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Mutligenerational chronic exposure to near future ocean acidification in European sea bass (*Dicentrarchus labrax*): Insights into the regulation of the transcriptome in a sensory organ involved in feed intake, the tongue

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Abstract :

In this study, we examined the effect of near future ocean acidification (OA) on the transcriptome of a sensory organ in contact with surrounding water, the tongue in adult European sea bass (Dicentrarchus labrax) by mean of RNAseq experiment. We acquired a total of 14.1 Mb quality-trimmed reads covering 18,703 expressed genes from the tongue of fish reared from two generations at actual (pH 8.0 condition) and predicted near-future seawater pH (pH 7.6 condition). Gene ontologies analyses of expressed genes support the evidence that the tongue exhibits biological processes related to the sensory system, tooth mineralisation and immune defences among others. Our data revealed only 295 OA-induced regulated genes with 114 up- and 181 down-regulated by OA. Functions over-represented encompass processes involved in organic substance metabolic process, RNA metabolism and especially RNA methylation which, combined with the regulation of some hsp genes expression, suggest a molecular response to stress which might contribute to lingual cell homeostasis under OA. The immune system process is also found enriched within OA-induced regulated genes. With the exception of one fatty acid receptor, known taste perception effectors were not impacted by OA in the tongue. However, a complementary droplet digital PCR approach dedicated to genes involved in gustatory signal transduction revealed the down regulation by OA of pyrimidinergic receptor (p2ry4) transcript expression in the gills of the fish. Combined with scanning electron microscopy analysis, our RNAseq data revealed that OA has no impact on processes related to teeth development and mineralization. Altogether, our data reveal that multigenerational exposure to OA has not a substantially effect on the tongue transcriptome but emphasis should be placed on investigating the potential physiological consequences related to the regulation of genes related to cell stress, immune system and fatty acid sensitivity to conclude on species resilience in face of OA.

Highlights

► Multigenerational exposure to OA has no impact on teeth mineralization in Sea bass. ► Of the 18703 genes expressed in the tongue, 295 exhibited OA-induced regulation. ► Genes related to cell stress, immune system and fatty acid sensitivity are regulated. ► OA impacts the branchial expression of *p2ry4* gene involved in gustatory system.

Introduction

Environment perception, particularly the detection of chemical cues, plays a crucial role in shaping community assemblies in marine systems and governs key physiological processes at the organismal level, including mate selection, fight-or-flight responses, and feeding behavior (Hay, 2009). Fish feeding behavior encompasses several sequential steps: foraging, detecting and capturing prey, and assessing the quality and palatability of food before ingestion. These behaviors rely on sensory capabilities (visual, auditory, gustatory) and oral functions (food processing), which are vital for fish survival (Kasumyan and Døving, 2003). Food processing is mediated by a few sensory structures that are in direct contact with the surrounding water (e.g., olfactory rosette, eyes, and oral epithelium) and are therefore highly susceptible to environmental fluctuations.

Despite phylogenetic divergence in tissue and cell composition, the tongue remains one of the primary organs involved in feeding, encompassing functions such as food capture, evaluation, and transport in fish (Yashpal et al., 2009). In European sea bass (*Dicentrarchus labrax*), the dorsal surface of the tongue is equipped with numerous curved, canine-like teeth, oriented posteriorly, which facilitate food processing and ingestion (Abbate et al., 2012). The tongue also plays a critical role in detecting chemical stimuli from the environment via taste buds, which are the principal organs of the gustatory system (Hara, 2006). Within the taste buds, chemical stimuli are transduced into electrical signals that convey taste information to the brain (Abe, 2008, Chaudhari and Roper, 2010). In European sea bass, the co-occurrence of teeth and taste buds on the tongue surface suggests a close correlation between food processing and taste perception processes (Abbate et al., 2012). Additionally, the mouth of vertebrates serves as a gateway for microbes from air, water, and food into the internal medium. Like mammals and birds, fish have evolved efficient innate and adaptive immune strategies to protect the mucosal regions of the buccal cavity, particularly through the activation of B cells and immunoglobulins within mucosa-associated lymphoid tissues (MALTs) (Xu et al., 2020, Yu et al., 2019). As a component of the buccal cavity mucosa, the tongue may contribute to this defense by expressing immune factors.

The tongue's direct exposure to the external environment within the oral cavity makes it particularly susceptible to environmental factors that can influence processes related to food tasting and processing, teeth formation and mineralization, and MALT-related pathways. Among the global changes affecting marine organisms' sensory perception, ocean acidification

(OA) is one of the most extensively documented (Sundin, 2023, Tresguerres and Hamilton, 2017, Munday et al., 2012, Hamilton et al., 2023, Wang et al., 2023). The impact of OA on fish olfactory-driven behavior has been widely associated with negative consequences for demographics and community structure changes (Dixson et al., 2015, Jiahuan et al., 2018, Porteus et al., 2021, Porteus et al., 2018, Munday et al., 2010, Leduc et al., 2013). Studies have shown that near-future CO_2-induced OA exposure disrupts the ability of various marine fish species to discriminate between odors of suitable and unsuitable settlement sites, as well as between predator and non-predator cues (Munday et al., 2009, Devine et al., 2012, Dixson et al., 2010, Munday et al., 2010). Although the impact of OA on fish behavioral traits remains controversial, primarily due to methodological biases in behavioral observations with small sample sizes (Clements et al., 2022), the regulation of cellular and molecular mechanisms associated with plasticity and neuronal activity in the olfactory epithelium and bulbs appears to be well-established, particularly in European sea bass (Porteus et al., 2018, Cohen-Rengifo et al., 2022).

While the gustatory senses of fish are less studied, they also appear to be sensitive to OA. For example, a study by Rong et al. (2020) revealed OA-induced regulation of neurotransmitter levels and the expression of genes involved in gustatory signal transduction, including transient receptor potential cation channel subfamily M member 5 (*trpm5*) and pyrimidinergic receptor (*p2ry4*) in black sea bream (*Acanthopagrus schlegelii*). However, it should be noted that this data was obtained from extraoral tissue (i.e., the gills), and may not be fully representative of oral taste buds, the primary organs responsible for the final selection and consumption of food (Morais, 2017).

Beyond sensory mechanisms, OA may physically impact canine-like teeth mineralization, potentially impeding food capture and processing. The mineralization of calcifying structures has been the subject of numerous studies in marine organisms, with effects varying among taxa, life stages, and OA intensity (Leung et al., 2022b). Depending on the species, OA may affect the size and density of otoliths, which are composed of calcium carbonate (CaCO3), due to increased CO2 flux into the endolymph (Bignami et al., 2013, Checkley et al., 2009, Mahé et al., 2023, Munday et al., 2011, Kwan and Tresguerres, 2022, Heuer and Grosell, 2014, Pimentel et al., 2014). Since most mineralized structures in fish are composed of cartilaginous material and calcium phosphate, the initial expectation was that OA-induced modifications in carbonate chemistry would have limited effects on fish (Munday et al., 2011). However, the accumulation

of plasma carbonate and non-carbonate buffer levels has also been shown to influence skeletal hydroxyapatite (Ca5(PO4)3OH) mineralization in the enameloid of various fish species, including Bucchich's goby (*Gobius bucchichi*), Senegal sole (*Solea senegalensis*), little skate (*Leucaria erinacea*) and Atlantic salmon (*Salmo salar*) (Di Santo, 2019, Mirasole et al., 2021, Pimentel et al., 2014). The effects of OA on teeth mineralization in fish have been scarcely studied. While sharks have been shown to modulate biomineralization (e.g., increasing fluoride content) to produce teeth more resistant to corrosion under OA, no data is currently available for teleost species, where teeth are primarily composed of hydroxyapatite and collagen (Leung et al., 2022a). Therefore, it would be particularly valuable to investigate the potential impact of OA on the expression of genes involved in enameloid mineralization in bony fish, particularly those in the SPARCL1-SCPP cluster (e.g. *sparcl1*, *odam*, *scpp1,9*) (Lv et al., 2017).

The impact of OA on the immune response in fish has received limited attention. However, some studies have demonstrated that OA affects physiological parameters related to innate immune function, which may enhance fish tolerance to pathogens. For instance, OA has been shown to increase lysozyme and complement system activity in Atlantic halibut (*Hippoglossus hippoglossus*) and to regulate the expression of factors involved in cell adhesion molecule production in seabream (*Sparus aurata*) (Araújo et al., 2018, Bresolin de Souza et al., 2016). In the olfactory epithelium of European seabass, a transcriptomic approach revealed that transgenerational exposure to OA stimulates the expression of genes involved in innate antiviral immunity, such as pathogen recognition receptors and interferon-stimulated genes. These changes in the olfactory MALT tissue were associated with increased resistance in fish challenged with betanodavirus (Cohen-Rengifo et al., 2022). To date, no information is available on the potential effects of OA on oral MALT.

The present study aimed to evaluate the impact of chronic and multigenerational exposure to ocean acidification on various biological processes related to tongue function in the European seabass, a fish species of economic importance. These processes include taste signal transduction, teeth formation and mineralization, and immune-related pathways. We employed a transcriptomic approach to investigate these effects. A two-generation rearing experiment was conducted, where fish were exposed to acidified seawater starting from the larval stage. We examined the effects of multigenerational OA exposure on the transcriptome of the apex part of the tongue, collected from fish reared in both current and projected near-future seawater pH conditions (–0.4 pH units). RNA sequencing (RNAseq) was performed on two groups of 4-

year-old F2 juveniles, which had been continuously exposed since spawning to the same OA conditions as have their parents (F1), under either current pH (pH 8.0) or the end-of-century IPCC SSP3-7 scenario (pH 7.6), representing a medium to high-end future forcing pathway (Intergovernmental Panel on Climate Change, 2019). Differential expression analysis and Gene Ontology (GO) enrichment analyses were conducted to identify key genes and associated biological processes regulated by OA. Based on the results related to gustative signal transduction obtained by Rong et al. (2020) in the gills of black sea bream exposed to OA, our RNAseq approach on tongue was completed by an analysis targeting the branchial expression of the *trpm5* and *p2ry4* genes in the gill tissue using droplet digital PCR (ddPCR). Furthermore, electron microscopy coupled with X-ray microanalysis was employed to assess the potential effects of OA on enameloid. Energy-dispersive spectrometry (EDS) was specifically used to analyze the mineral content of the curved, canine-like teeth located on the dorsal surface of the tongue.

Material and method

Animal husbandry and experimental setup

The experiment was performed within the facilities of the PHYTNESS Laboratory at the French Research Institute for Exploitation of the Sea (IFREMER) in Plouzané (Agreement number: B29-212-05) following the European Commission recommendation 2007/526/EC and Directive 2010/63/EU for the accommodation and care of animals used for experimental and other scientific purposes. The present experimental protocol was the subject of a specific authorization issued by a French Ethics Committee for animal testing [CEEA – 074: Comité d'éthique finistérien en expérimentation animale (CEFEA): Authorization APAFIS #2,018,032,209,421,223].

F2 fish were obtained from an *in vitro* fertilisation of 4 years old F1 parents (20 males x 6 females) exposed from larval to adult stages to control conditions (~ pH 8.0) or to OA conditions (~ pH 7.6). The individuals used in the present paper were originated from the same siblings but are different animals than the fish used in the work of Cohen et al. (2022). Sampling was performed from 4-year-old fish in the present study compared to 18-month-old in the Cohen study. F2 juveniles were reared in water at the same pH as the brood stock F1 they

originated from, in duplicate tanks for each pH condition (i.e. mean control condition: ~ pH 8.0, P_{CO2} ~ 700 µatm; mean OA condition: ~ pH 7.6, P_{CO2} ~ 1600 µatm, table 1). Rearing conditions in an open-circuit system during larval and juvenile stages were similar to those previously described (Cominassi et al., 2020). From juvenile stage, temperature and salinity followed seasonality of the Bay of Brest (documented at the following website: https://marc.ifremer.fr/en/results/temperature_and_salinity/mars3d_channel_bay_of_biscay_model#appTop). Seawater pumped 500 m off the coastline of the Bay of Brest at a depth of 20 m passed through a sand filter, a tungsten heater, a degassing column packed with plastic rings, a 2-µm filter membrane, and a UV lamp. For the OA condition, CO_2 was injected at constant flow in a header tank equipped with a degassing CO_2 column to favour mixing and adjusted by a flow-control unit. From there, seawater flow poured hydrostatically into each of the 3 replicate tanks following the adapted protocol of (Strickland and Parsons, 1972). pH and temperature were measured daily with a pH meter (WTW 3110; Xylem Analytics Germany, Weilheim, Germany, NBS scale) and total alkalinity was checked once a week. Summary data containing physico-chemical parameters of the rearing seawater is available in the SEANOE repository https://doi.org/10.17882/87395. Fish were fed ad libitum and no significant difference was observed in the growth rates between the two groups (Cohen-Rengifo et al., 2022).

Table 1. The physical and chemical parameters (mean ± SD) of the seawater (SW) in each F1 and F2 group. Psu: practical salinity units; NBS: Newborn screening.

	Physical and chemical parameters of the seawater (means of duplicated tanks for each group)				
Group	**Temperature (°C)**	**Salinity (psu)**	**Total alkalinity (µmol kg−1 SW)**	**pH (NBS scale)**	**pCO_2 (µatm)**
F1-pH 8.0	15.89 ± 2.07	33.29 ± 0.99	2425.85 ± 166.36	7.99 ± 0.07	671.55 ± 146.36
F1-pH 7.6	15.43 ± 2.03	33.34 ± 1.03	2427.35 ± 158.72	7.61 ± 0.04	1710.26 ± 244.45
F2-pH 8.0	16.06 ± 1.77	33.04 ± 0.83	2375.97 ± 113.48	7.98 ± 0.08	694.42 ± 145.01
F2-pH 7.6	16.25 ± 1.60	33.04 ± 0.83	2379.87 ± 105.06	7.64 ± 0.05	1598.63 ± 244.47

Sampling

After 4-year exposure period, tongues and gills for a total of 32 individuals have been sampled from F2 adult female European sea bass exposed from larval stage to control conditions (~ pH

8.0) or to OA conditions (~ pH 7.6) (n=8 per tank / per condition). From the 8 fish sampled per tank (16 per condition), all were sampled for gills, while four of the tongues per condition were used for RNA extraction (samples L23-30 for control group, L8-15 for OA group) and four for scanning electron microscopy analysis (SEM). All samples were taken on the same morning over a period of 2 hours. Fish were fasted for 24 h before sampling in order to empty the gastrointestinal system, as regurgitation and faeces pollute the water, which reduce the effect of anaesthesia and displace oxygen (Brønstad, 2022). Fish were anesthetized (20 mg L^{-1}), and then euthanized with a lethal dose (200 mg L^{-1}) of tricaine methane sulfonate 222 (MS222, Pharmaq, Fordingbridge, Hampshire, UK).

Tongues sampling consisted in isolating the apex parts of the tongues (the first 0.5 cm from the tip of the tongue, ~ 30 mg). Gills sampled consisted in dissecting ~30 mg of the lamellae of the third branchial arch. Samples for molecular analysis were immediately stored in RNA Stabilization Reagent (RNA later, Qiagen, Hilden, Germany) following supplier recommendations (and preserved at -20°C for downstream analysis). Samples for visual microscopic observations were fixed in 0.4% paraformaldehyde (PFA).

RNA extraction

Total RNA was extracted from each dedicated tongue and gill samples using Extract-All reagent (Eurobio, Courtaboeuf, Essonne, France) on Nucleospin RNA column according to the manufacturer's instructions (Macherey–Nagel, Düren, Germany). The protocol included a step dedicated to genomic DNA digestion. RNA concentration and purity were verified (260/280 ratio > 2; 260/230 ratio > 1.8) using a ND-1000 NanoDrop® spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA) and the integrity of RNA was verified using the TapeStation system (Agilent Technologies Inc., Santa Clara, CA, USA). All samples showed an RNA integrity (RIN) score > 8. RNA samples were stored at − 80 °C for further RNA sequencing (tongue) or Droplet digital PCR analysis (gills).

RNA-Sequencing analysis

RNA samples extracted from the apex part of the tongue were sent to the sequencing platform Montpellier GenomiX (MGX, Montpellier, France). RNAseq libraries were constructed using the TruSeq Stranded mRNA library prep (Illumina) following manufacturer's instructions. Sequencing was conducted on a single SP flow cell lane (Novaseq 6000) in paired end 2*50bp mode.

Transcriptomic and Gene Ontology (GO) analysis

Sequencing reads quality was assessed using seqkit v2.4.0, fastqc v0.11.9 and multiqc v1.15 (Shen et al., 2016, Andrews, 2010, Ewels et al., 2016, Manni et al., 2021). Reads were mapped to the European sea bass genome *dlabrax2021* [(Tine et al., 2014); GCF_905237075.1] using STAR v2.7.10b (Dobin et al., 2013) with standard parameters and 'quantMode GeneCounts' option. STAR provided matrices of raw counts which were used to perform differential analysis. Raw reads have been deposited to ENA, under the accession project number (PRJEB78433). Raw count matrices were filtered to exclude transcripts with very low expression (minimum counts > 1 in at least 3 samples) and normalized using DESeq2 v2.1.34 R package (Love et al., 2014). Differentially expressed genes (DEGs) between acidified and control samples were identified using DESeq2 v2.1.34 (Love et al., 2014) in R v4.1.2, employing a likelihood ratio test (LRT) with nested models including tanks as covariates (~tank + condition vs. ~tank). DEGs were considered significant when adjusted p-value < 0.05. Gene ontology (GO) enrichment analysis for DEGs was performed using GOEnrichment tool available on Galaxy version 2.0.1.

Droplet digital PCR

Droplet digital PCR (ddPCR) technology was used in the present study since it produces more precise, reproducible and statistically significant results than real time PCR for target genes of low expression levels (Taylor et al., 2017). Reverse transcription (RT) of cDNA was carried out in duplicate using 500 ng of RNA extracted from gills with an iScript™ cDNA Synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) following the protocol previously described (Mazurais et al., 2020). Negative RT consisting in RT reaction without retro-transcriptase enzyme were also performed for all samples. Primers specific to European sea

bass *trpm5* and *p2y4* cDNA sequences were designed using PrimerQuest™ program (IDT, Coralville, Iowa, USA. https://www.idtdna.com/SciTools) based on sequences available on the ncbi (https://www.ncbi.nlm.nih.gov/) and Max Planck Institute databases (http://seabass.mpipz.mpg.de/index.html). The sequences of the primers used in the present work are summarized in the table 2. For normalization of ddPCR, the amounts (copies per µl) of the target mRNA (*trpm5* and *p2y4*) within the samples were divided by the amounts (copies per µl) of the reference gene (*ef1α*).

Table 2. Primers used for relative quantification by ddPCR. Sequences used to design the primers are available in genbank databases.

Gene name	Ref seq	Forward primer (5'-3')	Reverse primer (5'-3')
trpm5	XM_051378910.1	CAGTGAAGACAGGGCTTATG	TTGTGAGGAGAGCCTGAA
p2ry4	XM_051419056.1	CCTACTCAGGGTGTTGTTATC	CAGGCAGCAGGATGTATTT
ef1α	AJ866727.1	CTGGAGGGCAGTGAAAAGAT	CATCAAGAGCCTCCAGCAGT

SEM analysis

The 16 apex parts of the tongue fixed in PFA were first dehydrated in successive ethanol immersion (from 30 to 100% saturated ethanol) and then dehydrated with a Leica EM CPD 300 Critical point dryer. The tissues were transferred onto a stub and then Gold/Palladium coated with a Polaron SC7640 sputter coater. The surface of the tongue was then examined under the Thermofischer Quanta SEM. Carbon (C), oxygen (O), sodium (Na), phosphorus (P), calcium (Ca) and iron (Fe) composition of teeth was determined by energy dispersive X-ray spectroscopy (EDS) under the Oxford Instruments X-Max80 detector. The surface of the teeth was scanned by an electron beam with an accelerating voltage of 20 kV to obtain the X-ray energy spectra. The elements were identified and semi-quantified in terms of weight percentage using the Aztec software (Oxford Instruments, UK). In order to compare the mineral contents on the enameloid regions of teeth between the two groups, energy-dispersive X-ray spectroscopy (EDS) was performed through energy spectrum analysis for 4 individuals from

each condition. Three to five teeth were analysed for each individual. For each tooth, three areas between the upper part of the length were analysed.

Results

General information on RNAseq and expressed genes

The libraries sizes were similar across conditions, reaching 24.29 M +/- 8.09 and 22.82 M +/- 5.48 of reads for control and OA groups, respectively (table 3). The mapping efficiencies per sample ranged from 60.92% to 75.35%. The numbers of reads overlapping genes per sample ranged from 8.29 M to 22.91 M for a total of 18 703 expressed genes (table 3, supplementary table 1).

Table 3. Sequence statistics of RNA-seq libraries mapped to the European sea bass reference genome. Nb: number.

Group	Samples	nb of reads	nb uniquely mapped	% uniquely mapped	nb overlapping genes	% overlapping genes
Control (pH 8.0)	L23	14,562,477	8,871,133	60.92	8,416,584	57.80
	L24	29,641,165	20,314,324	68.53	19,297,674	65.10
	L25	11,936,610	8,706,867	72.94	8,294,396	69.49
	L26	20,936,973	15,354,313	73.34	14,511,384	69.31
	L27	31,317,372	20,457,027	65.32	19,397,081	61.94
	L28	29,309,789	21,299,829	72.67	20,230,421	69.02
	L29	22,676,350	16,115,310	71.07	15,388,870	67.86
	L30	33,989,398	24,100,760	70.91	22,915,921	67.42
OA (pH 7.6)	L8	33,161,629	23,053,907	69.52	21,866,949	65.94
	L9	20,273,307	13,779,557	67.97	13,037,747	64.31
	L10	23,882,950	17,995,832	75.35	16,995,558	71.16
	L11	23,343,762	15,763,100	67.53	14,688,315	62.92
	L12	22,883,056	14,420,404	63.02	13,662,755	59.71
	L13	14,328,471	9,508,779	66.36	8,989,885	62.74
	L14	25,750,557	17,565,705	68.21	16,672,044	64.74
	L15	18,982,709	12,686,118	66.83	12,073,113	63.60

Table 4 lists some of the most highly represented GO in the transcriptome of the tongue apex (fully listed in supplementary table 2). It is worth noting the expression of 5637 genes (45% of

expressed genes) involved in the regulation of biological process including 2610 genes involved in the regulation of metabolic process, 1096 genes involved in the response to stimulus, 832 genes involved in cell surface receptor signalling pathway, 684 genes involved in intracellular signal transduction, 447 genes involves in G protein-coupled receptor signalling pathway including *p2ry4* and *trpm5*, 430 genes involved in the immune system (50 and 26 genes related to innate and humoral immune response, respectively), 32 genes involved in the sensory perception including 3 genes involved in the perception of taste and 7 genes involved in the regulation of bone mineralization (supplementary table 3). The genes included in the SPARCL1-SCPP cluster (*sparcl1*, *odam, scpp1, 5, 7, 8, 9*) involved in teeth mineralization are also part of the 18 703 expressed genes.

Table 4. List of some of the most highly represented GO in the transcriptome at the tongue apex as revealed by RNAseq experiment. Nb: Number, Freq.: Frequency.

GO Term	Nb of genes	Freq,	Name
GO:0050789	5637	45%	regulation of biological process
GO:0019222	2610	21%	regulation of metabolic process
GO:0071840	2277	18%	cellular component organization or biogenesis
GO:0048518	1357	11%	positive regulation of biological process
GO:0050896	1096	8.0%	response to stimulus
GO:0009058	1085	8.7%	biosynthetic process
GO:0009056	833	6.6%	catabolic process
GO:0007166	832	6.6%	cell surface receptor signalling pathway
GO:0051649	731	5.8%	establishment of localization in cell
GO:0006950	689	5.5%	response to stress
GO:0035556	684	2.5%	intracellular signal transduction
GO:0007186	447	3.6%	G protein-coupled receptor signaling pathway
GO:0002376	430	3.4%	immune system process

RNAseq differential expression analysis

Differential gene expression analysis shows a total of 295 DEGs (adjusted p-value < 0.05) differentiating OA from control groups. Among these DEGs, 114 and 181 were up-regulated and down-regulated, respectively, in OA compared to control (Supplementary table 3). GO enrichment analysis focused on biological functions revealed significant enrichment (qvalue < 0.05) within the DEGs (Table 5, supplementary table 4). Among them, numerous biological processes are related to the "organic substance metabolic" process which includes "cellular aromatic/ nucleobase-containing compound metabolic process", "RNA metabolic process", "positive regulation of transcription by RNA polymerase I", ribonucleoprotein complex biogenesis, tyrosyl-tRNA aminoacylation and "citrulline metabolic". Most of the genes involved in RNA, cellular aromatic or organic substance metabolic processes as well as in positive regulation of transcription by RNA polymerase I, methylation and in mitochondrial transcription were down-regulated by OA. Processes related to immune system including positive regulation of T cell activation as well as processes related to "inner ear receptor cell stereocilium organization" and "enteric nervous system development" were also significantly enriched within OA-regulated genes. Genes involved in immune related system were found both up and down-regulated (supplementary table 5). Among the up-regulated genes are found *LOC127360723* encoding Toll like receptor 2 (*tlr2*), *tnfsf11* and *LOC127360870* encoding tumor necrosis factor receptor superfamily members, *LOC127369170* encoding a C-C chemokine receptor and *LOC127359799* encoding a class II histocompatibility antigen. Down-regulated genes involved in immune system included among others, *LOC127359807*, *LOC127359808,* and *LOC127360937* encoding several class II histocompatibility antigens.

Biological processes related to teeth development and mineralization did not appear as significantly enriched within OA-induced regulated genes. None of the genes included in the SPARCL1-SCPP cluster (e.g. *sparcl1*, *spp1, odam, scpp1, 5, 7, 9*) involved in teeth mineralization showed differential expression. Similarly, *p2ry4* and *trpm5* transcripts involved in gustatory system did not exhibit any regulation in the tongue.

Among OA-induced regulated genes involved in GO:0009987 (cellular process) were down-regulated *si:dkey-211g8.9* encoding for a free fatty acid receptor. Regulated genes involved in cellular process included also *LOC127375993* encoding D-amino-acid oxidase and *LOC127364251* encoding "heat shock cognate 70 kDa protein-like" that displayed higher expression level under OA while *hsp90aa1.2, hspd1*and *hspa4a* encoding heat shock proteins exhibited lower expression.

334 *Table 5. List of the biological processes GO significantly enriched (p<0.05) within the DEGs*

GO Term	name	q-value	Number of regulated genes (up and down in OA)
GO:0016070	RNA metabolic process	2.78E-23	61 (3 up, 58 down)
GO:0006139	nucleobase-containing compound metabolic process	1.71E-18	73 (7 up, 66 down)
GO:0006725	cellular aromatic compound metabolic process	4.43E-18	74 (5 up, 69 down)
GO:0045943	positive regulation of transcription by RNA polymerase I	4.89E-6	4 (4 down)
GO:0071704	organic substance metabolic process	2.15E-4	107 (17 up, 90 down)
GO:0022613	ribonucleoprotein complex biogenesis	1.73E-3	6 (1 up, 5 down)
GO:0050870	positive regulation of T cell activation	5.03E-3	7 (2 up, 5 down)
GO:0006437	tyrosyl-tRNA aminoacylation	5.56E-3	2 (2 down)
GO:0009987	cellular process	6.9E-3	168 (58 up, 110 down)
GO:0032259	methylation	0.0118	7 (7 down)
GO:0006390	mitochondrial transcription	0.012	2 (2 down)
GO:0000054	ribosomal subunit export from nucleus	0.0201	3 (1 up, 2 down)
GO:0000052	citrulline metabolic process	0.0223	2 (1 up, 1 down)
GO:0002376	immune system process	0.0261	16 (7 up, 9 down)
GO:0060122	inner ear receptor cell stereocilium organization	0.0464	2 (1 up, 1 down)

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336 *Trpm5* and *p2y4* mRNA expression in the gills

337 ddPCR analysis of *p2ry4* and *trpm5* transcripts relative levels was performed in the gills from
338 the same fish than those used for RNAseq experiment (Figure 1). Data revealed significant
339 lower levels of *p2ry4* transcripts in the gills of OA-exposed fish compared to control group
340 while no significant differences was found for *trpm5* transcript levels.

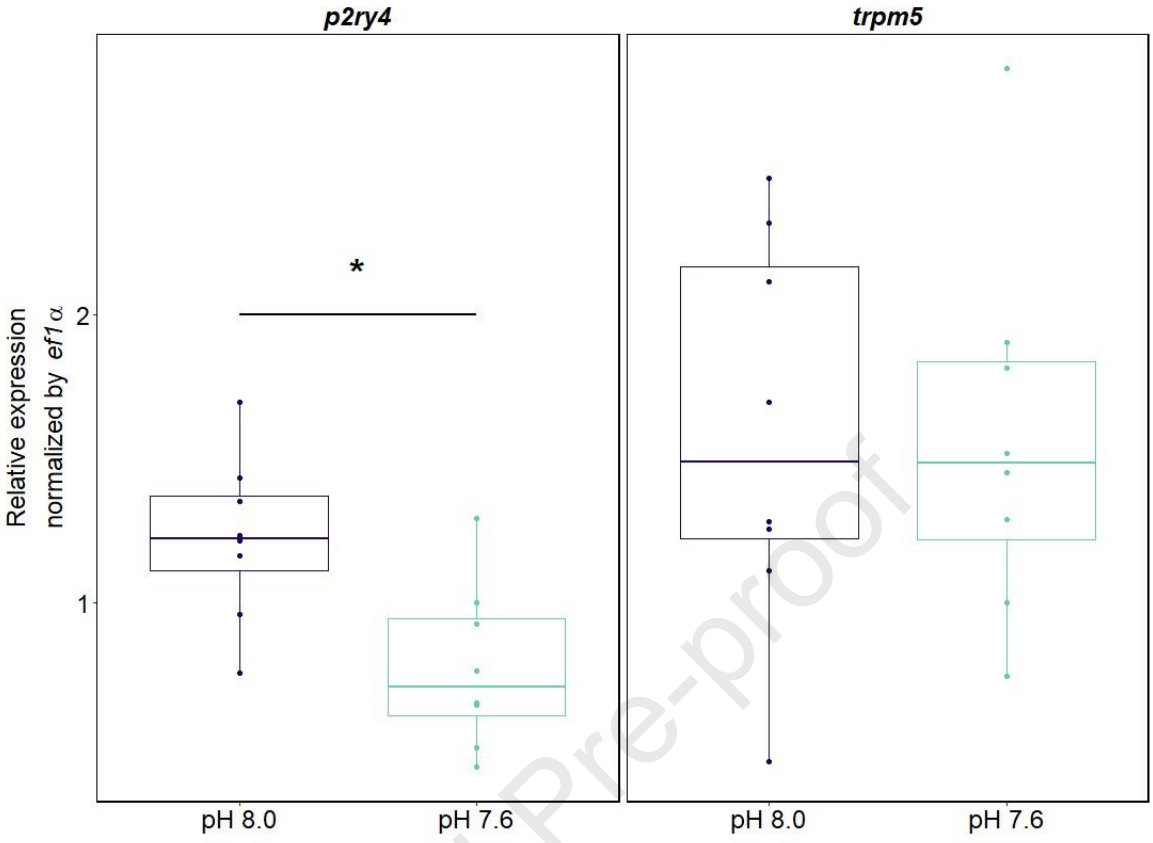
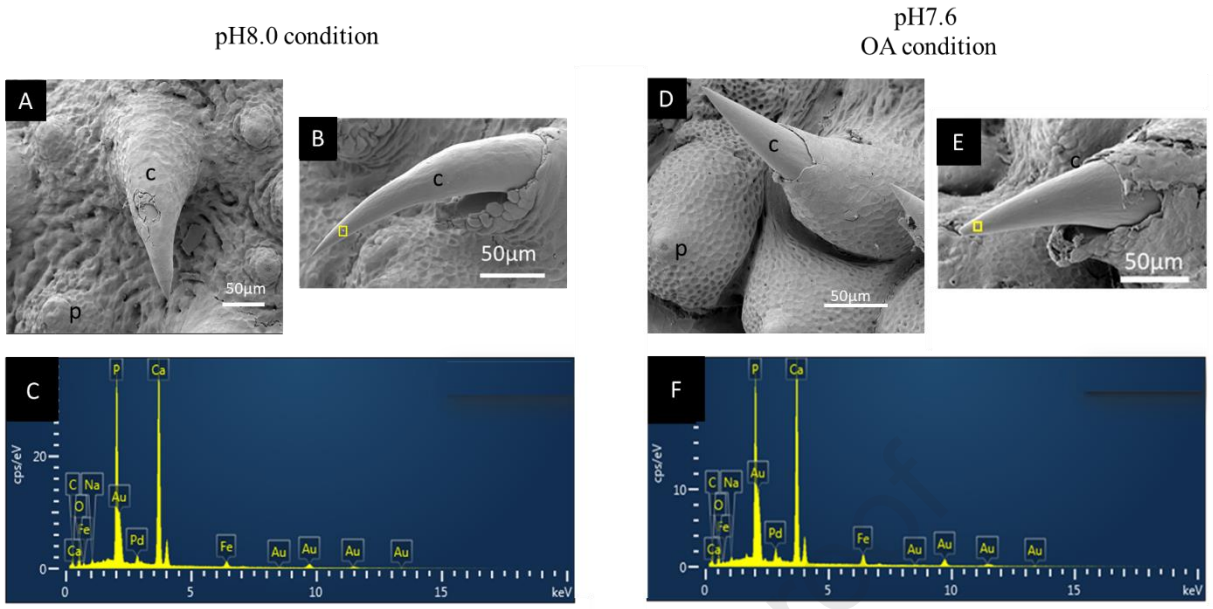


Figure 1. Droplet-digital PCR quantification of p2ry4 and trpm5 transcripts relative levels in response to transgenerational exposure to OA in the gills of European sea bass. Each transcript was normalized to elongation factor 1 alpha (ef1α) expression; Asterisk (*) denotes the significantly differentially expressed (P<0.05), tested by one factor ANOVA.

SEM analysis

To elucidate the potential effects of OA on teeth morphology and mineralization, SEM analysis was performed on the dorsal surface in the apex part of the tongue (Figure 2). Our analysis confirmed that the dorsal surface of this part consists of an epithelium dotted by numerous papillae including taste buds at the summit and covered by canine like teeth (Figure 2 A, B, D, E). Special attention was paid to examining the surface of enameloid in teeth. We found that the global surface of enameloid did not show any pits suggesting a normal mineralization process of the teeth in the fish under OA condition. Energy-dispersive X-ray spectroscopy (EDS) was performed on the enameloid regions of teeth to analyse the mineral contents for n = 4 individuals in each group (Figures 2 B, D). The major elements of European Sea bass enameloid were calcium, oxygen, phosphorus, carbon and iron (Fe), while trace amounts of sodium (Na) were also detected (Figure 2).

359



360 *Figure 2. (A, B, D, E) Example of SEM images showing canine-like teeth (c) and papilla (p)*
361 *containing taste buds localized on the tongue's dorsal surface of European sea bass exposed*
362 *to normal (A, B) and transgenerational OA (D, E) conditions. The tooth on panel A is still*
363 *covered by epithelium. The yellow square shows the scanning area at the apex part of the teeth.*
364 *Energy spectrum of corresponding areas were shown as images C and F. Gold (Au), palladium*
365 *(Pd), carbon (C), oxygen (O), sodium (Na), phosphorus (P), calcium (Ca) and iron (Fe).*

366

367 We focused our attention on relative mass proportion of Ca, P and Fe within mineral elements.
368 The relative mass proportion of each of these three mineral elements and deduced Ca/P and
369 Ca/Fe ratios were not significantly affected by OA (Figure 3).

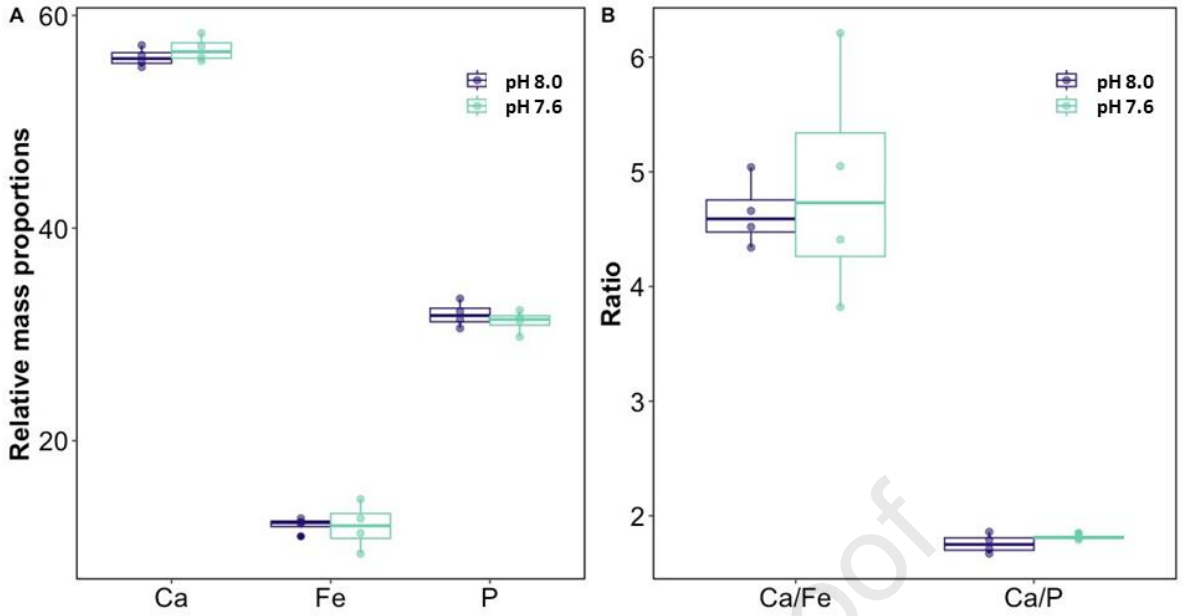


Figure 3. The relative mass proportion of Ca, P and Fe within mineral elements (A) and deduced Ca/P and Ca/Fe ratios (B) in the canine-like teeth localized on the tongue's dorsal surface of European sea bass exposed to control pH 8.0 and OA pH 7.6 condition. No significant differences were found between pH 8.0 and pH 7.6 groups.

Discussion

In this study, we characterized the transcriptome of the apex of the tongue in European sea bass and analyzed its regulation following multigenerational chronic exposure to OA. The originality of our work lies in both the specific tissue examined and the multigenerational dimension of the exposure, adding an ecological relevance to the study. Alterations in processes involved in prey detection and ingestion, as well as immune defenses, could have significant implications for population dynamics in natural environments. We employed RNAseq for this analysis, as it provides comprehensive insight into the potential for plastic responses at the transcriptional level to both short- and long-term environmental factors (Oomen and Hutchings, 2017).

The tongue, in direct contact with the external environment, supports various physiological functions (sensory, mechanical, and defense) that are likely to be impacted by environmental factors. Our SEM analysis confirmed that the dorsal surface of the tongue consists of an

389 epithelium punctuated by numerous papillate epithelial protrusions, including taste buds at their
390 summits, and covered by canine-like teeth (Abbate et al., 2012). As mentioned by Abbate et al.,
391 the presence of taste buds underscores the fundamental role of the tongue in food sensitivity,
392 while the dentition-equipped tongue likely facilitates the mechanical processing of food,
393 particularly given that European sea bass feeds on fish, crustaceans, and mollusks during its
394 juvenile and adult stages.

395 The number of reads obtained per sample varied between 11,936,610 and 33,989,398 with
396 mapping efficiencies ranging from 60.92 to 75.35% indicating good library qualities. Our
397 analysis revealed that a substantial proportion of the genes annotated in the European sea bass
398 genome (~78%) were constitutively expressed in the tongue. These genes encompassed various
399 biological functions, including sensory systems, tooth mineralization, and immune defenses,
400 among others. The most represented processes among the expressed genes were related to
401 metabolism and gene expression regulation, which is expected, as these genes are ubiquitously
402 and highly expressed across various vertebrate tissues (Ramsköld et al., 2009). However, our
403 RNAseq data also identified “response to stimulus” and “cell surface receptor signaling
404 pathway” as highly represented GO biological processes associated with the tongue
405 transcriptome, indicating a significant potential for regulation by extracellular factors in the
406 lingual epithelium, which is directly exposed to the environment. Surprisingly, only three genes
407 related to the GO term "sensory perception of taste," including one taste receptor gene (*gng13b*,
408 *gng13*, LOC127372407 encoding for *tas1r3*), were identified within the tongue transcriptome.
409 It should be noted, however, that this number may be influenced by the limitations of automatic
410 functional annotation, as other genes involved in taste signaling pathways, such as
411 LOC127377944 encoding for *tas1r1*, *si:dkey-211g8.9* encoding for a free fatty acid receptor,
412 Phospholipase C Beta 2 (*plcb2*), *p2ry4* and *trpm5* genes were listed in “G protein-coupled
413 receptor signalling pathway” or “signal transduction”. Ten genes annotated as “taste receptor
414 members” (2 genes) or “taste receptor members-like” (8 genes) were found in the European sea
415 bass genome. Only the “taste receptor members” (*tas1r1* and *tas1r3*) were found expressed in
416 the apex of the tongue while the 8 genes annotated as “taste receptor members-like” were not.
417 Interestingly, no gene annotated *tas1r2* nor *tas2r* were found in the genome of European sea
418 bass. In teleost, TAS1R2 plays species-specific functional role depending on habitats and diets
419 (Angotzi et al., 2018, Angotzi et al., 2020). We found also GOs related to “immune system
420 process”, including biological processes related to the innate and humoral immune response
421 within the most highly represented in the transcriptome which is consistent with the presence

of MALT in the fish tongue (Yu et al., 2019). Finally, we found the genes included in the SPARCL1-SCPP cluster (e.g. *sparcl1, spp1, odam, scpp1, 5, 7, 9*) expressed in the apex of the tongue indicating that they are used for enameloid mineralization of the canine-like teeth localized on the tongue's dorsal surface.

Among the 18,703 expressed genes, 295 (1.5%) were differentially expressed in the tongue of OA-exposed sea bass compared to controls. This low percentage of pH-regulated genes is consistent with findings from other studies on different tissues and species (Iguchi et al., 2024b). However, it is notably lower than the more than 6,000 transcripts found to be differentially expressed in the olfactory rosette of European sea bass, which were also multigenerationally exposed to the same level of OA (Cohen-Rengifo et al., 2022). These findings suggest tissue-specific sensitivity to OA, with the lingual epithelium exhibiting lower susceptibility to environmental acidification at the transcriptional level compared to the olfactory epithelium. Nevertheless, our GO enrichment analysis indicated that genes involved in "organic substance metabolic process," including "cellular aromatic compound metabolic process," "RNA metabolic process," and "ribonucleoprotein complex biogenesis," were overrepresented among OA-regulated genes, with most being downregulated, consistent with data obtained in the olfactory epithelium.

Studies have shown that exposure of eukaryotic organisms to environmental stresses, from yeast to humans, mobilizes essential intracellular metabolic processes, particularly those related to large macromolecular complexes, including RNA and ribonucleoproteins (Bond, 2006, Wilkinson et al., 2021, Griffiths et al., 2019). The transcriptomic responses we observed suggest a cellular stress response in the lingual tissue of the fish. Our data align with studies showing that OA induces downregulation of genes involved in ribosomal biogenesis in populations of *Balanophyllia elegans* corals sensitive to low pH (Griffiths et al., 2019). Furthermore, the OA-induced downregulation of genes involved in RNA metabolism observed in this study is consistent with data from the sea urchin *Strongylocentrotus droebachiensis* exposed to OA(Runcie et al., 2016).

Among the various RNA modifications that regulate RNA metabolism in response to stress, methylation of mRNA, tRNA, miRNA, and rRNA by members of the methyltransferase-like family is among the most abundant (Wilkinson et al., 2021). Given that we found "methylation" GO overrepresented among OA-regulated genes (*e.a. mettl3, mettl16, tarbp1, trmt1, ftsj3*), the present data suggest that epitranscriptomic regulation may play an active role in maintaining

cellular homeostasis in response to OA. Previous studies have highlighted the role of epigenetic regulation, such as DNA methylation or long non-coding RNA (lncRNA) expression, in the acclimation of marine organisms, including fish, to OA (Kang et al., 2024, Chandra Rajan et al., 2021, Downey-Wall et al., 2020). However, to our knowledge, our study is the first to indicate regulation occurring in RNA methylation processes. Additional analyses will be necessary to identify the mRNAs, lncRNAs, microRNAs, rRNAs, and tRNAs targeted by methylation and the biological pathways they are involved in, but our transcriptomic data suggest that OA-induced epitranscriptomic regulations may not have a strong impact on the tongue transcriptome. RNA modifications that occur after stress exposure are associated with the regulation of heat-shock mRNA encoding proteins involved in cellular recovery after stress (Bond, 2006). Consistent with this, we observed regulation of heat shock proteins (hsp) such as heat shock cognate 70 kDa protein-like, *hsp90aa1.2, hspd*, and *hspa4a* in the tongue of fish exposed to OA. Altogether, the observed regulations of genes involved in RNA modification, ribonucleoprotein biogenesis, and HSP synthesis suggest a molecular response to stress that may contribute to cellular homeostasis. We acknowledge that the hypothesis of cellular stress presented here will need to be further tested by additional cellular stress assays. If cellular stress occurs, it should be reflected in the regulation of other biological processes among the genes we found to be regulated.

We also found that "immune system" GO terms were overrepresented among OA-regulated genes. This finding is consistent with previous data from the olfactory rosette, revealing an effect of OA on immune status (Cohen-Rengifo et al., 2022). Particularly noteworthy was the up-regulation of the *tlr2* gene, which is essential for recognizing pathogen-associated molecular patterns (PAMPs) produced by viruses and bacteria, as it is the only TLR capable of forming functional heterodimers with more than two other TLR types (Pergolizzi et al., 2023). Up-regulation of key genes involved in the recognition of PAMPs can promote innate immune responses and influence antigen-specific adaptive immune responses. Consistent with this, among the 16 genes we found both up and down regulated by OA, 7 are involved in the positive regulation of T-cell activation and 4 are related to MHC class II protein complex. Modulation of key genes involved in PAMP recognition can promote innate immune responses and influence antigen-specific adaptive immune responses. Consistent with this, among the 16 genes we found to be both up- and downregulated by OA, seven are involved in the positive regulation of T-cell activation, and four are related to the MHC class II protein complex. OA-induced modulation of these genes suggests potential intricate regulation in antigen processing

and presentation, which are essential for triggering downstream cellular and/or humoral immune responses in fish, as in mammals (Levraud and Boudinot, 2009). Additionally, the OA-induced up-regulation of the D-amino acid oxidase gene, involved in innate immune response, is of particular interest, as it has been shown to be the most highly up-regulated gene in corals exposed to combined acidification and warming stress (Li et al., 2023). The precise molecular mechanisms involved in OA regulation of immune pathways in the tongue remain to be clarified, but it is noteworthy that studies have demonstrated that RNA modifications, particularly RNA methylation, participate in multiple biological processes of immune cells, including T cell activation (Cui et al., 2022, Han and Xu, 2023, Zha et al., 2022, Xia et al., 2021). It also remains to be determined whether the molecular regulations we observed in the tongue are associated with enhanced defense of the tissue against pathogens.

None of the 32 genes listed in the "sensory perception" GO exhibited a significant differential expression. Neither the specific genes encoding taste receptors including *tas1r3*, nor the *plcb2*, *trpm5* and *p2ry4* genes involved in the gustatory signal transduction genes are regulated by OA in the tongue. This result differs from those obtained in earlier experiments that have shown a decrease in the expression of *tas1r3*, *trpm5* and *p2ry4* transcripts in the gills of black sea bream during a short-term exposure (e.a. 15 days) to OA (Rong et al., 2020). Moreover, our targeted analysis by ddPCR of *trpm5* and *p2ry4* transcripts expression that we carried out in sea bass gills only partially confirmed the results obtained in sea bream, since only *p2ry4* expression was affected by OA. Apart from the species-specific aspect, the reduced transcriptional response on genes involved in taste sensitivity in the present study could be due to a multigenerational positive carryover effect whereby European seabass potentially acquire tolerance due to exposure of the parental generation to acidified water. Multigenerational acclimation resulting in a reduced transcriptome response to OA has been observed in marine organisms, including fish species (Iguchi et al., 2024a, Suresh et al., 2023). However, the list of genes significantly regulated by OA in the present study included *si:dkey-211g8.9* encoding for a free fatty acid receptor involved in the sensitivity to fatty acid (Baranek et al., 2024). Additional experiments, particularly focused on feeding behavior, would be necessary to determine whether this molecular regulation is associated with a modulation in fatty acid sensitivity.

Finally, the transcriptomic approach showed no significant changes in the expression of genes related to biomineralization. Specifically, none of the genes in the SPARCL1-SCPP cluster (e.g.

519 *sparcl1, spp1, odam, scpp1, 5, 7, 9*) exhibited differential expression based on pH conditions.
520 To confirm that the tooth phenotype, particularly their mineral structure, did not vary, we
521 examined the enameloid of the canine-like teeth using microscopy and EDS analysis.
522 Consistent with the transcriptomic results, we found no differences in form or mineral
523 composition, suggesting that the process of tooth mineralization is not affected in European sea
524 bass exposed to chronic OA.

525 **Conclusion**

526 In conclusion, our study represents the first comprehensive analysis using global RNAseq
527 profiles to identify all the genes expressed in the tongue of European sea bass, as well as those
528 and related biological processes that are regulated by multigenerational exposure to OA. The
529 transcriptomic profiles we obtained align with the known functions of the tongue, including
530 roles in food intake, sensory perception, and immune defense. While our RNAseq data indicate
531 that OA does not have a substantial impact on the global transcriptome—potentially due to
532 multigenerational adaptation—further research is needed to determine whether the suggested
533 epitranscriptomic regulations (e.g., RNA methylation) affect physiological processes in the
534 tongue, particularly those related to the immune system.

535 Moreover, when combined with electron microscopy analysis, our RNAseq results suggest that
536 OA does not have a long-term impact on processes related to tooth development and
537 mineralization. Although most key genes involved in taste sensitivity did not show differential
538 regulation, future research should focus on assessing the physiological consequences of OA-
539 induced regulation of the fatty acid receptor to better understand the capacity of European sea
540 bass to maintain foraging performance in future ocean conditions.

541

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Table 1. The physical and chemical parameters (mean ± SD) of the seawater (SW) in each F1 and F2 group. Psu: practical salinity units; NBS: Newborn screening.

	Physical and chemical parameters of the seawater (means of duplicated tanks for each group)				
Group	**Temperature (°C)**	**Salinity (psu)**	**Total alkalinity (µmol kg−1 SW)**	**pH (NBS scale)**	**pCO_2 (µatm)**
F1-pH 8.0	15.89 ± 2.07	33.29 ± 0.99	2425.85 ± 166.36	7.99 ± 0.07	671.55 ± 146.36
F1-pH 7.6	15.43 ± 2.03	33.34 ± 1.03	2427.35 ± 158.72	7.61 ± 0.04	1710.26 ± 244.45
F2-pH 8.0	16.06 ± 1.77	33.04 ± 0.83	2375.97 ± 113.48	7.98 ± 0.08	694.42 ± 145.01
F2-pH 7.6	16.25 ± 1.60	33.04 ± 0.83	2379.87 ± 105.06	7.64 ± 0.05	1598.63 ± 244.47

Table 2. Primers used for relative quantification by ddPCR. Sequences used to design the primers are available in genbank databases.

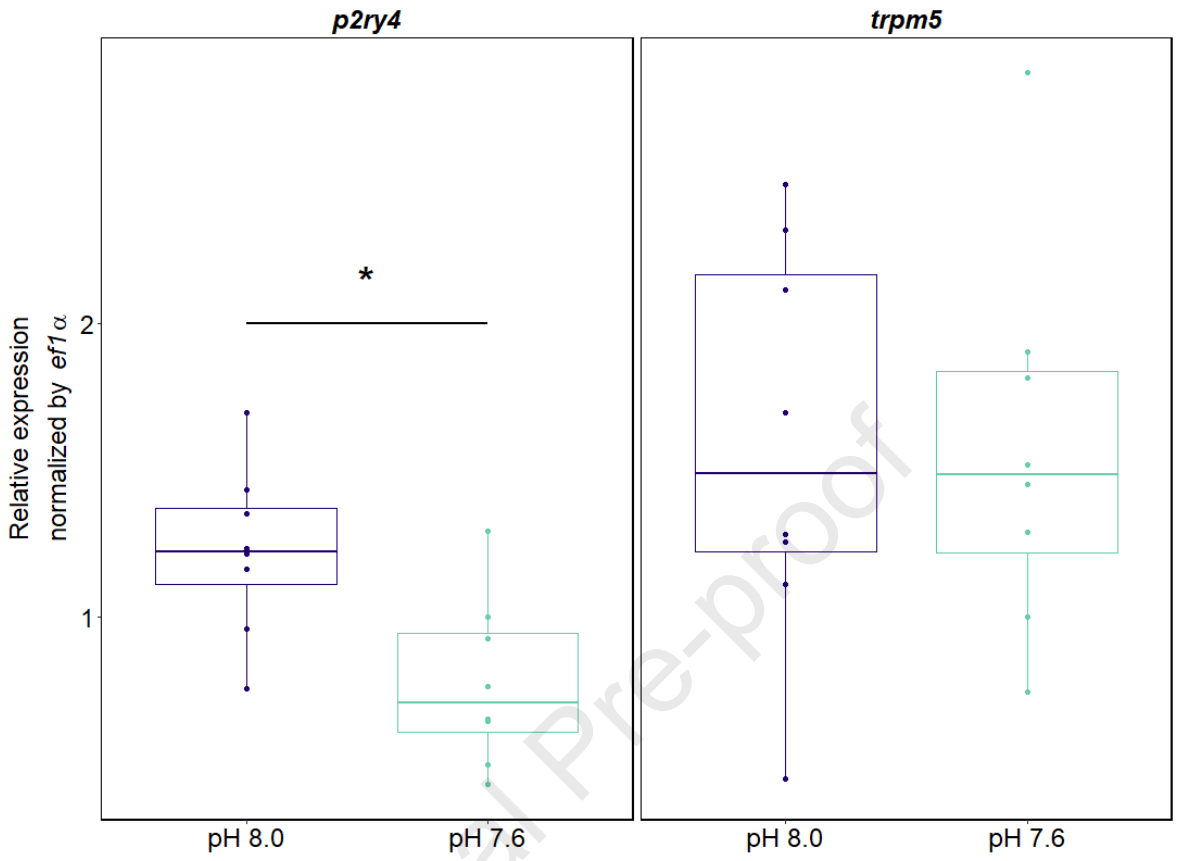
Gene name	**Ref seq**	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
trpm5	XM_051378910.1	CAGTGAAGACAGGGCTTATG	TTGTGAGGAGAGCCTGAA
p2ry4	XM_051419056.1	CCTACTCAGGGTGTTGTTATC	CAGGCAGCAGGATGTATTT
ef1α	AJ866727.1	CTGGAGGGCAGTGAAAAGAT	CATCAAGAGCCTCCAGCAGT

Table 3. Sequence statistics of RNA-seq libraries mapped to the European sea bass reference genome. Nb: number.

Group	Samples	nb of reads	nb uniquely mapped	% uniquely mapped	nb overlapping genes	% overlapping genes
Control (pH 8.0)	L23	14,562,477	8,871,133	60.92	8,416,584	57.80
	L24	29,641,165	20,314,324	68.53	19,297,674	65.10
	L25	11,936,610	8,706,867	72.94	8,294,396	69.49
	L26	20,936,973	15,354,313	73.34	14,511,384	69.31
	L27	31,317,372	20,457,027	65.32	19,397,081	61.94
	L28	29,309,789	21,299,829	72.67	20,230,421	69.02
	L29	22,676,350	16,115,310	71.07	15,388,870	67.86
	L30	33,989,398	24,100,760	70.91	22,915,921	67.42
OA (pH 7.6)	L8	33,161,629	23,053,907	69.52	21,866,949	65.94
	L9	20,273,307	13,779,557	67.97	13,037,747	64.31
	L10	23,882,950	17,995,832	75.35	16,995,558	71.16
	L11	23,343,762	15,763,100	67.53	14,688,315	62.92
	L12	22,883,056	14,420,404	63.02	13,662,755	59.71
	L13	14,328,471	9,508,779	66.36	8,989,885	62.74
	L14	25,750,557	17,565,705	68.21	16,672,044	64.74
	L15	18,982,709	12,686,118	66.83	12,073,113	63.60

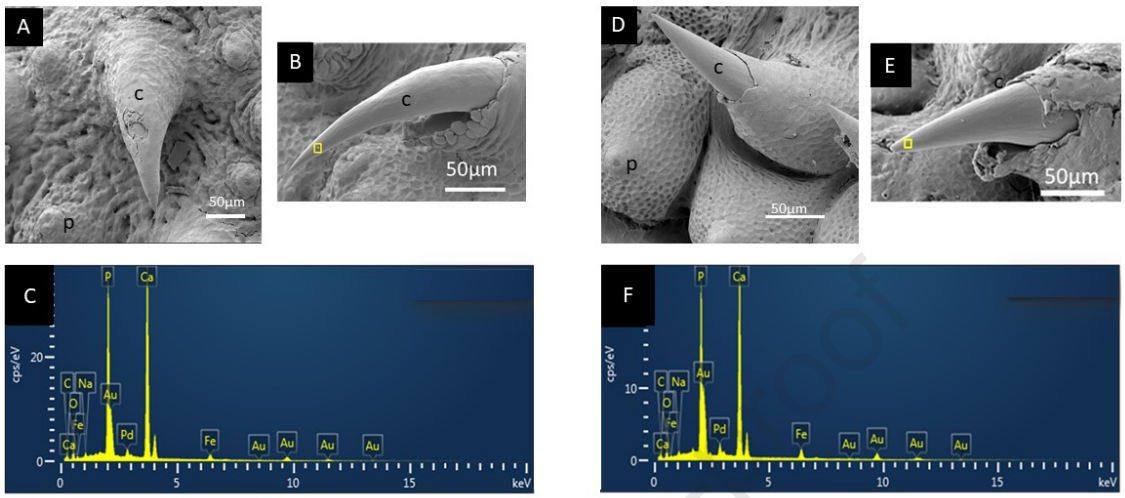
Table 4. List of some of the most highly represented GO in the transcriptome at the tongue apex as revealed by RNAseq experiment. Nb: Number, Freq.: Frequency.

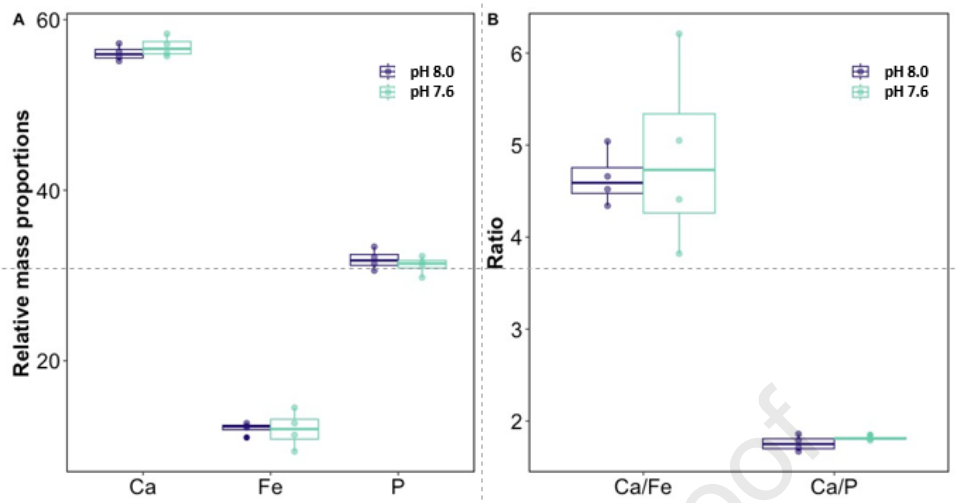
GO Term	Nb of genes	Freq,	Name
GO:0050789	5637	45%	regulation of biological process
GO:0019222	2610	21%	regulation of metabolic process
GO:0071840	2277	18%	cellular component organization or biogenesis
GO:0048518	1357	11%	positive regulation of biological process
GO:0050896	1096	8.0%	response to stimulus
GO:0009058	1085	8.7%	biosynthetic process
GO:0009056	833	6.6%	catabolic process
GO:0007166	832	6.6%	cell surface receptor signalling pathway
GO:0051649	731	5.8%	establishment of localization in cell
GO:0006950	689	5.5%	response to stress
GO:0035556	684	2.5%	intracellular signal transduction
GO:0007186	447	3.6%	G protein-coupled receptor signaling pathway
GO:0002376	430	3.4%	immune system process



pH8.0 condition

pH7.6
OA condition





- Multigenerational exposure to OA has no impact on teeth mineralization in Sea bass
- Of the 18703 genes expressed in the tongue, 295 exhibited OA-induced regulation
- Genes related to cell stress, immune system and fatty acid sensitivity are regulated
- OA impacts the branchial expression of *p2ry4* gene involved in gustatory system

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Mazurais reports financial support was provided by interdisciplinary graduate school for the blue planet ISBlue. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.