Physiological responses of European sea bass (Dicentrarchus labrax) exposed to increased carbon dioxide and reduced seawater salinities

Physiological responses of European sea bass (*Dicentrarchus labrax*) exposed to increased carbon dioxide and reduced seawater salinities

Warren G. Caneos, Jyotsna Shrivastava, Moses Ndugwa, Gudrun De Boeck

Author information

W. G. Caneos (✉) · J. Shrivastava · M. Ndugwa · G. De Boeck
ECOSPHERE, Department of Biology
University of Antwerp, Groenenborgerlaan 171
BE-2020 Antwerp, Belgium
e-mail: warren.caneos@msumain.edu.ph
ORCID: 0000-0003-0955-7414

J. Shrivastava
e-mail: jyotsna.ua@gmail.com

M. Ndugwa
e-mail: Moses.Ndugwa@uantwerpen.be
ORCID: 0000-0002-5717-3645

G. De Boeck
e-mail: gudrun.deboeck@uantwerpen.be
ORCID: 0000-0003-0941-3488

W. G. Caneos
Fisheries Department
College of Fisheries and Aquatic Sciences
Mindanao State University-Marawi
9700 Marawi City, Philippines

W. G. Caneos
Department of Biological Sciences
College of Science and Mathematics
Mindanao State University-Iligan Institute of Technology
9200 Iligan City, Philippines

Abstract

Background The iono- and osmoregulatory capacities of marine teleosts, such as European sea bass (*Dicentrarchus labrax*) are expected to be challenged by high carbon dioxide exposure, and the adverse effects of elevated CO_2 could be amplified when such fish migrate into less buffered hypo-osmotic estuarine environments. Therefore, the effects of increased CO_2 on the physiological responses of European sea bass (*Dicentrarchus labrax*) acclimated to 32 ppt, 10 ppt and 2.5 ppt were investigated.

Methods Following acclimation to different salinities for two weeks, fish were exposed to present-day (400 μatm) and future (1000 μatm) atmospheric CO_2 for 1, 3, 7 and 21 days. Blood pH, plasma ions (Na+, K+, Cl-), branchial mRNA expression of ion transporters such as Na^+/K^+–ATPase (NKA), $Na^+/K^+/2Cl^-$ co-transporters (NKCC) and ammonia transporters (e.g. Rhesus glycoproteins Rhbg, Rhcg1 and Rhcg2) were examined to understand the iono- and osmoregulatory consequences of elevated CO_2.

Results A transient but significant increase in the blood pH of exposed fish acclimated at 10 ppt (day 1) and 2.5 ppt (day 21) was observed possibly due to an overshoot of the blood HCO_3^- accumulation while a significant reduction of blood pH was observed after 21 days at 2.5ppt. However, no change was seen at 32 ppt. Generally, Na+ concentration of control fish was relatively higher at 10 ppt and lower at 2.5 ppt compared to 32 ppt control group at all sampling periods. Additionally, NKA was upregulated in gill of juvenile sea bass when acclimated to lower salinities compared to 32 ppt control group. CO_2 exposure generally downregulated NKA mRNA expression at 32ppt (day 1), 10 ppt (days 3, 7 and 21) and 2.5ppt (days 1 and 7) and also a significant reduction of NKCC mRNA level of the exposed fish acclimated at 32 ppt (1-3 days) and 10 ppt (7-21 days) was observed. Furthermore, Rhesus glycoproteins were generally upregulated in the fish acclimated at lower salinities indicating a higher dependance on gill ammonia excretion. Increased CO_2 led to a reduced expression of Rhbg and may therefore reduce ammonia excretion rate.
Conclusion Juvenile sea bass were relatively successful in keeping acid base balance under an ocean acidification scenario. However, this came at a cost for ionoregulation with reduced NKA, NKCC and Rhbg expression rates as a consequence.

Keywords: CO_2, salinity gradient, Na^+/K^+/ATPase, $Na^+/K^+/2Cl^-$ co-transporter, Rhesus glycoproteins, *Dicentrarchus labrax*

1. Introduction

Globally, the intensified, sustained and accelerating anthropogenic carbon dioxide (CO_2) emissions have resulted to the increased accumulation of CO_2 into the atmosphere which later dissolved in the oceans [1, 2, 3, 4, 5, 6]. This phenomenon, termed as ocean acidification, occurs when CO_2 dissolves in seawater generating carbonic acid (H_2CO_3) which dissociates into bicarbonate (HCO_3^-), carbonate (CO_3^{2-}) and hydrogen ions (H^+) [7, 8, 9]. The rise of atmospheric CO_2 partial pressures (P_{CO_2}) has quantifiable effects on the ocean's carbonate chemistry. Carbon dioxide acts as an acid in water, therefore, ocean pH declines as P_{CO_2} increases, which gave rise to the term of this phenomenon as a descriptor for ocean CO_2-related perturbation in the context of climate change [10]. By 2100, the P_{CO_2} levels are predicted to increase and may range from 420 to 940 μatm which is consistent with the average decrease of pH by 0.13 – 0.42 units in the surface waters of the ocean [11, 12, 13].

Along with ocean acidification, there is also an emerging and alarming concern about the future changes in seawater salinity levels due to global warming (causes the melting and disappearance of glaciers and ice caps) and frequent heavy rainfall events [14]. This has resulted to the gradual reduction of salinity gradient in some marine ecosystems, e.g. inshore waters, sheltered bays, estuaries and coastal lagoons. These aquatic systems also experience salinity fluctuations due to tidal and seasonal changes [15, 16]. These changes in salinity affect the species composition of the aquatic environments. These aquatic systems of unstable salinity are usually inhabited by euryhaline teleosts which have the ability to regulate dynamic changes in its osmoregulatory approach from active salt absorption to salt secretion and from water excretion to water retention [17, 27, 70]. Salinity adaptation of these species is largely an intricate process involving a cascade of responses, both in physiology and ion-regulation. For euryhaline teleosts (marine and freshwater), the mechanisms of ion-osmoregulation are reasonably well-understood which are coordinated by ion channels, co-transporters [e.g. $Na^+/K^+/2Cl^-$ (NKCC)], energy-dependent ATP-ases [e.g. Na^+/K^+–ATPase (NKA)] and ammonia transporters (e.g. Rhesus glycoproteins – Rhbg, Rhcg1 and Rhcg2) [17].

Several studies have demonstrated that the physiological and ion-regulatory responses of some euryhaline teleosts are directly and indirectly affected by the future CO_2 concentrations [5, 18, 19, 20, 21, 22, 23] and reduced seawater salinities [16]. High CO_2 showed significant effects on the physiological functions of the fish, such as acid-base regulation, respiration, circulation and metabolism affecting the growth rate, organ development, survival and behavior of the larvae [4, 11, 19, 21, 24, 25, 26]. Moreover, it is known that euryhaline teleosts, (e.g. diadromous and non-diadromous species) migrate between fresh and marine waters as part of their life cycle for spawning and foraging purposes, and thus, they can be physiologically affected and/ or osmotically challenged by salinity fluctuations [16]. However, these species have mechanisms of dynamic control of osmoregulation which include the ability to discern environmental salinity changes that perturb water body and salt homeostasis (osmo-sensing), signaling networks that encode information about the direction and magnitude of salinity change, and epithelial transport and permeability effectors [27]. The shift in the ambient salinity and elevation of CO_2 levels can affect the survival and alter overall fitness of the teleosts with severe challenges to physiology and ion-regulation.

Gills play an important role in maintaining intracellular homeostasis during osmotic challenges as they serve as the major osmoregulatory organs modulating the movement of ions between the internal and external environment and acid-base regulation in fish [16, 28, 29, 30, 31]. Since gills are directly exposed to changes in the external environment, it is evident that the effects of the individual stressors may differ from those induced by a combination of multiple stressors [31]. The transfer of marine teleosts to lower salinities allows the transport of Na^+ and Cl^- ions across the gill epithelia to switch from excretion to uptake [32]. Its branchial epithelium contains several transporters and systematic mechanisms which are involved in ion-regulation maintenance. NKA, present in the basolateral membrane of gill epithelium, is thought to provide the major energy source leading to the influx of Na^+. It produces a low intracellular Na^+ gradient; thus induce the transport of Na^+, K^+ and Cl^- ions into the cell through the presence of a basolateral NKCC co-transporter, apical Na^+/Cl^- cotransporter (NCC) [33, 34] and an apically located Cl^- channel similar to the human cystic fibrosis transmembrane conductance regulator (CFTR) [33, 34, 35, 36]. A high plasticity of the ion-transporting ability is therefore essential to deal with a wide range of salinity and varying CO_2 levels.

European sea bass (*Dicentrarchus labrax* L.) is an ecologically and commercially important marine teleost widely distributed in the temperate European coastal areas of the northeast Atlantic Ocean and Mediterranean Sea [16, 37, 38, 39]. Being euryhaline, sea bass can tolerate salinities ranging from freshwater to hypersaline conditions, credit to the efficiency of chloride cells (or mitochondria rich cells) in their gills [40, 76]. The biochemical and physiological adaptations of chloride rich cells including a substantial count of mitochondria and a well-developed endoplasmic canalicular system, complemented by a basolaterally positioned sodium pump. Such a distinctive ultrastructure makes gill chloride cells a conducive site for salt regulation. This structural similarity is observed in the distal tubular cells of the kidney, as well as in the salt glands of birds and the rectal glands of sharks [28].

However, despite their natural adaptations migrate seasonally between the open sea and hyposaline environments such as lagoons and estuaries, they are often challenged with hypo-osmotic stress [41, 42]. Additionally, this species is extensively used for aquaculture [16, 38] and most of the mariculture systems operate in relatively shallow coastal areas which are subjected to salinity changes [15]. Unlike wild individuals, captive fish have no means of

avoiding the potential environmental stressors and a deeper knowledge on how these species are physiologically challenged in fluctuating physico-chemical environments may benefit future aquaculture practices [15]. Hence, in the present study, European sea bass juveniles were used as a model species to determine its physiological responses when confronted simultaneously with different environmental stressors, e.g. high CO_2 (low pH) and low ambient salinity. Generally, it was hypothesized that the sea bass would be adversely affected by high CO_2 exposure, and the adverse effects of carbon dioxide would be amplified when fish are subjected to hypo-osmotic environments.

The aim of this study was to investigate the effects of high CO_2 during acclimation to experimental salinities (32, 10 and 2.5 ppt) on the physiological and ion-osmoregulatory processes that occur as compensatory mechanisms to maintain homeostasis. The research findings of the present work will help to identify the key environmental cues in which fluctuation of single and combined stressors might threaten the performance of marine teleost. The study will thereby assist in formulating guidelines for the regulation of certain environmental factors for the sustainability of ecological system as well as in aquaculture systems.

2. Materials and Methods

2.1. Chemicals

Unless otherwise indicated, all chemicals and enzymes were purchased from Sigma Aldrich (St Louis, MO, USA), Roche (Indianapolis, IN, USA) or Fisher Scientific (Pittsburg, PA, USA) and were of analytical grade or higher.

2.2. Test species

European sea bass (*Dicentrarchus labrax*) juveniles (10-14 g; <1-year-old) were used as test species to perform this experiment. They were obtained from Ecloserie Marine de Gravelines (Gravelines, France) and transported to the University of Antwerp (Antwerp, Belgium).

2.3. Acclimatization

Initially, 600 fish were kept and maintained in three 600-L holding tanks (200 fish per tank) of 32-ppt artificial seawater (hw-Marinemix® Professional, Wiegandt Germany) for four weeks prior to salinity reduction. These tanks were equipped with a recirculating water supply in a laboratory where the temperature was adjusted at 17±1ºC and photoperiod was 12-h light/12 h-dark cycle. Proper aeration was provided with air stones and three-layer biofiltration systems were installed on top of each tank, composed of synthetic wadding in the upper layer and activated lava stones in the two bottom layers. The former retained the crude waste while the latter were activated with ammonia-oxidizing bacteria (Sera Bio Nitrivec, Germany) on these substrates to keep the ammonia level minimal. Daily monitoring of ammonia level was done using an ammonia test kit (Tetra Test NH_3/NH_4^+) and 40-60% of the water was changed twice a week to remove uneaten foods and to minimize the build-up of nitrates and nitrites. When the total ammonia level was greater than 0.25 mg/L, water was changed immediately.

Fish were fed with commercial pellets (1.5 mm, Coppens Germany) at a rate of 2% of their body weight per day. Subsequently, the fish in one tank were continuously reared at a normal seawater salinity of 32 ppt. On the other hand, the fish in the other two tanks were progressively acclimated to two experimental salinities, i.e. 10ppt (~249 mOsm/Kg, pH 8.10) and 2.5ppt (~69 mOsm/Kg, pH 7.87). The experimental salinities in two tanks were adjusted by diluting the artificial seawater with deionized water and was measured using a hand-held refractometer.

Changes in salinity were progressed by reducing the salinity by 5% every three days until 10 ppt was reached in both tanks. Salinity reduction by 2% was continued in one of the tanks every three days until the desired salinity of 2.5 ppt was achieved. Fish were then transferred and acclimatized again in the climate chamber (Weiss Technik, Belgium) with the same conditions previously mentioned to three experimental salinities, i.e. 32, 10 and 2.5 ppt for four weeks.

Water quality parameters were maintained within the favorable range for sea bass i.e. dissolved oxygen was kept above 80% saturation or more by providing proper aeration, whereas pH values were set at 8.16, 7.89 and 7.47 for 32 ppt, 10 ppt and 2.5 ppt, respectively.

2.4. Experimental set-up and CO_2 treatment

Six experimental tanks were divided into two experimental groups: the exposed and control groups. Each group consists of three tanks at different salinities: 32 ppt, 10 ppt and 2.5 ppt. Thirty-five juveniles from each salinity treatment were randomly selected, weighed and equally distributed to each 200-L experimental tank. A total of 210 individuals were assigned to six experimental tanks in the climate chamber.

The control groups were subjected to the present-day atmospheric CO_2 concentration (P_{CO_2} = 400 µatm, pH total = 8.2 for 32 ppt, pH = 7.8 for 10ppt and pH =7.4 for 2.5 ppt depending on their dissolved inorganic carbon measurement and total alkalinity). On the other hand, the exposed group was supplied with high CO_2 [P_{CO_2} = 1000 µatm, pH total = 7.7 for 32 ppt, 7.4 ppt for 10 ppt and pH= 7.0 for 2.5 ppt based on ocean acidification calculator (Macro Version 2.2: CO_2 sys_v2.2xls)]. These conditions were established based on the projected P_{CO_2} scenarios for 2100 (IPCC 2014).

Seawater P_{CO_2} was maintained via a computerized feedback system which monitored seawater pH total scale (the total hydrogen concentration scale; the sum of free H^+ concentration plus the concentration of all complexes with the background ionic medium; H_2O, Na^+, Mg^{2+}, K^+, Ca^{2+}, Cl^- and SO_4^{2-}) which regulated the addition of CO_2 gas. Water's P_{CO_2} was increased by bubbling the CO_2 gas in the exposed tanks from an external CO_2 cylinder (Praxair NV, Schoten Belgium) with the aid of an automatic system (CapCTRL software) and a feedback controller/trigger box (DAQs, Loligo Systems) that monitored and controlled the pH setpoints in three different salinities to simulate the predicted end of the century ocean acidification scenario (1000 µatm). Three salinities were maintained at varying pH levels (total scale) corresponding to the dissolved inorganic carbon (DIC) and total alkalinity (TA) measurements (Table 1). Both DIC and TA were measured only once at the start of the experiment from triplicate samples collected from the three salinities. Daily monitoring of water salinity and pH was also done. The pH electrodes (WTW 3310, Germany) were calibrated with tris (tris hydroxymethyl aminomethane) buffers (GE Healthcare Bio-Sciences AB, Uppsala Sweden), with the same salinity and ionic strength as the experimental tanks. The Tris buffers thus served as the pH standard for calibration and daily maintenance of appropriate pH setpoints. Carbon dioxide dissolves rapidly in seawater, carbonate chemistry equilibrates less quickly (as a function of temperature, αCO_2) and thus an overshoot (hysteresis) of pH is created, thus in the present experiment, the pH set values were maintained within hysteresis ± 0.07 units.

Table 1 Water chemistry of experimental conditions

Parameter	Exposure					
Salinity	**2.5 ppt**		**10 ppt**		**32 ppt**	
Pco_2 (µatm)	**400**	**1000**	**400**	**1000**	**400**	**1000**
pH	7.4	7.0	7.8	7.4	8.2	7.7
T(°C)	17 ± 1	17 ± 1	17 ± 1	17 ± 1	17 ± 1	17 ± 1
TA (µmol kg-1 SW)	362.6±37.4	334.6 ± 37	1132.3±13.4	1081.8±12.9	3094.6±63.2	2874 ±57.6
HCO_3-(µmol kg-1 SW)	356.5±36.3	332.6±36.6	1060.9±11.9	1055.5±12.3	2472.7±43.2	2603.2±48.2
$CO_3$2-(µmol kg-1 SW)	3.2 ±0.6	1.1 ± 0.2	31.9 ± 0.7	12.6 ± 0.3	270.4 ± 9.4	119.8 ± 4.4
[CO_2] (µmolkg-1 SW)	16.2 ± 0.1	41.8 ± 0.1	14.1 ± 0.01	38.3 ± 0.03	11.9 ± 0.01	30.8 ± 0.04

Note: Salinity and temperature were determined in samples from experimental tanks at the beginning, middle and end of experimental exposure whereas other parameters were calculated using the Ocean acidification calculator at the beginning of the experiment (Macro Version 2.2: CO_2 sys_v2.2xls). Pco_2 represents the partial pressure of CO_2, T (°C) the water temperature, TA the total alkalinity, $CO_3$2- carbonates, HCO_3- bicarbonate and [CO_2] the sum of dissolved carbon dioxide [CO2 (aq)] and carbonic acid [H_2CO_3]. SW: seawater.

2.5. Sampling preparation and procedures

Two experimental groups were alternately sampled after 1, 3, 7 and 21 days of exposure. At each exposure time, eight fish per tank were sampled. Prior to sampling, a lethal dose of the sedative MS222 (Acros Organics, Geel, Belgium) was prepared by adding 1 g of ethyl 3-aminobenzoate methane-sulfonic acid to 1-L stock artificial seawater of the different salinity treatments. Potassium hydroxide (KOH) was added to adjust the pH back to the initial pH of the seawater. Syringe and labeled Eppendorf tubes were rinsed with heparin (2,500U/mL, Sigma, USA) to prevent blood clotting. A micro-centrifuge (Centrifuge 5415 R, Eppendorf) was fast-cooled to 4ºC to avoid changes of blood sample during the separation of plasma and red blood cells, and a pH probe (744 pH Meter, Metrohm) was calibrated for blood pH analysis.

During sampling, fish were individually removed from tanks and anaesthetized using the lethal dose of neutralized MS222, blotted dry and weighed. Subsequently, a blood sample was drawn from the caudal vein with the use of a heparin-washed syringe. Blood pH was measured using the calibrated pH meter and immediately centrifuged for 1 min at a speed of 16,000 rpm and a temperature of 4°C. Plasma aliquots were collected, frozen in liquid nitrogen and stored at -80°C for later physiological analysis. Fish were also dissected on ice and gills were removed, washed with physiological solution (0.9%), blotted dry, wrapped with labeled aluminum foil, frozen in liquid nitrogen and stored at -80°C for further molecular analysis.

2.6. Analytical techniques

2.6.1. Plasma ions

Plasma ions (Na^+, Cl^- and K^+) were analyzed using an AVL 9180 Electrolyte Analyzer (AVL, Roche Diagnostics, Belgium) which uses measurement by ion-selective electrodes. This instrument was calibrated using its internal standards before analyzing the samples. Then, 100-µL plasma sample aliquots were introduced, and ion concentrations were determined.

2.6.2. Molecular analyses

2.6.2.1. RNA extraction using TRIzol method

The gill filaments were dissected from the gill samples and put in the Biopur® bullet tube that was initially filled with 400µL TRIzol® Reagent (Invitrogen, Merelbeke, Belgium). This reagent facilitates the isolation of a variety of RNA species and maintains the RNA integrity

during tissue homogenization while disrupting and breaking down the cells and cell components at the same time. The bullet tubes were then loaded with sufficient amount of glass beads that permits optimal crushing of samples. Samples were homogenized using the TissueLyser LT (Qiagen, Germany) set at an oscillation of 50 Hz for 2 minutes. 600µL TRIzol® Reagent was added to the partially-homogenized samples and samples were subjected again to the procedure again until homogenization was completed and incubated. Subsequently, they were kept for 5 minutes at 15-30ºC (at room temperature) to permit complete dissociation of nucleoprotein complexes. 200-µL chloroform was then added and sample were shaken vigorously for 15 seconds. Samples were incubated again for 2-3 minutes at 15-30ºC (at room temperature) and centrifuged for 15 minutes at a speed of 12,000rpm at 4ºC to allow phase separation. The aqueous upper phase that contains RNA was transferred to the labeled Eppendorf tubes and 500µL 2-propanol was added. Samples were then mixed properly and incubated in the freezer at -20ºC overnight.

After overnight incubation, samples were centrifuged for 10 minutes at a speed of 12,000 rpm at 4ºC. The RNA formed a pellet at the bottom of the tube and the supernatants were removed from the tube. The RNA pellets were washed with 1mL 75% ethanol and centrifuged for 5 minutes at a speed of 7,600 rpm and a temperature of 4ºC. The ethanol was removed, and the remaining fluids were centrifuged and taken out. The samples were later dried through vacuum drying to allow evaporation of the remaining ethanol. The pellets were then dissolved in 30µL RNase-free water. RNA concentration was checked using NanoDrop spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA) to initially determine if sufficient RNA was extracted from the samples.

2.6.2.2. DNase treatment

After RNA extraction, DNase treatment was done using a DNase kit (Thermo Scientific, USA). A 30-µL RNA sample was mixed with 4µL 10x Reaction Buffer complexed with $MgCl_2$, 1µL RNase-free DNase and 1µL Riboblock ribonuclease (RNase) inhibitor. RNase-free DNase is an endonuclease that specifically cleaves the DNA strands in the sample by breaking the phosphodiester bonds. On the other hand, the RNase inhibitor added prevents RNase from degrading the RNA in the samples.

Afterwards, the samples were incubated in the thermomixer (Thermomixer Comfort, Eppendorf) for 30 minutes at 37ºC. Then, 164µL of RNase-free water and 200µL phenol-chloroform were added, and samples were mixed using a vortex and centrifuged at 14,000 rpm and 4ºC for 5 minutes for further purification of RNA. The clear, upper phase that contains the RNA was transferred to a new Eppendorf tube and 20µL sodium acetate was added and 400µL 100% ethanol, followed by overnight incubation in the freezer at –20ºC.

After the overnight incubation, samples were centrifuged at 13,200 rpm and 4ºC for 20 minutes, the supernatant was discarded, and the samples were briefly spun down using a microcentrifuge at 7,600 rpm and 4ºC. Residual fluids were continuously removed using a pipette and evaporated using a vacuum centrifuge for 30 seconds at 30ºC. Then, the RNA pellet was again resuspended in 30µL RNase-free water and mixed gently using a vortex. RNA concentration was checked again using NanoDrop spectrophotometry. Nucleic acids have optimal absorption at 260 nm and the 260/280 ratios should be between 1.8-2.0 and 260/230 ratios between 2.0-2.2. If they have lower values, it indicates the presence of contaminants such as proteins. If the values are not in these ranges, more purification steps should be taken as previously seen.

Based on the results, calculations were done to attain a 200 ng/µL RNA concentration which was used as working RNA for cDNA synthesis.

2.6.2.3. cDNA synthesis

The RevertAid H minus first-strand cDNA synthesis kit (Fermentas, Cambridge, UK) was used to transcribe 1µg RNA into the first-strand cDNA suitable for PCR amplification. The kit used for cDNA synthesis contains Random primer, RNase-free water, RevertAid H Minus reverse transcriptase, 5x Reaction buffer, Ribolock Ribonuclease inhibitor (20 U/µL) and 10 mM dNTP mix (which provides deoxyribonucleotides for making cDNA).

Prior to cDNA synthesis, a 12-µL reaction mixture composed of template RNA (1µg), Random primer and RNase-free water was prepared. It was gently vortexed and spun down in a microcentrifuge for 3 minutes at 7,600 rpm and 4°C. It was then incubated in a thermomixer at 37°C, cooled on ice and centrifuged for 2 minutes at the same speed and temperature.

During synthesis, 4µL 5x Reaction buffer, 1µL Ribolock Ribonuclease inhibitor and 2µL 10mM dNTP mix were added to the mixture, gently vortexed and centrifuged for few seconds at 4°C. The whole mixture was then incubated for 5 minutes at 37°C in a thermomixer and added with 1µL of RevertAid H minus reverse transcriptase. The reverse transcriptase is an enzyme that generates the first strand complementary DNA (cDNA) from a RNA template. After vortexing, the whole mixture was incubated at 25°C and then at 42°C for 10 minutes and 60 minutes, respectively, in a thermomixer. The reaction was stopped by heating the mixture at 70°C for 10 minutes in a thermomixer, chilled on ice and diluted with 80µL of RNase-free water.

Afterwards, DNA concentration was checked using NanoDrop spectrophotometry. Based on the results, working cDNA stock of 10 ng/µL was prepared for qPCR analysis by diluting it with RNase-free water.

2.6.2.4. qPCR

The qPCR analysis on the mRNA expression of Na^+/K^+-ATPase α-subunit 1 (NKA), $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and Rhesus family glycoproteins (Rhbg, Rhcg1 and Rhcg2) in the gills was quantified by Mx3000P QPCR System (Agilent Technologies, Belgium) using the primers listed in Table 2.

The samples were initially vortexed gently and spun down in a microcentrifuge at 7,600 rpm and 4ºC for few seconds. The qPCR reaction per well includes 5µL of 5x diluted cDNA plus 15µL Master mixture prepared from the PCR kit (Agilent Technologies, USA). The Master mixture contains 10µL of each 10pmol forward and reverse primers, 0.3µL ROX dye (1:500 dilution) and 10µL Brilliant II SYBR Green qPCR. The well plates with the reaction mixture were then centrifuged using Centrifuge 5415 R (Eppendorf) for 1 minute at 2400 rpm and 4°C.

The qPCR analyses were performed in a four-step experimental run protocol: denaturation program (3 min at 95 °C), amplification and quantification program (15 s at 95°C and 20 s at 60°C, respectively) repeated 40 times, melting curve program (60 s at 95 °C, 30 s at 60°C, 30 s at 95°C) and finally a cooling step. Melt curve analyses of the target genes and reference genes resulted in single products with specific melting temperatures. In addition, 'no-template' controls (i.e. with water sample) for each set of genes were also run to ensure no contamination of reagents, no primer–dimer formation, etc.

Comparison of two reference genes (elongation factor-1α and 18s rRNA) showed that elongation factor-1α (EF-1α) was the most stable gene across the samples (20 random samples were tested) and was used as endogenous standard to calculate relative mRNA expression by the standard curve method. Standard curves were generated by serial dilution of a random mixture of control samples.

Table 2 qPCR primer list and accession numbers of target and reference genes, and efficiencies of the PCR reactions.

Gene	Sequence of Primer (5' → 3')	Efficiency (%)	References	Accession No.
Na^+/K^+-ATPase	F: CTG-GAG-TGG-AAG-AAG-GTC R: GAT-GAA-GAG-GAG-GAA-GG	109.8	[43]	AY532637
Na-K-Cl cotransporter	F: TCA-TCA-CTG-CTG-GAA-TCT-T R: AGA-GAA-ACC-CAC-ATG-TTG-TA	113.4	[44]	AY954108
Rhbg	F: CCT-CAT-GGT-GAC-CCG-AAT-CC R: TAT-GTG-GAC-AGA-GTG-CAG-GC	101.7	[45]	
Rhcg1	F: TCA-GGG-AAT-TGT-GTG-ACC-GC R: CCC-AGC-GTG-GAC-TTG-ATT-CT	97.0	[45]	
Rhcg2	F: TGG-CTA-CCT-GTT-TGT-CAC-GC R: TAT-AAA-GCC-GCC-GAG-CAT-CC	105.1	[45]	
18S rRNA	F: CGC-TAG-AGG-TGA-AAT-TCT-TGG-A R: GAT-CAG-ATA-CCG-TCG-TAG-TTC-C	107.8	[46]	AM419038
EF 1α	F: GCT-TCG-AGG-AAA-TCA-CCA-G R: CAA-CCT-TCC-ATC-CCT-TGA-AC	97.5	[47]	AJ866727

2.7. Statistical analysis

All data have been presented as mean values ± standard error (SE). For comparisons between different experimental groups, a one-way analysis of variance (ANOVA) was performed followed by the Tukey's test. Student's two-tailed t-test was used for single comparisons. Main effects of salinity and CO_2 exposure and their interactions were analyzed by three-way ANOVA. The data were analyzed using Statistical Package for the Social Sciences (SPSS) version 21.0.

3. Results

3.1. Blood pH

The blood pH measurements of European sea bass (*Dicentrarchus labrax*) from the two CO_2 levels, control (400µatm) and exposed groups (1000µatm), acclimated at seawater (32 ppt), brackish water (10 ppt) and hyposaline water (2.5 ppt), are presented in Figure 1 and Table 3. It shows that there was no significant decrease ($p>0.05$) of blood pH on fish reared at 32 ppt. On the other hand, when fish were acclimated at 10 ppt, a transient but significant increase ($p<0.05$) was observed after 1-day exposure with high CO_2. Interestingly, a significant decrease ($p<0.001$) was observed after 21 days of CO_2 exposure of fish reared at 2.5 ppt.

No general pattern for blood pH to differ between salinities was noted, however, minor differences have existed between few sampling points. Blood pH was relatively higher in the control fish reared at normal seawater (32 ppt) at all sampling days, brackish water (10 ppt) only on the 3rd day and hyposaline water (2.5 ppt) on the 7th and 21st sampling days compared to high CO_2 exposed fish. A significant difference between 2.5 ppt and 32 ppt control groups was observed on the 1st and 7th day of exposure. On the contrary, no significant differences were noted in all salinity treatments following the 3rd and 21st day of exposure. Meanwhile, a significant difference was seen in 2.5 ppt compared to 32 ppt after the 7th and 21st day of exposure to high CO_2, however, no significant difference was observed in the exposed groups

on the 1st and 3rd day of exposure. Overall, a significant interaction was observed between salinity, elevated CO_2 and exposure time on blood pH (Table 4).

3.2. Plasma ion concentration

A significant reduction of sodium concentration was only noted in the elevated CO_2-exposed fish acclimated at brackish water (10 ppt) compared to control fish after 1 day of exposure (Fig. 2 and Table 3). Like blood pH, no clear patterns on the changes of sodium concentration were noted, especially in the exposed group. Control fish showed a relatively higher plasma [Na^+] concentration at 10 ppt and relatively lower at 2.5 ppt compared to 32 ppt throughout the exposure period. Increase in plasma [Na^+] ions at 10 ppt was observed in most sampling periods, except on the 7th day while a reduction at 2.5 ppt was noted after 1-7 days. Significant differences were only observed on the control fish reared at 10 ppt after one-day exposure. In the exposed group of fish acclimated at 10 ppt, Na^+ concentration was significantly higher on the 3rd day, but relatively lower on the 7th day of high CO_2 exposure compared to the 32 ppt control group. Generally, a significant interaction was completely seen between salinity and length of exposure (Table 4).

Chloride (Cl^-) and potassium (K^+) ions were also measured. However, due to the dilutions used to obtain sufficient volume (100 µL), results showed that the concentrations of these ions fell out of the detection range.

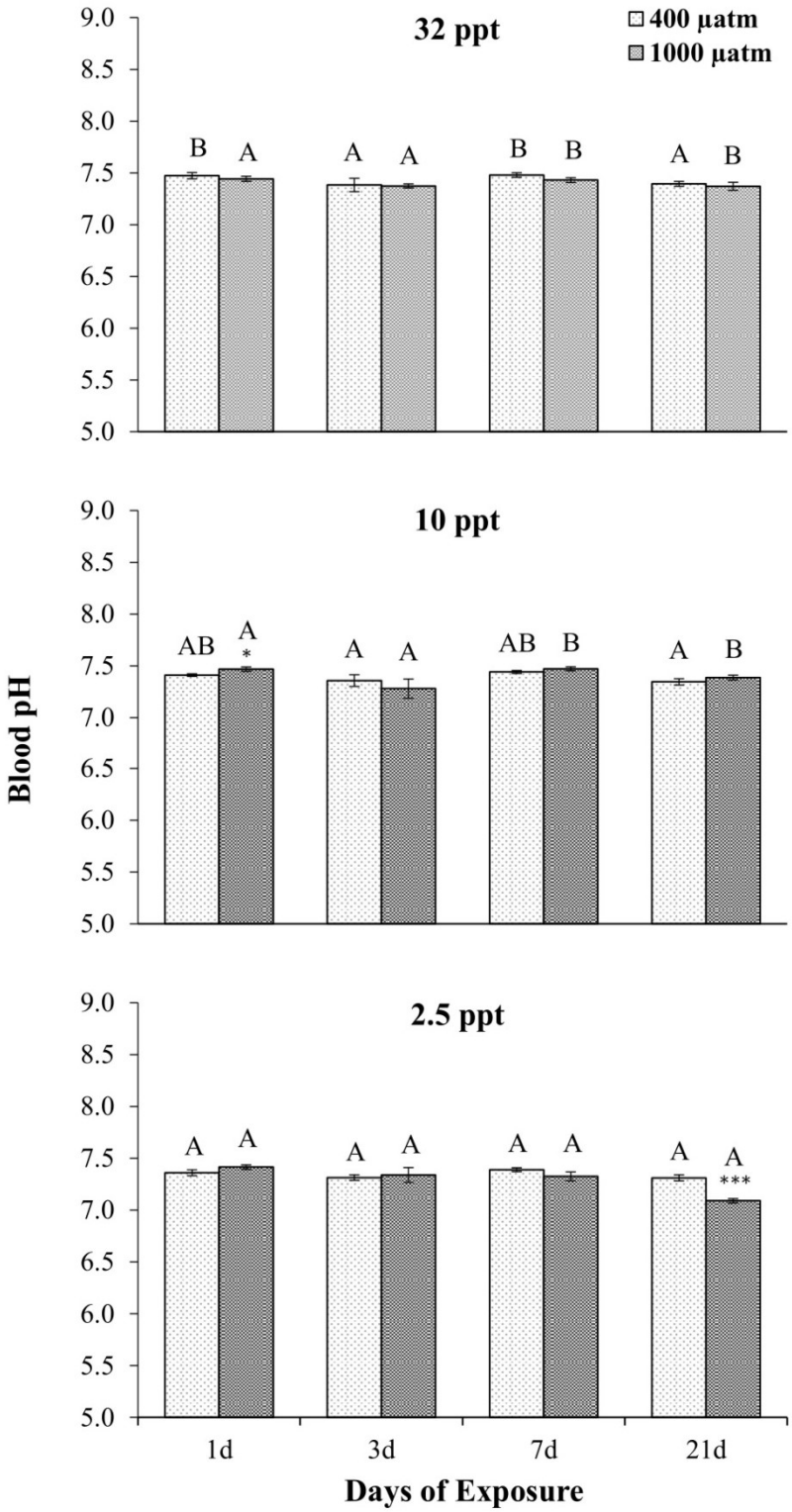

Fig. 1 Blood pH of European seabass acclimated to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to high P_{CO_2} (1000 µatm). Values are mean ± S.E. Asterisk (*) indicates a significant difference between high P_{CO_2} exposed fish and its respective control (400 µatm) at each salinity ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; by *t-test*) for each sampling point. Different letters (A-B; by *ANOVA*) indicates a significant difference ($p < 0.05$) between the fish acclimated to different experimental salinities (32 ppt, 10 ppt and 2.5 ppt) at the same sampling period under control or high P_{CO_2} conditions.

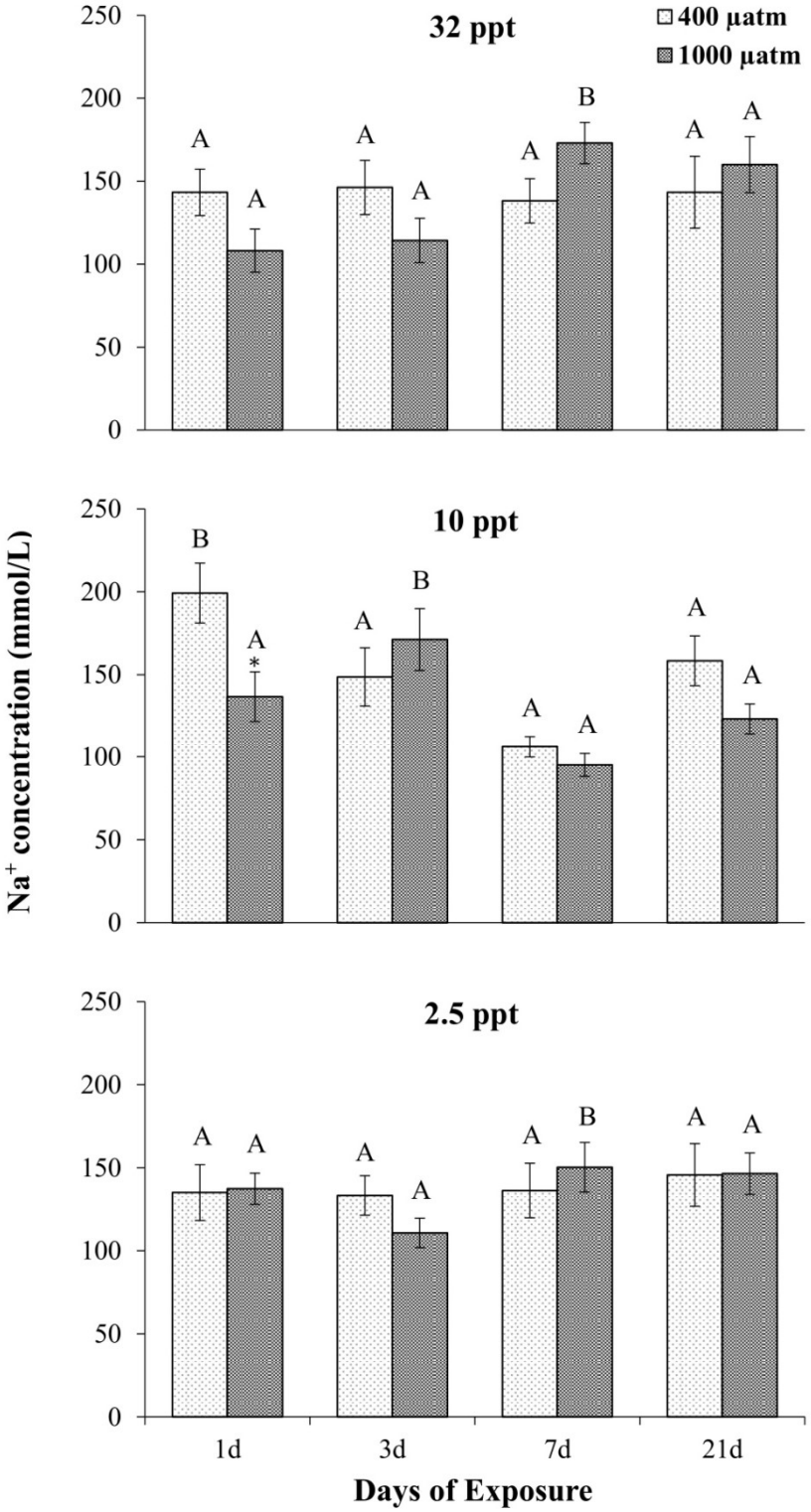

Fig. 2 Sodium (Na^+) ion concentration in the plasma of European seabass acclimated to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to high P_{CO_2} (1000 µatm). Values are mean ± S.E. Asterisk (*) indicates a significant difference between high P_{CO_2} exposed fish and its respective control (400 µatm) at each salinity ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; by *t-test*) for each sampling point. Different letters (A-B; by *ANOVA*) indicates a significant difference ($p < 0.05$) between the fish acclimated to different experimental salinities (32 ppt, 10 ppt and 2.5 ppt) at the same sampling period under control or high P_{CO_2} conditions.

3.3. Gene expression of *NKA*

In the present study, the level of NKA mRNA in the gill tissue of *D. labrax* exposed to elevated CO_2 level and reared at 32 ppt was significantly lower on the 1st ($p<0.001$) and 3rd ($p<0.05$) than those fish exposed to present-day CO_2 concentration (Fig. 3 and Table 3). Furthermore, no significant difference between control and exposed fish was noted on the 7th and 21st day of exposure. Control fish acclimated at 32 ppt showed an increase on the 1st and 3rd day, however, a decreasing trend was observed from 7th day until the end of the experiment. At 10 ppt, it showed that the transcript level of *NKA* of control fish increased on the 1st to 7th day of exposure but decreased towards the last day of exposure. Significant reduction of NKA mRNA level in the gill tissues of exposed fish was observed on the 3rd ($p<0.001$), 7th ($p<0.01$) and 21st ($p<0.001$) day of exposure. In the hyposaline water (2.5 ppt), an increasing transcript level was observed on the control groups from the 1st day towards the end of exposure period. However, NKA mRNA level of exposed fish was significantly lower on the 1st and 7th ($p<0.05$) day of exposure compared to the control fish and showed an alternating increase-decrease pattern of transcript levels on the whole duration of exposure period.

The results showed that the NKA mRNA level was relatively higher in control fish acclimated at lower salinities (10 ppt and 2.5 ppt) than those reared at 32 ppt in all sampling periods. A significant difference was observed on the control fish at 2.5 ppt throughout the experiment but only on 7th and 21st day of exposure for control fish at 10 ppt. Generally, upregulation of *NKA* as evidenced by increasing level of NKA mRNA was noted when the fish were acclimated at a decreasing salinity level. Unlike in the control groups, both upregulation and downregulation of NKA mRNA level were observed in the CO_2-exposed fish acclimated at 10 ppt and 2.5 ppt. Exposed fish acclimated at 2.5 ppt have significantly higher transcript levels of *NKA*, except on the 7th day of exposure. On the contrary, exposed fish acclimated at 10 ppt showed a numerically lower transcript level than those fish reared at 32 ppt. Overall, a significant interaction was observed between salinity, CO_2 exposure and length of exposure (Table 4).

3.4. Gene expression of *NKCC*

A significant reduction of the transcript levels of NKCC mRNA was noted in the gills of exposed fish acclimated at 32 ppt, specifically on the 1st and 3rd ($p<0.001$) day of exposure (Fig. 4 and Table 3). Exposed fish acclimated at 10 ppt showed also a significant decrease but on the 7th ($p<0.01$) and 21st ($p<0.05$) day of high CO_2 exposure.

NKCC mRNA level in the gills of control fish reared at 10 ppt and 2.5 ppt was relatively lower than those acclimated at 32 ppt in all sampling periods. A significant difference was observed in control fish reared at 10 ppt on the 1st day. Control fish from all salinity treatments also showed a significant difference on the 3rd day, however, no significant difference was noted on the 7th and 21st day of exposure. Meanwhile, *NKCC* in the gill tissue of exposed *D. labrax* was significantly upregulated at 10 ppt and 2.5 ppt on the first day, however, on the third day, it was only upregulated at 2.5 ppt. No significant difference was observed in all salinities on the later days of exposure. Overall, a significant interaction was observed between salinity, CO_2 exposure and length of exposure (Table 4).

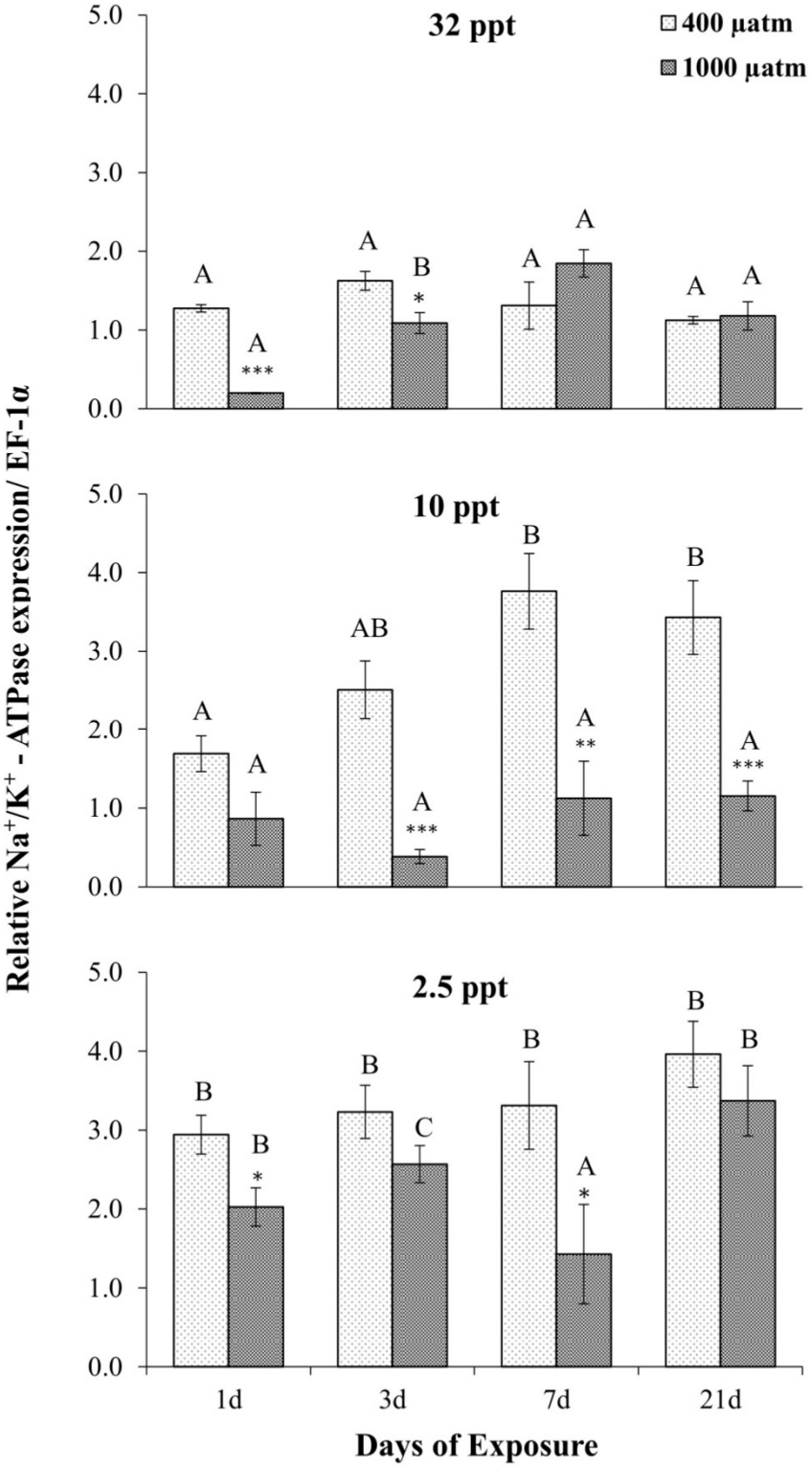

Fig. 3 Relative gene expression of Na^+/K^+-ATPase in the gills of European seabass acclimated to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to high P_{CO_2} (1000 µatm). Values are mean ± S.E. Asterisk (*) indicates a significant difference between high P_{CO_2} exposed fish and its respective control (400 µatm) at each salinity ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; by *t-test*) for each sampling point. Different letters (A-C; by *ANOVA*) indicates a significant difference ($p < 0.05$) between the fish acclimated to different experimental salinities (32 ppt, 10 ppt and 2.5 ppt) at the same sampling period under control or high P_{CO_2} conditions.

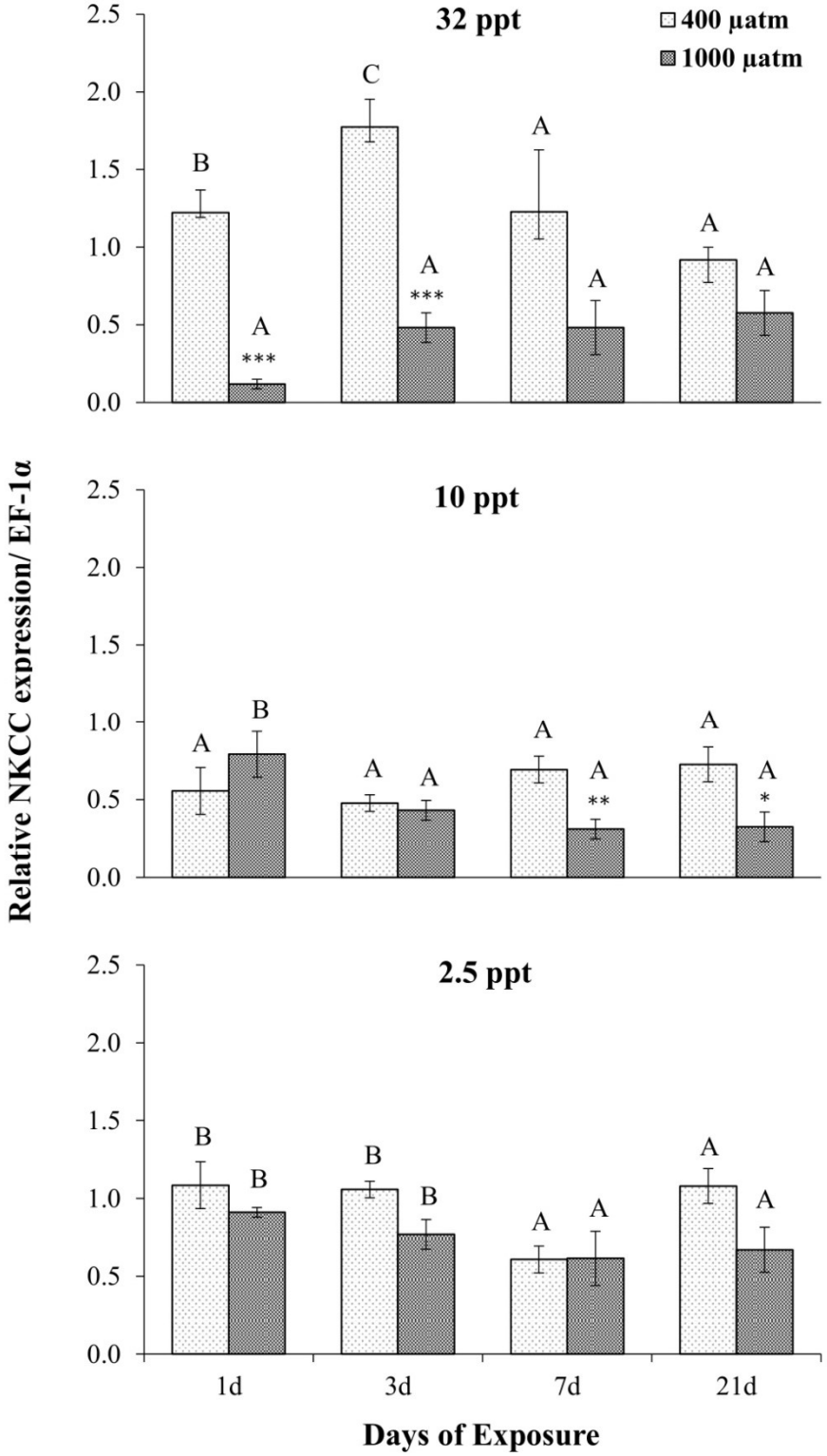

Fig. 4 Relative gene expression of Na-K-Cl cotransporter in the gills of European seabass acclimated to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to high P_{CO_2} (1000 µatm). Values are mean ± S.E. Asterisk (*) indicates a significant difference between high P_{CO_2} exposed fish and its respective control (400 µatm) at each salinity ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; by *t-test*) for each sampling point. Different letters (A-C; by *ANOVA*) indicates a significant difference ($p < 0.05$) between the fish acclimated to different experimental salinities (32 ppt, 10 ppt and 2.5 ppt) at the same sampling period under control or high P_{CO_2} conditions.

3.5. Gene expression of *Rhbg*

At 32 ppt, the transcript level of *Rhbg* in the gills of fish exposed to elevated CO_2 was significantly reduced on the 1st ($p<0.01$), 3rd ($p<0.01$) and 7th ($p<0.01$) day relative to the control fish (Fig. 5 and Table 3). However, no significant difference was observed on the last day of exposure. Exposed fish acclimated at 10 ppt showed a significant decrease of Rhbg mRNA only on the 7th ($p<0.05$) and 21st ($p<0.01$) day of exposure. Meanwhile, at 2.5 ppt, the transcript level of *Rhbg* in the exposed fish was significantly lower on the 1st ($p<0.001$) and 21st ($p<0.05$) day of exposure.

Upregulation and downregulation of Rhbg mRNA level in the gills of European sea bass were both observed in all salinity treatments at all sampling periods. In the control group, a significant difference in the transcript level of *Rhbg* of *D. labrax* acclimated at three salinity treatments was only observed on the 1st day of exposure. Reduced expression of *Rhbg* was noted at 10 ppt and 2.5 ppt on the 1st to 3rd day and 7th day, respectively relative to the 32 ppt control group. Meanwhile, in the exposed groups, the fish acclimated at 10 ppt showed a significant difference in transcript levels compared to 32 ppt on the 1st day. *Rhbg* was significantly upregulated at 10 ppt on the last day and 2.5 ppt on the 3rd day and the last day of exposure. No significant difference was noted on the 7th day. Generally, a significant interaction was observed between salinity, CO_2 exposure and length of exposure (Table 4).

3.6. Gene expression of *Rhcg1*

The transcript level of *Rhcg1* in the gill tissue of high CO_2-exposed *D. labrax* acclimated at 32 ppt and 2.5 ppt showed an increasing trend but decreasing at 10 ppt (Fig. 6 and Table 3). Meanwhile, the control fish at 32 ppt exhibited an alternating transcript level of *Rhcg1* but after the first day an increasing trend was seen at 10 ppt. At 32 ppt, exposed fish have significantly lower Rhcg1 mRNA after one ($p<0.001$), three ($p<0.05$) and seven ($p<0.05$) days of exposure than those control fish. Interestingly, the exposed fish acclimated at 10 ppt have significantly higher ($p<0.001$) transcript level of *Rhcg1* on the first day of exposure.

Both control and exposed fish showed that *Rhcg1* was upregulated at reduced salinities (2.5 ppt and 10 ppt) in all sampling periods, except in control fish acclimated at 10 ppt which were relatively lower than 32 ppt on the 1st day. A significant difference on transcript levels of *Rhcg1* was observed in the control fish and the exposed fish acclimated at 10 ppt after 7-21 and 1-7 days, respectively. Similarly, transcript levels of *Rhcg1* in the gill tissues of both exposed and control fish acclimated at 2.5 ppt showed a significant difference on the whole duration of exposure. Overall, a significant interaction was observed between salinity, CO_2 exposure and length of exposure (Table 4).

3.7. Gene expression of *Rhcg2*

Rhcg2 mRNA level showed no significant difference between control and exposed groups at any salinity treatment, 32 ppt, 10 ppt and 2.5 ppt (Fig. 7 and Table 3). Between salinities, control fish acclimated at 10 ppt exhibited a significant increase in Rhcg2 mRNA level only on the last day of exposure and, in the exposed fish, on the 3rd to 7th day of exposure. At 2.5 ppt, a significant difference in mRNA level was also observed on the 1st and 7th day of exposure of those control fish while only on the 7th day for the exposed fish. Overall, augmentation of Rhcg1 mRNA expression was observed in most sampling periods, except in exposed fish reared at 2.5 ppt on the last day of exposure. Generally, a significant interaction was observed between salinity, CO_2 exposure and length of exposure (Table 4).

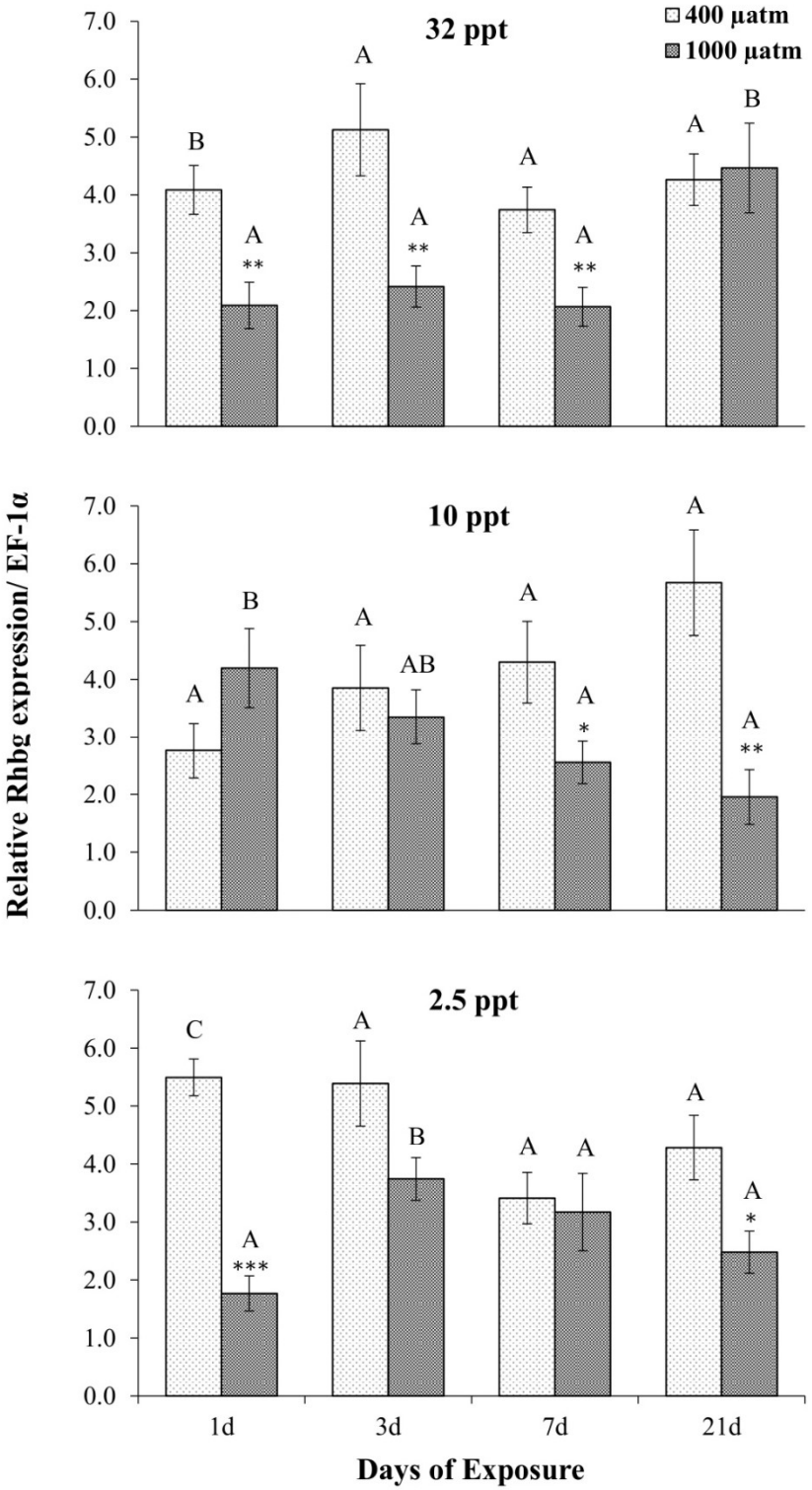

Fig. 5 Relative gene expression of Rhbg in the gills of European seabass acclimated to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to high P_{CO_2} (1000 µatm). Values are mean ± S.E. Asterisk (*) indicates a significant difference between high P_{CO_2} exposed fish and its respective control (400 µatm) at each salinity ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; by *t-test*) for each sampling point. Different letters (A-C; by *ANOVA*) indicates a significant difference ($p < 0.05$) between the fish acclimated to different experimental salinities (32 ppt, 10 ppt and 2.5 ppt) at the same sampling period under control or high P_{CO_2} conditions.

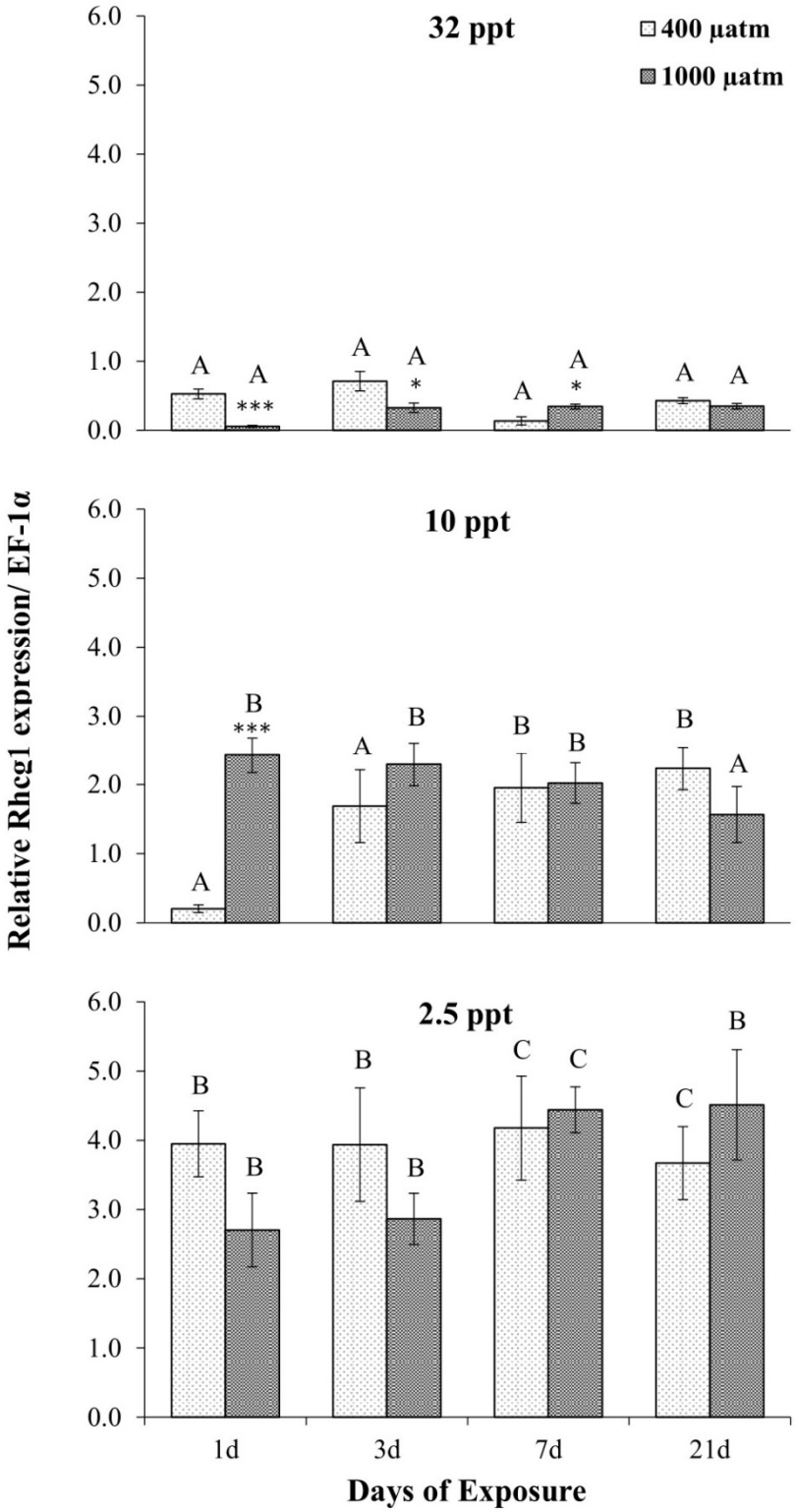

Fig. 6 Relative gene expression of Rhcg1 in the gills of European seabass acclimated to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to high P_{CO_2} (1000 µatm). Values are mean ± S.E. Asterisk (*) indicates a significant difference between high P_{CO_2} exposed fish and its respective control (400 µatm) at each salinity ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; by *t-test*) for each sampling point. Different letters (A-C; by *ANOVA*) indicates a significant difference ($p < 0.05$) between the fish acclimated to different experimental salinities (32 ppt, 10 ppt and 2.5 ppt) at the same sampling period under control or high P_{CO_2} conditions.

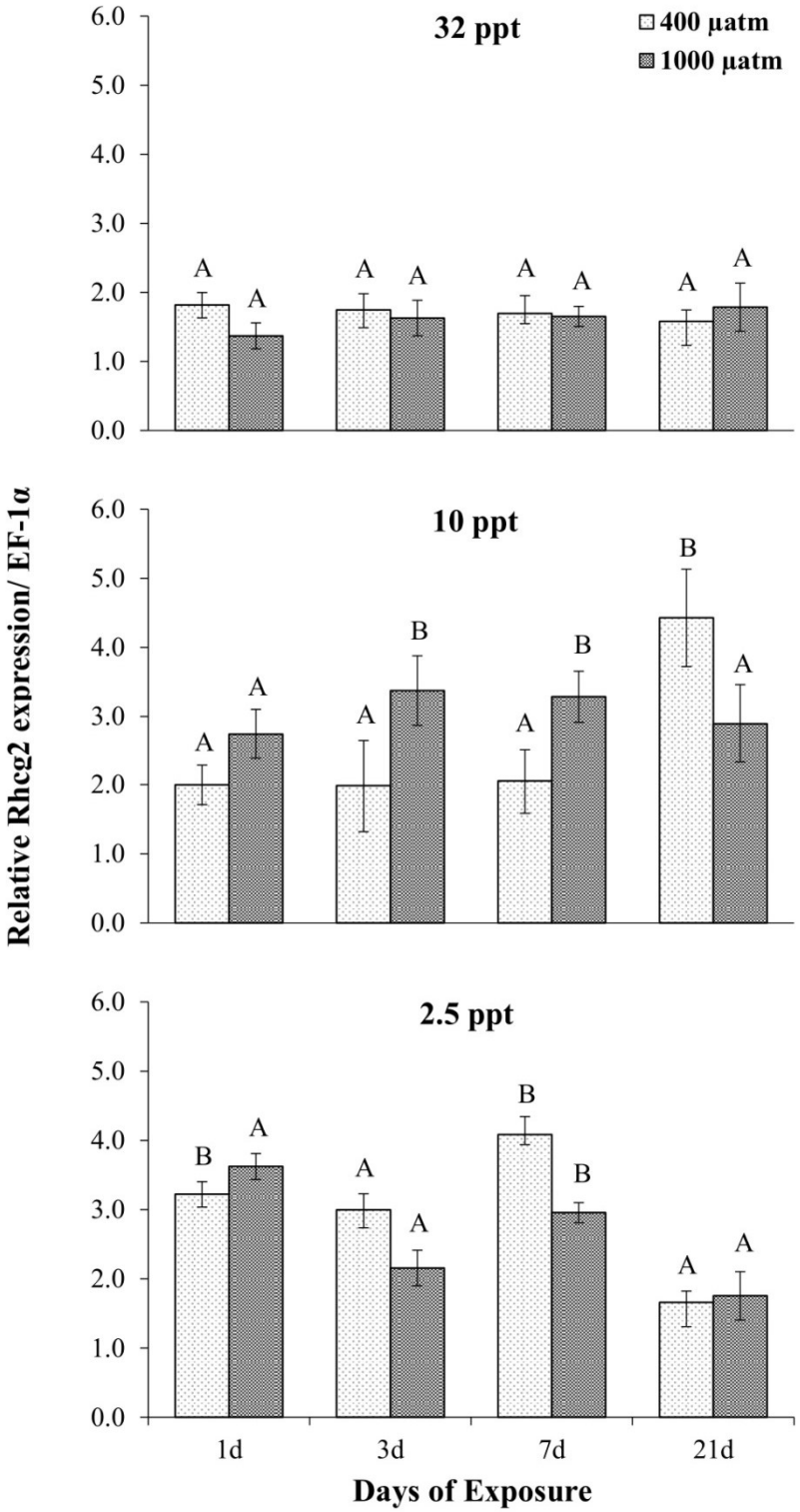

Fig. 7 Relative gene expression of Rhcg2 in the gills of European seabass acclimated to different salinities (32 ppt, 10 ppt and 2.5 ppt) and exposure to high P_{CO_2} (1000 µatm). Values are mean ± S.E. Asterisk (*) indicates a significant difference between high P_{CO_2} exposed fish and its respective control (400 µatm) at each salinity ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; by *t-test*) for each sampling point. Different letters (A-B; by *ANOVA*) indicates a significant difference ($p < 0.05$) between the fish acclimated to different experimental salinities (32 ppt, 10 ppt and 2.5 ppt) at the same sampling period under control or high P_{CO_2} conditions.

Table 3 Blood pH, Na^+ concentrations (mmol/L) in plasma, and gene expressions of branchial ion and ammonia transporters of European sea bass under different treatments

Treatments			Blood pH	Na^+	NKA	NKCC	Rhbg	Rhcg1	Rhcg2
32 ppt	1d	Control	7.47±0.03	143.32±13.98	1.27±0.05	1.22±0.15	4.09±0.42	0.53±0.07	1.82±0.18
		Exposed	7.44±0.02	108.15±12.98	0.20±0.01	0.12±0.03	2.09±0.40	0.06±0.01	1.37±0.19
	3d	Control	7.38±0.06	146.22±16.34	1.62±0.12	1.77±0.18	5.13±0.80	0.71±0.14	1.75±0.23
		Exposed	7.37±0.02	114.24±13.40	1.09±0.13	0.48±0.10	2.42±0.36	0.33±0.07	1.63±0.26
	7d	Control	7.48±0.02	138.14±13.35	1.31±0.30	1.23±0.40	3.74±0.39	0.14±0.06	1.69±0.26
		Exposed	7.43±0.02	173.01±12.46	1.85±0.17	0.48±0.17	2.06±0.34	0.34±0.03	1.65±0.15
	21d	Control	7.39±0.02	143.35±21.70	1.12±0.05	0.92±0.08	4.26±0.45	0.43±0.04	1.58±0.16
		Exposed	7.37±0.04	159.99±16.92	1.18±0.18	0.58±0.14	4.46±0.77	0.35±0.04	1.78±0.35
10 ppt	1d	Control	7.41±0.01	199.21±18.07	1.69±0.23	0.56±0.15	2.76±0.48	0.20±0.06	1.99±0.29
		Exposed	7.47±0.02	136.69±15.04	0.86±0.34	0.79±0.15	4.20±0.68	2.43±0.26	2.74±0.36
	3d	Control	7.36±0.06	148.65±17.56	2.51±0.37	0.48±0.05	3.85±0.74	1.69±0.53	1.98±0.67
		Exposed	7.28±0.09	171.19±18.66	0.38±0.09	0.43±0.06	3.35±0.47	2.29±0.31	3.37±0.50
	7d	Control	7.44±0.01	106.21±6.37	3.76±0.48	0.69±0.09	4.30±0.71	1.95±0.50	2.05±0.47
		Exposed	7.47±0.02	95.06±6.92	1.12±0.47	0.31±0.06	2.55±0.37	2.02±0.29	3.28±0.37
	21d	Control	7.35±0.03	158.39±14.98	3.43±0.47	0.73±0.11	5.67±0.91	2.23±0.31	4.43±0.71
		Exposed	7.39±0.02	123.31±9.03	1.15±0.19	0.32±0.10	1.95±0.47	1.56±0.40	2.89±0.57
2.5 ppt	1d	Control	7.36±0.03	135.04±16.80	2.94±0.25	1.08±0.13	5.50±0.32	3.95±0.48	3.22±0.46
		Exposed	7.41±0.02	137.74±9.40	2.03±0.24	0.91±0.31	1.77±0.30	2.70±0.53	3.62±1.30
	3d	Control	7.31±0.03	133.31±16.41	3.23±0.34	1.06±0.13	5.39±0.73	3.94±0.82	2.99±0.59
		Exposed	7.34±0.07	110.75±8.86	2.57±0.23	0.77±0.10	3.74±0.37	2.86±0.37	2.16±0.28
	7d	Control	7.39±0.02	136.27±16.41	3.31±0.56	0.61±0.12	3.41±0.44	4.18±0.75	4.08±0.64
		Exposed	7.32±0.04	150.30±14.93	1.43±0.63	0.61±0.12	3.17±0.67	4.44±0.33	2.95±0.45
	21d	Control	7.31±0.03	145.68±18.84	3.96±0.42	1.08±0.20	4.28±0.56	3.67±0.53	1.66±0.18
		Exposed	7.09±0.02	146.39±12.51	3.37±0.45	0.67±0.16	2.48±0.36	4.51±0.80	1.75±0.36

Table 4 The effects of salinity, CO_2 exposure, time and their interactions on physiological and ion-regulatory parameters in European sea bass.

Treatment	Salinity		CO_2		Time		Salinity x CO_2		Salinity x Time		CO_2 x Time		Salinity x CO_2 x Time	
	F value	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*	*F value*	*P value*
Non-invasive measurements														
Blood pH	17.295	0.000	2.252	0.135	14.795	0.000	1.669	0.192	2.356	0.033	1.746	0.160	2.804	0.013
Na^+ ions	0.301	0.741	2.278	0.133	1.001	0.394	1.131	0.325	5.552	0.000	2.502	0.061	1.925	0.080
Branchial ion transporters														
NKA expression	47.210	0.000	57.958	0.000	6.596	0.000	11.744	0.000	3.404	0.004	0.408	0.748	2.696	0.017
NKCC expression	11.434	0.000	44.804	0.000	1.546	0.205	13.386	0.000	2.606	0.020	0.522	0.668	2.950	0.009
Ammonia transporters														
Rhbg expression	0.260	0.771	45.800	0.000	2.832	0.040	0.873	0.420	1.406	0.216	0.299	0.826	6.396	0.000
Rhcg1 expression	140.90	0.000	0.020	0.887	1.965	0.122	2.603	0.077	1.106	0.361	0.426	0.734	3.629	0.002
Rhcg2 expression	13.882	0.000	0.000	0.987	0.456	0.713	1.444	0.239	4.017	0.001	0.478	0.698	2.204	0.046

4. Discussion

In order to gain a deeper understanding of how fish adapt to stress caused by a combination of salinity challenges and increased carbon dioxide levels, this study aimed to examine the temporal effects of individual and combined exposures to these waterborne variables on a range of adaptive responses in European sea bass. The investigation focused on different levels of biological organizations to unravel the intricate mode of interaction between these stressors.

4.1. Blood acidosis

Fish are known to its ability to respond effectively and restore blood pH when exposed to high CO_2 causing acid-base balance disturbance [48]. This characteristic might explain why there were no significant changes of blood pH of both control and CO_2-exposed European sea bass acclimated at 32 ppt. Esbaugh et al. (2012) found that gulf toadfish have been able to defend blood pH after exposure to high CO_2 (1900 μatm) by sustaining increased levels of HCO_3^- in the face of higher pCO_2 in the blood [49]. This could be similar to *D. labrax* experiencing increased CO_2 in a normal seawater environment. The compensatory response of fish during exposure to elevated CO_2 levels to correct blood pH may lead to negative downstream consequences or induce tradeoffs [23, 50, 51]. Furthermore, the compensatory responses of teleosts to pH are linked to a sustained increase of extra and intracellular concentration of HCO_{3-} in response to the elevated P_{CO_2} [29, 49, 52, 53, 54].

Nilsson et al. (2012) revealed that these chronic alterations in intra- and extracellular HCO_3^- and Cl^- affect the gamma-aminobutyric acid type A ($GABA_A$) receptor functionality, leading to behavioral disturbances of many aquatic organisms [51]. Several acute behavioral modifications include olfactory disturbance and olfaction preference reversal in larval clownfish [55]; effects on boldness and exploratory behavior, in the three-spined stickleback (*Gasterosteus aculeatus*) [22]; visual impairment in juvenile damselfish (*Pomacentrus amboinensis*) [56]; reduced auditory response in clownfish (*Amphiprion percula*) [57]; reduced olfactory prey detection, reduced feeding, and increased activity in brown dottyback (*Pseudochromis fuscus*) [58]; reduced lateralization and prey detection in a larval *Neopomacentrus azysron* [51, 59]; and effects on learning clownfish (*Amphiprion percula*) [57] and three-spined stickleback (*Gasterosteus aculeatus*) [22].

However, a sudden but transient increase of blood pH of fish acclimated in the brackish water could be due to an overshoot of the accumulation of blood HCO_3^- followed by Cl^- loss [60], which was compensated following prolonged exposure. The increased blood pH might indicate hyperventilation which also coincides with the reduced Na^+. Loss of Na^+ could be a consequence of hyperventilation as well. Both increased plasma CO_2 or reduced plasma pH could lead to hyperventilation which then could affect ion regulation. Hyperventilation would lead to an increased plasma/blood pH which was observed at 10 ppt after 1st day of exposure. On the contrary, the significant blood pH reduction of fish reared at hyposaline environment on the last days of high CO_2 exposure could be attenuated and attributed to the chemical buffering of low-molecular weight substances, such as proteins [61]. Consequently, the gill transfer of acid-base ions with the ambient water recuperated such transient depression [61].

4.2. Ion-regulatory adjustments

The study conducted by Lin et al. (2003) revealed that there was an increase in branchial NKA activity in milkfish (*Chanos chanos*) exposed in freshwater environment (FW; 0‰), which in turn enhanced the driving force for ion transport [74]. This suggests that there was a

higher demand for ion uptake or secretion in the gills. In vitro activity assays conducted on euryhaline teleosts such as Mozambique tilapia [75], European seabass [40], milkfish and spotted green pufferfish [75] demonstrated that the branchial NKA of these species was influenced by the ionic strength of the assay medium. In this study, lower salinities (10 ppt and 2.5 ppt) stimulated the mRNA expression of Na^+/K^+ ATPase (NKA) significantly which could be a compensatory mechanism to counteract ion loss in control fish [16]. Elevated expression of *NKA* is usually observed in marine teleosts during high CO_2 exposure [23, 62]. This could be attributed to the requirement for increased activity of acid-base transporters such as Na^+/H^+ exchanger, anion exchangers and co-transporters, aided by the Na^+/K^+ ATPase enzyme [10, 23]. The enhanced activity of *NKA* in both control and exposed fish aided to regulate their ion status and was reflected by the Na^+ retention even in a hypo-osmotic environment [16]. It indicates the regulatory capacity of fish in maintaining acid-base balance when exposed to environmental stressors.

In contrast, the elevated CO_2 level has induced the downregulation of *NKA* in the exposed fish at 10 ppt which could be a physiological response of fish to lower the Na^+ gradient by increasing the intracellular Na^+. This reduces the back movement of protons to the plasma via Na^+/H^+ exchanger 1 aided by the gradient for HCO_{3-} transport via HCO_3^- cotransporter (NBC). Similarly, Esbaugh et al. (2012) also revealed that there was downregulation of NKA mRNA expression in the Gulf toadfish (*Opsamus beta*) [49]. The reduced *NKA* expression suggests that estuarine teleost could be mostly affected by ocean acidification.

Like *NKA*, the NKCC mRNA expression considerably links to salinity the upregulation at normal seawater salinity and downregulation under low salinities as demonstrated by euryhaline teleosts [25]. Sinha et al. (2015) reported a downregulation of NKCC expression as adaptive response to foster ion retention under hyposaline environment in seabass exposure to low salinity and high environmental ammonia [16, 38].

Aside from NKA and NKCC, Na+/H+ exchanger 1 (NHE-1), a transmembrane protein, also enables the transfer of a sodium ion (Na+) in exchange for a hydrogen ion (H+) across the cellular membrane. Additionally, these proteins possess the ability to detect and respond to changes in the intracellular pH levels. During acute hypercapnia, there was a decrease in NHE-1 mRNA levels observed in both the gill and kidney, whereas an increase in NHE-1 mRNA levels was noted in the kidney during chronic hypercapnia exposure [71].

4.3. Roles of *Rhesus (Rh) glycoproteins* in ammonia transport of fish exposed to high CO_2 acclimated at lower salinities

The excretion of ammonia from the gill in teleost fish is vital for nitrogen elimination [63]. Recent studies revealed that the transport of ammonia may be mediated in part *via* ammonia gas (NH_3) channels called Rhesus (Rh) glycoproteins [62, 64, 65]. *Rhbg* appears to be localized in the basolateral membranes while *Rhcg* in the apical membranes of the branchial epithelium [66]. In the present study, upregulation of *Rhbg, Rhcg1* and *Rhcg2* was generally observed at reduced salinities in most sampling periods. It suggests that the presence of environmental stressor, such as increased CO_2, may increase ammonia excretion rate at lower salinities. A dual NH_3 / CO_2 transport function of Rhesus glycoproteins is attributed to their upregulation following high CO_2 exposure. This could be linked to acid-base regulation and probably a sign of changes in conditions caused by elevated CO_2 and/or a sign of change in conditions for ammonia excretion dictated by elevated P_{CO_2} [23, 66, 67]. In the branchial epithelium, *Rhbg*

was found to be highly upregulated and play a significant role in ammonia excretion in freshwater teleosts, such as zebrafish larvae [68] and goldfish (*Carassius auratus*) [69].

The upregulation of Rhesus glycoproteins may indicate high efficiency of the juvenile European sea bass when exposed to hypercapnic environment at reduced salinities. Such elevated expression of the putative Rh glycoproteins is the ideal pattern in enhancing ammonia excretion rates in marine fish [62, 64, 66, 72, 73].

5. Conclusion

The effects of high CO_2 on European sea bass acclimated to experimental salinities from full strength seawater (32 ppt) to hyposaline water (2.5 ppt) were determined. The results showed that the physiological and ion-osmoregulatory performance of euryhaline teleost were strongly affected when acclimated at reduced salinities and exposed in a hypercapnic environment. Fish reared at normal seawater showed no significant difference of blood pH between control and CO_2-exposed fish which could be attributed to the compensatory responses of fish to pH linked to the sustained increase of extra and intracellular concentration of HCO_{3-} as a response to the increased P_{CO_2} levels. High CO_2 had caused the transient but significant increase in the blood pH of juvenile sea bass acclimated at 10 ppt but a significant decrease of blood pH was observed after 21 days of exposure at 2.5ppt. This sudden but transient increase of blood pH might be due to an overshoot of the blood HCO_{3-} accumulation followed by Cl^- loss. Furthermore, a significant reduction of plasma $[Na^+]$ concentration was only observed in the elevated CO_2- exposed fish reared at 10 ppt on the 1st day of exposure compared to control fish. Nevertheless, control fish acclimated at 10 ppt have higher plasma $[Na^+]$ and lower plasma $[Na^+]$ at 2.5 ppt relative their 32 ppt control group throughout the experiment. Upregulation of *NKA* was observed in some gill tissues of the fish. NKA mRNA level was generally higher in the gill tissue of control fish at lower salinities (10 ppt and 2.5 ppt) relative to normal seawater-acclimated control fish at all sampling periods. The elevated expression could be attributed to the increased activity of acid-base transporters, aided by NKA enzyme and may indicate the ability of the fish to regulate their ion status. Significant reduction of NKA mRNA level was noted in the exposed fish reared at 32 ppt (1 day), 10 ppt (3-21 days) and 2.5 ppt (1 and 7 days) relative to the control fish at same salinities. This downregulation pattern of basolateral NKA might be a physiological response of the fish to lower the Na^+ gradient by increasing the intracellular Na^+, which reduces the backflow of protons into the plasma via Na^+/H^+ exchanger (NHE), aided by the gradient for HCO_{3-} transport via NBC. Moreover, NKCC mRNA level was significantly reduced in the exposed fish than control fish at 32 ppt (1 and 3 days) and 10 ppt (7 and 21 days). Control fish at lower salinities (10 ppt and 2.5 ppt) also had relatively lower NKCC mRNA level compared to 32 ppt control group. The reduced expression of *NKCC* could be an adaptive response to foster ion retention during hypercapnia in sea bass acclimated at lower salinities. A significant difference of *NKCC* in the gills of *D. labrax* acclimated at different salinities was noted on the 3rd day of exposure. Rhesus (Rh) glycoproteins (e.g. *Rhbg*, *Rhcg1* and *Rhcg2*) were generally upregulated in marine teleosts acclimated at reduced salinities. It suggests that environmental stressors, such as increased CO_2, may enhance ammonia excretion rate. Long-term exposure may be necessary to better understand the effects of elevated CO_2 level on the physiological responses of European sea bass.

Acknowledgments The authors would like to thank Dr. Steven Joosen, Prof. Raewyn Town and Dr. Amit Kumar Sinha for the technical assistance and data analysis and interpretation. Grateful acknowledgment is also given to Systemic Physiological and Ecotoxicological Research (SPHERE) research group.

Author contribution J.S. and G.D.B. conceptualized the project and acquired the research funding. W.C., J.S., M.N. and G.D.B. developed the materials and methods, conducted the experiment and processed the samples. J.S. and G.D.B. managed project administration and supervised the project. W.C. analyzed the data and generated the visuals. W.C. wrote the manuscript. All authors read, reviewed, and approved the final manuscript.

Funding This study was funded by a PhD Grant (BOF-DOCPRO-31444) from the University of Antwerp Research Council awarded to J. Shrivastava. W. Caneos is a recipient of *Vlaamse Interuniversitaire Raad - Universitaire Ontwikkelingssamenwerking* (VLIR-UOS) scholarship.

Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Code availability Not applicable

Declarations

Ethics approval All procedures related to animal handling, exposure and sampling were approved by the local ethics committee of the University of Antwerp (ECD 2014-23) and performed according to the guidelines of the Federation of European Laboratory Animal Science Associations.

Consent to participate All authors gave their consent to participate.

Consent for publication All authors gave their consent to publish this manuscript.

Competing interests The authors declare no competing interests.

Conflict of interests The authors declare no competing interests.

References

1. Doney SC (2010) The growing human footprint on coastal and open-ocean biogeochemistry. Science 328: 1512–1516. https://doi.org/10.1126/science.1185198
2. Franke A, Clemmesen C (2011) Effect of ocean acidification on early life stages of Atlantic herring (*Clupea harengus* L.). Biogeosciences Discuss 8: 7097–7126. https://doi.org/10.5194/bg-8-3697-2011
3. Lönnstedt OM, Munday PL, McCormick MI, Ferrari MCO, Chivers DP (2013) Ocean acidification and responses to predators: Can sensory redundancy reduce the apparent impacts of elevated CO_2 on fish? Ecol Evol 3: 3565–3575. https://doi.org/10.1002/ece3.684
4. Bresolin de Souza K, Jutfelt F, Kling P, Förlin L, Sturve J (2014) Effects of increased CO_2 on fish gill and plasma proteome. PLoS One 9: e102901. https://doi.org/10.1371/journal.pone.0102901
5. Green L, Jutfelt F (2014) Elevated carbon dioxide alters the plasma composition and behaviour of a shark. Biol Lett 10: 20140538. https://doi.org/10.1098/rsbl.2014.0538

6. Azevedo LB, De Schryver AM, Hendriks AJ, Huijbregts MAJ (2015) Calcifying species sensitivity distributions for ocean acidification. Environ Sci Technol 49: 1495–1500. https://doi.org/10.1021/es505485m
7. Checkley DM, Dickson AG, Takahashi M, Radich JA, Eisenkolb N, Asch R (2009) Elevated CO_2 enhances otolith growth in young fish. Science 324: 1683. https://doi.org/10.1126/science.1169806
8. Doney SC, Fabry VJ, Feely RA, Kleypas JA (2009) Ocean Acidification: The Other CO_2 Problem. Ann Rev Mar Sci 1: 69–92. https://doi.org/10.1146/annurev.marine.010908.163834
9. Logan CA (2010) A Review of Ocean Acidification and America's Response. Bioscience 60: 819–828 https://doi.org/10.1525/bio.2010.60.10.8
10. Esbaugh AJ (2017) Physiological implications of ocean acidification for marine fish: emerging patterns and new insights. J Comp Physiol B 188:1-13. https://doi.org/10.1007/s00360-017-1105-6
11. Hurst TP, Fernandez ER, Mathis JT (2013) Effects of ocean acidification on hatch size and larval growth of walleye pollock (*Theragra chalcogramma*). ICES J Mar Sci 70: 812–822. https://doi.org/10.1093/icesjms/fst053
12. Kreiss CM, Michael K, Lucassen M, Jutfelt F, Motyka R, Dupont S, Pörtner HO (2015) Ocean warming and acidification modulate energy budget and gill ion regulatory mechanisms in Atlantic cod (*Gadus morhua*). J Comp Physiol B Biochem Syst Environ Physiol 185: 767–781. https://doi.org/10.1007/s00360-015-0923-7
13. Kwan GT, Hamilton TJ, Tresguerres M (2017) CO_2-induced ocean acidification does not affect individual or group behaviour in a temperate damselfish. R Soc Open Sci 4: 170283 https://doi.org/10.1098/rsos.170283
14. IPCC (2013) Summary for Policymakers. *Clim Chang 2013 Phys Sci Basis Contrib Work Gr I to Fifth Assess Rep Intergov Panel Clim Chang* 33.
15. Magill SH, Sayer MDJ (2004) The effect of reduced temperature and salinity on the blood physiology of juvenile Atlantic cod. J Fish Biol 64: 1193–1205. https://doi.org/10.1111/j.0022-1112.2004.00383.x
16. Sinha AK, Dasan AF, Rasoloniriana R, Pipralia N, Blust R, De Boeck G (2015) Hypo-osmotic stress-induced physiological and ion-osmoregulatory responses in European sea bass (*Dicentrarchus labrax*) are modulated differentially by nutritional status. Comp Biochem Physiol -Part A Mol Integr Physiol 181:87–99. https://doi.org/10.1016/j.cbpa.2014.11.024
17. Sinha AK, Liew HJ, Diricx M, Blust R, De Boeck G (2012) The interactive effects of ammonia exposure, nutritional status and exercise on metabolic and physiological responses in gold fish (*Carassius auratus* L.). Aquat Toxicol 109: 33–46. https://doi.org/10.1016/j.aquatox.2011.11.002
18. Dixson DL, Munday PL, Jones GP (2010) Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. Ecol Lett 13: 68–75. https://doi.org/10.1111/j.1461-0248.2009.01400.x
19. Baumann H, Talmage SC, Gobler CJ (2012) Reduced early life growth and survival in a fish in direct response to increased carbon dioxide. Nat Clim Chang 2: 38–41. https://doi.org/10.1038/nclimate1291
20. Briffa M, de la Haye K, Munday PL (2012) High CO_2 and marine animal behaviour: Potential mechanisms and ecological consequences. Mar Pollut Bull 64: 1519–1528. https://doi.org/10.1016/j.marpolbul.2012.05.032
21. Frommel AY, Maneja R, Lowe D, Malzahn AM et al (2012) Severe tissue damage in Atlantic cod larvae under increasing ocean acidification. Nat Clim Chang 2: 42–46 https://doi.org/10.1038/nclimate1324

22. Jutfelt F, Bresolin de Souza K, Vuylsteke A, Sturve J (2013) Behavioural disturbances in a temperate fish exposed to sustained high-CO_2 levels. PLoS One 8: 6–11. https://doi.org/10.1371/journal.pone.0065825
23. Heuer RM, Grosell M (2014) Physiological impacts of elevated carbon dioxide and ocean acidification on fish. AJP Regul Integr Comp Physiol 307: R1061–R1084 https://doi.org/10.1152/ajpregu.00064.2014
24. Ishimatsu A, Kikkawa T, Hayashi, M, Lee KS, Kita J (2004) Effects of CO_2 on Marine Fish: Larvae and Adults. J Oceanogr 60:731–741. https://doi.org/10.1007/s10872-004-5765-y
25. Evans DH, Piermarini PM, Choe KP (2005) The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol Rev 85:97–177 https://doi.org/10.1152/physrev.00050.2003
26. Ishimatsu A, Hayashi M, Lee KS, Kikkawa T, Kita J (2005) Physiological effects on fishes in a high-CO_2 world. J Geophys Res C Ocean 110:1–8. https://doi.org/10.1029/2004JC002564
27. Kultz D (2015) Physiological mechanisms used by fish to cope with salinity stress. J Exp Biol 218:1907–1914. https://doi.org/10.1242/jeb.118695
28. Zadunaisky JA (1996) Chloride cells and osmoregulation. Kidney international, 49(6), 1563-1567. https://doi.org/10.1038/ki.1996.225
29. Claiborne JB, Edwards SL, Morrison-Shetlar AI (2002) Acid-base regulation in fishes: cellular and molecular mechanisms. J Exp Zool 293:302–319. https://doi.org/10.1002/jez.10125
30. Perry SF, Gilmour KM (2006) Acid-base balance and CO_2 excretion in fish: Unanswered questions and emerging models. Respir Physiol Neurobiol 154:199–215. https://doi.org/10.1016/j.resp.2006.04.010
31. Shrivastava J, Ndugwa M, Caneos W, De Boeck G (2019) Physiological trade-offs, acid-base balance and ion-osmoregulatory plasticity in European sea bass (*Dicentrarchus labrax*) juveniles under complex scenarios of salinity variation, ocean acidification and high ammonia challenge. Aquatic Toxicology 212:54-69. https://doi.org/10.1016/j.aquatox.2019.04.024
32. Marshall WS, Grosell M (2006) Ion transport, osmoregulation, and acid–base balance. Physiol Fishes 3: 179–214.
33. Hiroi J, Yasumasu S, Mccormick SD, Hwang P, Kaneko T (2008) Evidence for an apical Na–Cl cotransporter involved in ion uptake in a teleost fish. 2584–2599. https://doi.org/10.1242/jeb.018663
34. Hsu H, Lin L, Tseng Y (2014) A new model for fish ion regulation : identification of ionocytes in freshwater- and seawater-acclimated medaka (*Oryzias latipes*). Cell and Tissue Research 357:225–243. https://doi.org/10.1007/s00441-014-1883-z
35. Hwang PP, Lee TH, Lin LY (2011) Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. AJP Regul Integr Comp Physiol 301: R28–R47 https://doi.org/10.1152/ajpregu.00047.2011
36. Hiroi J, Mccormick SD (2012) New insights into gill ionocyte and ion transporter function in euryhaline and diadromous fish. Respir Physiol Neurobiol 184:257–268. https://doi.org/10.1016/j.resp.2012.07.019
37. Louro B, Power DM, Canario AVM (2014) Advances in European sea bass genomics and future perspectives. Mar Genomics 18:71–75. https://doi.org/10.1016/j.margen.2014.06.010
38. Sinha AK, Rasoloniriana R, Dasan AF, Pipralia N, Blust R, De Boeck G (2015) Interactive effect of high environmental ammonia and nutritional status on ecophysiological performance of European sea bass (*Dicentrarchus labrax*) acclimated to reduced seawater

salinities. Aquatic Toxicology 160:39–56. https://doi.org/10.1016/j.aquatox.2015.01.005
39. Duteil M, Pope EC, Pérez-Escudero A, de Polavieja GG, Fürtbauer I, Brown MR, King AJ (2016) European sea bass show behavioural resilience to near-future ocean acidification. R Soc Open Sci 3:160656 https://doi.org/10.1098/rsos.160656
40. Jensen MK, Madsen SS, Kristiansen K (1998) Osmoregulation and salinity effects on the expression and activity of Na+,K(+)-ATPase in the gills of European sea bass, *Dicentrarchus labrax* (L.). J Exp Zool 282: 290–300. https://doi.org/10.1002/(SICI)1097-010X(19981015)282:3<290::AID-JEZ2>3.0.CO;2-H
41. Barnabe G, Boulineau-Coatanea F, Rene F (1976) Chronology of morphogenesis in *Dicentrarchus labrax* (L.) (Pisces, Serranidae) obtained by artificial reproduction. *Aquaculture* **8**: 351–363. https://doi.org/10.1016/0044-8486(76)90117-4
42. Fiol DF, Kültz D (2007) Osmotic stress sensing and signaling in fishes. FEBS J 274:5790–5798. https://doi.org/10.1111/j.1742-4658.2007.06099.x
43. Giffard-Mena I, Lorin-Nebel C, Charmantier G, Castille R, Boulo V (2008) Adaptation of the sea-bass (*Dicentrarchus labrax*) to fresh water: role of aquaporins and Na+/K+-ATPases. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 150(3):332-338. https://doi.org/10.1016/j.cbpa.2008.04.004
44. Lorin-Nebel C, Boulo V, Bodinier C, Charmantier G (2006) The Na+/K+/2Cl-cotransporter in the sea bass *Dicentrarchus labrax* during ontogeny: involvement in osmoregulation. J Exp Biol 209(24):4908-4922. https://doi.org/10.1242/jeb.02591
45. Blondeau-Bidet E, Hiroi J, Lorin-Nebel C (2019) Ion uptake pathways in European sea bass *Dicentrarchus labrax*. Gene 692*:*126-137. https://doi.org/10.1016/j.gene.2019.01.006
46. Hakim Y, Harpaz S, Uni Z (2009) Expression of brush border enzymes and transporters in the intestine of European sea bass (*Dicentrarchus labrax*) following food deprivation. Aquaculture 290(1-2):110-115. https://doi.org/10.1016/j.aquaculture.2009.02.008
47. Geay F, Culi ESI, Corporeau C, Boudry P, Dreano Y, Corcos L et al (2010) Regulation of FADS2 expression and activity in European sea bass (*Dicentrarchus labrax*, L.) fed a vegetable diet. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 156(4):237-243. https://doi.org/10.1016/j.cbpb.2010.03.008
48. Heuer RM, Grosell M (2016) Elevated CO_2 increases energetic cost and ion movement in the marine fish intestine. Scientific Reports 6:1–8. https://doi.org/10.1038/srep34480
49. Esbaugh AJ, Heuer R, Grosell M (2012) Impacts of ocean acidification on respiratory gas exchange and acid-base balance in a marine teleost. Opsanus beta. J Comp Physiol B Biochem Syst Environ Physiol 182:921–934. https://doi.org/10.1007/s00360-012-0668-5
50. Pörtner HO, Langenbuch M, Reipschläger A (2004) Biological Impact of Elevated Ocean CO_2 Concentrations: Lessons from Animal Physiology and Earth History. Journal of Oceanography 60:705-718. https://doi.org/10.1007/s10872-004-5763-0
51. Nilsson GE, Dixson DL, Domenici P, McCormick MI, Sørensen C, Watson SA, Munday PL (2012) Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. Nat Clim Chang 2:201–204. https://doi.org/10.1038/nclimate1352
52. Toews DP, Holeton GF, Heisler N (1983) Regulation of the acid-base status during environmental hypercapnia in the marine teleost fish *Conger conger*. J Exp Biol 107:9–20. https://doi.org/10.1242/jeb.107.1.9
53. Brauner CJ, Baker DW (2009) Patterns of acid-base regulation during exposure to hypercarbia in fishes. In Cardio-Respiratory Control in Vertebrates: Comparative and Evolutionary Aspects. 43–63. https://doi.org/10.1007/978-3-540-93985-6_3
54. Esbaugh AJ, Ern R, Nordi WM, Johnson AS (2016) Respiratory plasticity is insufficient to alleviate blood acid–base disturbances after acclimation to ocean acidification in the

estuarine red drum, *Sciaenops ocellatus*. J Comp Physiol B Biochem Syst Environ Physiol 186:97–109. https://doi.org/10.1007/s00360-015-0940-6
55. Munday PL, Dixson DL, Donelson JM, Jones GP, Pratchett MS, Devitsina GV, Døving KB (2009) Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. Proc Natl Acad Sci 106:1848–1852. https://doi.org/10.1073/pnas.0809996106
56. Ferrari MCO, McCormick MI, Munday PL, Meekan MG, Dixson DL, Lönnstedt O, Chivers DP (2012) Effects of ocean acidification on visual risk assessment in coral reef fishes. Funct Ecol 26:553–558. https://doi.org/10.1111/j.1365-2435.2011.01951.x
57. Simpson SD, Munday PL, Wittenrich ML, Manassa R, Dixson DL, Gagliano M, Yan HY (2011) Ocean acidification erodes crucial auditory behaviour in a marine fish. Biol Lett 7:917–920. https://doi.org/10.1098/rsbl.2011.0293
58. Cripps IL, Munday PL, McCormick MI (2011) Ocean acidification affects prey detection by a predatory reef fish. PLoS One 6. https://doi.org/10.1371/journal.pone.0022736
59. Domenici P, Allan B, McCormick MI, Munday PL (2012) Elevated carbon dioxide affects behavioural lateralization in a coral reef fish. Biol Lett 8:78–81. https://doi.org/10.1098/rsbl.2011.0591
60. Petochi T, Di Marco P, Priori A, Finoia MG, Mercatali I, Marino G (2011) Coping strategy and stress response of European sea bass *Dicentrarchus labrax* to acute and chronic environmental hypercapnia under hyperoxic conditions. Aquaculture 315:312–320. https://doi.org/10.1016/j.aquaculture.2011.02.028
61. Heisler N (1984) Acid-base regulation in fishes. In Fish Physiology. Academic Press 10:315–401.
62. Nawata CM, Wood CM, O'Donnell MJ (2010) Functional characterization of Rhesus glycoproteins from an ammoniotelic teleost, the rainbow trout, using oocyte expression and SIET analysis. J Exp Biol 213:1049-1059. https://doi.org/10.1242/jeb.038752
63. Nakada T, Westhoff CM, Kato A, Hirose S (2007) Ammonia secretion from fish gill depends on a set of Rh glycoproteins. The FASEB Journal 21:1067–1074. https://doi.org/10.1096/fj.06-6834com
64. Nawata CM, Hirose S, Nakada T, Wood CM, Kato A (2010) Rh glycoprotein expression is modulated in pufferfish (*Takifugu rubripes*) during high environmental ammonia exposure. J Exp Biol 213:3150–3160. https://doi.org/10.1242/jeb.044719
65. Cooper CA, Wilson JM, Wright PA (2013) Marine, freshwater and aerially acclimated mangrove rivulus (*Kryptolebias marmoratus*) use different strategies for cutaneous ammonia excretion. Am J Physiol Integr Comp Physiol 304:R599–R612. https://doi.org/10.1152/ajpregu.00228.2012
66. Wright PA, Wood CM (2009) A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. J Exp Biol 212:2303–2312. https://doi.org/10.1242/jeb.023085
67. Tseng Y, Hu MY, Stumpp M, Lin L, Melzner F, Hwang P (2013) CO_2-driven seawater acidification differentially affects development and molecular plasticity along life history of fish (*Oryzias latipes*). Comp Biochem Physiol Part A 165:119–130. https://doi.org/10.1016/j.cbpa.2013.02.005
68. Braun MH, Steele SL, Ekker M, Perry SF (2009) Nitrogen excretion in developing zebrafish (*Danio rerio*): a role for Rh proteins and urea transporters. AJP Ren Physiol 296:F994–F1005 https://doi.org/10.1152/ajprenal.90656.2008
69. Sinha AK, Giblen T, AbdElgawad H, De Rop M, Asard H, Blust R, De Boeck G (2013) Regulation of amino acid metabolism as a defensive strategy in the brain of three freshwater teleosts in response to high environmental ammonia exposure. Aquatic Toxicology 130–131:86–96. https://doi.org/10.1016/j.aquatox.2013.01.003
70. Edwards SL, Marshall WS (2012) Principles and patterns of osmoregulation and

euryhalinity in fishes. In *Fish physiology*. Academic press 32:1-44. https://doi.org/10.1016/B978-0-12-396951-4.00001-3

71. Rimoldi S, Terova G, Brambilla F, Bernardini G, Gornati R, Saroglia M (2009) Molecular characterization and expression analysis of Na+/H+ exchanger (NHE)-1 and c-Fos genes in sea bass (Dicentrarchus labrax, L) exposed to acute and chronic hypercapnia. *Journal of experimental marine biology and ecology*, *375*(1-2), 32-40. https://doi.org/10.1016/j.jembe.2009.05.002
72. Nawata CM, Hung CCY, Tsui TKN, Wilson JM, Wright PA, Wood CM (2007) Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. *Physiol. Genomics* 31,463-474. https://doi.org/10.1152/physiolgenomics.00061.2007
73. Hung CYC, Tsui KNT, Wilson JM, Nawata CM, Wood CM, Wright PA (2007) Rhesus glycoprotein gene expression in the mangrove killifish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air. *J. Exp. Biol*. 210, 2419-2429. https://doi.org/10.1242/jeb.002568
74. Lin YM, Chen CN, Lee TH (2003) The expression of gill Na, K-ATPase in milkfish, *Chanos chanos*, acclimated to seawater, brackish water and fresh water. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *135*(3), 489-497. https://doi.org/10.1016/S1095-6433(03)00136-3
75. Lin CH, Lee TH (2005) Sodium or potassium ions activate different kinetics of gill Na, K-ATPase in three seawater-and freshwater-acclimated euryhaline teleosts. *Journal of Experimental Zoology Part A: Comparative Experimental Biology*, *303*(1), 57-65. https://doi.org/10.1002/jez.a.130
76. Saroglia M, Caricato G, Frittella F, Brambilla F, Terova G (2010) Dissolved oxygen regimen (PO_2) may affect osmo-respiratory compromise in European sea bass (*Dicentrarchus labrax*, L.). *Italian Journal of Animal Science*, *9*(1), e15. https://doi.org/10.4081/10.4081/ijas.2010.e15