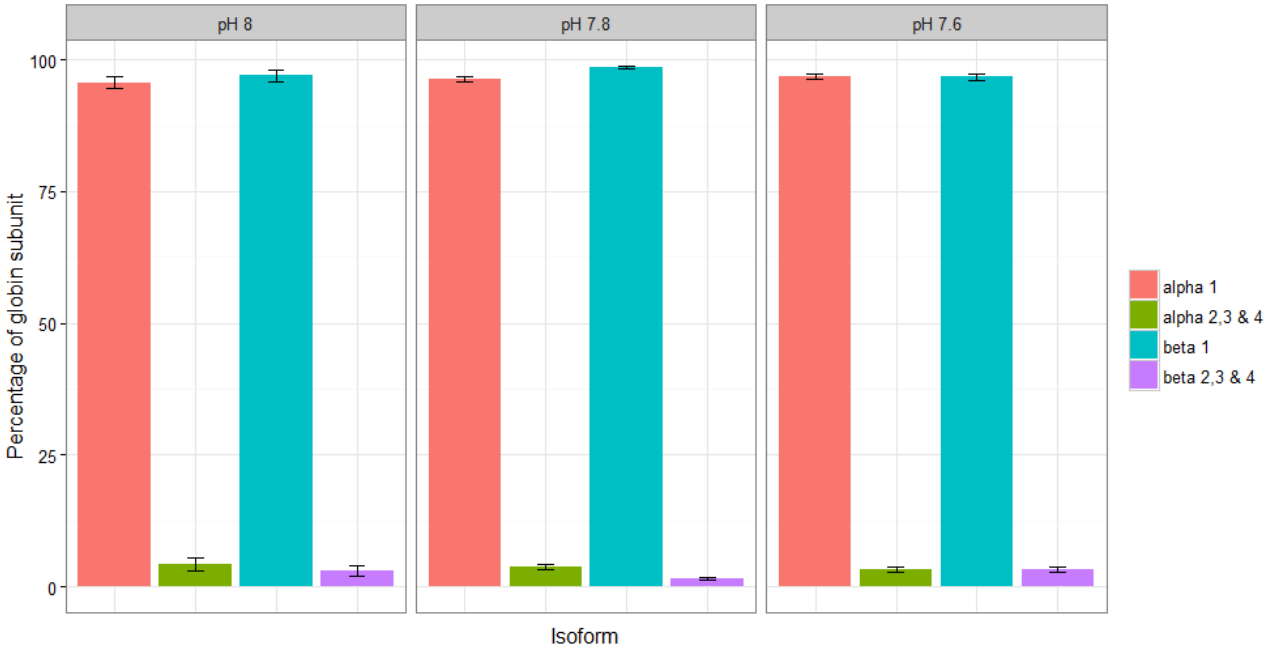

Figure 12: *Mean percentage ± standard deviation of globin subunits expressed in sea bass (Dicentrarchus labrax) blood, reared under three different pH environments. Alpha 1 and beta 1 are expressed as percentage of total alpha globins and beta globins respectively. Remaining percentage of subunit isoforms are summed up.*

To display the expression level change of the Hb subunits among the treatments, the data are depicted as fold-change from an endogenous control i.e. the mean of the control treatment pH 8 of the respective isoform (Figure 13). For clarification, a fold-change of 1 would indicate no change in expression levels, a fold-change of +2 would show a doubling of the translated product.

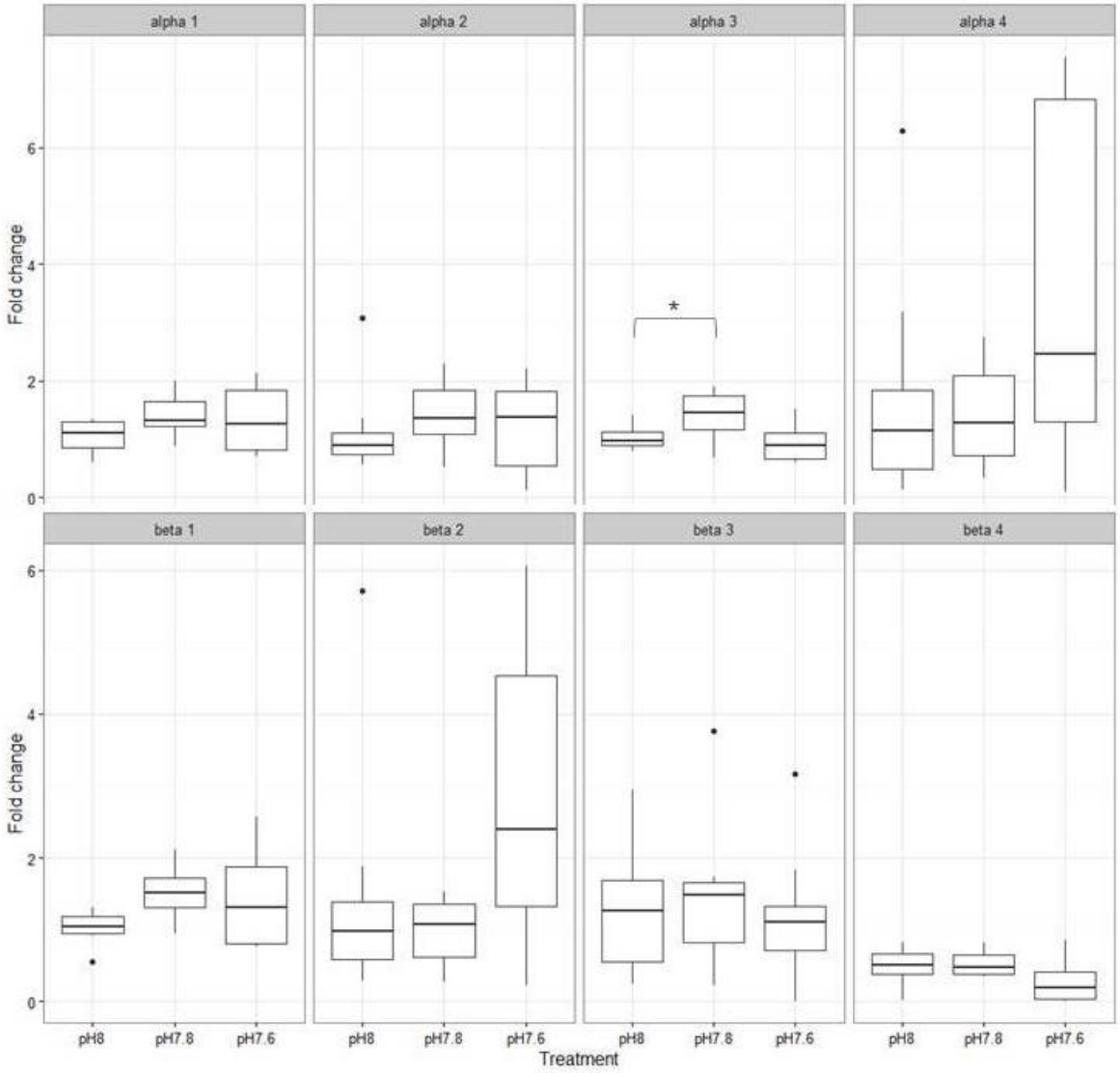

Figure 13: *Expression fold-change of 8 haemoglobin isoforms of sea bass (Dicentrarchus labrax) blood, normalised by the housekeeping gene 28S and by an endogenous control (mean ΔCt of control group pH 8). Significant change is marked by an asterisk.*

Despite a significant change in the expression of alpha 3 between treatment pH 8 and pH 7.8 (Pr (>F) = 0.034), no other significant changes in expression fold change were found. For fold-changes of expression of all isoforms, see Appendix I. The expression levels of alpha 1, 2, and 4 as well as beta 1 and 2 show a minute increase at the two low pH treatments, although masked by a high variability within groups. This variability seems to increase for some isoforms with treatments (see Figure 13: alpha 1, 2 and 4; beta 1, 2). A minor decrease in expression fold change with decreasing pH is seen in beta 4, all other treatments show the same expression as the pH 8 control group or a slight increase as mentioned afore.

5 Discussion

5.1 Linking subunit expression levels to functional properties of Hb

In spite of a significant increase in expression level of Hb subunit alpha 3 at water pH 7.8, there was no other treatment dependant change in expression level found. As subunit expression levels of alpha 3, 4, and beta 3, and 4 are comparatively low (high Ct values up to 28), this result has to be treated with caution, as low target mRNA content (indicated through high Cts) can increase result variability (Pfaffl *et al.* (2004); e.g. see Figure 13. alpha 4). The interindividual variability was found to be quite high (up to 4 Cts difference in between one treatment, also in the HKG, which would translate into 16 times more target mRNA), possibly due to technical problems, caused by soot from a fire in the laboratory several months before. This problem occurred during other studies conducted in this laboratory as well, and therefore the high variability of the Cts is likely not a biological effect. If the variability would have been smaller, a clearer picture of changes in expression levels would be conceivable (e.g. alpha 1, 2 and beta 1, 2; Figure 13). But with the current data obtained, changes in blood oxygen binding parameters cannot be directly linked to expression levels. Therefore, the data do not support the assumption that the detected changes in pH sensitivity (Figure 11), are due to changes in isoform expression levels. Therefore, other regulatory mechanisms of Hb-pH sensitivity are discussed below.

5.2 Haemoglobin mRNA composition

Despite technical problems, results show that total haemoglobin mRNA composes mainly of alpha 1 and beta 1 (up to 96.78 ± 1.47% and 98.51 ± 0.51%, respectively). Being from the same gene cluster (Mn), it is reasonable that the main part of sea bass Hb is composed of an Hb isoform consisting two alpha 1 and two beta 1 globin chains ($\alpha^1{}_2\beta^1{}_2$). Since beta 1 expression levels are slightly higher than those for alpha 1, it can be assumed that there is at least one other Hb isoform present that contains beta 1 globins. The remaining Hb isoforms could be a composition of the remaining globin subunits, also including alpha 1 and beta 1. Those other Hb isoforms can only represent up to 4.32±3.18% (highest percentage measured for isoforms excluding alpha 1 and beta 1; Figure 12) of total isoform composition, and are likely to play a

secondary role in tissue oxygen supply, compared to $\alpha^1{}_2\beta^1{}_2$. It is imaginable that those Hb isoforms fulfil other functional roles, e.g. they may show a stronger Root-Effect to liberate oxygen against high pressures within the swim bladder as seen in trout (de Souza & Bonilla-Rodriguez, 2007). Perez & Maclean (1976) found 5 distinct Hbs in sea bass, consisting of only 4 globin subunits. The amount of globin subunits does not match the finding in this study, as in adult fish, at least 8 globin subunits were expressed. As molecular analyses where not possible at the time, only main components of sea bass blood might have been detectable in the study of Perez & Maclean (1976). Moreover the number of Hb isoforms detected can vary depending on the analytical method used as seen in trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) *(de Souza & Bonilla-Rodriguez, 2007).* Therefore, it is uncertain whether Perez & Maclean (1976) found the correct number of Hb isoforms, as they only applied a preparative starch gel electrophoresis. In order to assess the Hb isoform composition and their functional relevance in sea bass blood, further analyses on haemoglobin components, including binding properties of individual haemoglobin isoforms, are necessary. However, this study laid the foundation for future research on sea bass Hb isoform composition, as the different globin subunit expression levels were displayed.

5.3 Acclimation of sea bass blood parameters to environmental hypercapnia

Under a chronic exposure to water pH levels of 7.8 and 7.6 most blood parameters (haematocrit, P50, n50) measured did not show any significant changes, compared to normocapnic control conditions (pH 8). Although not significant, due to a high interindividual variability, P50 was found to slightly increase with lowered water pH. A possible explanation for the unaffected binding properties could be that sea bass frequently inhabits estuaries, and creeks (Kennedy & Fitzmaurice, 1972), were abiotic parameters, such as water pH can fluctuate strongly (Selleslagh & Amara, 2008). Sea bass could be equipped with an Hb inventory, which maintains its functions at changing water pH levels, and thus no changes in haematocrit and n50 are seen. Furthermore, one has to bear in mind that fish used for this study originate from a domestic population. Being bred in an aquaculture, where water pH easily is well below average sea water pH, *D. labrax* can thrive at water pH as low as 7.17

(Papoutsoglou *et al.*, 1998). Keeping this species over numerous generations in such facilities might have led to a selective breeding towards individuals whose Hbs are less sensitive towards fluctuating pH. This theory of evolutionary adaptation could be tested by comparative experiments on wild animals, with a possibly higher sensitivity to such low water pH levels.

pH50: Hb half saturation was found at higher blood pH under environmental hypercapnia (Figure 11) at 15 °C experimental temperature. When blood pH is not regulated, elevated water P_{CO2} leads to an increase in arterial blood CO_2, accompanied by a transient blood acidosis, as seen as immediate response in rainbow trout (Perry *et al.*, 1987), Japanese flounder, yellowtail (Hayashi *et al.*, 2004) and cod (Melzner *et al.*, 2009). The drop in extracellular pH (pHe) leads to qualitatively similar but less severe pattern in the intracellular pH (pHi) in red blood cells (Brauner & Baker, 2009). This would cause a lowered Hb-oxygen affinity, due to the Bohr effect, and sufficient tissue oxygen supply would not be warranted anymore. Therefore most studied teleosts compensate for a hypercapnic acidosis in their blood with acid-base relevant ion transfer (Larsen & Jensen, 1997), namely by non-respiratory accumulation of bicarbonate through the gills (Brauner & Baker, 2009). The accumulation of plasma [HCO_3^-] buffers the excess protons in the plasma, facilitating pHe regulation. This regulation is aided by intracellular carbonic anhydrase activity, enabling the excretion of excess CO_2 across the gills (Henry, 1996). In most fresh water teleosts studied so far, the uptake of bicarbonate is closely correlated to an equimolar loss of [Cl^-] (Goss *et al.*, 1998; Petochi *et al.*, 2011) as well as increased [Na^+] in the extracellular space. Transports against the electrochemical gradient are realised by Cl^-/HCO_3^- exchanger and the Na^+/HCO_3^- cotransporter, with latter's expression levels being upregulated during hypercapnia in some fish (Deigweiher *et al.*, 2008). [HCO_3^-] accumulation can even lead to an overcompensation of the blood acidosis, which results in a shift of pHe towards more alkaline values as seen in *Notothenia rossii* (Strobel *et al.*, 2012), carp (*Cyprinus carpio;* Claiborne & Heisler (1986)) and Atlantic cod (*Gadus morhua,* Melzner et al. (2009)). Indeed, sea bass was found to produce a compensatory blood alkalosis under environmental hypercapnia as well, significantly elevating the blood pH above normal levels after short term exposure towards graded environmental hypercapnia (down to water pH of 6.48) (Cecchini *et al.*, 2001). Assuming a similar over-compensation on a longer time scale, as in this study, it would seem likely that Hb-

oxygen affinity is regulated, as a response to chronic drops in water pH. To ensure tissue oxygen supply, the Hb-pH sensitivity would need to increase, already discharging oxygen at a higher pH, to match the shift towards a higher blood pH, possibly induced through elevated [HCO_3^-]. This response might be seen in this study (Figure 10 a), and although not significant (due to a high SD), but still marginally different in b)). Bicarbonate itself is only an allosteric effector in crocodile (Bauer *et al.*, 1981) and hagfish (Fago, Giangiacomo*, et al.*, 2001) blood, and does not affect binding affinity of teleost Hb directly. Furthermore, bicarbonate concentrations equilibrate quickly with the surrounding medium (e.g. air in the diffusion chamber). Therefore [HCO_3^-] levels are likely to be similar in samples during measurements, and OECs are being shifted to the right. Higher pH sensitivity might be achieved by a change in other allosteric effectors concentrations such as ATP and GTP. Particularly GTP, which exerts a greater effect on Hb-O_2 affinity than ATP (Weber & Fago, 2004), might play a role in the intraspecific acclimation to an elevated plasma pH. Generally, organic phosphates decrease Hb-oxygen affinity by binding at specific amino acid residues and stabilising the low–affinity T-state of Hb as reviewed by Nikinmaa (1997) and Jensen *et al.* (1998). Erythrocyte GTP and ATP levels have been found to adjust to environmental variables, such as changing temperature, and dissolved oxygen availability (Somero & Hochachka, 1976; Wood & Johansen, 1972). Studies on the effect of water pH on organic phosphate concentrations in the erythrocytes are scarce. Nevertheless, a species specific increase in erythrocyte [ATP], and/or [GTP] was found in some fresh water fish under hypercapnia, which is assumed to be an acclimation towards more acidic environments (Ramirez-Gil *et al.*, 1998; Val *et al.*, 1998). In some fish, the Bohr effect of haemoglobin is augmented by the presence of organic phosphates (Gillen & Riggs, 1971; Weber & Lykkeboe, 1978). Presuming similar responses in marine species, like *D. labrax*, Hb affinity could be adjusted to higher pH50 by a chronic increase in erythrocyte organic phosphate levels. Chronically high levels of erythrocyte organic phosphates, however, are energetically costly and would ultimately result in a lowered fitness of those animals. Yet, in order to support these theoretical explanations, measurements of blood pH (pHe and pHi), blood [HCO_3^-] and [CO_2], and erythrocyte organic phosphate levels need to be carried out.

Haematocrit: Haematocrit values were quite variable (from 31 ± 5.43% - 34.25 ± 3.07%, table 2), and seemed not be affected by hypercapnia. The high variability

within groups could be a result of an individual stress response. Due to the sampling design, some fish were anesthetised longer than others, which might have resulted in a higher stress level, and a concomitant elevation in haematocrit, as found in other fish species (Biron & Benfey, 1994). Furthermore, haematocrit values could have been altered by the differing sedation time of the fish with MS-222, as the sedative has been reported to increase haematocrit due to erythrocyte swelling (Ryan (1992); for review see Topic Popovic *et al.* (2012)). Petochi *et al.* (2011) found similar haematocrit percentages as the present study, and that sea bass haematocrit only slightly increases under severe hypercapnia (water pH of 6.8 to 6.4), probably induced by erythrocyte swelling, as found in Atlantic salmon smolts (Fivelstad *et al.*, 1999; 2003). The relatively high haematocrit values compared to other fish species (e.g. turbot 16.5±0.3%; Pichavant *et al.* (2003)), might be an adaption to high swimming activity and muscle perfusion requirements. High erythrocyte concentrations are closely linked to a high haematocrit, which lead to an increase of the total oxygen carrying capacity of sea bass blood, and therefore to an increased oxygen delivery to the tissues (Pichavant *et al.*, 2003). The haematocrit levels measured in this study were unaffected by any treatment, matching the findings of Petochi *et al.* (2011).

P50: The values for Hb half saturation (P50) found in this study, lie between 2.4 ± 0.56 and 3.09 ± 1.12 kPa at a venous P_{CO2} of 1.3 kPa. Those values are almost two times higher than values of sea bass P50 found at venous P_{CO2} = 0.27 kPa (1.7±0.13 kPa; Pichavant *et al.* (2003)). A lower venous P_{CO2}, however, elevates Hb-oxygen affinity, due to a decreased Bohr effect. Oxygen binding parameters of teleost Hb can be linked to O_2 availability. Fish inhabiting environments which frequently become hypoxic (e.g. carp and tench) show the highest O_2 affinity range (0.77 kPa (10°C, pH 8) – 0.83 kPa (15°C, pH 8.05) (Albers *et al.*, 1983; Jensen & Weber, 1982), while lowest oxygen affinity ranges are found in fishes living in well-oxygenated environments, such as salmonids (2.97 kPa, (12°C, 0.3% CO_2) – 3.21 kPa (20°C, pH 7.8) (Maxime *et al.*, 1990; Tetens & Lykkeboe, 1981). Nevertheless, a P50 down to 2.4± 0.56 kPa at an *in vitro* pCO_2 of 1.3 kPa indicates a rather high oxygen affinity of sea bass Hb. Living in well-aerated water, a lower oxygen affinity was expected (Pichavant *et al.*, 2003; Powers, 1980). The low P50 at venous P_{CO2} might maintain high blood P_{O2} to be used under exercise. Then the tissue oxygen demand increases

and blood pH decreases due to a hypercapnic metabolic acidosis, and the excess oxygen can be unloaded. Furthermore the myocardial oxygen supply might be ensured with the high O_2 affinity (Farrell & Clutterham, 2003). For a highly active species such as sea bass, having a blood oxygen reserve to supply tissues, and especially the heart, during exercise, would thus be favourable.

There was no treatment effect on P50s detectable, as the differences were not significant between the pH groups. Nevertheless, a slight increase in P50, which translates to a slightly lowered Hb- oxygen affinity, can be seen at 15°C (Figure 9), but is masked by a high interindividual variability. When assuming higher levels of organic phosphates in erythrocytes (as mentioned above), the increased blood pH sensitivity would be accompanied by a decrease in Hb-oxygen affinity. No temperature sensitivity of the Hb-oxygen affinity was detectable. The blood samples originate from animals living at a water temperature of 16.4°C, which lies closer to the lower experimental temperature of 15°C. Even an experimental increase of temperature on blood samples to 20°C had no effect on *in vitro* Hb-oxygen affinity. As Hb oxygenation is an overall exothermic reaction, increasing temperatures normally lead to a decrease in Hb O_2 affinity, with an accompanied shift of the OEC to the right (Heldmaier & Neuweiler, 2013). Nevertheless, if allosteric factors such as protons, or organic phosphates are bound to deoxygenised Hb, a endothermic release of them upon Hb oxygenation is required, which might pose a compensation for the heat released during Hb oxygenation, and therefore cause a reduction or even a reversion of temperature sensitivity (Barlow *et al.*, 2017). As *D. labrax* inhabits environments where fluctuations in temperature are quite frequent (estuarine marine water as adults; Claireaux *et al.* (2006)), a temperature insensitivity in a certain range makes sense, in order to maintain O_2 tissue supply when entering warmer/colder waters, as seen in tuna (Rossi-Fanelli & Antonini, 1960).

n50: Sea bass blood shows positive cooperativity with values of n50 between 1.57 ± 0.38 and 1.87 ± 0.34. No effect of chronic CO_2-exposure or *in vitro* temperature changes was detectable. Cooperativity is slightly less pronounced than in other fish such as cod (2.9 ± 0.2; 10°C, 0.2% CO_2; Petersen & Gamperl (2011)) and rainbow trout (2.05, 20°C, pH7.8; Tetens & Lykkeboe (1981)), but similar to salmon (1.823, 12°C, 0.3% CO_2; Maxime *et al.* (1990)), resulting in a lower sigmoidicity of the OEC compared to other fish. Hence, Hb-oxygen affinity is relatively high even at low P_{O2},

which correlates with the comparatively low values for P50 mentioned above. The n50 values found do not match those by Pichavant *et al.* (2003) (1.1 ± 0.09), which could again be due to the different *in vitro* P_{CO2} chosen for the studies. Sea bass blood can have venous P_{CO2} of 1.3 kPa (Petochi *et al.*, 2011); therefore this value was chosen for the *in vitro* experiments in this study, in order to gain an understanding of the ability of sea bass Hb to deliver O_2 to tissues. In some species, the largest cooperativity was found to close to the range of *in vivo* blood pH, decreasing with both decreasing and increasing pH (Portner, 1990). In carp (*Cyprinus carpio*) the Hill coefficient was found to also be dependent on blood pH, with a maximum at pH 7 (Tan *et al.*, 1972) decreasing to both sides. Assuming similar responses of cooperativity towards pH in sea bass, a higher P_{CO2} of 1.3 kPa (resembling an *in vivo* venous blood pH) leads to a more acidic blood pH compared to that at P_{CO2} of 0.27 kPa (resembling more an *in vivo* arterial blood pH) and thus could explain the increase in n50 found in the present study. Combined with dropping O_2 tensions, an increase in cooperativity would likely facilitate tissue oxygen supply, by liberating more oxygen over a narrower pO_2 range at venous blood pH than at a more arterial blood pH. However, the relatively low cooperativity, compared to other fish species (Bushnell *et al.*, 1984; Pichavant *et al.*, 2003), might be an indication for an Hb-oxygen unloading capacity over a wider range of oxygen tensions (Pichavant *et al.*, 2003).

6 Conclusion

In this study, the functional properties of sea bass (*D. labrax*) Hb reared at three environmentally relevant water P_{CO2}, were investigated. The results were linked to the globin isoform expression levels of those fish. Comprising the current data, environmental hypercapnia led to a significant increase of the pH50 at 15°C experimental temperature. Since expression levels did not change, an acclimation to an over-compensatory blood alkalosis, caused by elevated blood bicarbonate levels is hypothesised. Nevertheless, due to potentially biased results caused by technical problems during qPCR experiments, a change in isoform expression levels upon hypercapnic acclimation cannot be fully ruled out. To strengthen the presented

hypothesis, measurements on pHe and pHi, as well as blood bicarbonate and organic phosphate levels, are essential. Hypercapnic acclimation did neither significantly influence most of the blood binding parameters measured (n50 and haematocrit), nor most isoform expression levels. Also P50 was not significantly different, but a trend towards higher P50 with increasing water pH was detectable, with supports the hypothesis mentioned above. Furthermore there was no temperature effect on those parameters measurable, indicating a temperature insensitivity of *D. labrax* Hb in the measured temperature window. The presented data could indicate a broad functional range of sea bass blood at various pH, which would be beneficial in the fluctuating pH environments *D. labrax* inhabits. Moreover, selective breeding could have intensified the adaption of sea bass to changing $P_{CO2.}$ Furthermore, data on Hb inventories, including oxygen binding parameters of the individual isoforms are necessary to reveal the functional characteristics of sea bass blood.

Although domesticated *D. labrax* seems to be well equipped for natural fluctuations in water pH levels, regulatory responses of wild animals might be different. Therefore further investigations are needed in order assess the acclimation of natural occurring populations. But even for sea bass originating from a domesticated broodstock, lowered pH might induce regulatory mechanisms on a non-transcriptional level of Hb, such as constantly elevated concentrations of organic phosphates, which are energetically costly and consequently might reduce the overall fitness of individuals exposed to long term environmental hypercapnia.

7 Methological considerations

General Sea bass blood used for this study was taken from fish derived from a captive brood stock. In order to rule out possible cross-generational adaptations towards low water pH due to selective breeding, fish originating from a wild brood stock should be considered for further studies. Comparing these results with the ones presented here could not only assess the acclimation capacity of wild populations to changing environments, but also be a first step to investigate evolutionary adaptations of *D. labrax* to a life in captivity.

Due to problems during mRNA extraction, blood samples for molecular analyses had to be retaken two months later from the same fish. During this time, the water temperature increased by 1.5°C. Tun & Houston (1986) found that environmental factors, such as temperature, influence Hb isoform abundance in rainbow trout. Although the temperature increase is not a lot, simultaneous sampling of blood for mRNA and blood binding parameters is necessary to minimize possible effects of temperature change upon isoform expression levels in sea bass.

In order to fully assess acclimation capacity of sea bass blood to environmental hypercapnia, measurements on blood parameters, such as blood bicarbonate and organic phosphates levels, as well pHe and pHi would be needed. Furthermore, the determination of Hb isoform composition and the assessment of blood oxygen binding parameters from stripped Hb isoforms individually, would yield additional information about the functional purpose of these pigments during acclimation to hypercapnia.

Blood binding properties Upon thawing of the full blood and the plasma samples, some samples slightly coagulated. Therefore 5 µl of heparin (5000 u/ml) had to be added when mixing haemolysate with the equal amount of plasma, slightly diluting the haemolysate. Still, partial coagulation of haemolysate could not be avoided, and might have disturbed the equal distribution of Hb throughout the sample. Sea bass is known for fast and easy coagulation rates of its blood (personal communication), therefore a higher concentration of heparin (e.g. 10000 u/ml) should be used to heparinise the syringes in future studies.

During analysis of the OECs it became clear that the sample did not fully equilibrate in between the 10 min intervals. This was not noticed during measurements, but is visible for example in the inability of the sample to saturate fully at high pH (Figure 11). To improve the current protocol, it is advised to either reduce the sample volume, to reach blood-gas equilibrium quicker, or to elongate equilibrium steps (e.g. 15 min). It should be considered for the latter, that one experiment alone can take up to 4 ½ h (protocol 2; 10 min; without calibration of the pH optode). Further reducing the sample volume could also prove difficult, as the pH optode was already difficult to introduce in a sample volume of 10 µl. During some experimental runs, the optode was not properly covered by the sample droplet, and consequently those pH values had to be excluded from analysis.

Samples were measured in the following order: first treatment pH 8, pH 7.8, and then pH 7.6. Throughout the measurement period, some degree of pH optode photo bleaching was inevitable, possibly reducing the optode quality along the measurement period. When bleached, the phase range of the optode becomes smaller, which leads to more unstable pH readings. Although there is no evidence for this phenomenon in the current study (Figure 11: variability does not become greater within the treatments), a random order of sample processing is advised for further experiments.

Molecular analyses Problems during RNA extraction included contamination with gDNA. Therefore a DNase treatment, followed by a RNA precipitation had to be included in the protocol. The RNA precipitation is a rather complicated and time consuming step; furthermore the risk of losing the RNA is present (as experienced for one sample in this study). Commercial RNA clean-up kits are available (e.g. NucleoSpin® RNA Clean-up, Macherey-Nagel GmbH & Co. KG, Düren, Germany, or Invitrogen Ambion TURBO DNA-free Kit, Thermo Fisher Scientific Inc, Waltham, USA), which are easier to use and faster, moreover the risk of losing the sample is minimised.

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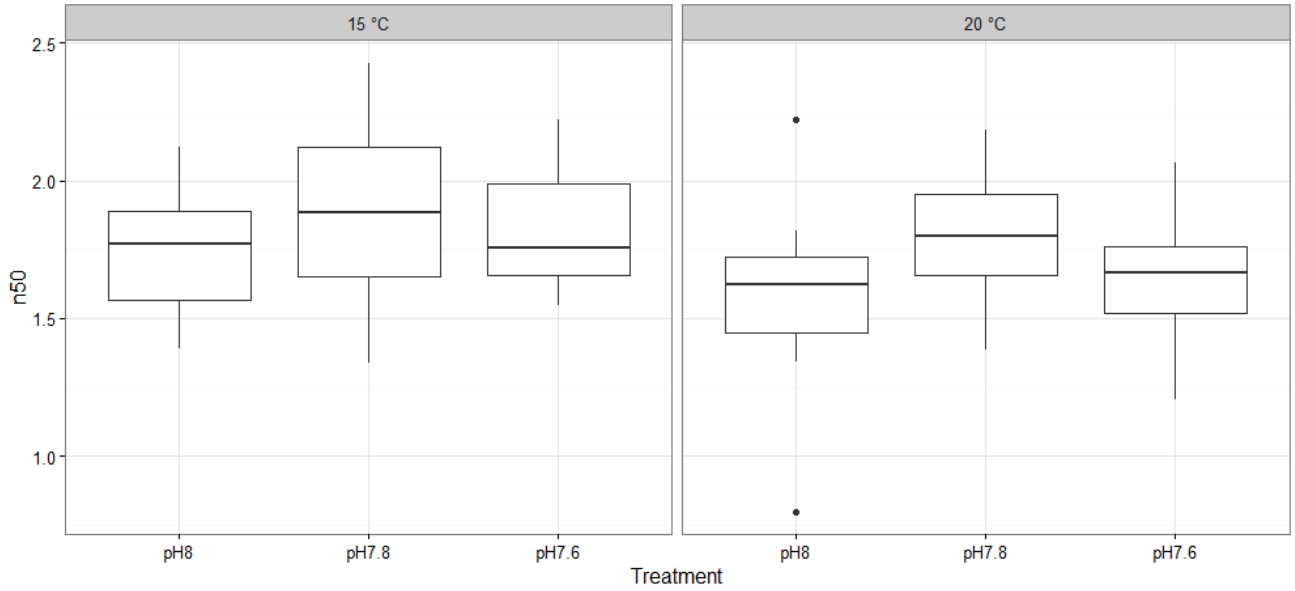
10 Appendix

Appendix I: *Fold changes and percentage of respective globin subunit (alpha and beta) of 8 Haemoglobin isoforms from sea bass (Dicentrarchus labrax) ± SD. Fold changes are normalised by the housekeeping gene 28S and an endogenous control (mean ΔCt of treatment pH 8).*

Treatment	alpha 1		alpha 2		alpha 3		alpha 4		beta 1		beta 2		beta 3		beta 4	
	fold change	%	fold change	%	fold change	%	fold change	%	fold change	%	fold change	%	fold change	%	fold change	%
pH 8	1.05 ± 0.28	95.68 ± 3.18	1.16 ± 0.76	3.01 ± 2.83	1.02 ± 0.19	1.17 ± 0.28	1.77 ± 1.92	0.14 ± 0.18	1.03 ± 0.22	97.01 ± 2.84	1.52 ± 1.65	2.55 ± 2.74	1.28 ± 0.82	0.27 ± 0.18	1.02 ± 0.2	0.17 ±0.02
pH 7.8	1.41 ± 0.34	96.31 ± 1.21	1.43 ±0.52	2.47 ± 1.02	1.41 ± 0.43	1.15 ± 0.34	1.42 ± 0.84	0.07 ± 0.03	1.52 ± 0.34	98.51 ± 0.6	0.98 ± 0.67	1.11 ± 0.59	1.49 ± 1.04	0.20 ± 0.16	1.56 ± 0.33	0.18 ± 0.04
pH7.6	1.33 ± 0.56	96.78 ± 1.47	1.22 ± 0.72	2.19 ± 1.31	0.93 ± 0.29	0.87 ± 0.22	3.56 ± 2.91	0.16 ± 0.1	1.43 ± 0.66	96.73 ± 1.46	2.82 ± 1.95	2.9 ± 1.37	1.21 ± 0.89	0.21 ± 0.21	1.34 ± 0.54	0.16 ± 0.02



Appendix II *Hill coefficients (n50; at 1.3 kPa P_{CO2}) of sea bass (D. labrax) haemolysate measured at 15 and 20°C experimental temperature. Blood originated from fish reared under different CO_2 partial pressures resulting in three pH treatments.*