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Research Article

Olfactory specialization in the Senegalese sole (*Solea senegalensis*): CO_2 acidified water triggers nostril-specific immune processes

Rita A. Costa [a], Peter C. Hubbard [a], Manuel Manchado [b], Deborah M. Power [a], Zélia Velez [a,*]

[a] Comparative Endocrinology and Integrative Biology Group, Centre for Marine Sciences, University of Algarve, Campus of Gambelas, Building 7, 8005-139 Faro, Portugal

[b] IFAPA Centro El Toruño, Junta de Andalucía, Camino Tiro Pichón s/n, 11500 El Puerto de Santa María, Cádiz, Spain

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ABSTRACT

Increased carbon dioxide (CO_2) in the ocean is changing seawater chemistry. Behavioural alterations in CO_2 exposed fish have been linked to changes in the central nervous system (CNS). However, we hypothesise that receptor cells in direct contact with the environment are more susceptible to changes in water chemistry than the CNS. Electrophysiology, histology, and transcriptomics were used to explore the effect of exposure to CO_2 acidified water on the olfactory epithelium (OE) of the Senegalese sole (*Solea senegalensis*). The upper and lower OE of this flatfish detect different odorants and are in contact with different environments. Acute exposure to acidified water decreased olfactory sensitivity more in the upper than in the lower OE. After chronic exposure to high CO_2 there were no histological changes in the upper OE; however, in the lower OE, there was a massive infiltration of melanomacrophage (MMC) and tissue disorganization. In addition, in the upper OE, differential expressed gene transcripts (DETs) were related to inflammation and innate immune processes whereas in the lower OE, DETs were related to the adaptative immune response. Differential regulation of genes related to neurogenesis and plasticity occurred in both epithelia.

The effects of ocean acidification in sole OE depends on the nostril; however, the occurrence of an exacerbated immune response, OE remodelling and reduced sensitivity indicate that ocean acidification is likely to have significant and unpredictable consequences for behaviour.

1. Introduction

Since the Industrial Revolution, atmospheric carbon dioxide (CO_2) has increased from 280 p.p.m. to 420 p.p.m. CO_2 (Doney et al., 2009), an increase that would have been much greater if the ocean did not absorb a large part of anthropogenic CO_2. Oceanic pH is estimated to have dropped by 0.1 units since preindustrial times and is predicted to fall 0.3–0.4 units by 2100 (Caldeira and Wickett, 2003). This drop in pH arises from an increase in the ocean CO_2 pressure (PCO_2) from around 400 μatm to 1100 μatm (Caldeira and Wickett, 2003).

Aquatic organisms are directly affected by changes in water pH; marine fish in water with a PCO_2 predicted for the end of this century have altered behaviour (Ferrari et al., 2012; Wisenden, 2012). The $GABA_A$ theory has linked altered fish behaviour under ocean acidification (OA) (Clark et al., 2020; Munday et al., 2020) to modified neurotransmitter function due to a shift in acid-base regulation (Chung et al., 2014). It was recently proposed that such altered behaviour may also be due to direct effects of pH and/or PCO_2 on olfactory perception (Porteus et al., 2021). Fish perceive odour through the olfactory epithelium (OE), which is usually organised in leaf-shaped rosettes with a long central raphe and rows of lamellae on both sides. Some flatfish have specilized OE on the ocular and blind sides. In sole, the lower OE, in contact with the interstitial water, is slightly smaller and has fewer shorter lamellae than the upper OE (Rodríguez-Gómez et al., 2001). While the upper OE is often pigmented, the lower OE rarely is, and is more concave and lies deeper within the head (Velez et al., 2005). CO_2-acidified seawater causes an immediate and reversible reduction in olfactory sensitivity to some odorants in the gilthead seabream (Velez et al., 2019), sea bass (Porteus et al., 2018) and crabs (Roggatz et al., 2016). This has been explained, in part, by conformational changes in the odorant and/or binding domain of olfactory receptors (due to increased protonation), which reduces receptor-ligand binding affinity (Velez et al., 2019) and

* Corresponding author.
E-mail address: zvelez@ualg.pt (Z. Velez).
@Manchado_M (M. Manchado)@InnovacionIFAPA (M. Manchado)

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the transduction efficiency due to decreased intracellular pH (Velez et al., 2021). The olfactory system is critical for survival; it mediates food selection and predator identification, but also impacts social interactions, reproductive function, and other aspects of behaviour (Laberge and Hara, 2001). The decreased sensitivity of the OE under elevated CO_2, means that fish must be up to 42 % closer to the odorant source to detect it (Porteus et al., 2018). However, the consequences of CO_2 induced decreased sensitivity may be more pronounced since olfaction is affected by internal stimuli such as hormones, neurotransmitters, and paracrine signals (Herz, 1998) which may be affected by exposure to OA. In addition, low stimulation of olfactory receptor neurons may induce neuromodulation; indeed, plasticity which was thought to occur only in the central nervous system (CNS), can also take place in the peripheral nervous system, including the OE (McGann, 2015). Recent studies have shown activity-dependent modulation of the olfactory sensory neurons in the OE (Wang et al., 2017a; Bryche et al., 2021), which is mediated by changes at the level of olfactory sensory neurons (Zhao and Reed, 2001; Watt et al., 2004) and by changes in the efficiency of transduction pathways at the level of single neurons (Bryche et al., 2021; Kerr and Belluscio, 2006). In addition, infection of rainbow trout with *Flavobacterium columnare* caused histopathological changes in the olfactory organ (OO) (Dong et al., 2020), suggesting that immune changes may also alter olfactory perception (Ullah et al., 2024). In marine invertebrates OA is known to alter immune system function (Cao et al., 2018; Mackenzie et al., 2014; Culler-Juarez and Onthank, 2021), in fish few studies have focused on the effects of acidification in the immune system. A study carried with the European sea bass (*Dicentrarchus labrax*) showed that transgenerational exposure to CO_2 acidified water induced up-regulation of genes involved in innate antiviral immunity in the olfactory epithelium (Cohen-Rengifo et al., 2022). Although F2 exposed to OA showed superior viral resistance, their odour transduction programs were altered, raising the hypothesis that odour-mediated behaviours might be impacted (Cohen-Rengifo et al., 2022).

The current study takes an integrative approach to assess the effects of high PCO_2/low pH water on the OE of Senegalese sole (*Solea senegalensis*; hereafter 'sole'). To this end, the effects of acute exposure to acidified water on olfactory sensitivity and the consequences of chronic exposure on OE histology and gene transcription were assessed. The sole was chosen because of its functional asymmetry in olfactory perception (Velez et al., 2005). It typically displays nocturnal feeding and reproductive pattern (Morais et al., 2016) while it spends most of its time half-buried in sand with only its eyes and upper nostril exposed to open water, and feeds on benthic invertebrates. Therefore, the two OEs detect cues in two distinct environments; the upper nostril samples open water whilst the lower nostril samples interstitial water. It was previously shown that (Velez et al., 2005) the upper OE is more sensitive to bile acids, body fluids (bile and intestinal fluid) and some amino acids (Velez et al., 2005) while the lower OE is more sensitive to aromatic amino acids such as 1-methyl-L-tryptophan (Velez et al., 2011). The current study, therefore, investigated the effects of CO_2-induced acidification on the two olfactory epithelia of the sole, in contact with different environments, at functional, structural and transcriptomic levels.

2. Material and methods

2.1. Fish maintenance

Sole were cultivated under aquaculture protocols from hatching to juvenile stages at IFAPA Centro El Toruño (Junta de Andalucía, Cadiz, Spain). For the experiments, animals were transported to Ramalhete Marine Station, Faro, Portugal, and maintained in a RAS system under controlled temperature (18 °C) and photoperiod (12 L/12 D) in 800 L tanks and fed daily with commercial pellets (Sparos, Olhão, Portugal).

2.2. Exposure to ocean acidification

Animal maintenance and experimentation were carried out in certified experimental facilities at Ramalhete Marine Station (Centre of Marine Sciences, Portugal), and followed Portuguese national legislation (DL 113/2013) under a "group-1" license from the Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal. The behaviour and health of all animals were monitored daily by evaluating changes in food intake, changes in skin colouration and alteration in movement patterns; no behavioural alterations, changes in food intake, infection or mortality were observed.

For fish exposed to ocean acidification over four weeks, seawater was pumped from the ocean into two 2000 L header tanks. In one tank, seawater was aerated with ambient air (control) and in the other CO_2 was bubbled through the water to achieve the desired pH (elevated-CO_2 treatment). The PCO_2 in the seawater of the header tanks was maintained at the target values of 400 μatm and 1900 μatm using a pH probe connected to an internal controller (EXAxt PH450G, Yokogawa Iberia). Control and high CO_2 seawater was supplied from each header tanks to 100 L tanks at 2 L·min^{-1}. The pH (Orion star A221, Thermo Scientific), temperature (Orion star A221, Thermo Scientific) and salinity (WTW, cond3310) of the seawater were recorded daily in each tank. Total alkalinity in water samples was analysed twice a week by Gran titration (DL15 titrator, Mettler Toledo) using a certified acid titrant (0.1 M HCl, Fluka Analytical, Sigma-Aldrich). Carbonate chemistry parameters (Supplementary file – table S1) were calculated in CO2SYS (Pierrot et al., 2011) using the constants K1 and K2 from Mehrbach et al. 1973 (Mehrbach et al., 1973) refit by Dickson (Dickson and Millero, 1987; Dickson, 1990).

2.3. Electrophysiology

2.3.1. Experimental animals

Sole (n = 10; 432 ± 33 g; 27.3 ± 1.1 cm) were anesthetized in seawater containing 300 mg L^{-1} MS222 (3-aminobenzoic acid ethyl ester; Sigma-Aldrich, Portugal) until the response to a tail pinch stopped. An intramuscular injection of the neuromuscular blocker gallamine triethiodide (Sigma-Aldrich, Portugal; 10 mg/kg in 0.9 % NaCl) was then given. Fish were placed on a padded surface (with a slight backward tilt) and the gills continuously flushed with aerated seawater containing 150 mg L^{-1} MS222, via a plastic tube in the mouth. The pH of the seawater flushing the gills was around 8.2, therefore, only the OE experienced high PCO_2 conditions during electrophysiology experiments; water chemistry parameters during the experiments are summarized in a Supplementary file – table S1. The olfactory rosette was exposed by cutting the skin and connective tissue overlying the nasal cavity. The nostril was constantly irrigated with filtered sea water (without anaesthetic) under gravity (flow rate: 6 mL min^{-1}) via a glass tube.

2.3.2. Odorant selection and preparation

Test solutions were delivered to the tube irrigating the nasal cavity via a computer-operated three-way solenoid valve for four seconds. Charcoal-filtered seawater was used to make up odorant solutions and to irrigate the olfactory rosette during experiments. This water was either bubbled with air (control) or CO_2 (low pH) until the desired pH_{NBS} was reached. Amino acid solutions were prepared from frozen aliquots of stock solutions at 10^{-2} M; bile fluid was taken directly from the gall bladder of adult sole sampled as part of another study. All stimuli tested on the OE were diluted in charcoal-filtered sea water (either control or high CO_2 as appropriate) immediately prior to use. The order in which odorants were given was randomized, but each odorant was always given starting from the lowest to the highest concentration.

The effect of acute exposure to high PCO_2 on olfactory sensitivity was tested for amino acids and bile fluid. For this study, odorants that are better detected in the upper (L-arginine and bile fluid) (Velez et al.,

2005), lower OE (1-methyl-L-tryptophan) (Velez et al., 2011) or equally detected by the OEs of both sides (L-leucine, L-cysteine) (Velez et al., 2005) were selected, and the effects of elevated PCO_2/low pH on the olfactory response to these odorants was evaluated.

2.3.3. Electrolfactogram (EOG)

The EOG was recorded as previously described (Velez et al., 2011). The recording electrode was placed in a position that resulted in the largest response to the "standard" stimulus (10^{-3} M L-cysteine). The reference electrode was placed lightly on the skin of the head nearby.

The fish was grounded with an Ag/AgCl pellet electrode placed under the head. The d.c. signal was amplified using a NeuroLog headstage NL100 and NL109 bridge amplifier passed through an NL125 filter with low pass set at 30 Hz (Digitimer Ltd., https://www.digitimer.com/). The signal was digitized (Digidata 1440 A, Molecular Devices, https://www.moleculardevices.com/) and stored on a personal computer running AxoScope software (version 10.6,Molecular Devices). Once all stimuli had been applied to one epithelium (usually the lower because it gives a smaller response), the fish was turned over and the stimuli were applied to the opposite OE in the same manner. Response amplitudes were normalized to the amplitude of the response to 10^{-3} M L-cysteine (the "standard"). The response of the OE to the standards was recorded regularly at the beginning and end of each group of samples (every three to five samples) throughout the recording sessions.

In the electrophysiological studies, only sole kept in control conditions were used, given that animals exposed to CO_2 acidified water for four weeks died during anaesthesia.

2.3.4. Data and statistical analysis

All statistical analyses and calculations were carried out on blank-subtracted normalized data. To test for the effects of concentration and treatment a two-way repeated-measures analysis of variance was performed for all the odorants (Prism 9 for macOS). A Holm–Sidak post hoc test was used to test for differences from the control and between different concentrations. A significance level of $P < 0.05$ was used.

2.4. Histological analysis of the olfactory rosettes

To characterize the morphology of the two OE, the upper and lower OE from the same individual were collected after four weeks in control ($n = 6$) and in low pH/high PCO_2 (n = 6) seawater, fixed in 4 % PFA and stored in 70 % ethanol until paraffin embedding. Tissue samples were dehydrated in a graded ethanol series (70 %, 96 % and 100 %), saturated in xylene and impregnated and embedded in low melting point paraffin wax (Histosec, Merck). Serial 5 μm sections of each tissue were cut with a rotary microtome (Leica RM2125 RT), mounted on poly-L-lysine (Sigma-Aldrich) coated glass slides and stained with Alcian blue – Periodic Acid Schiff technique (Mowry, 2008) to highlight the goblet/mucous cells. For staining, tissue sections were dewaxed and rehydrated and then immersed in an Alcian blue solution (3 % in acetic acid) for 30 min, oxidized with 1 % periodic acid solution for 10 min and covered in Schiff reagent for 15 min in the dark. Stained histological sections were observed under a microscope (Leica DM2000), digital images were captured (Leica DFC480; IM50-software) and analysed.

The goblet cells and melanomacrophages (MMC) in sections of the upper and the lower olfactory rosettes from three fish per group (control and low pH/high PCO_2), were counted in three lamellae per section, from three sections per individual spaced at 15–20 μm. A total of 27 measurements per individual was analysed for the upper and lower OE. The thickness of the sensory epithelium (SE) was measured in three lamellae per section; the ratio between the non-sensory epithelium (NSE) vs SE within the olfactory lamella was assessed by dividing the length of the apical NSE by the length of the olfactory lamellae (from the top to the central raphe) in three individuals of the two experimental groups (three lamellae *per* section). The software ImageJ was used to perform this histomorphometric study, as previously described (Velez et al., 2019).

Statistically significant differences between the number of goblet cells, the thickness of the SE and the ratio between NSE and SE in control animals and experimental animals was assessed using a Student's *t*-test (Prism 9).

2.5. Transcriptomics

Total RNA was extracted from the upper ($n = 12$) and lower (n = 12) OE of control ($n = 6$) and high PCO_2 (n = 6) after 4 weeks in the experimental conditions. Total RNA of tissue samples previously fixed in RNA*later*®(Sigma-Aldrich) was subsequently extracted with an E.Z.N.A® Total RNA Kit (R6834, Omega) according to the manufacturer's instructions. Tissue samples (upper OE ~12 mg; lower OE ~6 mg average wet weight) were placed in lysis buffer with mercaptoethanol and were disrupted with two iron beads in a tissue grinder (Mixer Mill MM400, Retsch) at 30 Hz, for 3 × 30 seconds. The homogenates were centrifuged and the supernatant mixed with an equal volume of 100 % ethanol. RNA extracts were purified and treated with DNase on a column provided with the E.Z.N.A® RNase-Free DNase Set I (E1091, Omega) kit according to the manufacturer's instructions. RNA quality and concentration were assessed using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) and the integrity verified by electrophoresis on 2 % agarose gels.

The RNA integrity value (RIN) of all the extracted RNAs was verified with a Bioanalyzer before the construction of 24 RNAseq libraries (Control upper ($n = 6$), Control lower (n = 6), high PCO_2 upper (n = 6) and high PCO_2 lower (n = 6)). Libraries were constructed using a Stranded mRNA Library Preparation Kit. Each library was sequenced with paired-end 150 bp reads to a minimum depth of 40 million reads on an Illumina NovaSeq platform at STAB VIDA, LDA. (Caparica, Portugal).

2.5.1. Data analysis

Primary analysis of the RNA-seq raw data was performed using the public server at usegalaxy.org (Afgan et al., 2016). The quality of the raw data was evaluated using FastQC (Andrews, 2010) and then cleaned using Trimmomatic (Bolger et al., 2014). Cleaned data were mapped onto the *S. senegalensis* representative transcriptome v4. (*Solea senegalensis* v4.1 – global assembly) downloaded from the SoleaDB database (Benzekri et al., 2014) in October 2020, using Bowtie2 (Bolger et al., 2014). Potential transcripts and gene abundance were assembled with StringTie (Kovaka et al., 2019; Pertea et al., 2015) and read counts were obtained with featureCounts (Liao et al., 2014). A summary of the sequencing statistics for the RNAseq analysis obtained for each library from the upper and lower OE is presented in supplementary file – table S3.

Manual curation of the data was performed to exclude gene transcripts represented in three or fewer of the biological replicates of each experimental group, independent of the total read counts. The number of gene transcripts present in at least four biological replicates/group/tissue, are presented in supplementary file - table S4, and were used in all subsequent analysis. The multidimensional scaling analysis demonstrated the distance and/or similarities between the sets of objects and is presented in supplementary file – fig. S1.

Annotation of gene transcripts in each library was performed using the biomaRt 2.46.3 package in RStudio, and the ensembl database for the zebrafish *Danio rerio* as the reference. Briefly, the external gene name (gene symbol) for each transcript was determined in bulk using the zebrafish orthologues and the data previously provided from the reference transcriptome (*Solea senegalensis* v4.1 – global assembly) using the identifiers: ensembl_peptide_id, refseq_peptide_predicted and refseq_peptide. The total number of gene transcripts annotated (with gene symbol) and those that were not annotated (not identified) in each experimental group is presented in supplementary file – table S5.

Differentially expressed gene transcripts (DETs) were determined in the upper and lower OE of sole and under the two experimental

conditions, control vs. low pH/high PCO_2 seawater, using edgeR (Robinson et al., 2010) running in usegalaxy.eu edgeR tool (Liu et al., 2015) between April and June 2021. The estimated *p*-values (0.05) for the threshold were adjusted using Benjamin and Hochberg's method (Yoav and Hochberg, 1995) for controlling the false discovery rate (FDR) and library sizes were normalized using the TMM method. The edgeR quasi-likelihood test for biological replicates was used with robust settings to protect against outlier genes. DETs were selected with the criteria FDR ≤ 0.05 and $|\log FC| = 0$. The functional analysis of DETs was performed with the ClueGO Cytoscape plug-in (Bindea et al., 2009; Shannon et al., 2003) using as the input the zebrafish *Danio rerio* orthologues for each Sole DET. Enrichment analysis (right-sided, hypergeometric test) was run using Biological Process (GO-BP) and KEGG pathway (Kanehisa et al., 2010) terms for zebrafish *Danio rerio* between levels 1–4 (between July and October 2021). Terms were considered significantly enriched at an FDR (Benjamin-Hochberg) < 0.05 and a minimum of three genes/4 % of the GO-BP/KEGG genes represented in the list. Enriched GO-terms were grouped into functionally related networks using i) an initial group size 1, ii) a group merging setting of 50 % and iii) a Kappa score of 1. KEGG enrichment was used to identify putative functions and pathways of DETs.

3. Results

Electrophysiological records showed that, under acute exposure to high CO_2/low pH water, L-arginine and bile fluids had decreased olfactory potency in the upper OE (Fig. 1 A and C), but not in the lower OE (Fig. 1 B and D). Concerning L-arginine in the upper OE, significant differences ($n = 6$; $P < 0.0001$) were found only at 10^{-3} M; while for conspecific bile fluids (Fig. 1 C) there were significant differences between the olfactory responses at the dilutions 1:1000000 (n = 6, $P = 0.04$), 1: 100000 (n = 6, $P = 0.0005$) and 1: 10000 (n = 6, $P = 0.008$). No differences in the olfactory potency of conspecific bile were detected at the highest concentration tested (1: 1000).

For 1-methyl-L-tryptophan (Fig. 2), there was a significant decrease in olfactory responses only at 10^{-3} M in the lower nostril (n = 6, $P < 0.0001$) at pH 7.7.

Olfactory responses to L-cysteine and L-leucine decreased at pH 7.7, especially in the upper OE (Fig. 3); responses to L-cysteine were significantly higher in water pH 8.2 at the concentrations 10^{-4} M ($n = 7$, $P = 0.03$) and 10^{-3} M (n = 7, $P = 0.029$). In the lower OE, the olfactory responses to 10^{-3} M L-cysteine was significantly higher ($n = 6$; $P = 0.01$) at pH 8.2 than 7.7; there were no differences between the olfactory responses at the other concentrations tested. For L-leucine (Fig. 3 C and D), significant differences were found between olfactory responses at pH 8.2 and 7.7 only in the upper nostril. Olfactory responses to 10^{-4} and 10^{-3} M L-leucine was significantly higher (n = 7; $P = 0.03$ and $P = 0.004$, respectively) at pH 8.2.

Like other teleosts, the olfactory organs of the sole are composed of several olfactory lamellae (olfl). Each lamella has two layers of epithelium that enclose a central core (cc), which is separated from the epithelium by a basement membrane. The non-sensory epithelium (NSE) consisted of a stratified epithelium covering the outer margins of the lamella. The sensory epithelium (SE) consists of pseudostratified epithelium covering the lateral sides of the lamella to the central raphe (cr). Goblet cells (gl) were abundant throughout the SE and the lamina propria of the olfactory lamellae was melanin (mln) rich with extensive dark, pigmented regions in the upper olfactory rosette (Fig. 4 D and F). In the lower olfactory rosette (Fig. 5) mucous cells were less regular or abundant and melanin in the lamina propria was less abundant.

No significant differences were observed in the upper OE of fish kept in control and high PCO_2 seawater except for the number of MMC; the number of MMC was significantly higher ($P = 0.03$) in PCO_2 exposed fish (92 ± 38.8 cells *per* 3 lamallae) than in control animals (no MMC were detected). Similarly, the proportion of NSE in the olfactory lamella was not significantly different between the control and PCO_2 exposed fish. Finally, the thickness of the SE were similar in the control and experimental groups (Fig. 4).

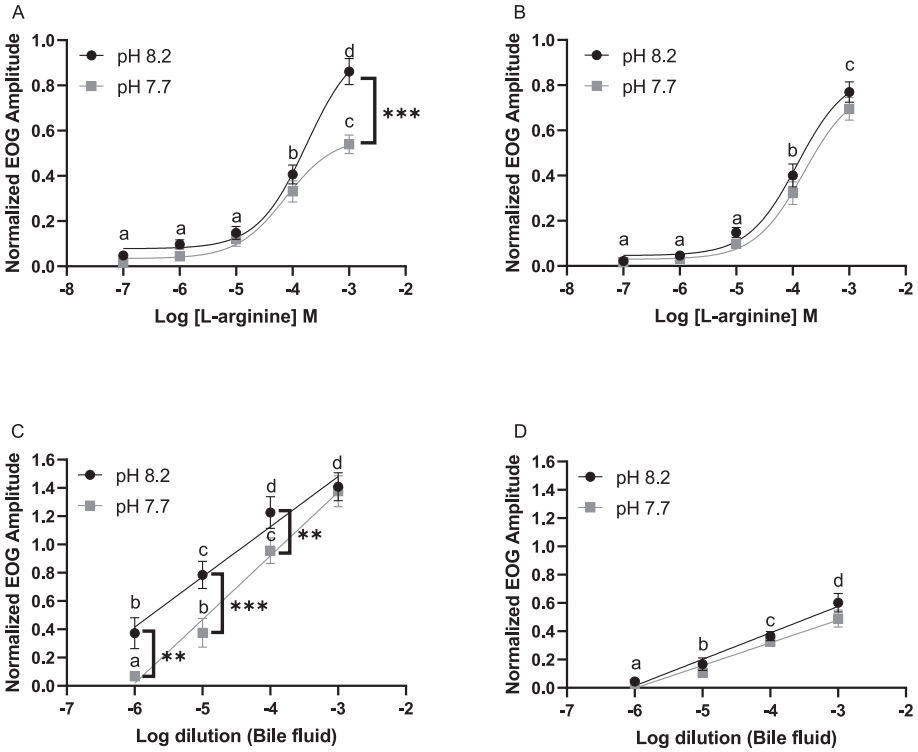

Fig. 1. Normalized EOG responses of sole OE to L-arginine (A and B) and conspecific bile fluid (C and D), recorded in the upper (A and C) and lower (B and D) olfactory epithelium, under control (black circles; odorant pH 8.2) and elevated PCO_2 conditions (grey squares; odorant pH 7.7). Values are shown as the mean ± S.E. M $^{**}P < 0.01$, $^{***}P < 0.001$.

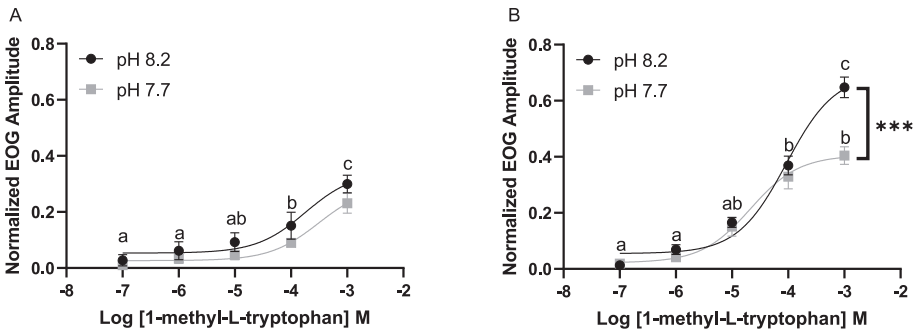

Fig. 2. Normalized EOG responses of sole to 1-methyl-L-tryptophan recorded in the upper (A) and lower (B) olfactory epithelium, under control (black circles; odorant pH 8.2) and elevated PCO_2 conditions (grey squares; odorant pH 7.7). Values are shown as the mean ± S.E.M. ***$P < 0.001$.

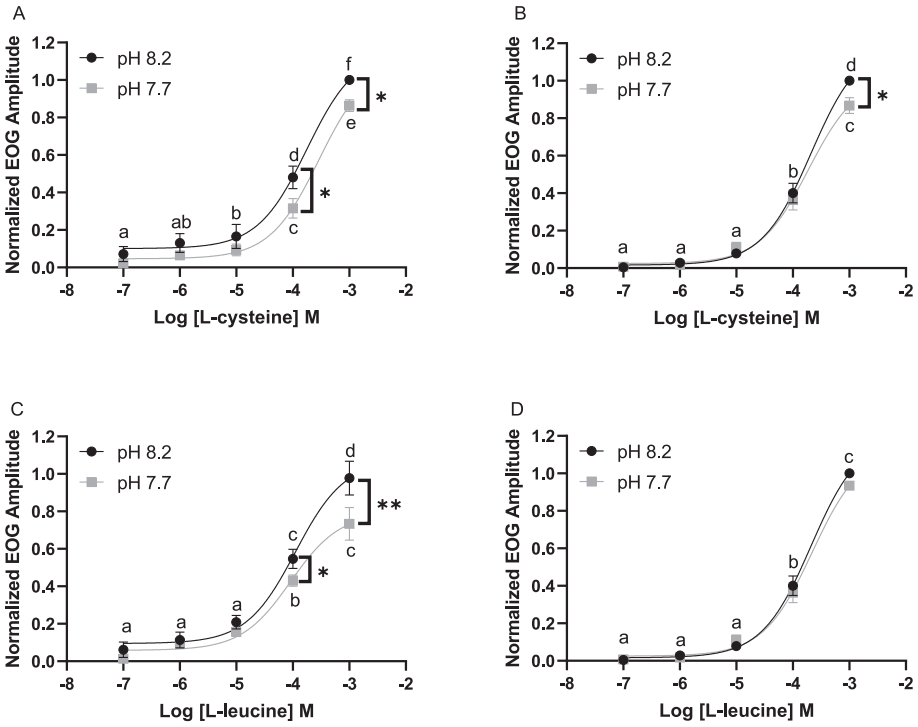

Fig. 3. Normalized EOG responses of sole to L-cysteine (A and B) and L-leucine (C and D), recorded in the upper (A and C) and lower (B and D) olfactory epithelium, under control (black circles; odorant pH 8.2) and elevated PCO_2 conditions (grey squares; odorant pH 7.7). Values are shown as the mean ± S.E.M. *$P < 0.05$, **$P < 0.01$.

In contrast, in the lower OE, four-weeks exposure to high PCO_2 seawater caused a significant decrease in the number of goblet cells ($P = 0.007$) and an increase in the thickness of the SE ($P = 0.003$), but no change in the non-sensory/sensory area ratio (Fig. 5 A, B and C).

Furthermore, a massive infiltration by pigmented phagocytes (melanomacrophages; MMCs) was observed in the lower OE accompanied by the formation of several proliferative lesions not seen in the upper OE; intraepithelial lesions grew further and formed masses (Fig. 5 E). There were significant differences between the number of MMCs found in the control and experimental fish ($P < 0.0001$); while in control animals no MMCs were found, in CO_2 exposed fish MMCs were found in all samples (5126 ± 4923). MMCs are found primarily in poikilotherm lymphoid tissues and they are darkly pigmented due to their high lipofuscin, melanin, and haemosiderin content, making them histologically distinguishable by light microscopy (Fig. 5 D and E). In the proliferative lesions basal cells formed clusters along the basal lamina (Fig. 5 E). The melanomacrophage were larger than the normal basal cells. Cell degeneration was evident with the formation of vacuoles. Abnormal proliferation of basal cells altered the organization of the epithelium; columnar sensory and non-sensory cells were partially disorganized, and the epithelium was occupied by the proliferating undifferentiated cells.

In the upper OE of control and PCO_2-exposed fish (Fig. 4) goblet cells-stained deep pink/blue (more acidic) at the base of the OE (close to the central raphe) and became progressively pink/purple along the olfactory lamellae, being deep pink/purple (more neutral) on the edge of the lamellae. In the lower OE of PCO_2-exposed fish, goblet cells-stained pink/purple throughout the entire lamellae, indicating the contents of goblet cells had a neutral pH, whereas in the control fish the mucins were more acidic and the cells stained a bluish colour. The lower OE in the control fish resembled the upper OE, and goblet cells became progressively more pink/purple along the olfactory lamellae, and were deep pink/purple on the edge (Fig. 5).

Lastly, considering the histological and sensory changes caused by exposure to high PCO_2/low pH water, RNA sequencing of the OE was performed to understand the molecular mechanisms involved. Comparison of the lower and upper OE in control conditions revealed 32,075 expressed gene transcripts (76.2 % expressed in both the upper and lower OE, 18.8 % only in the upper OE and 4.9 % exclusively in the

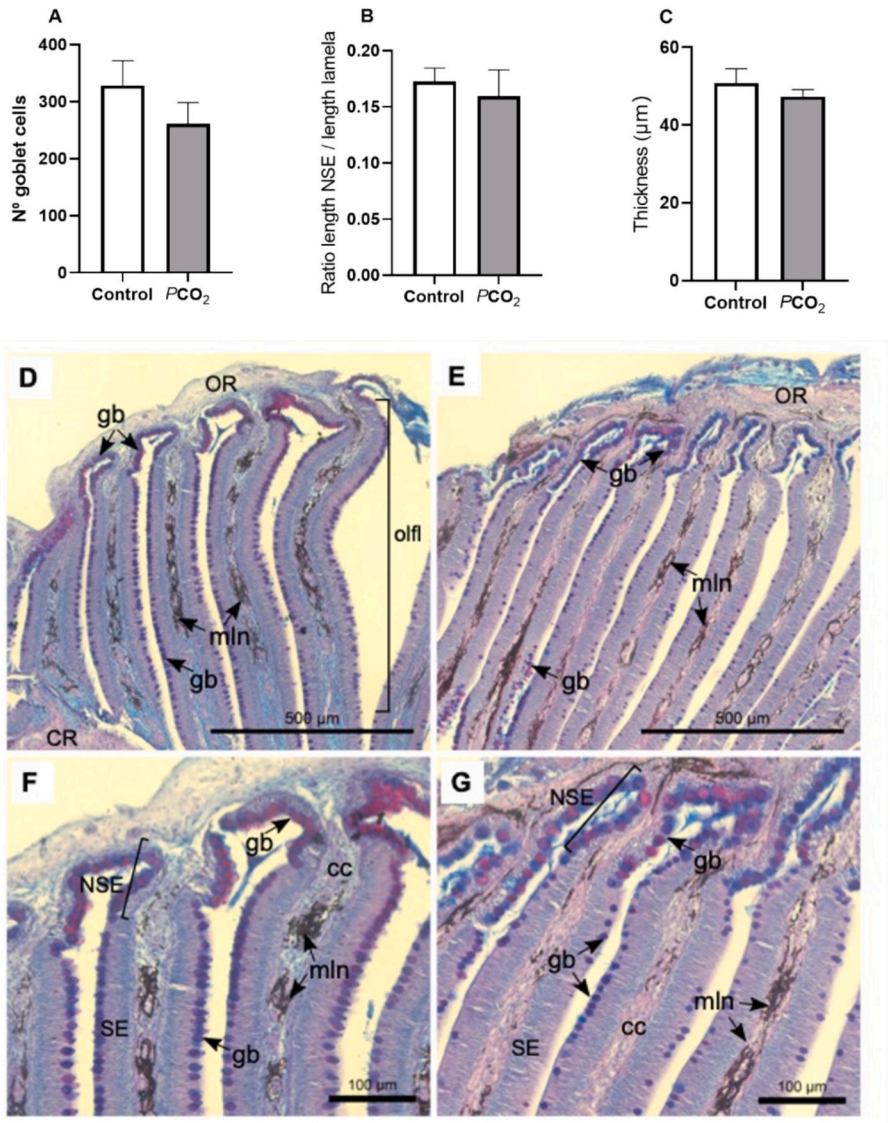

Fig. 4. A-C) Histomorphometry of the upper OE of *S. senegalensis* after four weeks of exposure to low pH/high PCO_2 seawater. A) Number of goblet cells *per* 3 lamelae, B) Ratio of the non-sensory epithelium (NSE) length / lamella length and C) Thickness of the OE. The results are presented as mean ± S.E.M. ($N = 6$/ group). D-G) Histological sections of the sole OE stained with AB-PAS (Alcian blue – Periodic Acid Schiff). D) Upper OE of Control fish. E) Upper OE of experimental fish. F) Higher magnification of D. G) higher magnification of E. OR: olfactory rosette, gb: goblet cells, mln: melanin, olfl: olfactory lamellae, CR: central raphe, NSE: non-sensory epithelium, SE: sensory epithelium, cc: central core/lamina propria. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lower OE; supplementary file – table S6). Of the genes expressed in both OEs (76.2 %; 24,459), 28 % were equally expressed in both OEs, and 36 % were significantly upregulated in the lower OE and 36 % in the upper OE, respectively (supplementary file - table S7). Analysis of gene transcripts with gene symbols revealed upregulation of 8 olfactory receptors in the upper OE (*or115–1, or115–12, or115–15, or134–1, or126–3, or126–4, or126–7, or127–1*), three of which are exclusive to the OE (underlined). In the lower OE there was upregulation of three olfactory receptors (*or108–1, or121–1, or126–1)*, two of which were only expressed in this epithelium (underlined).

Although a high number of gene transcripts were shared between experimental groups (control and high PCO_2) in the upper and lower OE (94.7 % in the upper OE and 93.8 % in lower OE), there were a considerable number of gene transcripts specifically expressed in each experimental condition (Fig. 6). The results indicate that high PCO_2 seawater changed the steady state transcriptome in the OE and that in the upper and lower OE-specific gene transcripts were expressed and the response of the nostrils to high PCO_2 seawater differed from the control.

For DETs estimation highly stringent criteria were used to make the analysis more robust; differential expression analysis identified 561 DETs (346 up- and 215 down-regulated) in the upper OE and 193 (99 up- and 94 down-regulated) in the lower OE (supplementary data – DETs and supplementary file – table S8-S12, Figs. S4 and S5).

Functional annotation revealed that genes related to ion transport were mainly downregulated (*kcnj14*, *slc13a1, slc9a1b, si:ch211-225b11.1, cacna1ia, abcc9, Piezo, slc26a10, abcc1)* in the upper OE of PCO_2-exposed fish, with *ahcyl1* the only exception since it was upregulated. Downregulated genes were involved in sodium, potassium, calcium and chloride ion transport, and the upregulated gene was involved in bicarbonate secretion (supplementary data – DETs). In the lower OE, we found four DETs related to potassium ion transport; *nkcc2, kcnj14 and kcna2a,* were downregulated and *nalcn* which was upregulated and codes for a voltage-gated sodium and calcium channel that regulates the resting membrane potential and excitability of neurons.

Several pathways related to neuroplasticity and neuromodulation were regulated by exposure to high PCO_2/low pH water (Table 1).

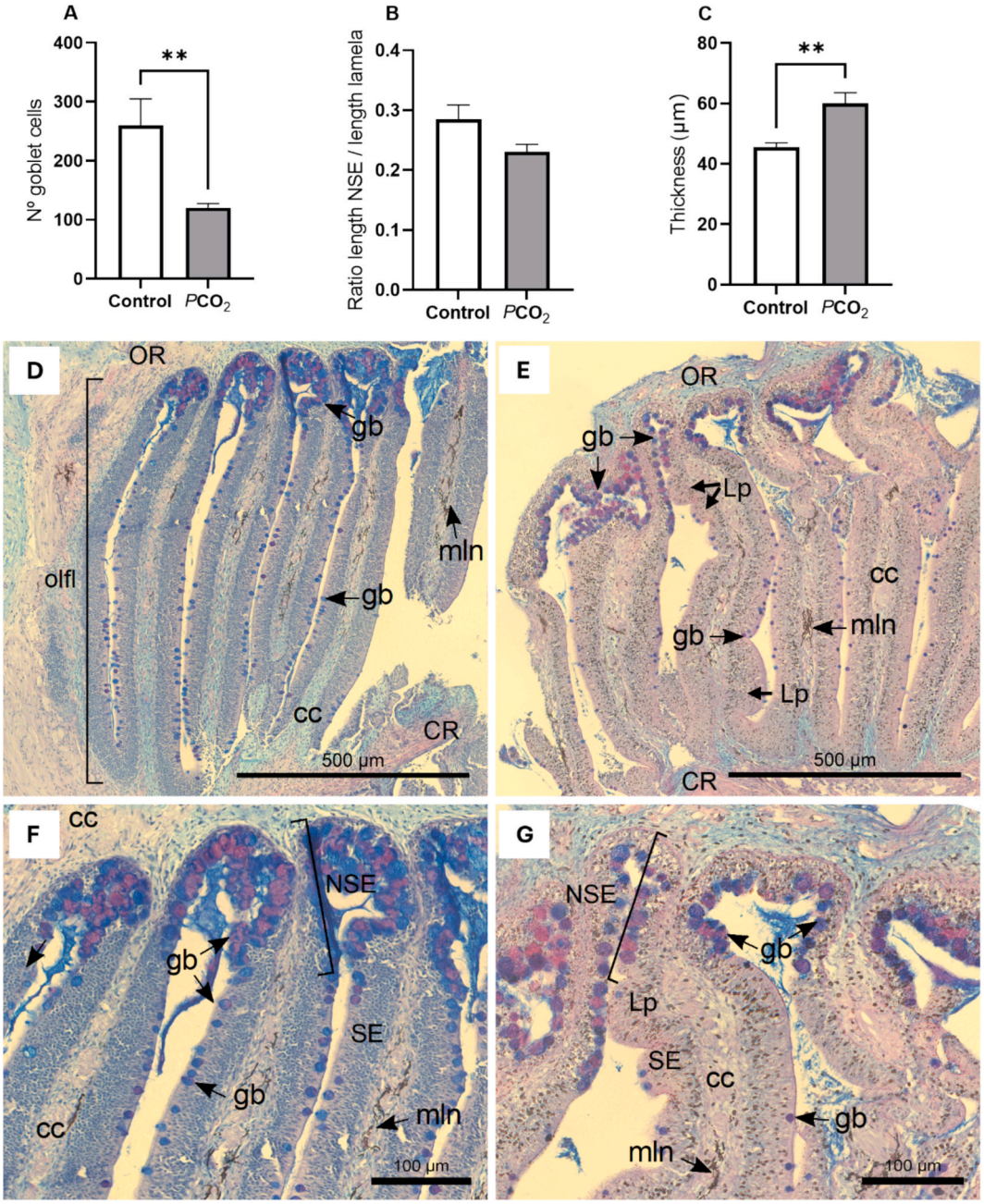

Fig. 5. Histomorphometry of the lower OE of sole after four weeks of exposure to low pH/high PCO_2 seawater. A) Number of goblet cells, B) proportion of NSE vs SE and C) thickness of the OE. (D-G) - Histological sections of the lower olfactory rosette of *S. senegalensis* stained with AB-PAS (Alcian blue – Periodic Acid Schiff). A) Lower OE of control fish. B) Lower OE experimental fish. C) Higher magnification of A. D) Higher magnification of B. OR: olfactory rosette, gb: goblet cells, mln: melanin, olfl: olfactory lamellae, CR: central raphe, NSE: non-sensory epithelium, SE: sensory epithelium, cc: central core/lamina propria, Lp – tissue mass. Results are presented as the mean ± S.E.M. ($N = 6$ *per* group). $^{**}P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DETs related to neurogenesis were found only in the lower OE (Table 1). DETs related to neurite morphogenesis were upregulated in the upper OE and downregulated in the lower OE. In both OEs there was a significantly lower expression of genes related to myelination. Genes related to neuron migration were differentially regulated only in the upper OE. Genes involved in synaptic assembly were mainly upregulated in both OEs. Genes related to GABAergic innervation, neurotransmitter activity and GPCR signaling were upregulated in the lower OE and up and downregulated in the upper OE (Table 1).

A large portion of DETs in the upper OE of fish exposed to low pH/high PCO_2 water were related to immune processes (Table 2).

In both OEs, exposure to high PCO_2 water induced mainly higher expression of genes involved in the inflammatory response (*ccl38.6, csf1ra, cxcl8a, gbp3, si:ch211-195 h23.3, tlr21, tirap, pla2g10)*. Genes involved in adaptative immune processes and macrophage activation were diferentialy regulated only in the lower OE (supplementary data – DETs) while in the upper OE we found mainly upregulation of genes related to innate immunity (*ccl38.6, cd226, csf1ra, gbp3, si:ch211-195 h23.3, stat6, tlr21*).

In the upper OE several genes related to ErbB signaling (*cblb, eif4ebp1, erbb3a, pik3r1*), MAPK signaling (*fosaa, pbx1a, ppp5c, met, tlr21, tmem106a, tirap, mapk12b, MAP4K1, csf1ra, max, mapkap1, hspa8,*

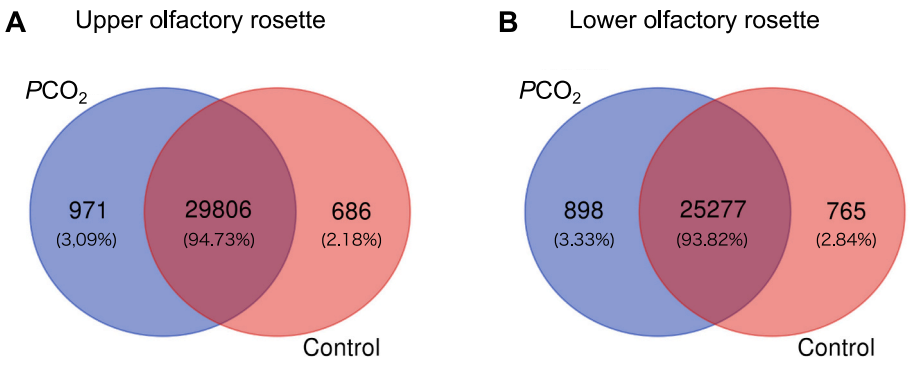

Fig. 6. Venn diagrams displaying the number of gene transcripts in the A) upper olfactory rosette and B) lower olfactory rosette of Control and PCO_2-exposed fish after 4 weeks of exposure to low pH/high PCO_2. There was a high number of gene transcripts shared between the OE of the control and high PCO_2-exposed fish. However, there were also specific gene transcripts that were expressed in each experimental condition, confirming that the exposure to high PCO_2 seawater changed the transcriptome of the tissues.

Table 1
Differentially expressed genes related to neuroplasticity and neuromodulation in the upper and lower olfactory epithelium of PCO_2 exposed sole, genes in blue are downregulated, and genes in black are upregulated.

Function	Process	Nostril	DET
Neuroplasticity	Neurogenesis	Lower	*ednrba, meis3, mpp5, hcfc1, foxn4, gdf3, rxraa*
	Apoptosis and neuron death	Upper	*kdm5c, bbc3, si:ch211-195h23.3, tmem117*
	Myelination	Upper	*adcy6a, nsfa*
		Lower	*mpp5*
	Neurite morphogenesis and extension	Upper	*cr854980.1, nrcama, adora2b, bloc1s6, ptprd*
		Lower	*prkcsh, caprin1a, micall2a, col15a1b*
	Neuron migration	Upper	*gata3, hdac1, met*
	Synaptic assembly	Upper	*mpp2b, vamp4, prepl, setd5, syt8, si:ch73-364h19.2, ptprd, actb2, atad1b, nxph3*
		Lower	*gphna, abhd17aa, epb41l3a*
Neuromodulation	GABAergic innervation	Upper	*gabrr1, zgc:158427, clptm1*
		Lower	*slc6a13, nalcn*
	Neurotransmitter receptor activity	Upper	*dlgap4b, klhl24b, cblb, gria2a*
		Lower	*gria3a*
	GPCR signaling	Upper	*or126-1, gpr116, rgs7bpb, atad1b, adcy6a, gucy2f*
		Lower	*pde11a, olfcb1, or127-1, adgrf3b*

erbb3a, cacna1ab, mapk12b) and FoxO signaling (*ccnb2, mapk12b, pik3r1, prkag2a*) were upregulated. In addition, there were several DETs involved in cell proliferation and differentiation but mainly of neuronal and immune processes (supplementary data – DETs).

Interestingly, in both OEs there was downregulation of green-sensitive opsin-3 a visual pigment (supplementary data – DETs).

4. Discussion

We found diferential changes in olfactory sensitivity in the upper and lower OE as a consequence of CO_2 acidification and this is matched by asymmetry in gene expression and histological changes in the OE.

The physiological impact of exposure to high PCO_2/ low pH was compared in the two OE of the sole because of their functional asymmetry (Velez et al., 2005). At the functional level, comparison of the olfactory sensitivity of sole under acute exposure to seawater at pH 7.7 (CO_2 acidified water) caused an immediate but reversible reduction in olfactory responses; this was odorant- and nostril-dependent and was most marked in the upper OE. Diminished olfactory sensitivity under high PCO_2 water has previously been described in seabream (Porteus et al., 2018) and seabass (Velez et al., 2019). This was proposed to be due to reduced receptor-ligand binding affinity (Velez et al., 2019) and lowered intracellular pH in olfactory receptor neurons caused by carbonic anhydrase activity (Velez et al., 2021). In sole, the lower OE was more resilient to changes in environmental PCO_2/pH; in addition, the carbonic anhydrase (*cahz*) gene was down regulated in the lower OE compared to the upper OE. Lower expression of carbonic anhydrase in the lower OE may lead to the decrease in intracellular pH in olfactory neurons mediated by carbonic anhydrase, being less relevant in the lower than the upper nostril, explaining its resilience when exposed to CO_2 acidified water and this may reflect a physiological adaptation to the more rapidly changing substrate chemistry compared to the water column.

Although, we were not able to test the effects of long term exposure

Table 2
DETs related to immune processes in the upper and lower OE of PCO_2 exposed sole; genes in blue are downregulated, and genes in black are upregulated.

Function	Nostril	DET
Immune system development	**Upper**	*adora2b, csf1r, cxcl8a, f2r, gata3, gpr137c, hdac1, max, nop14, pbx1a, prdm16, slc7a7, tlr21*
Immune receptor activity	**Upper**	*cmklr1, lepr, lifrb*
Innate immune response	**Upper**	*ccl38.6, cd226, csf1ra, gbp3, si:ch211-195h23.3, stat6, tlr21*
	Lower	*polr3f, tirap*
Regulation of immune system process	**Upper**	*gpr137c, hhla2a.2, ripor2, cd226, csf1r, gata3, cxcl8a*
Response to cytokine	**Upper**	*ccl38.6, cmklr1, csf1ra, cxcl8a, lepr, lifrb, ripor2, stat6*
Lymphocyte migration	**Upper**	*ccl38.6, ch25h, ripor2*
Lymphocyte activation	**Upper**	*gata3, hhla2a.2, ripor2*
	Lower	*sh2d1ab*
Leukocyte activation	**Upper**	*cd226, cxcl8a, gata3, hhla2a.2, ripor2*
Leukocyte differentiation	**Upper**	*csf1ra, gata3, gpr137c, pbx1a, slc7a7*
Defence response to biotic organisms	**Upper**	*ccl38.6, cd226, ch25h, csf1ra, gbp3, si:ch211-195h23.3, stat6, tlr21*
Antimicrobial humoral response	**Upper**	*cxcl8a, tlr21*
Phagocytosis	**Upper**	*met, rab34a, scarb1*
	Lower	*Itgam*
Toll signaling pathway	**Upper**	*cxcl8a, fosaa, mapk12b, pik3r1, tirap, tlr21*
	Lower	*Tirap*
Adaptative immune response	**Lower**	*cd8b, sh2d1ab, cd109*

to ocean acidification on the olfactory sensitivity of sole (they died when they were anesthetized), data from histology suggests that the olfactory response may be compromised especially in the lower OE. At the histological level, in the upper OE no overt histological changes occurred after exposure to high PCO_2/low pH seawater, while in the lower OE there were notable changes including a decrease in the number of goblet cells, an increase in the thickness of the SE and a massive infiltration of melanomacrophages (MMC). A study carried with raimbow trout showed that bacterial infection induces increases the density of goblet cells in the OE (Dong et al., 2020); in the current study we found that, in sole, exposure to OA induces a deacrease in the number of goblet cells. Fewer goblet cells may lead to a consequent decrese in the production of mucus, the presence of mucus may decrease olfactory sensitivity, thus we hypothesis that the decrease in goblet cells and most likely mucus production may be an adaptative response to increase sensitivity. The increase in the thickness of the SE seems to be due to proliferation of basal cells, the same alterations were previous reported in OE of raimbow trout under bacterial infection (Dong et al., 2020), in OA exposed sole proliferation of basal cells was associated with deorganization of the OE, which may also compromise sensitivity. MMC are phagocytic cells rich in melanin, lipofuscin and hemosiderin, which are thought to play dual roles, immunological and normal non-immunological physiological processes (Stosik et al., 2019). Like other macrophages, the primary function of MMCs is phagocytosis, participating in clearance of debris and long-term storage of indigestible and/or toxic material (Sayed and Younes, 2017; Lamers and De Haas, 1985; Ellis et al., 1976; Mackmull and Michels, 1932). It has been proposed that MMCs are analogous to mammalian germinal centers of the adaptative immune response (Stosik et al., 2019), indeed, following immunization or infection MMC-adjacent Ig^+ cells (presumed to be B cells) increased in size and/or number (Bermúdez et al., 2006; Press et al., 1994).

At the molecular level, only 21.3 % of the common genes were equally expressed in both OEs, and the other genes had a divergent expression. Furthermore, functional analysis of DETs showed higher diversity in the expression of genes related to neuronal processes in the upper OE than in the lower OE. Thus, in addition to the previously described differences in odorant detection between the upper and lower OE of sole (Velez et al., 2005), we suggest that transcriptional differences underpin the divergent functions of the OE in sole. Analysis of the biological function of DETs in the upper and lower OE indicated that differences were not only related to neuronal function since the expression of genes related to cellular metabolism, regulation of transcription and immune processes were also different. Interestingly, a previous study demonstrated the occurrence of sexual disparities between the expression of immune related genes, but not olfactory receptors in the blunt snout bream (*Megalobrama amblycephala*) (Lv et al., 2021). Considering the crucial role that olfactory mucosa immunity plays in fish health, the functional and transcriptional differences between the upper and lower OE of sole, should be analised in the future considering the sex of the fish.

Concerning DET in control and high CO_2 exposed fish, in general we found differential expression of genes related to ion transport, neuroplasticity and neuromodulation and immune processes; our results align with the previous results in European sea bass (*Dicentrarchus labrax*) where transcriptomic analysis of the olfactory rosette of F2 exposed to long-term transgenerational OA acclimation showed differential regulation of transcripts related to regulation of pH, bicarbonate transport and chloride homeostasis, neuronal plasticity and activity, metabolism (downregulated) and innate immunity and anti-viral response (Cohen-Rengifo et al., 2022).

The differential regulation of genes related to neuronal renewal and neuroplasticity in CO_2 exposed fish together with changes in olfactory sensitivity under acute exposure to CO_2 acidified water suggests the occurrence of activity dependent survival/differentiation of olfactory receptor neurons. Indeed, previous work has suggested that activity dependent survival/differentiation of olfactory receptor neurons can occur within the OE (Wang et al., 2017b); however, this seems to differ according to the olfactory receptor being expressed (Ibarra-Soria et al., 2017). After prolonged exposure to OA conditions, seabream still have reduced olfactory sensitivity compared to control fish (Velez et al., 2019). Unfortunately, it was not possible to test the effects of long-term exposure of sole to acidified water on olfactory sensitivity, as the exposed fish died under anaesthesia.

Unexpectedly, one of the most strongly downregulated genes in both OEs was green-sensitive opsin-3, that has non-visual functions and is expressed in immune cells (macrophages, primary T cells and dendritic cells); altered IL-2 production by T cells with opsin-3 knockout, and polymorphisms associated with asthma suggest it has a role in inflammation (White et al., 2008). The functional importance of opsin-3 in the OE is unknown; however, the expression of this gene in T cells raises the hypothesis that diferential regulation of opsin-3 in the olfactory organ (OO) of sole exposed to acidified water may be related to immune responses and emphasises the importance of further studies. Indeed, in Raimbow trout, nasal delivery of rhaddovirus induces apoptosis in crypt olfactory neurons via the interaction of olfactory neurons TrkA receptor with the viral glycoprotein, this signal takes to electric activation of neurons and very rapid proinflammatory responses in the OO (Sepahi et al., 2019). CD8α + T cells infiltrate the OO within minutes of nasal viral delivery (Sepahi et al., 2019), this study showed a function of olfactory sensory neurons in nasal antiviral immune responses and the involvement of T cells.

In sole, in agreement with previously described in the European sea bass (Cohen-Rengifo et al., 2022) we found differences in the expression of genes related to immune processes in the upper and lower OE of CO_2 exposed fish. Strongly upregulated genes were related to innate immune processes and inflammation in the upper OE, while in the lower OE this was less notable and DETs were mainly related to the adaptative immune response, more specifically, T lymphocytes (e.g., *cd8b* and *cd109*). For example, the *cd8b* expresses the CD8 antigen which is a cell surface glycoprotein found on most cytotoxic T lymphocytes. The *cd109* gene encodes a glycosyl phosphatidylinositol (GPI)-linked glycoprotein that localizes to the surface of platelets, activated T-cells in which it works as an activation antigen (Lin et al., 2002). Combining the results of the transcriptome with the histology of the lower OE leads us to propose that MMC infiltration was accompanied by T cell infiltration. The immune cell infiltration and immune modulation of the OE is reported to be partly from nasal associated lymphoid tissue (NALT) (Tacchi et al., 2014). Whether the change in the immune response in the OE reflects a change in the whole body immune response remains to be established and highlights a further mechanism by which OA could affect fitness.

The transcriptional response in the upper OEs seem to be at odds with the absence of change in the histology of the upper OE. No MMCs were found in the upper or lower olfactory epithelia of control fish. In fish exposed to high CO_2, all samples analysed in the lower nostril showed a high amount of MMCs, while in the upper nostril, MMCs were only detected in two samples and in a small number. However, this suggests that the changes observed in the lower nostril (including a massive infiltration of MMC) may be ongoing (although in an early stage) in the upper nostril. Future work will be required to address this issue and to establish the basis of this difference between nostrils. However, since the analysis of the OEs occurred after four weeks exposure to acidified seawater, it is possible that the observed changes in tissue morphology in the lower OE occurred early on, and the gene expression at week four represents a new "homeostatic steady state" under acidified conditions. Further work will be required to fully establish the temporal characteristic of the OEs response to acidified seawater.

Although in light of current knowledge our results are difficult to interpret, they clearly show a differential immune reaction in the upper and lower OE in response to ocean acidification, and the consequences of this are unpredictable. Being at the interface of the environment and the CNS, the OE comprises a direct route by which harmful agents can damage the CNS. This neuro-immune interface has been characterized in several vertebrates including teleost fishes (Yu and Zhang, 2014). Herein we clearly show the effects on the peripheral olfactory system of exposure to future predicted OA conditions. There are marked differences in the response of the upper and lower OE to CO_2 acidified water at the molecular, functional, and histological levels. The different response of the upper and lower OE agrees with previously reported functional differences that are associated with differential gene expression in both OEs. We propose that exposure to acidified water causes an odorant-dependent decrease in olfactory sensitivity. This activates mechanisms of neurogenesis and plasticity with unpredictable behavioural consequences. In addition, the lower OE is significantly modified with a massive infiltration of MMCs accompanied by the formation of proliferative lesions and vacuoles in the SE, indicative of degeneration. The disorganization of the lower OE is likely to compromise olfactory perception leading to unpredictable behavioural alterations. There is a large body of literature linking OE inflammation and olfactory disorders and several studies have shown that olfactory cleft inflammatory cytokines/chemokines are correlated with olfactory performance (Wu et al., 2018). Furthermore, olfactory receptor neurons project directly to the olfactory bulb in the forebrain via the olfactory nerves and the olfactory forebrain is intimately associated with the limbic system, that is involved in learning, memory, and emotions through interactions with the endocrine system. Thus, changes in olfactory perception are probably also associated with endocrine and behavioural changes in fish.

5. Conclusion

Acute exposure to high CO_2 water decreases the olfactory sensitivity of sole to all odorants tested; however, the effects more marked in the upper OE. We propose that the resilience of the lower OE to high PCO_2/low pH water is an ecological adaptation. Lower sensitivity is linked to regulation of genes related to neuromodulation and neuroplasticity suggesting that exposed sole activated compensatory molecular mechanisms to overcome lower olfactory sensitivity in acidified water. However, in the lower OE, long-term exposure to acidification cased substantial tissue modifications that may seriously compromise olfactory sensitivity with unpredictable behavioural consequences.

CRediT authorship contribution statement

Rita A. Costa: Investigation, Formal analysis, Conceptualization. **Peter C. Hubbard:** Conceptualization. **Manuel Manchado:** Data curation. **Deborah M. Power:** Writing – review & editing, Conceptualization. **Zélia Velez:** Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare no competing or financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2025.111820.

Data availability

No data was used for the research described in the article.

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