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Neuronal effects of ocean acidification in gilthead seabream (*Sparus aurata*)

Mestrado em Biologia Marinha

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ABBREVIATIONS

OA: Ocean Acidification

GABA: γ-Aminobutyric acid

pCO2: Partial pressure of CO_2

GPCRs: G-protein-coupled receptor family

OR: Olfactory receptor

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ABSTRACT

As atmospheric CO_2 increases, so does the amount of CO_2 dissolved in the ocean; this causes ocean acidification. The impact of ocean acidification on marine biodiversity and ecosystems has received considerable attention; however, study of its effects on fish physiology and behaviour is just beginning. Although there is evidence that the atmospheric CO_2 concentrations predicted to occur by the end of this century have mal-adaptive effects on olfactory-mediated behaviour of reef fish, the cellular mechanism(s) involved is unclear. In the current study, we recorded the olfactory responses of gilthead seabream (*Sparus aurata*) to explore the effects of high pCO_2 and low pH – separately - on olfactory sensitivity. Exposure to elevated pCO_2 (but at normal pH) significantly decreased olfactory sensitivity to some odorants, such as L-serine, L-leucine and L-arginine. Moreover, low pH (but at normal pCO_2) also decreased olfactory sensitivity to L-serine, L-leucine and L-arginine and L-glutamine. At the histological level, medium-term exposure to ocean acidificaiton increased the ratio between non-sensory epithelium/total length of the lamella significantly at one week, three weeks and four weeks. Furthermore, the number of mucous cells increased significantly after four weeks of exposure to high pCO_2 water. These structural changes suggest that the olfactory epithelium can respond to the changes in low pH and/or high CO_2 levels, but cannot fully counteract the effects of acidification on olfactory sensitivity. Together, these results show that both high pCO_2 and low pH can independently reduce olfactory sensitivity in marine fish, and that although acidification can evoke structural changes in the olfactory epithelium, these changes cannot fully restore olfactory sensitivity.

Keywords: Ocean acidification, olfaction, carbon dioxide, amino acids, olfactory epithelium, neuronal impairment, gilthead seabream (*Sparus aurata*)

RESUMO

Desde a revolução industrial que as atividades antropogénicas, como a utilização de combustíveis fósseis, liberta grandes quantidades de dióxido de carbono (CO_2) para a atmosfera, levando ao aumento substancial da pressão de CO_2 atmosférico (pCO_2). Uma grande parte deste CO_2 atmosférico (aproximadamente 1/3 a 1/4) é absorvido pelo oceano, o qual, apesar de mitigar o aumento do CO_2 na atmosfera, diminui o pH à superfície da água do mar e leva à sua acidificação. Comparando com os níveis na época pré-industrial, o pH da água do mar já diminuiu 0.1 unidades e estima-se que ainda diminua 0.3 – 0.4 unidades até ao final do século, e 0.7 – 0.8 unidades até ao ano 2300. O impacto da acidificação oceânica na biodiversidade e nos ecossistemas marinhos tem vindo a receber mais atenção, porque, por exemplo, afeta negativamente a formação das estruturas calcárias nos organismos que fazem calcificação e limita o crescimento dos recifes de coral. Contudo, o estudo do efeito da acidificação na fisiologia e comportamento dos peixes está apenas a começar. O olfato é um dos sistemas quimiorrecetores principais que os peixes utilizam para detetar químicos no ambiente, o que afeta o comportamento dos organismos marinhos. O epitélio olfativo é uma estrutura muito importante do sistema olfativo que contém recetores neuronais olfativos, cujos axónios transmitem a informação sensorial diretamente para o cérebro. Há estudos que demonstram que a concentração de CO_2 prevista para o final do século altera o comportamento mediado pelo olfato nos peixes de recife de coral, mas os mecanismos celulares envolvidos não são foram ainda elucidados. Um estudo recente demonstrou que níveis elevados de CO_2 afetam o comportamento mediado pelo olfato porque afeta a função dos recetores GABA no cérebro. Outro estudo refere que a sensibilidade olfativa da dourada (*Sparus aurata*) a aminoácidos (e outros odorantes) é reduzida devido à alteração no estado de protonação do odorante e/ou recetor, devido à alteração do pH da água (de ph 8.1 para pH 7.7). A exposição a médio-prazo (4 semanas) a água com elevada pCO_2 pode induzir um aumento do rácio entre o comprimento do epitélio não sensorial *vs*. comprimento da lamela e um aumento no número de células de muco por lamela; houve ainda uma alteração do pH do muco de acídico para neutro. Contudo, os efeitos diretos da elevada pCO_2 e pH baixo em

separado no epitélio olfativo não foram ainda testados. No presente trabalho, foram registadas as respostas olfativas da dourada (*Sparus aurata*) para explorar os efeitos da elevada pCO_2 e pH baixo, em separado, na sensibilidade olfativa. Os aminoácidos são odorantes para os peixes, e a sua ação depende da sua polaridade e da cadeia lateral. Foram selecionados cinco aminoácidos: L-serina, L-arginina, L-leucina, L-glutamato e L-ácido glutâmico. Todos os cinco aminoácidos são α-aminoácidos, o que significa que todos têm o grupo funcional ligado ao α-carbono. Cada estímulo foi diluído e testado na gama de deteção da resposta olfativa de 10^{-3} M a 10^{-7} M. Neste estudo utilizou-se o registo a partir do nervo olfativo para testar a resposta aos aminoácidos. No epitélio olfativo exposto a água com elevada pCO_2, a resposta olfativa diminuiu significativamente para três dos cinco odorantes testados: L-serina, L-arginina e L-leucina, e o limite de deteção destes três odorantes aumentou. No epitélio olfativo exposto a água com pH baixo, a resposta olfativa diminuiu significativamente para quatro dos cinco odorantes testados: L-serina, L-arginina, L-leucina e L-glutamina, e o limite de deteção para a L-serina, L-arginina e L-leucina aumentou. Comparando a amplitude das respostas nas duas situações experimentais, a resposta à L-serina, L-arginina e L-leucina foi mais afetada pela elevada pCO_2 do que pelo pH baixo. A amplitude da resposta ao L-ácido glutâmico foi a mesma em ambas as condições experimentais de elevada pCO_2 e pH baixo. Ao nível histológico, verificou-se nos peixes expostos durante 4 semanas a condições de acidificação, um aumento no rácio entre o comprimento do epitélio não sensorial / comprimento da lamela, após uma, três e quatro semanas em condições de acidificação. O número de células de muco aumentou significativamente após 4 semanas de exposição a água com elevada pCO_2. Estas alterações estruturais sugerem que o epitélio olfativo pode responder à alteração dos níveis de pH baixo e/ou elevada pCO_2, mas não consegue compensar totalmente o efeito da acidificação na sensibilidade olfativa. Estes resultados demonstram que tanto a pressão de CO_2 elevada como o pH baixo podem, independentemente, reduzir a sensibilidade olfativa dos peixes marinhos, e, apesar da acidificação provocar alterações estruturais no epitélio olfativo, estas não são suficientes para compensar a perda de sensibilidade olfativa. É possível que o efeito do pH se deva à diminuição na afinidade entre os odorantes e os seus recetores como consequência da alteração do seu estado de protonação

e, consequentemente, à forma do odorante e/ou do seu local de ligação no recetor. O(s) mecanismo(s) pelo(s) qual(ais) a pCO_2 afeta a sensibilidade olfativa é(são) menos claro(s). É possível que o pH intracelular esteja diminuído como consequência do pH extracelular mais baixo; ou pela difusão de mais CO_2 para os neurónios (reduzindo o pH intracelular), e que este fenómeno possa reduzir a atividade das enzimas envolvidas nas vias de transdução, afetando a sua eficiência. É também possível que o excesso de CO_2 se ligue diretamente a alguns recetores, atuando como um antagonista alostérico. Contudo, o mecanismo específico precisa de ser mais explorado no futuro. O presente estudo sugere que a redução da sensibilidade olfativa – como consequência do pH baixo e/ou elevada pCO_2, não estará confinada à espécie em estudo, mas poderá ser verificado em outros organismos marinhos. Sendo este o caso, as consequências da acidificação dos oceanos serão generalizadas, complexas, e difíceis de prever.

Palavras-chave: Acidificação do oceano, olfato, dióxido de carbono, aminoácidos, epitélio olfativo, alterações neuronais, dourada (*Sparus aurata*)

1. INTRODUCTION

1.1. Ocean acidification background

The oceans play an extremely important role on Earth; they cover 71% of the Earth's surface, account for 97% of the Earth's water and 90% of habitat [1] including a variety of ecosystems for many different taxa. Furthermore, oceans are believed to have the capacity to absorb most of the anthropogenic CO_2 emissions [2]. Before the Industrial Revolution, atmospheric carbon dioxide (CO_2) levels were approximately 280 ppm (parts per million volume) [2], and the oceans were a net source of CO_2 to the atmosphere [3]. Over the past 250 years, however, carbon dioxide levels in the atmosphere have increased by nearly 40% to approximately 384 ppm (in 2007) [4], mainly caused by the burning of fossil fuels, but also because of land-use practices such as deforestation [2]. Nearly a third of CO_2 (about 525 billion tons over the past 200 years) [5] added to the atmosphere has been absorbed by the oceans, which makes them a CO_2 sink; and the atmospheric CO_2 level would be approximately 450 ppm today without this oceanic uptake [4].

It was thought that oceanic uptake of CO_2 might even be a good thing because it would help moderate future climate change. However, the scale of anthropogenic CO_2 production is so vast that the oceans' buffering capacity has been exceeded and the pH of seawater is beginning to drop; 'ocean acidification'.

1.1.1 How does ocean acidification happen

Carbon dioxide is a natural constituent of the Earth's atmosphere: animals exhale it when they breathe, plants and other photoautotrophs use CO_2 to produce carbohydrates by photosynthesis [6]. Every atmospheric gas, including carbon dioxide, is in equilibrium with that gas dissolved in ocean water, which obeys Henry's law - the amount of dissolved gas in a liquid is proportional to its partial pressure above the liquid - that means if CO_2 levels increase in the atmosphere, the concentrations of CO_2 in the ocean surface would also increase [7]. The oceans are a major sink for atmospheric carbon dioxide. However, the

oceans are no longer able to absorb CO_2 that human-released into the atmosphere without changing its acidity. Even though it is a chemically unreactive gas but, when dissolved in water, it becomes more reactive and takes part in several chemical reactions [1]; it will first react with seawater to form a chemical equilibrium with carbonic acid (H_2CO_3) [8]. However, not all the CO_2 that dissolved into seawater will react to form carbonic acid and therefore dissolved gaseous CO_2 is contained in the seawater.

$$[CO_2] + [H_2O] \Rightarrow [H_2CO_3]$$

As carbonic acid is a weak acid, which may not act as quick as hydrochloric acid or sulfuric acid, but it works the same way as all acids; it releases hydrogen ions (H^+) which bond with other molecules, and form bicarbonate (HCO_3^-) by losing hydrogen ions and carbonate ions (CO_3^{2-}) [4].

$$[H_2CO_3] \rightleftharpoons [H^+] + [HCO_3^-]$$

The increased H^+ in the seawater causes some carbonate ion (CO_3^{2-}) to react with H^+ to form HCO_3^-. These seawater carbonate chemical reactions are reversible and near equilibrium [8].

$$[H^+] + [CO_3^{2-}] \rightleftharpoons [HCO_3^-]$$

The acidity level of the ocean is expressed as its pH. The square bracket refers to the concentration of H^+, and negative logarithm of hydrogen ion concentration (moles per liter) in the seawater is defined as the pH which can describe the ocean acidity:

$$pH = -\log 10\ [H^+]$$

1.1.2. pH decreasing in the ocean

Over the past 300 million years, oceanic pH has been slightly basic, averaging about 8.2. Since the industrial revolution, the ocean has absorbed nearly one third of the CO_2 human activity has released into the atmosphere. In the recent past (1990s), the pH of the ocean surface is around 8.1 (Fig. 1.1); a drop of slightly more than 0.1 pH units. However, when converted into H^+ concentration, this represents a 29% increase over the past two centuries.

Based on the probable pre-historical changes during the last 300 million years, if we release carbon dioxide at current rates, Caldeira and Wickett predicted that by the end of this century, a further drop of 0.5 units pH, to 7.7 or 7.8, will occur [2]. The ocean pH is going to be more acidic than ever seen for the past 300 million years. With a pH 7.7 or 7.8 of seawater is not actually acidic *per se*, but the changes are still dramatic.

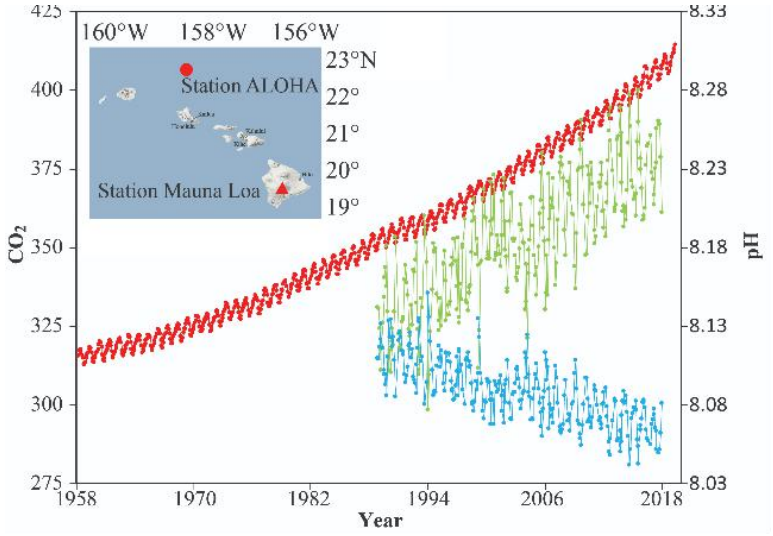


Fig. 1.1 Time series data of atmospheric pCO_2 (red), oceanic pCO_2 (green) and pH (blue) from the surface ocean in North Pacific (Ref. Dore et al., 2009).

1.1.3. Partial pressure of CO_2 increasing in the ocean

Another essential component to study ocean acidification is to quantify the amount of carbon dioxide in seawater. Over the past 20-30 years, scientists have measured the seawater pH in the North Pacific. The observed increase in acidity agrees with estimates of the oceanic uptake of CO_2 from human activity, suggesting that seawater is actively exchanging CO_2 with the atmosphere (Fig. 1.1) [9]. It shows that rising atmospheric CO_2 is not caused by oceanic outgassing, otherwise the partial pressure of the oceanic CO_2 would be falling if the amount of CO_2 in the ocean was falling. Global mean atmospheric CO_2 values are expected to reach 1000 μAtm by the year 2100, and 1900 μAtm CO_2 by 2300 [2]. Even though decreasing pH is the hallmark of the ocean acidification, the inorganic carbon system in the ocean and total alkalinity are both affected by the increase of atmospheric pCO_2.

1.1.4. Effects of ocean acidification on calcifying organisms

Normally, marine organisms can adapt well to the normal fluctuations of oceanic pH. Primary producers, such as seagrasses, can adapt to higher CO_2 concentrations [10]. But many organisms will suffer, and there may be extinction, especially of calcifying organisms such as molluscs, foraminifera (protist plankton), coccolithophores (calcifying phytoplankton), crustaceans, echinoderms, and corals [1]. To maintain their calcareous structures, such as shells, plates or exoskeletons, seawater has to be supersaturated with calcium (Ca^{2+}) and carbonate (CO_3^{2-}) ions to ensure that $CaCO_3$ does not dissolve once it forms. However, as the pH decreases, the concentration of carbonate ions required for saturation increases. Whilst ocean acidification reduces the pH, this causes undersaturation of carbonate, and then causes the difficulty of calcification [11]. Ocean acidification limits the growth of coral reefs as well, but when the pH returns to normal, skeletons can regrow [12]. If CO_2 continues to be emitted at the current rate, around 70% of North Atlantic cold-water corals will live in corrosive waters by 2050 [13]. And by 2080, the acidity level will even make coral reefs erode faster than they can recover [14].

1.1.5. OA effects on fish behaviour

The effects of ocean acidification on calcifying species have been relatively well studied; however, its effects on fish are just beginning to be addressed. Prior to 2009, fish - as effective acid-base regulators - were thought to be less affected by ocean acidification. However, many recent studies have documented significant impacts on both neurosensory and behavioural endpoints of fish by levels of CO_2 predicted to occur at the end of the 21st century [15]. Recently, most of the research on the effects of ocean acidification on fish are focused on sensory systems and behavior. Robust and consistent interferences by CO_2 have found in a range of sensory systems including olfaction [16-23], hearing [24], vision [25, 26], and have also been implicated in processes related to general cognitive function including undesirable changes in lateralization [27, 28], and learning [28-30]. Except for Atlantic cod [31], impairments to olfactory-driven behaviour have been noted in several species at

numerous life stages. Several studies have shown increased mortality was directly linked to sensory disruptions [22, 29]. These impairments could affect dispersal [21], social interactions [19], connectivity [21] , predator-prey dynamics [16, 18, 25, 32-35] population replenishment [20, 22], biodiversity [21, 22], habitat preference [36, 37] and settlement timing [36]; all of which are expected to affect population and ecosystem dynamics. As mentioned in some studies, disruptions of endpoints represent that broad cognitive impairment on lateralization and learning suggest that ocean acidification will affect not only individual sensory systems but also central neuronal processing [25, 27, 36, 38, 39]. Multiple sensory systems in a single damselfish species (*Pomacentrus amboinensis*) [25, 33, 34] and in the orange clownfish (*Amphiprion percula*) [22, 24] disrupted by the ocean acidification further support this idea. Recent studies have shown that ocean acidification impairs the olfactory preferences of fish and behavioural responses to odorants. For example, settlement-stage larvae of the orange clownfish at pH 7.8 and 1000 ppm. CO_2 are attracted to, rather than avoid, the smell of a predator [20]. Juvenile damselfish (*Pomacentrus wardi*) exposed to 850 μAtm of CO_2 and released to the wild suffered an 8-fold increase in predation-related mortality, compared to control fish exposed to present-day levels of CO_2 (440 μAtm CO_2) [22]. Both studies suggest that mal-adaptive behavioural responses are not simply due to impaired olfactory perception at lower pH but alteration of sensory processing by the central nervous system. However, a direct effect of CO_2 alone or pH alone on the olfactory sensitivity has never been tested.

1.1.6. The importance of olfactory system

Chemical cues are omnipresent in marine ecosystems and influence critical aspects of the behaviour of marine organisms across the phylogenetic tree [40]. Such cues determine foraging strategies, feeding choices, prey selection, commensal associations, selection of mates and habitats and competitive interactions, which will strongly affect marine population and communities [41]. Chemoreception is the biological process whereby marine organisms detect these chemical cues. Chemical senses are the most ancient sensory systems, which have thought to appear 500 million years ago [42], and obtain information from the

environment. Olfaction (smell) is one of the most principle chemoreception systems of fish to detect chemicals in the environment [42]. Chemical cues help fish to distinguish conspecific individuals, determine their species and population identity, and result into different behavioural patterns: reproductive, schooling, defensive, migration, parental, agonistic, territorial among others [43-45]. Since a range of behaviours of marine species is mediated by the olfactory system, and a range of behaviours of marine species were demonstrated to be impaired by OA [16,21,22,33,35,36,46,47], therefore, the impacts on olfactory impairment caused by OA are profound.

1.2. The olfactory system

1.2.1. The olfactory organ

The olfactory organ of fish consists of a pair of structures on each side of head situated in the snout. In most fish species it is well developed and located on the dorsal surface of the head to the rostrum and medial to the eyes [48].

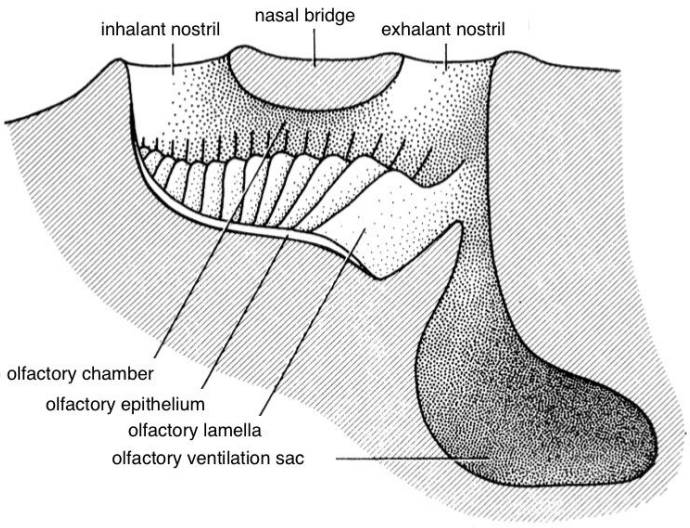


Fig. 1.2 Schematic median section of the olfactory organ of a generalized fish (from Zeiske et al., 1992).

It consists of an olfactory chamber, olfactory rosette, olfactory epithelium, olfactory lamellae, and olfactory ventilation sac. The olfactory chamber (cavity or nasal) connects with the exterior through the inhalant and exhalant nostrils, two openings are separated by the

nasal bridge [49]. Water enters the olfactory cavity through the anterior (inhalant) nostril and exits via the posterior (exhalant) nostril [48]. The olfactory rosette is usually located on the bottom of the olfactory chamber. The olfactory epithelium which contains the olfactory receptor neurons covers the most of the olfactory lamellae. The olfactory ventilation sac is an extension of the olfactory chamber (Fig. 1.2) [49]; however, there are some variations between different taxonomic group of fish depending on their life mode and behaviour [48].

1.2.2. Olfactory rosette

The olfactory epithelium located on the multilamellar mucosal folds which basic function is to increase the area of the olfactory epithelium bearing the receptor neurons that detect chemical stimuli [48]. These lamellar folds form a flower-like structure called the olfactory rosette (Fig. 1.3). Secondary lamellae are present in acipenseriforms, lepisosteids, and some advanced teleosts [50], and are on the surface of the primary lamellae and perform the same function as the primary lamellae. And tertiary lamellae are found in *Acipenser oxyrhynchus* [50]. Enormous diversity exists in different fish regarding the shape, number, and arrangement of olfactory lamellae and distribution of sensory and non-sensory epithelium on the olfactory lamellae depending upon various factors including food searching, migration, predator avoidance, and reproduction [51].

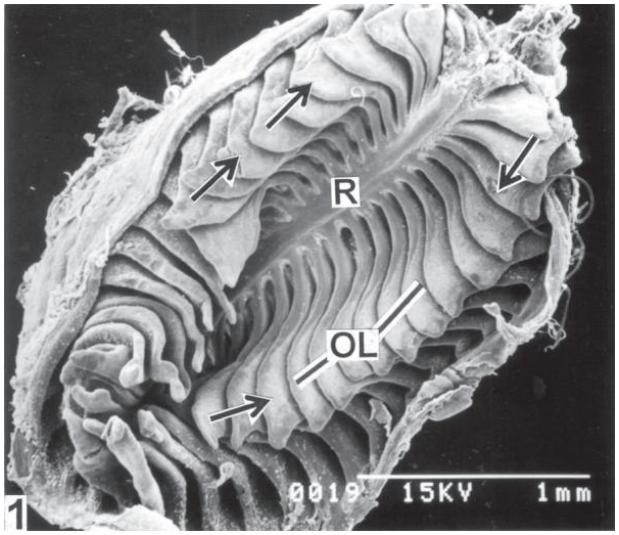


Fig. 1.3 Photomicrographs of the olfactory epithelium of fish (*Scatophagus argus*) (from Chakrabarti et al., 2011). Elongated olfactory rosette exhibiting different shapes of olfactory lamellae (OL) radiating from median raphe (R). Arrows indicate tongue shaped apical part of the OL.

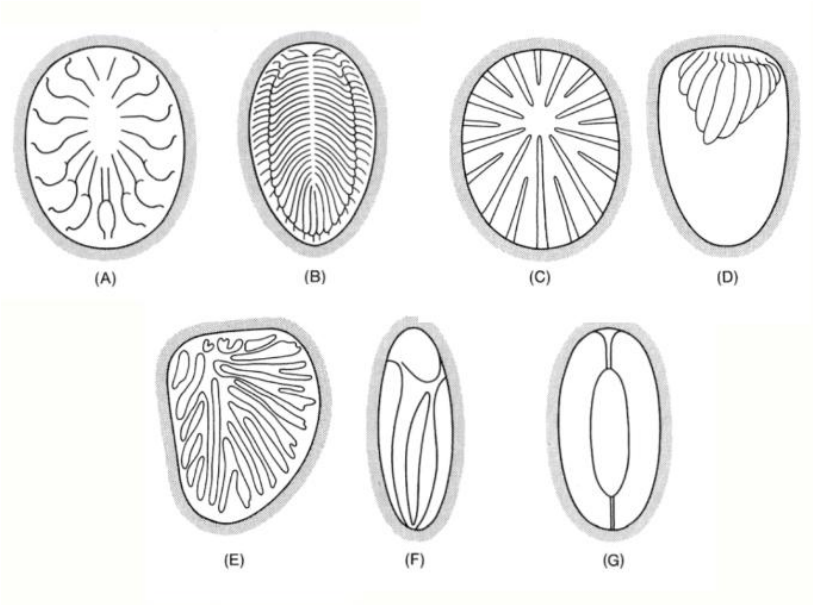


Fig. 1.4 Different shapes of olfactory rosette (from Zeiske et al., 1992).

The rosette of most fish is oval and consists of a row of lamellae on each side of a midline raphe (Fig. 1.4A, B). Some lamellae are arranged in a circle (Fig. 1.4C) or in a semicircle (snakefish, *Trachinocephalus myops*) [52], lie parallel to one another (Fig. 1.4D) or form irregular patterns (Fig. 1.4E). Rosettes may have few (Fig. 1.4F) or many lamellae, and lamellae may also be totally absent, as in the pipefish, *Siphonostoma typhle* [53] or in clingfish [54]. The sand eel, *Ammodytes*, possesses a kidney-shaped bulge (Fig. 1.4G). In some forms, the lamellae are only slightly raised folds, but in most species, they are truly different shape lamellae which are correlated with the different types of ventilation. Olfactory rosettes grow by increasing the number and size of their lamellae [49].

1.2.3. Olfactory epithelium and olfactory receptor cells

The olfactory epithelium covers the olfactory lamellae [48]. There are two different types of olfactory epithelium: sensory and non-sensory. These epithelia show great variability in their arrangement and distribution. Both epithelia are covered with a mucous layer and rest on a basal lamina and the underlying lamina propria [49]. The sensory epithelium is a columnar pseudostratified epithelium. The surface of the sensory epithelium is composed of

three main components: olfactory receptor cells, supporting cells, and basal cells. All cells are closely packed in the olfactory epithelium (Fig.1.5) [49].

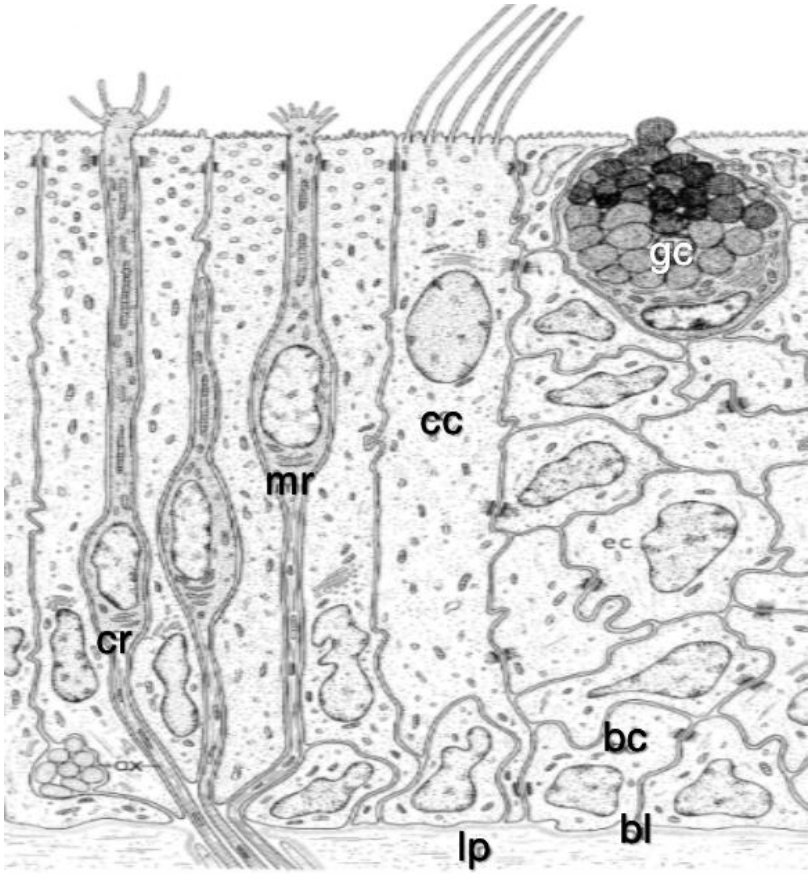


Fig. 1.5 Simplified diagram of the olfactory epithelium of teleosts, showing sensory and non-sensory epithelium (from Zeiske et al., 1992). Abbreviations: ax, axon; bc, basal cell; bl. basal lamina; cc, ciliated non-sensory cell; cr, cilated receptor cell; ec, epithelial cell; gc, goblet cell; lp, lamina propria; mr, microvillous receptor cell; sc, supporting cell.

Olfactory receptor neurons are the primary sensory cells of the olfactory epithelium. They are bipolar neurons with their cell bodies (perikarya) arranged in layers throughout the broad mid-region of the epithelium. They cover the side surface of olfactory lamellae, are absent on ridges of lamellae and in the vaults of the olfactory cavity [48]. There are three different types of receptor cells in fish olfactory epithelium: ciliated, microvillous, and crypt receptor cells (Fig. 1.6) [49, 55]. Recently, a fourth type of cells has been found in the olfactory epithelium of zebrafish: ‘kappe’ cells [56]. Crypt cells are relatively rare and their knob does not reach the surface of the epithelium but opens in a small cavity located directly at the surface [57-59].

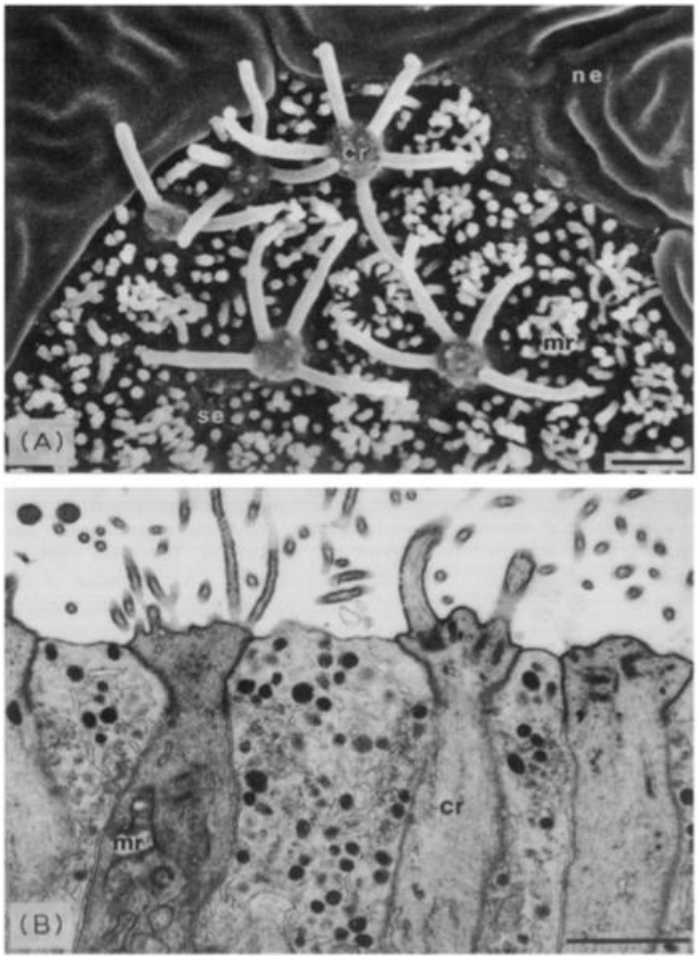


Fig. 1.6 Ciliated (cr) and microvillous (mr) receptor cells (from Zeiske, 1992). (A) Scanning and (B) transmission electron micrographs; ne, non-sensory epithelium; se, sensory epithelium. Scale bars, 1 μm.

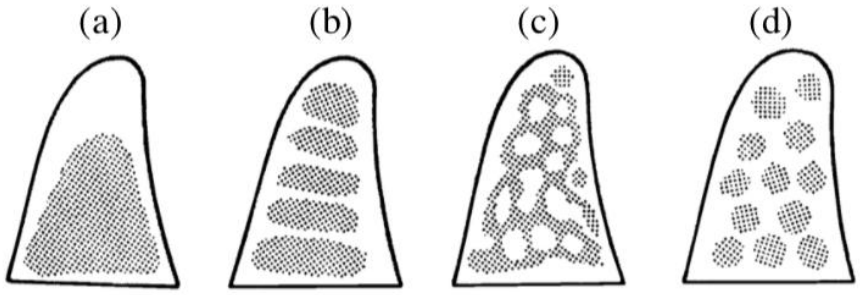


Fig. 1.7 Four distribution of the receptor cells on lateral side of olfactory lamellae (from Yamamoto, 1982) (a) continuous; (b) large-zonal; (c) irregular; (d) small-zonal.

The distribution of receptor cells may be different at the lateral surface of lamellae in different fish species. The most common distribution types are continuous, large-zone, fine-zone and irregular types (Fig. 1.7) [55, 74].

Two different types of supporting cells help the receptor cells to separate from each other [48, 49]: the supporting cells with a free surface not bearing any specialized structures; and ciliated cells which bearing numerous kinocilia on the surface facing the cavity of the nasal sac.

1.2.4. G-protein coupled receptors

The detection of odorants by olfactory sensory neurons in teleost fish is mediated by different families of olfactory receptor proteins [60], which all belong to the G-protein coupled receptor super-family (GPCRs), mediate most cellular response to photons, pheromones, neurotransmitters and odorants [61]. The GPCRs consist of seven hydrophobic transmembrane regions connected by three extracellular and intracellular loops regions, respectively [62]. The exact size of the GPCR superfamily is unknown, but it is divided into three main classes (A, B and C). By far, the largest class is class A, which is rhodopsin-like class and over half of this class are predicted to encode olfactory receptors, while the remaining receptors are liganded by known endogenous compounds [61].

Rhodopsin-like related G-protein coupled receptors exist in one of two main conformations: an inactive conformation, and an active conformation that interacts productively with an intracellular heterotrimeric G protein complexes, which is made up of alpha (α), beta (β) and gamma (γ) subunits (Fig. 1.8). The receptors display the affinity for a range of ligands rather than binding specific ligands, and conversely, a single ligand may bind to a number of receptors with varying affinities [63], which depend on physio-chemical properties of the molecules [64]. When an agonist stabilizes the active state of the receptor, conformational changes in the receptor allow it to couple with the G-protein [65]. The odorant receptors are likely to follow the rules of rhodopsin-like related G-protein coupled receptors.

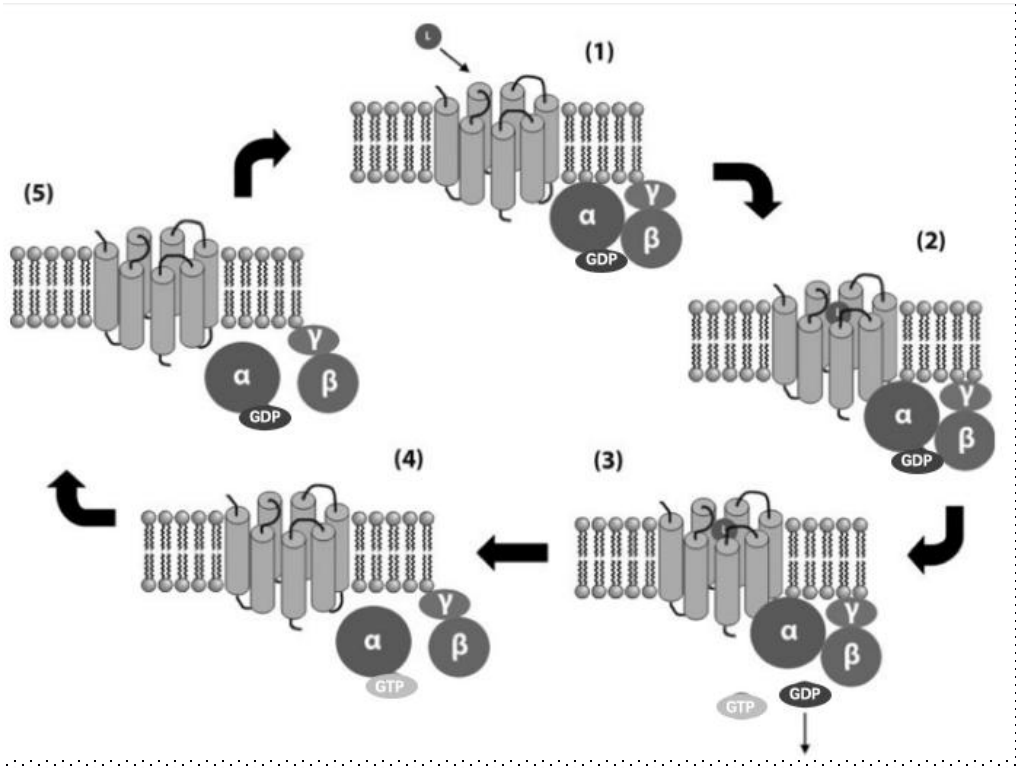


Fig. 1.8 The activation cycle of heterotrimeric G proteins (from Khafizov et al., 2009). At the inactive state, G proteins exist as heterotrimers, and GDP bounded with the Gα-subunit (1). Then the receptors undergo a conformational change when they interact with ligands (L) (2). This results in an increased binding affinity of GPCR to the G protein in its inactive trimeric state. After GPCR is conformationally changed, G protein release GDP (3), a GTP bound to the G protein subsequently which causes the dissociation of the Gα-subunit from the Gβγ dimer (4). Finally, GTP hydrolyzed to GDP by inherent enzymatic activity of Gα-subunit, that leads to reverse structural changes of Gα (5) and, consequently, to an increase of its binding affinity to Gβγ. Binding of the monomeric inactive state Gα to Gβγ closes the cycle (1).

In fish, it is generally accepted that olfactory transduction occurs through both AC/cAMP and PLC/IP_3 pathways as, for example, in carp [66], goldfish [67] and Atlantic salmon [68, 69]. After Gα dissociates from the Gβγ dimer and binds to GTP (Fig. 1.9), this specific G-protein (Golf and/or Gs, which are the subtypes of Gα) [70] in turn activates the lyase - adenylate cyclase Ⅲ (AC3) to converts ATP into cyclic AMP (cAMP). The cAMP opens cyclic nucleotide-gated ion channels (CNGC) which allow monovalent (mainly Na^+ and K^+) and divalent cations (including Ca^{2+}) to enter into the cell, which depolarizes the neuronal

cell membrane and eventually evokes an action potential which carries the information to the brain.

In the PLC/IP_3 pathway, the odorant binds to OR and activates phosphoinositide 3-kinase (PI_3K) through the β/γ subunit of the same or as yet unknown G-protein. Activated PI_3K produces phosphatidylinositol (3,4,5) trisphosphate (PIP_3), which negatively regulates sensitivity of the CNGC to cAMP and reduces the net output of the ORNs (Fig. 1.9) [71]. The PLC/IP_3 pathway is much less understood than the AC/cAMP pathway. And mechanisms of the phospholipase C (PLC) to produce inositol-1,4,5-phosphate (IP_3) and diacylglycerol (DAG) in the transduction pathway need to be further studied.

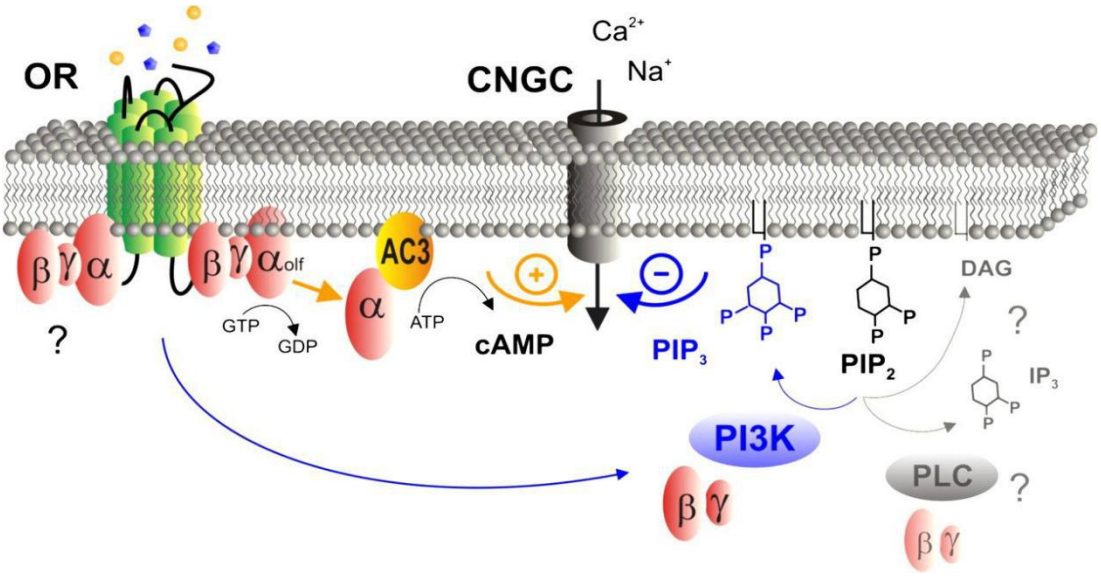


Fig. 1.9 Potential model for AC/cAMP and PLC/IP3 pathways in rodent olfactory receptor neurons (from Ache et al., 2010). The ligand is a mixture of binary odorant which the yellow circles excite the cell through the AC/cAMP pathway, and the blue stars inhibit the cell through the PLC/IP3 pathways.

Special G-protein-coupled receptor molecules are contained in the cellular membrane of cilia and microvilli. They play a critical role in recognizing thousands of odorant molecules in the olfactory sensory system [72]. It has been demonstrated that pH changes will alter the GPCR-ligand affinity *in vitro* [73].

1.2.5. The Olfactory nerve and the olfactory bulb

In vertebrates, olfactory sensory neurones (OSN) are the only ones whose axons carry sensory information directly to the brain [75]. Axons of all receptor cells are joined into the single nerve (Fig. 1.10), and run to the olfactory bulbs where they make a synaptic contact with the second-order bulbar neurons in the form of glomeruli (Fig. 1.11) [48, 76].

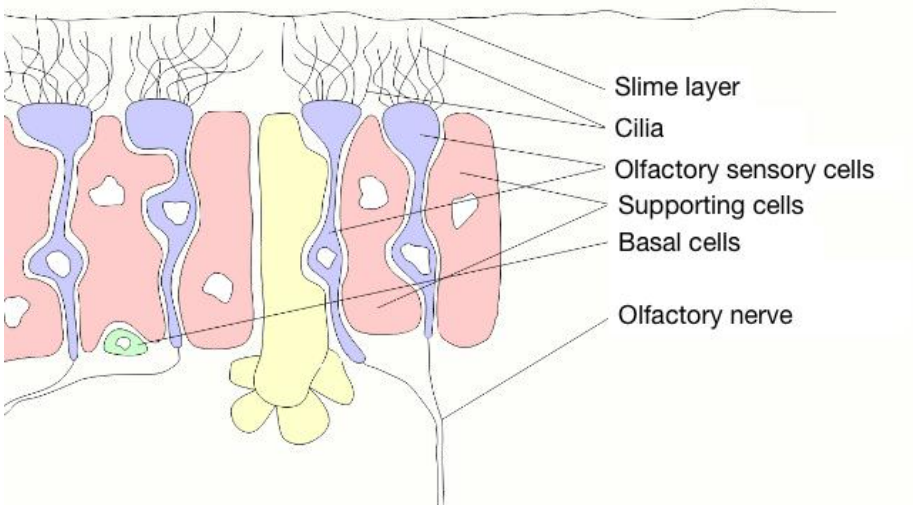


Fig. 1.10 A Schematic Representation of the Olfactory receptor neuron composition. (from MarianSigler , 2006)

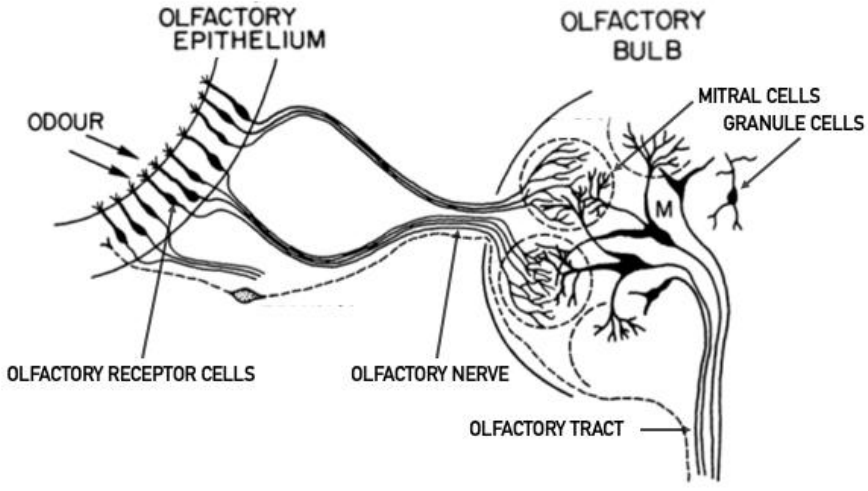


Fig. 1.11 A Schematic Representation of the Cellular Anatomy of the Peripheral Olfactory System and the Neural Organization in the Olfactory Bulb of Teleost. (from Hara, 1986)

The olfactory bulb is the first relay station in the olfactory system of vertebrates. Here, the axons of the sensory neurons terminate, and the transmission of olfactory information is conveyed by a new set of secondary neurons called relay cells [77]. Starting from the surface, the olfactory bulb can be divided into four layers: the olfactory nerve layer, the olfactory glomeruli layer, the mitral cell layer, and the internal cell layer (Fig.1.12) [77].

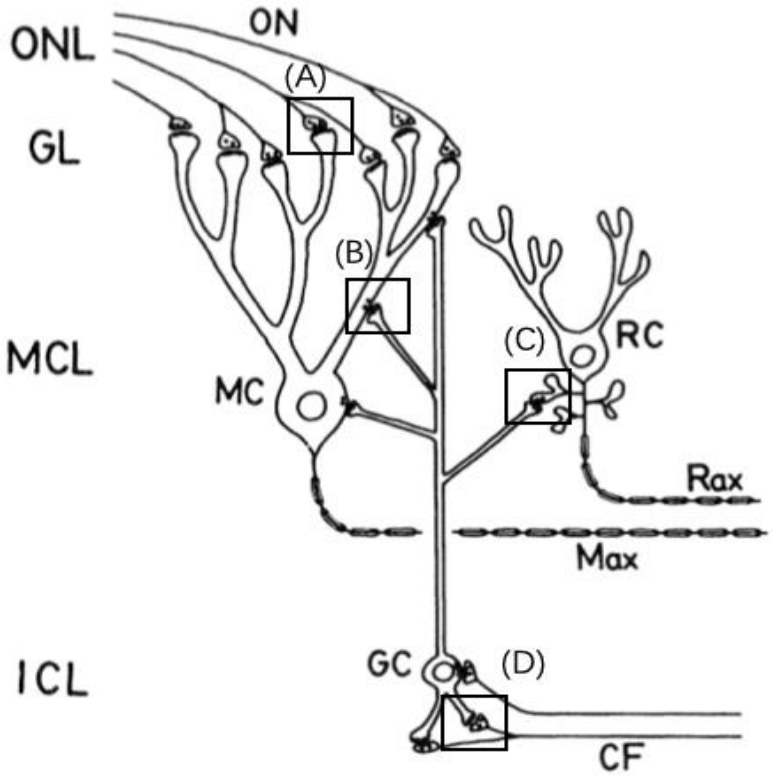


Fig. 1.12 Scheme of the teleost olfactory bulb (from Satou, 1992). Abbreviations: ONL. olfactory nerve layer; GL. glomerular layer; MCL. mitral cell layer; ICL. internal cell layer; ON. olfactory nerve; MC. mitral cell; RC. ruffed cell; MC. mitral cell; CF. centrifugal fiber. GC. granule cell; Max. mitral cell axon; Rax. ruffed cell axon. The synaptic organization and signal transfer in the olfactory bulb (A) Synaptic inputs from olfactory nerve fibers to mitral cell dendritic tufts; (B) dendro-dendritic reciprocal synapse between mitral cell dendritic shaft and granule cell peripheral dendrite; (C) reciprocal synapse between the pedunculated protrusion of the initial segment of ruffed cell axon and granule cell peripheral dendrite; (D) synaptic inputs from centrifugal fibers to granule cell deep dendrite.

1.3. OA effects on olfactory system

Up until now, there were some studies describing behavioural alterations in fish exposed to low pH/high CO_2 water; a possible explanation for this behavioural disruption could be the occurrence of disruptions in specific neuronal cells/regions [78, 79] or brain hemisphere

communication errors [27]. For example, the anxiety behaviour of the fish is increased when they are kept in acidified water [78], a study showed that the antagonist of $GABA_A$ receptor (gabazine), can restore proper discrimination of predator odour [96] suggesting that $GABA_A$ receptors are involved in behavioural disruptions induced by ocean acidification. Recent studies showed that behavioural changes are due to alterations in both olfactory perception (at the level of the olfactory epithelium) and olfactory integration (at the level of central brain function) [80]. The olfactory sensitivity can be affected by short and medium-term exposure to acidified water, and the effect act directly on the olfactory epithelium; it was proposed that changes in the charge distribution of odorant molecules due to the reduced pH lead to decreases in the affinity of the odorant to its receptor [81].

γ-Aminobutyric acid (GABA) is a well-known amino acid present in bacteria and plants, was first recognized as a major inhibitory neurotransmitter in the adult mammalian [80] brain [82]. GABA can interact with three types of receptors: $GABA_A$, $GABA_B$, $GABA_c$. $GABA_A$ and $GABA_c$ receptors are members of the ligand-gated Cl^- channel superfamily and mediate the fast-inhibitory activity of GABA [83]. When GABA binds to the $GABA_A$ receptor, it opens the associated Cl- channel and allows Cl^- flow into the neuron, therefore, GABA hyperpolarizes the neuronal membrane and makes the cell less reactive to excitatory neurotransmitters [82]. An important step to understand the mechanisms of neurosensory disruptions in fish was reported by Nilsson and colleagues in 2012 [38], who suggested that GABA and its accompanying $GABA_A$ receptors in the vertebrate nervous system were associated with the disruption in olfaction and lateralization during CO_2 exposure, which was an important step to understanding the mechanisms of neurosensory disruptions in fish. The addition of gabazine, a $GABA_A$ receptor antagonist, can re-establish adaptive olfactory-mediated behavior and lateralization in CO_2-exposed fish [38]. Since then, other studies have demonstrated that gabazine is able to restore much of the high pCO_2 related altered behaviors [29, 78, 84].

To date, disruption in brain neurotransmitter function has been regarded as the main mechanism of the fish behavioral alteration under high pCO_2 [85]; however, the direct effect(s) of high pCO_2/low pH on the olfactory system may be underestimated. A recent study showed that the olfactory sensitivity of the sea bass (*Dicentrarchus labrax*) to specific

odorants was directly reduced by acute exposure to acidified seawater [80], suggesting a direct effect on the olfactory epithelium; however, the mechanism remains unknown. Medium-term (four weeks) exposure also decreased the olfactory sensitivity of seabream (*Sparus aurata*) and the fish were unable to compensate for elevated pCO_2 water [81]. Protonation was demonstrated to change the charge distribution of odorant molecules, an essential component for ligand-receptor interaction, under the low pH seawater; the number of mucus cells increases when fish medium-term expose to the high CO_2 seawater [81].

Assessing the alteration of morphology and physiology in fish under high pCO_2 is essential to predict the effects of ocean acidification on marine organisms. Thus, the main objective of the current work was to evaluate the effects of high pCO_2 and/or low pH water independently on the olfactory sensitivity of the gilthead seabream (*Sparus aurata*). Structural change process of olfactory epithelium under high pCO_2 seawater during the medium-term experimental period (four weeks) was also evaluated in this study.

2. MATERIAL AND METHODS

2.1. Fish Maintenance

Animal maintenance and experimentation were carried out in certified experimental facilities and followed Portuguese national legislation (DL 113/2013) under a "group-1" license by the Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal. Gilthead seabream (*Sparus aurata*), hereafter "seabream", 2 years old, were originally obtained from the IFAPA Centro EI toruno, Junta de Andalucia, Camino Tiro Pichon sln, 11500 EI Puerto Santa Maria, Cadiz, Spain, then were kept in the Ramalhete experimental station (Universidade do Algarve) and fed with commercial pellets (Sparos, Olhão, Portugal) everyday. The weight and length of each seabream were measured before the experiment (Table.2.1). Fish were firstly maintained in 1000 L tanks with continuously running natural seawater under natural photoperiod and temperature. The experimental design of both hiostology and electrophysiology are shown in Fig. 2.1.

Table 2.1 Average weight and length of the seabream used for histology and electrophysiology. (Data are given as "Mean ± SEM")

	Weight (g)	Length (cm)
Histology	221.7 ± 3.5	21.7 ± 0.13
Electrophysiology	396.2 ± 29.56	27.23 ± 0.79

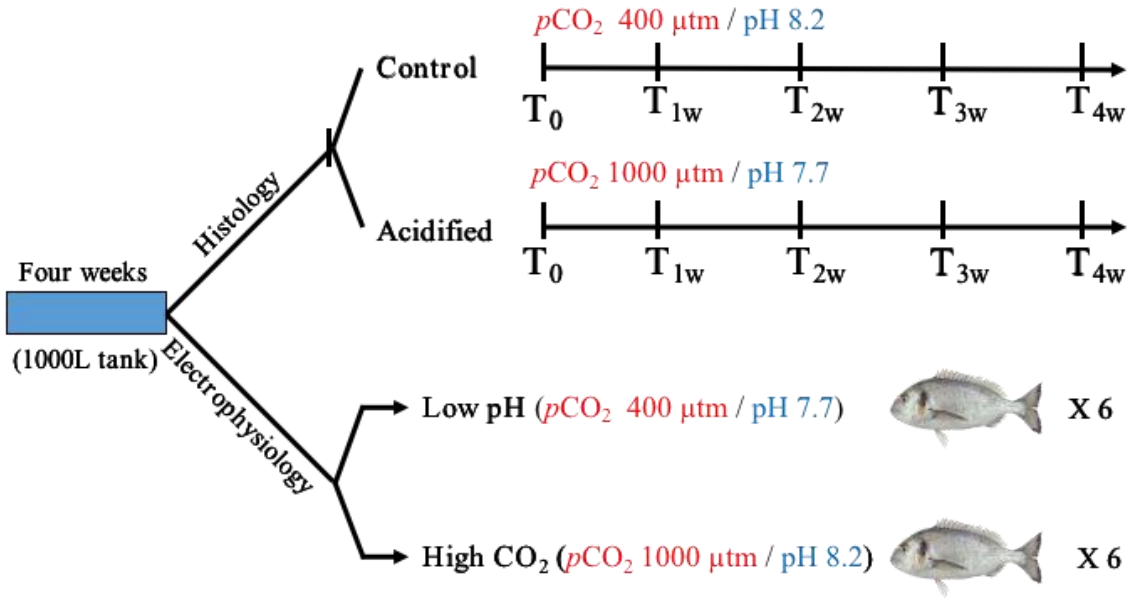


Fig. 2.1 Schematically representation of the experimental design. Two different sets of experiments were performed: one focusing on the histological analysis of the olfactory epithelium (OE) of seabream when exposed to control (pH 8.2, 400 µAtm) and low pH (pH 7.7, 1000 µAtm) seawater; and another one focusing on the separate effect of low pH (pH 7.7) and high pCO_2 (pH 8.2) using electrophysiology. For histological analysis, fish were sampled (n=3) after the quarantine period (T_0), and then after one week (T_{1w}), two weeks (T_{2w}), three weeks (T_{3w}) and four weeks (T_{4w}) in the experimental conditions. No mortality was observed during this period. For electrophysiology, six experiments were conducted for both high pCO_2 (pH 8.2) and low pH (pH 7.7), respectively.

2.2. Histological study

After four weeks of quarantine in 1000 L open circuit system tank kept at natural temperature and photoperiod, seabreams were randomly distributed into six sampling tanks (100L). Three tanks were kept in control conditions ("control fish") at pH 8.2 and 400 µAtm pCO_2; while the other three were kept at pH 7.7 and 1000 µAtm by bubbling CO_2 gas into the water ("acidified fish") (Fig. 2.1).

2.2.1. Seawater Chemistry

Seawater was pumped from the ocean into two header tanks (2000L), which were aerated with ambient air (control) or CO_2 to achieve the desired pH (elevated-CO_2 treatment), respectively. The pCO_2 of the elevated-CO_2 treatment header tank was maintained at a target value of 1,000 µAtm by using pH probe connected to an internal controller (EXAxt $PH_{450}G$,

Yokogawa Iberia, Portugal). Water in the header tanks was supplied to the 100 L tanks at 2 L/min. Seabream for histology were kept in these six 100L tanks. The pH (Orion star A221, Thermo Scientific, Portugal), temperature (Orion star A221, Thermo Scientific, Portugal), and salinity (WTW, cond3310, Spain) of seawater in each tank were recorded every day at the same time, 14h30min. Total alkalinity of water sample was analyzed twice a week by using Gran titration (DL15 titrator, Mettler Toledo, Portugal) with a certified acid titrant (0.1 M HCl, Fluka Analytical, Sigma-Aldrich). Water chemistry parameters of header tanks were used to estimate the pCO_2 using 'CO2SYS' software [86] with constant K_1 and K_2 from Mehrbach et al. [87] and refited by Dickson and Millero [88] and Dickson [89] for $KHSO_4$. The water chemistry parameters of control and high CO_2 of the header tanks during the 4 weeks experimental trial are summarized in Table 2.2.

Table 2.2 Water chemistry parameters of control and high pCO_2 header tanks. (Data are given as "Mean ± SEM")

Parameter	Control	High pCO_2
pH_{NBS}	8.179 ± 0.0115	7.705 ± 0.0151
Temperature (℃)	16.97 ± 0.37	17.04 ± 0.36
Salinity (ppt)	34.83 ± 0.14	34.82 ± 0.14
Total Alkalinity (μmol/kg SW)	2445 ± 22	2466 ± 43
pCO_2 (μAtm)	369.4 ± 12.33	1281 ± 57.08

2.2.2 Sampling of olfactory epithelium

Seabream were killed rapidly using an anesthetic overdose (2-phenoxyethanol, Sigma-Aldric). The weight and length of each seabream were measured (Table.2.2) Olfactory rosettes were sampled by opening the nostrils and removing the connecting tissues under a stereo-microscope with sterile scalpels and tweezers and fixed in the 4% paraformaldehyde (4% PFA). Fixed samples were stored at 4 ℃ overnight and washed 3 times (15 minutes each) with phosphate-buffered saline (PBS), and then 15 minutes with sterile water. Finally, the

tissues were transferred to 70% ethanol (prepared with sterile water) and stored at -20 ° C until use.

2.2.3. Paraffin embedding of sampled tissues

Fixed tissues were put into labeled histo-cassettes and placed in the automatic tissue processor (Leica TP1020). The tissues were processed overnight by the following serial steps: 70% ethanol for 10 min; 95% ethanol for 30 min; 95% ethanol for 30 min; 100% ethanol for 1 h; 100% ethanol for 1 h; ethanol: xylene (1:1) for 1 h; xylene for 1 h; xylene for 1,5 h; xylene: paraffin for 2 h and paraffin for 2 h. Paraffin blocks were then prepared by placing the tissues properly oriented in a metallic mold with liquid paraffin , in order to support the fixed tissue with a harder medium which allows cutting it into thin tissue slices, and were then left on a cooling plate until the paraffin solidified.

2.2.4. Coating of histological slides and sectioning

Histological glass slides were coated with diluted 0.01% poly-L-lysine solution before use. The polycationic nature of poly-L-lysine allows interaction with the anionic sites of tissue sections resulting in strong adhesive properties. First, the slides were immersed in 1% acid/alcohol for 5 minutes to clean the slides and washed under running tap-water for one minute. Next, the slides were immersed into 0.01% poly-L-lysine for 5 minutes and finally were allowed to dry at 37 ℃ overnight. The paraffin blocks of with the olfactory rosettes were sectioned longitudinally (5 μm) using a sliding microtome (Leica RM2135) until the complete olfactory organ with central raphe was visible.

2.2.5. Staining methods

2.2.5.1. Haematoxylin and Eosin (H&E) stain

Haematoxylin and eosin (H&E) staining was used to show detail of cellular components at an intra-nuclear level and in the cytoplasmic matrix and therefore to study their distribution "*in situ*".

Paraffin was removed from the tissue sections by exposing them to xylene for 2 x 15 minutes. Tissue sections were then hydrated by immersion in a graded ethanol series: 10 minutes in 100% ethanol followed by immersion in 95% and 70% ethanol, 5 minutes each, and finally an immersion in distilled water for 5 minutes. Hydrated sections were immersed in Harris haematoxylin for 5 min, blued in running tap water for 5 min and briefly washed in distilled water before immersed in eosin Y for 2 minutes. Excess dye was washed by the distilled water with a few drops of acetic acid. Finally, the stained tissues were dehydrated through a graded ethanol of increasing concentration (70%, 95%, and 100%), 5 minutes each. A final clearing was done with xylene, for 2 x 15 minutes, and final histological preparations were mounted with DPX (BioChemika, Sigma-Aldrich, Madrid, Spain) and cover slips.

2.2.5.2. Masson's trichrome stain

Masson's trichrome is a three-colour staining used to distinguish cells from surrounding connective tissue. The collagen fibers will be stained into blue and the nuclei will be stained into dark brown to black and the cytoplasm, muscle, erythrocytes are stained into light red or pink.

Tissue sections were hydrated following the same steps as previously described. Hydrated sections were immersed in Mayer`s acid haematoxylin for 10 min, blued in running tap water for 10 min and briefly rinsed in distilled water before immersed in Xylidine ponceau for 2 minutes. The Xylidine ponceau working solution was prepared by adding equal volumes of Solution A composed by 0.5% Xylidine ponceau in 1% acetic acid, and Solution B, composed by 0.5% acid fuchsin in 1% acetic acid. Excess dye was washed by the distilled water. Then, the sections were immersed in 1% phosphomolybdic acid for 4 minutes and rinsed in distilled water. Tissue sections were immersed directly into the light green for 90 seconds. The light green working solution was prepared by diluting the stock solution (2% light green SFY in 2% citric acid) 1/10 in distilled water before use. Staining sections were quickly dehydrated through ethanol series and cleared with xylene for 2 x 15 minutes. Histological preparations were mounted with DPX (BioChemika, Sigma-Aldrich, Madrid, Spain) and coverslips.

2.2.5.3. Periodic acid-Schiff (PAS)-Alcian blue stain

Periodic acid–Schiff (PAS) is a staining method used to detect polysaccharides such as glycogen, and mucosubstances such as glycoproteins, glycolipids, and mucins in tissues. Alcian blue which is a cationic dye, that is used to stain acidic polysaccharides such as glycosaminoglycans in cartilages, some types of mucopolysaccharides, sialylated glycocalyx of cells, etc [90]. Here, we use this staining to to highlight the goblet cells.

Tissue sections were hydrated following the same steps as described above. Hydrated sections were immersed in the Alcian blue solution for 30 minutes, gently washed in running tap water for 2 minutes and rinsed in distilled water. The tissues were them oxidized with 1% periodic acid solution for 10 minutes, washed in distilled water and immersed in Schiff reagent for 15 minutes in the dark. Sections were then rinsed in running tap water for 10 minutes and lightly stained with Harris haematoxylin for 2 minutes. After being rinsed in running tap water for 1 minute, tissue sections where dehydrated as previously described and mounted with DPX and coverslip.

2.2.6. Histomorphometry of the olfactory epithelium

Stained sections were observed under a microscope (Leica DM 2000, Famalicão, Portugal) coupled to a digital camera (Leica DFC 480) linked to a computer for digital images acquisition (IM50-software). For histological analysis, we focused on the three lamellae in the middle of each olfactory rosette. The software Fiji Image J (National Institutes of Health, USA) [91] was used to estimate the total length of the lamella as well as the length of the apical non-sensory epithelium. These two measurements were used to establish the ratio non-sensory epithelium / total length of the lamella (NSE/Total length). The number of goblet cells (mucus-producing) on the apical non-sensory surface of the lamellae was also estimated using the same software. Statistically significant differences between control fish and high CO_2 fish were considered at $p<0.05$ and assessed using Student's t-test with Prism, version 8 (GraphPad Inc., La Jolla, CA, USA). Results are presented as mean ± standard error of the mean (SEM).

2.3. Olfactory Nerve Recording

The weight and length of the each seabream used for electrophysiology were measured (Table 2.3). Prior to recording, seabream were anesthetized by immersion in aerated natural seawater containing 300 mg/L MS222 (ethyl-3-aminobenzoate methanesulfonate salt, Sigma-Aldrich, Portugal) until the response to a tail pinch had stopped; then neuromuscular blocker gallamine triethiodide (Sigma-Aldrich, Portugal; 10 mg/kg in 0.9% NaCl) was injected into the muscle. Anesthetized fish were then placed on a padded V-clamp and gills were irrigated with a constant flow of aerated seawater containing 150 mg/L MS-222, whilst a tube containing same seawater with approximately 100 ml/min/100g body-weight flow rate was placed in the mouth to irrigate the gills. Then the high CO_2 or low pH water went over the olfactory epithelium. And the water chemistry parameters of high pCO_2 conditions and its control during experiments were summarized in Table 2.3, and the ones of low pH condition and its control were in Table. 2.4.

Table 2.3 Water chemistry parameters of high pCO_2 conditions and its control for fish during electrophysiology experiment.

Parameter	Control	High pCO_2
pH_{NBS}	8.178 ± 0.0135	8.178 ± 0.0172
Temperature (℃)	23.62 ± 0.93	23.4 ± 0.85
Salinity (ppt)	34.5 ± 0.72	34.5 ± 0.72
Total Alkalinity (μmol/kg SW)	2930 ± 162	3000 ± 84
pCO_2 (μAtm)	323.1 ± 55.82	1504 ± 243.8

Table 2.4 Water chemistry parameters of low pH conditions and its control for fish during electrophysiology experiment.

Parameter	Control	low pH
pH_{NBS}	8.175 ± 0.0235	7.725 ± 0.0138
Temperature (℃)	24.25 ± 0.94	24.25 ± 0.94
Salinity (ppt)	35.17 ± 0.75	35.17 ± 0.75
Total Alkalinity (μmol/kg SW)	2803 ± 101	2767 ± 132

Exposed parts of the fish were covered with wet tissues to keep moist throughout the experiment. The olfactory rosette was exposed by cutting the skin and connective tissue covering the epithelium. The nostril was constantly irrigated with charcoal filtered seawater (without anesthetic) under gravity (flow rate: 6 ml/min) via a glass tube. Test solutions were delivered to the tube irrigating the nasal cavity via a computer-operated three-way solenoid valve for 4s. Charcoal-filtered seawater was used to make up the odorants 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, and then re/adjusted to 8.2 with 1 MNaOH. Low pH seawater was prepared by adding 1 M HCl until the desired pH (7.7) was reached. Amino acid solutions were prepared from frozen aliquots of 10^{-2} M; all stimuli were diluted into 10^{-3} M to 10^{-7} M with the appropriate (control, high CO_2 or low pH) seawater immediately before use. The order of each stimulus was always from the lowest to the highest concentration, but the order of each odorant was varied. 10^{-3} M L-serine was used as the standard solution to test, periodically, the stability of the preparation. The stimulants used were L-amino acids; at least one from each group of basic, acidic, polar and non-polar, (L-leucine, L-glutamine, L-glutamic acid, L-arginine, and L-serine); fish, in general, have a well-defined olfactory sensitivity to L-amino acids [94].

Fish were connected to earth via a copper wire inserted in the flank. The olfactory nerves were exposed by removal of the skin, connective tissue, and overlying bone [92, 93]. Olfactory nerve activity was recorded using tungsten micro-tungsten electrodes (0.1 MΩ,

World Precision Instruments, UK) (Hubbard et al., 2000) which were placed in the olfactory nerve where the maximal response to 10^{-3} M L-serine was recorded. The raw signal was amplified (x20,000; AC pre-amplifier, Neurolog NL104; Digitimer Ltd., Welwyn Garden City, UK), filtered (high pass: 200 Hz, low pass: 3,000 Hz; Neurolog NL125, Digitimer Ltd.) and integrated (time constant 1 s; Neurolog NL703, Digitimer Ltd.). Raw and integrated signals were digitized (Digidata 1440A, Molecular Devices, San Jose, CA, USA) and recorded on a PC running AxoScope™ software (version 10.6, Molecular Devices).

Each stimulus was applied for 4 s, with at least 1 min between each stimulus to allow wash-out from the nasal chamber and the receptors to recover completely. Responses to the 10^{-3} M L-serine standard were recorded regularly at the beginning and end of every four stimuli throughout the recording period. All integrated response amplitudes were blank-subtracted (using the slight response to background water) and normalized to the integrated response amplitudes of 10^{-3} M L-serine (similarly blank-subtracted).

2.4. Data and Statistical Analysis

All the amplitudes of electrophysiological response were normalized to that of 10^{-3} M L-serine and all statistically analyses were carried out on these normalized data. Olfactory nerve responses to L-serine, L-leucine, L-arginine were analyzed by linear regression of semi-logarithmic plots, and both of the slopes and elevations were compared between control and high CO_2 or low pH (Prism, version 8). The slope of the curve indicates the relation between odorant concentration and the amplitude of response which depends on the binding affinity between ligand (odorant in this case) and receptor. Responses to L-glutamic acid and L-glutamine were described by a three-parameter Hill curve as previously described [95]. EC_{50} and E_{max} were calculated for each fish, and were also compared using Student's t-test (Prism, version 8). Detection thresholds, which also depend on ligand-receptor affinity, were determined for each independent experiment, from the intercept with the x-axis of the linear regression fit to individual concentration-response curves for each odorant and each treatment (Prism, version 8). Differences between detection thresholds were converted to a normal

distribution using logarithmic mathematics, Student's t-test was used to test the paired data, and two-way ANOVA were used the analyse the histology data.

3. RESULTS

L-serine from the high CO_2 group and L-leucine from low pH group are selected as examples of the olfactory response incontro and high CO_2 or low pH water. Both L-serine and L-leucine were concentration-dependent response as shown in Fig. 3.1 under both control and treatment group, and all five amino acids which were tested showed the same clear concentration-dependent response. Response of the olfactory nerve to L-serine under high pCO_2 and L-leucine under low pH were both lower than that under control. (Fig. 3.1).

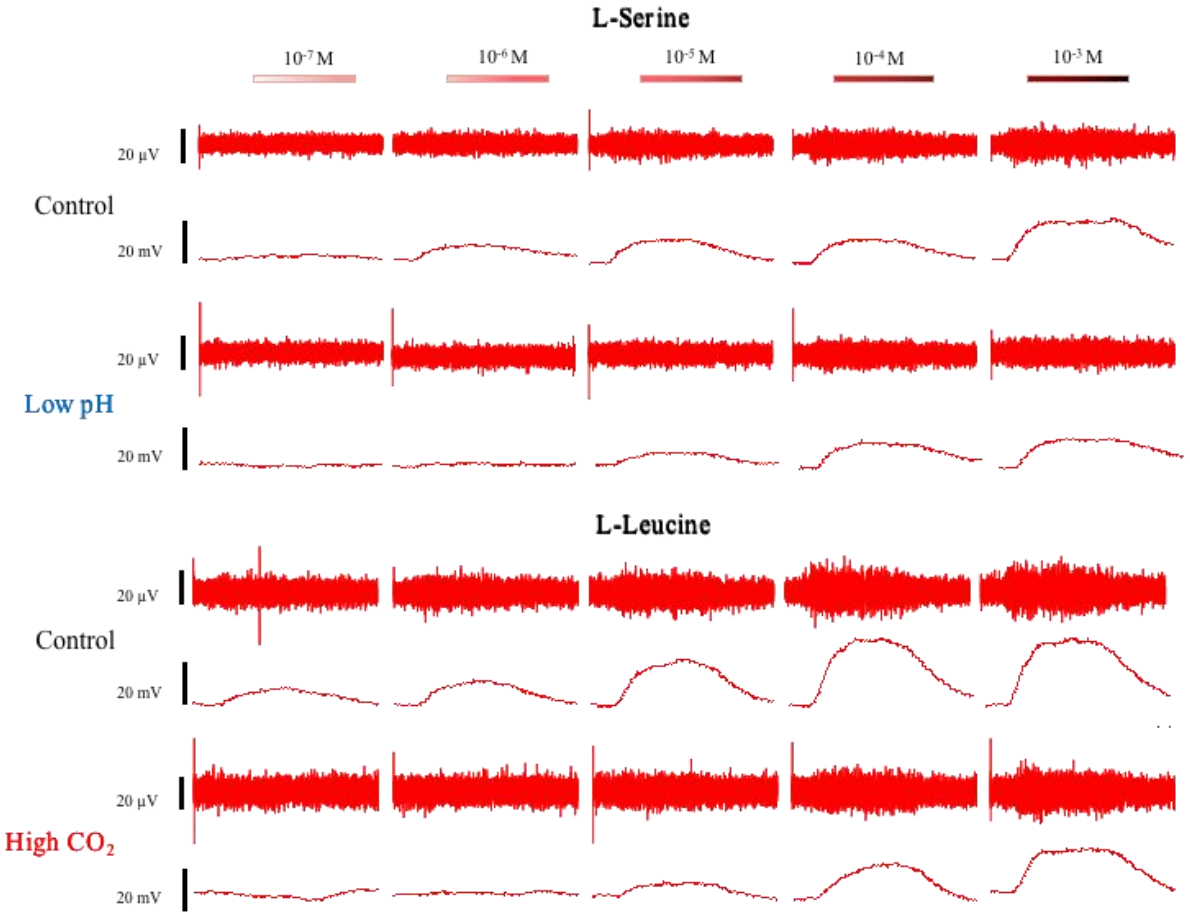


Fig. 3.1 Olfactory responses of the gilthead seabream (*Sparus aurata*). Increasing concentrations of amino acids (horizontal bars) were assessed by multi-unit recording from the olfactory nerve. (upper trace) with 20µV scale for all treatments, the integration of this activity (lower trace). Scale bar = 20 mV. L-Serine was selected for high pCO_2 group and L-Leucine was selected for low pH group.

3.1. Effects of high CO_2/ low pH on olfactory sensitivity

The concentration-response curves of L-serine, L-leucine, and L-arginine under high pCO_2 fit linear regression, and there were no differences among the slope of these regressions between control and high pCO_2 (Fig. 3.2A–C). However, the elevations of the control were significantly higher than those under high pCO_2 (Fig. 3.2A–C). In contrast, the concentration-response curves of L-glutamine and L-glutamate under high pCO_2 fitted with three-parameter Hill curve instead of linear regression (Fig. 3.2D, E). Olfactory nerve responses of seabream to L-glutamine and L-glutamate under control and high pCO_2 were statistically equivalent (Fig. 3.2D, E).

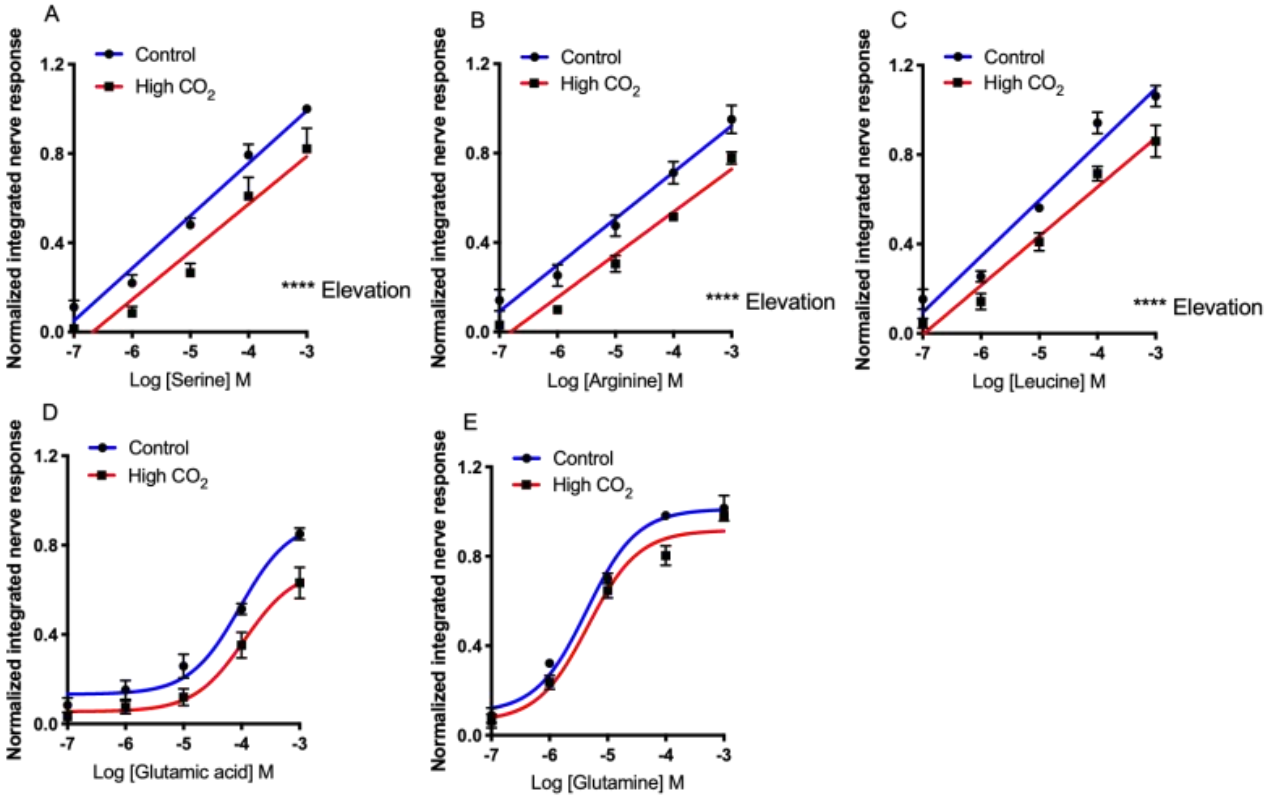


Fig. 3.2 Normalized olfactory nerve responses of gilthead seabream (*Sparus aurata*) to (A) L-serine, (B)L-leucine, (C) L-arginine, (D) L-glutamine, and (E) L-glutamic acid; under control (blue) and high pCO_2 (red). (Values are shown as mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001; n = 5)

The concentration-response curves of L-serine, L-leucine, and L-arginine under low pH fitted linear regression, and the slope of these three amino acids concentration-response curves between control and high pCO_2 had no difference (Fig. 3.3A–C). However, the elevations of the control condition were significantly higher than those under low pH (Fig. 3.3A–C). By contrast, the concentration-response curves of L-glutamine and L-glutamate under low pH were three-parameter Hill curve (Fig. 3.3D, E). Olfactory nerve responses of seabream to L-glutamine and L-glutamate under control and low pH were statistically equivalent (Fig. 3.3D, E). There were no significant differences between EC_{50} and E_{max} of L-glutamic acid (Fig. 3.3D). For glutamine, the E_{max} between control and low pH had no statistical difference; however, the EC_{50} had significant difference and the EC_{50} under control was lower than that under low pH (Fig. 3.3E).

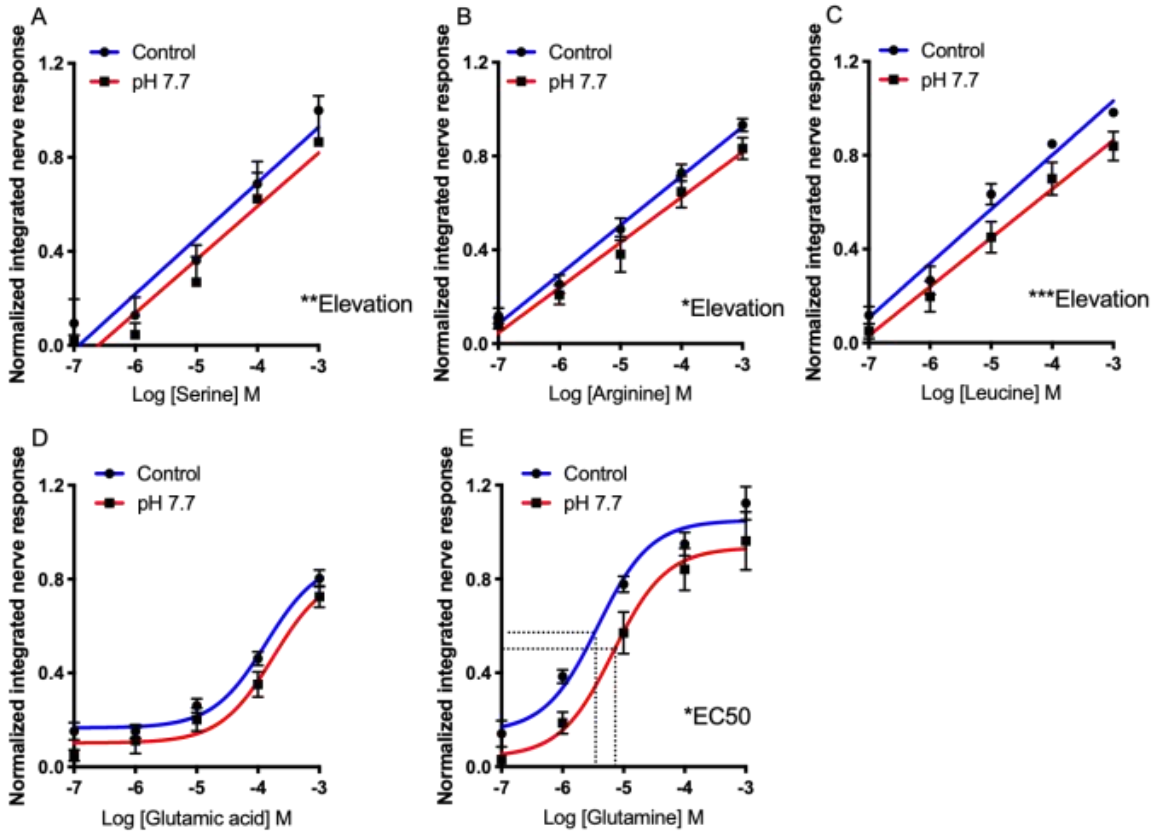


Fig. 3.3 Normalized olfactory nerve responses of gilthead seabream (*Sparus aurata*) to (A) L-serine, (B)L-leucine, (C) L-arginine, (D) L-glutamine, and (E) L-glutamic acid; under control (blue) and low pH (red). (Values are shown as mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001; n = 5)

Besides, the detection thresholds of L-serine, L-leucine, and L-arginine under both low pH / high pCO_2 were significantly increased (Fig. 3.4A,B). Moreover, the detection threshold of L-glutamine and L-glutamate under both low pH / high pCO_2 did not change compared controls.

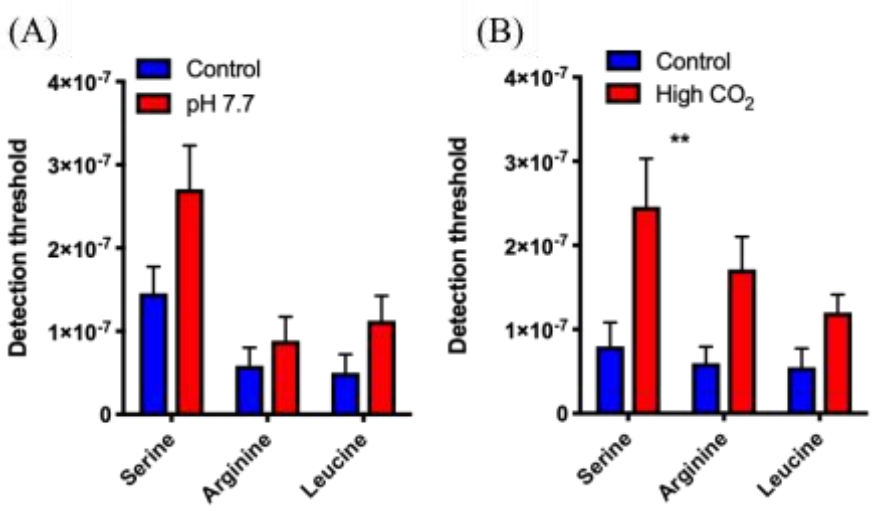


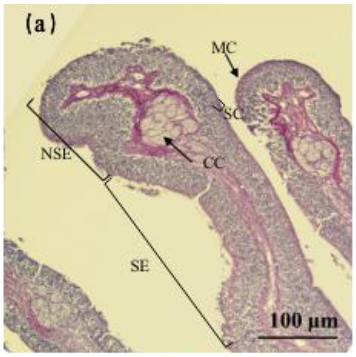
Fig. 3.4 Effects of low pH / high pCO_2 in the olfactory detection threshold of L-serine, L-leucine, and L-arginine (A) low pH, (B) high pCO_2. (Values are shown as mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001; n = 5)

3.2. Histology

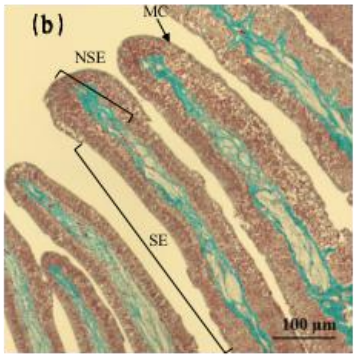
Based on the observation of the stained sections under the microscope, the gross anatomy of the olfactory epithelia of fish kept in high CO_2 water showed no significant differences with the control fish. Each lamella has two different types of epithelium which are the non-sensory epithelium (NSE) located on the top of the lamellae, and the sensory epithelium (SE), extending downwards into the central raft. The goblet cells that produce mucus are on the apical surface of the lamella. In the middle of each lamellae is the central core which is separated from the epithelium by the basal cell (BC). (Fig. 3.5 and 3.6). The length of non-sensory epithelia and the number of the mucous cells had an increase shown in Fig. 3.6.

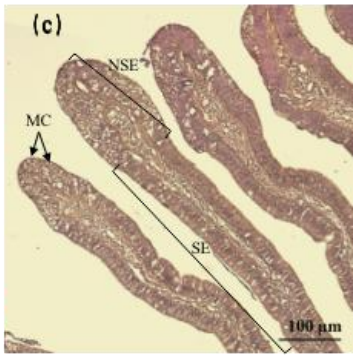
Control High pCO_2

T_0

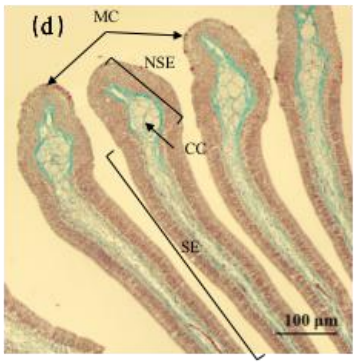


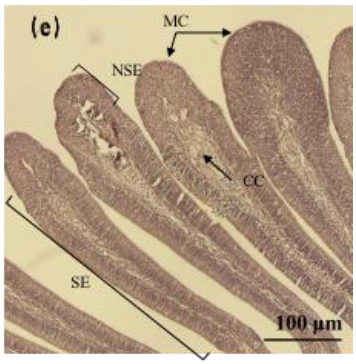
T_{1w}



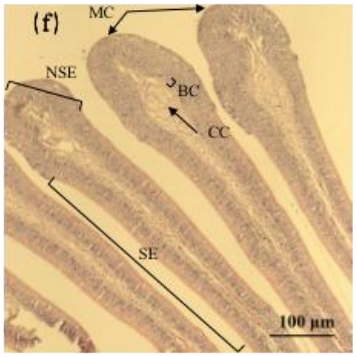


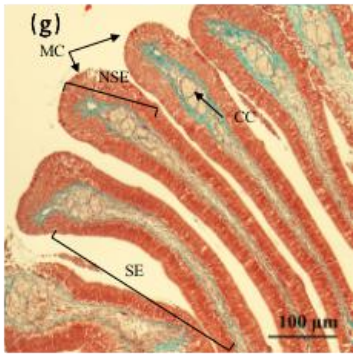
T_{2w}



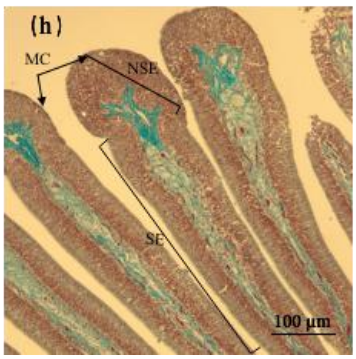


T_{3w}





T_{4w}



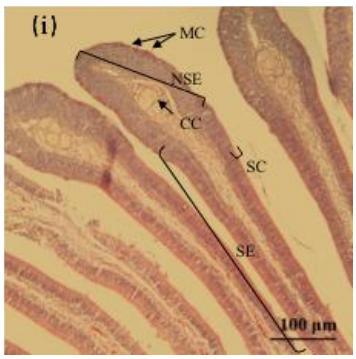


Fig. 3.5 Representative histological sections of the olfactory lamellae of the gilthead seabream exposed to control and high pCO_2 along the experimental trial (T_0, T_{1w}, T_{2w}, T_{3w} and T_{4w}). (a) Tissue stained with periodic acid-Schiff/Alcian blue; (b) (d) (g) (h) Tissues stained with Masson's trichrome; (c) (e) (f) (i) Tissues stained with Hematoxylin and eosin. Legend: NSE: non-sensory epithelium; SE: sensory epithelium; MC: Mucous cell; CC: Central core; BC: basal cell, SC: support cell.

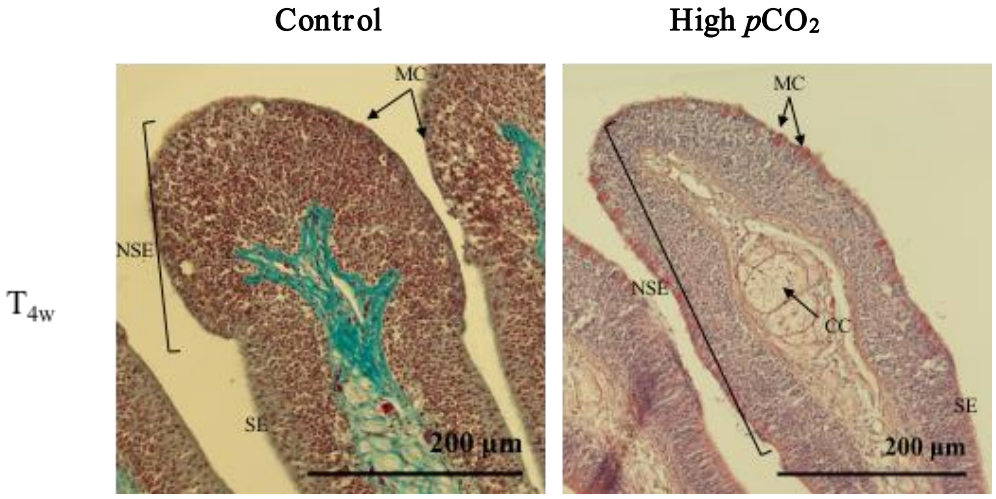


Fig. 3.6 Representative histological sections of the olfactory lamellae of the gilthead seabream sampled at four weeks. In (a) the tissue was stained with Masson's trichrome and in (b) was stained with Hematoxylin and eosin. Legend: NSE: non-sensory epithelium, SE: sensory epithelium, MC: Mucus cell, CC: Central core.

The statistical results of the histomorphometric analysis are shown in Fig. 3.7. The number of mucous cells per lamellae was higher in the high pCO_2 fish than in the control group along the experimental trial (Fig. 3.7A). However, statistically significant differences ($p<0.0001$) were only found after 4 weeks of exposure to high pCO_2/low pH, when the number of mucous cells (23.78±2.2) was increased compared to the control group (7.0±1.4).

The ratio between the length of the non-sensory epithelium / length of the lamella was significantly increased ($p<0.05$) in fish exposed to high pCO_2/low pH after 1w, and at 3w and 4 weeks of exposure (Fig. 3.7B). As the mucous cells are present only in the non-sensory epithelium, the increase of the number of mucous cells in high pCO_2 kept seabream may be related to the increased ratio of the non-sensory epithelium.

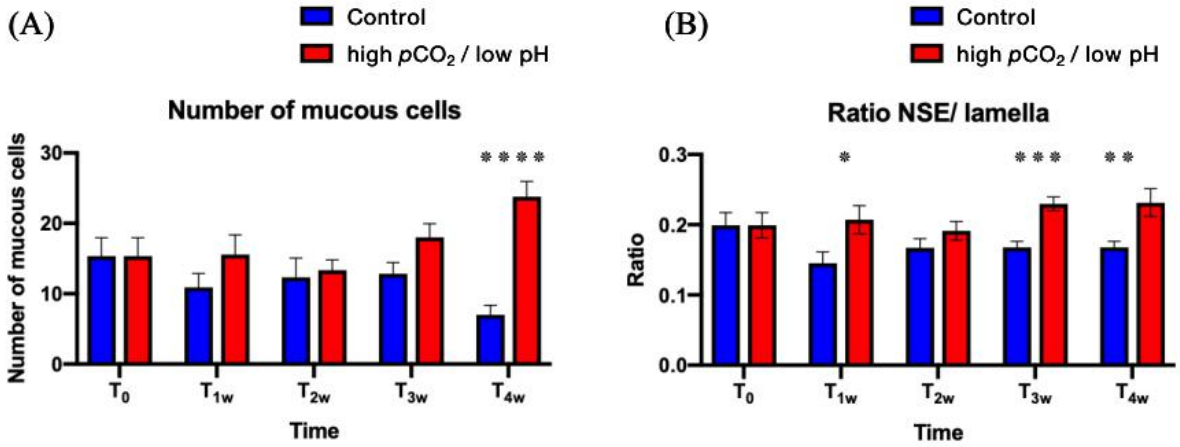


Fig. 3.7 Scatter dot plot of (A) number of mucous cells and (B) Ratio length of NSE/total length of lamella along the experimental trial (T_0, T_{1w}, T_{2w}, T_{3w} and T_{4w}). T0 was only treated under control for both (A) and (B). Legend: NSE: non-sensory. (Values are shown as mean ± S.E.M, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

4. DISCUSSION

The current study shows that the direct effects of ocean acidification on olfactory sensitivity to some, but not all, odorants in gilthead seabream are mediated by the reduction in pH and the increase in pCO_2 acting independently. The effects on all the amino acids that were tested, except for L-glutamine, had similar tendency when exposed to high pCO_2 or low pH. The effect of pH is likely due to reduced odorant-receptor affinity as a consequence of change in protonation - and therefore shape - of the odorant and/or binding site [81]. The mechanism(s) by which pCO_2 affects olfactory sensitivity is less clear. Moreover, the current study shows that during four weeks' exposure to high pCO_2/low pH water there were morphological changes in the olfactory epithelium, the morphological changes were comparable to a previous study [81], indicate that exposure to high pCO_2/low pH evokes modification of olfactory epithelium.

Overall, for the olfactory epithelium exposed to high pCO_2 seawater, olfactory response significantly reduced for three out of five odorants: L-serine, L-arginine and L-leucine, and the detection threshold of those three odorants also increased. When the olfactory epithelium exposed to low pH seawater, olfactory response significantly reduced for four out of five odorants: L-serine, L-arginine, L-leucine, and L-glutamine, and the detection threshold of three odorants (L-serine, L-arginine and L-leucine) increased. For those responses to the amino acids increase significantly in the detection threshold and decrease significantly in the amplitude under both high pCO_2 and low pH conditions means the olfactory sensitivity of fish to some odorants decreased in the acidified water and they would need to be closer to those odor sources to detect them, which could result in a decrease of the efficiency of foraging and predator-avoidance behaviour. It maybe also explain the impairment of olfaction-mediated homing ability [19]. Compared to the different reduced amplitude of response between two seawater conditions of each L-serine, L-arginine, and L-leucine, all of them were affected more by the high pCO_2 condition than low pH, which suggests that exposure to high CO_2 or low pH cause different changes in the olfactory sensitivity to these odorants *per se.* The responses amplitude of L-glutamine reduced significantly under low pH but did not change

significantly under high pCO_2; a previous study showed that the olfactory sensitivity of seabream to L-glutamine does not change in high CO_2/low pH water [81], suggesting that the effect of both factors together is different from that of each factor isolated. It was suggested that, decrease in the olfactory sensitivity is due in part to the protonation of the odorant at lower pH [81]; change of the protonation states of amino acids (and other odorants) caused by low seawater pH may change affinity of the odorants to their receptor(s). With the current study we showed that high CO_2 is also contributing to the described decrease in sensitivity in acidified water. Currently, it is believed that elevated CO_2 levels would impair the olfactory-mediated behaviour by affecting GABA receptor function in the brain [29, 96-98]. A recent study showed that both GABA and ACh were decreased under elevated pCO_2 suggest that both ACh and GABA may participate in the regulation of OA induced behavioral changes. And also, possibly, the intracellular pH may be decreased caused by the lower external pH, or by diffusion of more CO_2 into the neuron (and thereby reducing the intracellular pH), and this may reduce the activity of enzymes involved in the transduction pathway, and therefore the efficiency of such transduction. However, these inference requires further experimental confirmation.

No significant difference was observed in the gross anatomy of the olfactory epithelium between fish kept in high pCO_2 and control during four weeks. However, the number of mucous cells per lamellae began to increase significantly after four weeks of exposure to high pCO_2 seawater. Mucus can protect the epithelium from the noxious chemicals, and removal of odorants, and provide the biochemical microenvironment for olfactory binding and/or transduction [99]; therefore, the increase of mucus secretion may be an adaptation to the changes in water chemistry (high pCO_2/or low pH). However, the number of odorants molecules that can reach to the site of olfactory detection (the cilia of the olfactory receptor neurons) may be reduced by the thicker mucus layer, therefore, lowering the olfactory sensitivity of the fish. The ratio between the length of non-sensory epithelium/total length of the lamella increased significantly at one, three and four weeks's exposure. A similar increase in this ratio had already been reported in a previous study [81]. Since the non-sensory epithelium is the upper and outer part of the olfactory lamellae, it is possible (but not supported by the electrophysiology results) that the increase in the length of the non-sensory

epithelium relative to the total length of the lamella could lead lead the sensory area less exposed to the external environment, and ultimately contribute to a decrease of olfactory sensitivity. Initial studies have indicated that ocean acidification induces the changes in gene expression of the olfactory system [80, 100]. Further studies are required to evaluate the factors that increase mucous cells number and non-sensory epithelium proportion.

In conclusion, while the current study determined both high pCO_2 and low pH predicted by the end of the century could reduce the olfactory sensitivity of seabream to some odorants by acting directly - and independently - on the olfactory epithelium, little is known about the underlying mechanisms. Nevertheless, that the shape of odorant and/or binding sites are also likely to change due to the reduced pH caused by OA, as previously reported [81], combined with the current study, suggests that the morphological changes and the direct reduction of olfactory sensitivity - whether due to low pH and/or high pCO_2 - are acting via separate mechanisms. Especially, these effects may not be confined to the species under study, but may be generally applicable to other marine organisms. If so, the consequences of ocean acidification are likely to be widespread, complex, and difficult to predict.

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