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Near-future CO₂ levels impair the olfactory system of a marine fish

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Supplementary File 1

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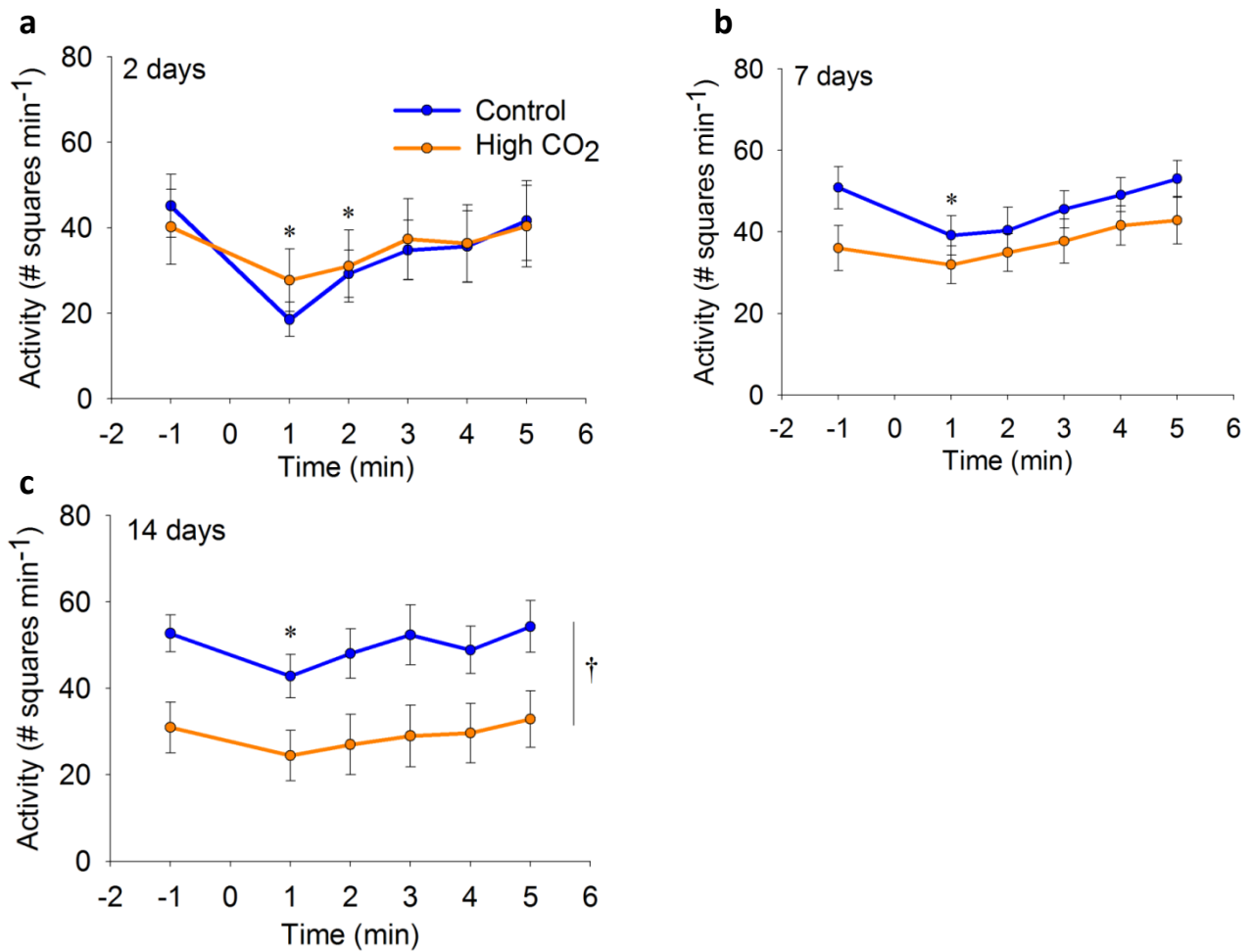


Figure S1. Behaviour responses of European sea bass (*Dicentrarchus labrax*) to a 5 min exposure to a predator odor after exposure to control (~420 μ atm) or elevated CO₂ (~950 μ atm) for 2, 7, and 14 days. Activity after 2 (a), 7 (b), and 14 (c) days exposure. Monkfish bile (dilution 1:1,000,000) was added at time point 0 for 5 minutes.

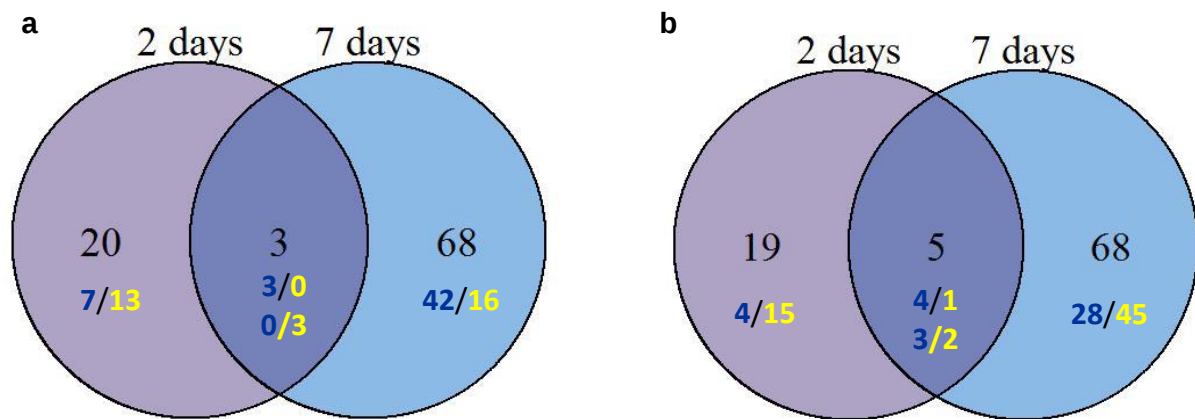


Figure S2. Venn diagrams displaying the numbers of differentially expressed genes (FDR<0.1) in each tissue at both time points. a, olfactory epithelium. b, olfactory lobe. Blue and yellow numbers represent up- and down-regulated genes, respectively.

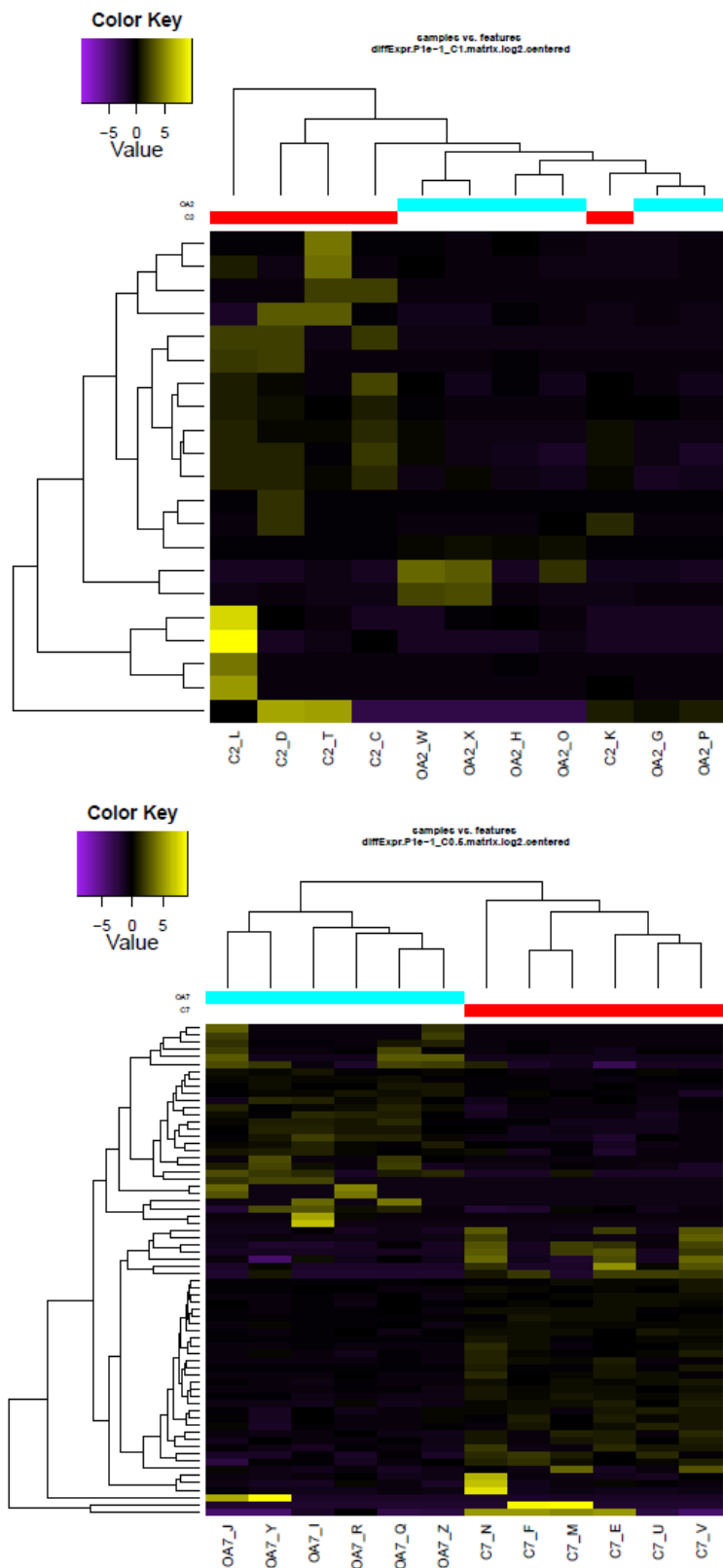


Figure S3. Heat map illustrating changes in expression level of all differentially expressed genes across treatments groups at both time points in olfactory epithelium. a, 2 days. b, 7 days. Data presented are the mean log₂ fold change in expression level in each treatment group compared to the control. Hierarchical trees were generated using the Trinity pipeline. Red bars represent control samples and light blue bars represent high CO₂ exposed fish.

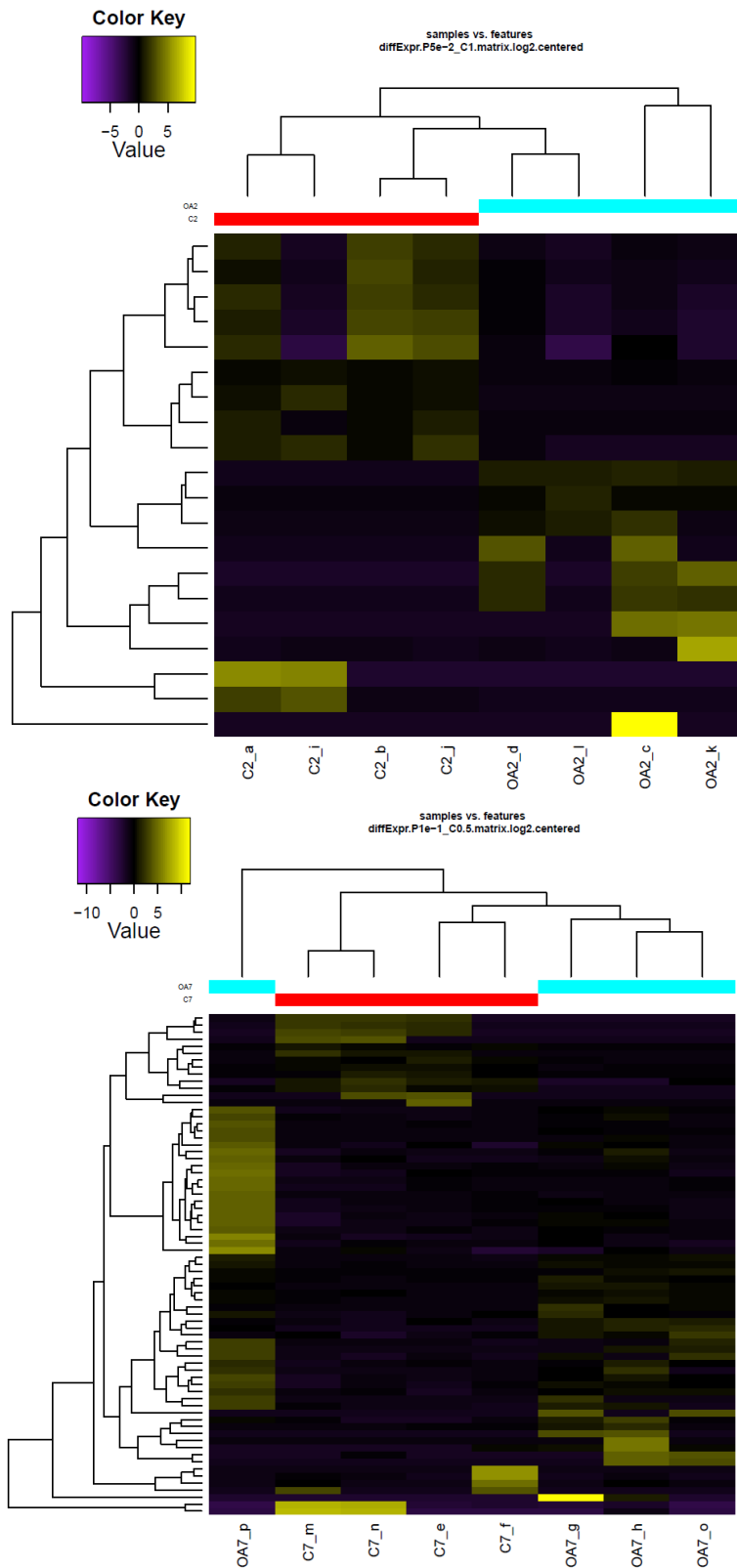


Figure S4. Heat map illustrating changes in expression level of all differentially expressed genes across treatments groups at both time points in the olfactory lobe. a. 2 days. b. 7 days. Data presented are the mean log₂ fold change in expression level in each treatment group compared to the control. Hierarchical trees were generated using the Trinity pipeline. Red bars represent control samples and light blue bars represent high CO₂ exposed fish.

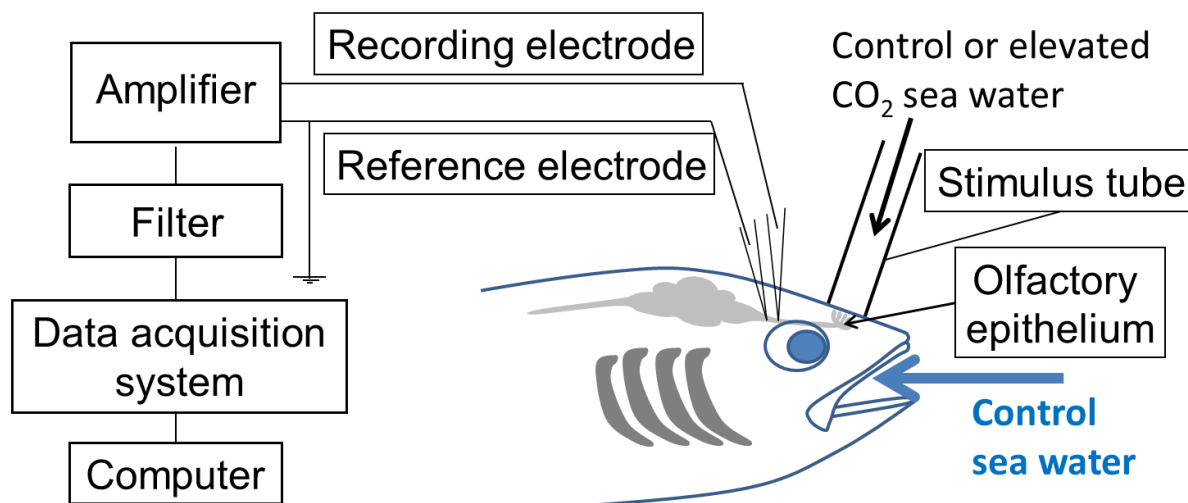


Figure S5. Activity was recorded from the olfactory nerve, while the fish gills were irrigated with control sea water containing anesthetic. The olfactory epithelium ("nose") was bathed with control or elevated CO₂ sea water.

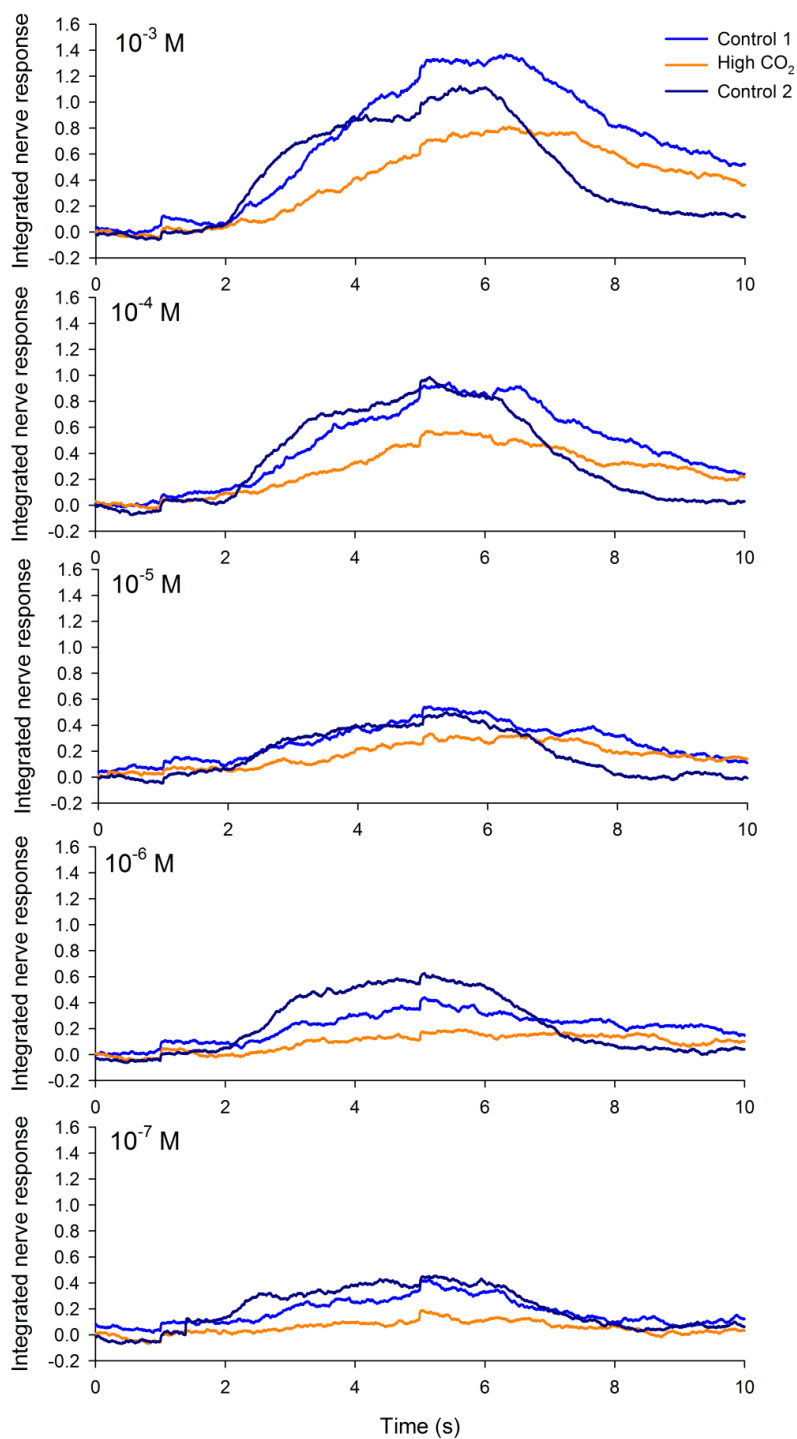


Figure S6. Representative traces of integrated nerve responses of one European sea bass (*Dicentrarchus labrax*) to L-glutamate in control (pH 8.10 ± 0.01 , 537 ± 34 μ atm) and high CO₂ (pH 7.80 ± 0.01 , 1187 ± 44 μ atm) sea water showing the response was reversible. For each concentration the odorant was applied for 4 seconds (second 2 to 6).

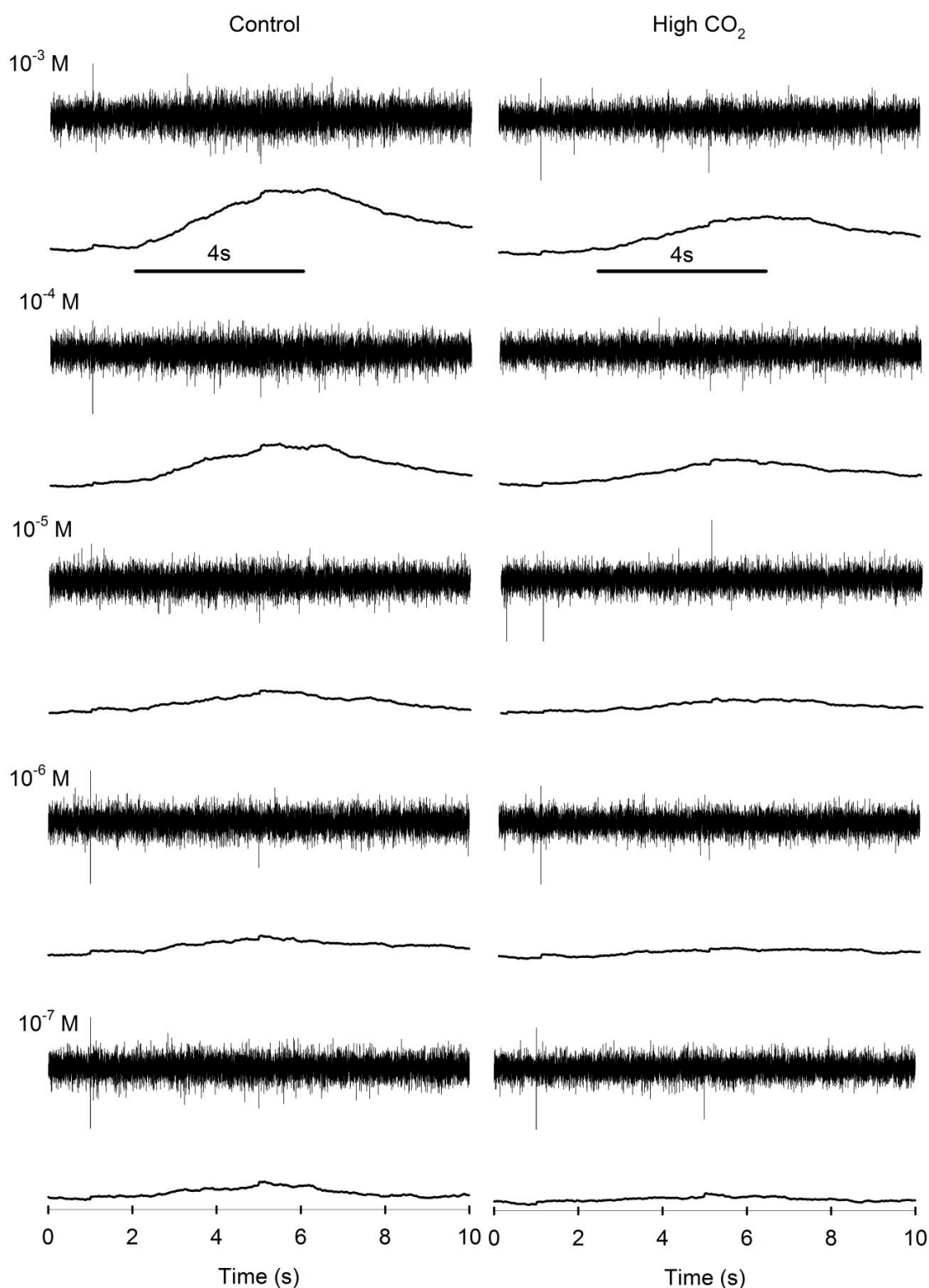


Figure S7. Sample raw (upper) and integrated (lower) traces of olfactory nerve activity of European sea bass (*Dicentrarchus labrax*) in control (pH 8.10 ± 0.01 , 537 ± 34 μ atm) and high CO₂ (pH 7.80 ± 0.01 , 1187 ± 44 μ atm) sea water to increasing concentrations (10^{-3} to 10^{-7} M) of L-glutamate. For each concentration the odorant was applied for 4 seconds (second 2 to 6) as indicated by the black bars.

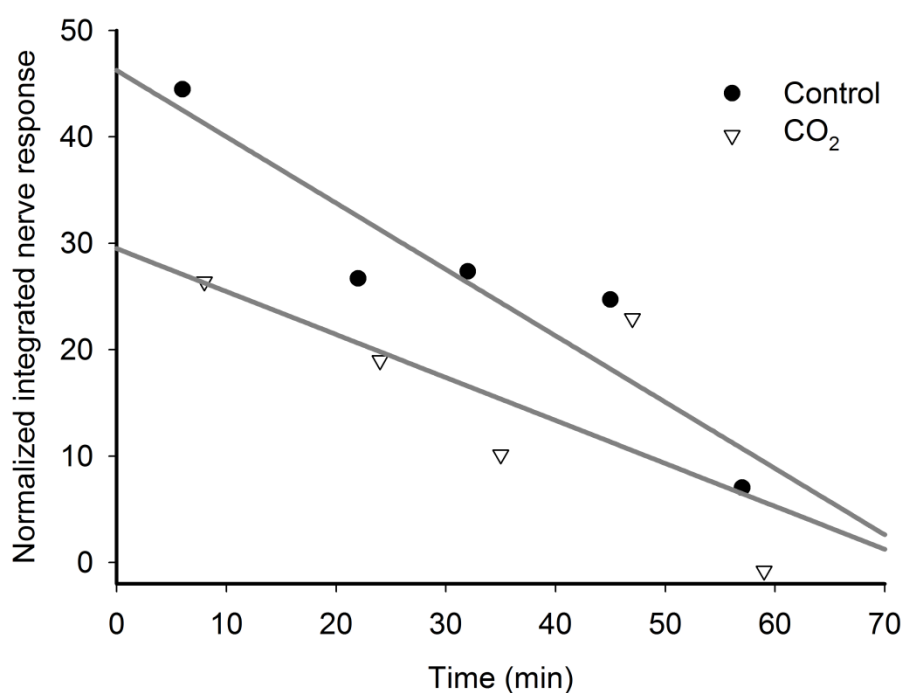


Figure S8. Temporal decay of the olfactory nerve responses to alarm cue of one European sea bass (*Dicentrarchus labrax*) under control (●) (pH 8.18 ± 0.01 , 428 ± 4 μ atm) and elevated CO₂ (▽) (pH 7.83 ± 0.01 , 1065 ± 5 μ atm). The olfactory response to freshly prepared alarm cue declined in a time-dependent manner with half-maximal responses reached after about 30 min. This is a similar time scale to that previously reported and reflects the natural decay of the active alarm cue molecule(s) in sea water⁶⁹, providing evidence that the response was to the alarm cue rather than other compounds, such as amino acids, that degrade much slower.

Table S1. Water chemistry parameters for control and elevated CO₂ treatments in exposure tanks for behavior and gene expression experiments. pH_{NBS}, temperature and salinity data are direct measurements. pCO₂ and total alkalinity (TA) were calculated from these parameters in CO2SYS. Data are presented as mean ± SE.

Parameter	Control CO ₂			Elevated CO ₂		
	2 days	7 days	14 days	2 days	7 days	14 days
pH _{NBS}	8.12±0.01	8.17±0.003	8.15±0.004	7.84±0.00	7.84±0.00	7.85±0.01
Temperature(°C)	15.5±0.06	15.4±0.09	15.5±0.01	15.5±0.09	15.4±0.02	15.4±0.02
Salinity(ppt)	34.8±0.06	35.0±0.07	34.9±0.05	34.9±0.03	35.2±0.06	35.1±0.05
TA(μmol/kgSW)	2227±5.2	2305±14.0	2300±9.2	2316±1.8	2416±14.3	2394±8.8
pCO ₂ (μatm)	459±1.1	449±5.2	478±13.8	994±0.8	1022±9.8	1001±5.4

Table S2. Water chemistry parameters for control and elevated CO₂ treatments in behavior flume. pH_{NBS}, temperature and salinity data are direct measurements. pCO₂ and total alkalinity (TA) were calculated from these parameters in CO2SYS. Data are presented as mean ± SE.

Parameter	Control CO ₂	Elevated CO ₂
pH _{NBS}	8.11±0.02	7.84±0.01
Temperature(°C)	15.1±0.04	15.0±0.08
Salinity(ppt)	35.3±0.03	35.4±0.03
TA(μmol/kgSW)	2367±57.2	2474±57.6
pCO ₂ (μatm)	521±22	1019±26

Table S3. Dynamic range in transcript expression profiles for all individual olfactory epithelium samples included in this study, calculated based on the measurements for ERCC spike-in controls. Values presented are log₂ transformed maximum-minimum FPKM values calculated for ERCC spike-in control transcripts. R² values represent the relationship between the calculated expression level (FPKM) and the expected concentration of each control transcripts.

Treatment	Time	Sample	R ²	Log ₂ dynamic range (FPKM)
Control	2 days	1	0.91	15.6
		2	0.96	17.1
		3	0.92	14.8
		4	0.96	17.0
		5	0.92	16.6
		6	0.97	16.2
Elevated CO ₂	2 days	1	0.95	15.3
		2	0.93	18.4
		3	0.91	15.2
		4	0.95	16.5
		5	0.95	15.9
		6	0.96	15.6
Control	7 days	7	0.95	15.6
		8	0.95	17.0
		9	0.95	15.5
		10	0.95	16.3
		11	0.92	15.0
		12	0.95	15.8
Elevated CO ₂	7 days	7	0.95	16.4
		8	0.95	16.3
		9	0.92	15.4
		10	0.95	15.4
		11	0.94	16.4
		12	0.95	16.7

Table S4. Dynamic range in transcript expression profiles for all individual olfactory bulb samples included in this study, calculated based on the measurements for ERCC spike-in controls. Values presented are $\log_2$ transformed maximum-minimum FPKM values calculated for ERCC spike-in control transcripts. R^2 values represent the relationship between the calculated expression level (FPKM) and the expected concentration of each control transcripts.

Treatment	Time	Sample	R^2	$\log_2$ dynamic range (FPKM)
Control	2 days	1	0.93	14.5
		2	0.95	14.6
		3	0.90	14.0
		4	0.96	13.7
Elevated CO ₂	2 days	1	0.92	14.4
		2	0.95	13.6
		3	0.94	13.6
		4	0.95	13.9
Control	7 days	7	0.92	15.1
		8	0.95	14.6
		9	0.94	13.3
		10	0.94	13.2
Elevated CO ₂	7 days	7	0.95	12.6
		8	0.95	15.0
		9	0.90	14.7
		10	0.93	13.9

Table S5. Comparative summary statistics describing the *de novo* transcriptome assemblies for the olfactory epithelium and olfactory lobe constructed using the Trinity pipeline. Data presented include the number and length of transcripts produced for each assembly and the percentage of transcripts annotated using Blastx against Ensembl protein databases using an e-value cut off $< 1e^{-15}$.

	Olfactory epithelium	Olfactory bulb
No. transcripts	393,368	268,812
No. loci	211,264	209,404
Mean length (bp)	1288	1242
N50 (bp)	2709	2634
% annotated	42%	47%
No. zebrafish transcripts annotations	20,504	19,349

Table S6. Number of partial olfactory receptor (OR), trace amine-associated receptors (TAARs), and vomeronasal receptors 1 and 2 (VR1 and VR2) genes and transcripts in sea bass compared to genes from other teleost species. Table modified from Churcher et al 2015⁷¹.

	<i>D. labrax</i>		<i>Anguilla anguilla</i>		<i>Danio</i>	<i>Takifugu rubripes</i>	<i>Oryzias latipes</i>	<i>Tetraodon nigroviridis</i>	<i>Gasterosteus aculeatus</i>
Gene family	Genes	Transcripts	Genes	Transcripts	Genes	Genes	Genes	Genes	Genes
ORs	115	652	112	429	142	44	53	42	95
TAAR	32-50	234	13	113	109-112	13-18	25	17-18	48-49
VR1*	≥ 2	3	6	29	6	5	6	5	6
VR2*	≥ 27	312	38	299	40-54	30	22	12	23

Table S7. Gene Ontology Terms over-represented in the list of differentially expressed transcripts in the olfactory bulb at 2 days. Values presented are the P-values and FDR associated with this over-representation. Analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8) using the *de novo* olfactory bulb transcriptome assembly as a background.

Category	Term	P Value	Fold Enrichment	Benjamini
BIOLOGICAL PROCESS (BP ALL)				
GO:0034622	cellular macromolecular complex assembly	3.01E-02	9.5	9.97E-01
GO:0065003	macromolecular complex assembly	4.80E-02	7.3	9.92E-01
KEGG PATHWAY				
dre04914	progesterone-mediated oocyte maturation	9.90E-02	16.2	9.18E-01

Table S8. Gene Ontology Terms and Kegg Pathways over-represented in the list of differentially expressed transcripts in the olfactory bulb at 7 days. Values presented are the P-values and adjusted P-values (Benjamini) associated with this over-representation. Analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8) using the *de novo* olfactory bulb transcriptome assembly as a background.

Category	Term	P Value	Fold Enrichment	Benjamini
BIOLOGICAL PROCESS (BP ALL)				
GO:0043207	response to external biotic stimulus	2.45E-02	11.7	1.00E+00
GO:0051707	response to other organism	2.45E-02	11.7	1.00E+00
GO:0009607	response to biotic stimulus	2.77E-02	10.9	9.97E-01
GO:0009615	response to virus	4.93E-02	37.8	9.99E-01
GO:0006952	defense response	6.01E-02	7.1	9.99E-01
GO:0016032	viral process	6.52E-02	28.4	9.97E-01
GO:0044764	multi-organism cellular process	6.75E-02	27.4	9.93E-01
GO:0044403	symbiosis	6.97E-02	26.5	9.87E-01
GO:0044419	interspecies interaction between organisms	6.97E-02	26.5	9.87E-01
GO:0051704	multi-organism process	7.26E-02	6.4	9.81E-01
CELLULAR COMPONENT (CC ALL)				
GO:0005634	nucleus	9.50E-02	1.7	1.00E+00
MOLECULAR FUNCTION (MF ALL)				
GO:0035639	purine ribonucleoside triphosphate binding	1.81E-02	2.4	9.21E-01
GO:0001883	purine nucleoside binding	1.82E-02	2.4	7.22E-01
GO:0032549	ribonucleoside binding	1.84E-02	2.4	5.77E-01
GO:0001882	nucleoside binding	1.85E-02	2.4	4.77E-01
GO:0017076	purine nucleotide binding	1.96E-02	2.4	3.67E-01
GO:0032553	ribonucleotide binding	2.02E-02	2.4	3.33E-01
GO:0097367	carbohydrate derivative binding	2.64E-02	2.3	3.71E-01
GO:1901363	heterocyclic compound binding	4.12E-02	1.6	4.78E-01
GO:0097159	organic cyclic compound binding	4.44E-02	1.6	4.68E-01
GO:0005524	ATP binding	5.16E-02	2.4	4.88E-01
GO:0032559	adenyl ribonucleotide binding	5.44E-02	2.4	4.77E-01
GO:0030554	adenyl nucleotide binding	5.47E-02	2.4	4.52E-01
GO:0000166	nucleotide binding	6.29E-02	1.9	4.75E-01
GO:1901265	nucleoside phosphate binding	6.29E-02	1.9	4.75E-01
GO:0036094	small molecule binding	7.32E-02	1.9	5.05E-01
GO:0008066	glutamate receptor activity	9.54E-02	19.2	5.81E-01

Table S9. Gene Ontology Terms over-represented in the list of differentially expressed transcripts in the olfactory epithelium at 2 days. Values presented are the P-values and adjusted P-values (Benjamini) associated with this over-representation. Analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8) using the *de novo* olfactory epithelium transcriptome assembly as a background.

Category	Term	P Value	Fold Enrichment	Benjamini
BIOLOGICAL PROCESS (BP ALL)				
GO:0050801	ion homeostasis	1.53E-02	13.8	9.80E-01
GO:0048878	chemical homeostasis	2.27E-02	11.2	9.47E-01
GO:2000021	regulation of ion homeostasis	2.59E-02	68.6	8.93E-01
GO:0065008	regulation of biological quality	4.56E-02	4.2	9.08E-01
GO:0055085	transmembrane transport	9.58E-02	5.1	9.74E-01
MOLECULAR FUNCTION (MF ALL)				
GO:0022857	transmembrane transporter activity	8.40E-02	5.3	8.35E-01

Table S10. Gene Ontology Terms and Kegg Pathways over-represented in the list of differentially expressed transcripts in the olfactory epithelium at 7 days. Values presented are the P-values and adjusted P-values (Benjamini) associated with this over-representation. Analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8) using the *de novo* olfactory epithelium transcriptome assembly as a background.

Category	Term	P Value	Fold Enrichment	Benjamini
CELLULAR COMPONENT (CC ALL)				
GO:0098796	membrane protein complex	1.19E-02	5.1	7.15E-01
GO:0032991	macromolecular complex	8.74E-02	1.9	9.92E-01
MOLECULAR FUNCTION (MF ALL)				
GO:0003008	system process	1.02E-02	5.5	9.97E-01
GO:0050877	neurological system process	6.46E-02	6.9	1.00E+00
GO:0050954	sensory perception of mechanical stimulus	6.98E-02	26.6	1.00E+00
GO:0033036	macromolecule localization	7.86E-02	2.9	1.00E+00
GO:0048598	embryonic morphogenesis	9.04E-02	3.6	1.00E+00

Table S11. List of real-time PCR primer details used for qRT-PCR validation of RNAseq. Genes sequences were derived from the RNA-Seq analysis in this study.

Gene	FP sequence	RP sequence	Product size (bp)	Ta	Efficiency (%)	R ²
rpl8	TAGTTCTCCTTCTCCTGTA	CAAACCCTCAACAATCAG	114	56.5	106.1	0.998
slc4a8	TGAGAAGAACAAGATGGTAG	TCAAGGAGTGTATCAGTCT	107	60.0	102.9	0.999
samhd1	TATCTGTCAGCTTGGTGTA	AAATTAGGGAAGAAGGTGAG	78	61.0	111.3	0.988
klc8	GCAGTACCATGTCCAGAT	AGTGAGAAGCAGTGATGA	92	59.5	108.9	0.989
CACNA2D	CGGAAGATTGTGGAGAAG	CTGGCTAACCTATCAAGTG	80	58.5	109.5	0.979
tmp4	CATTCTCCTTGTCCAGTT	GACTGAATCTCCTACATCTC	153	60.0	97.3	0.982

Table S12. Comparison between the fold changes in expression in the olfactory epithelium for selected transcripts generated based on RNAseq and on the individual gene quantification (qRT-PCR). Values presented are mean expression of transcripts for fish exposed to elevated CO₂ (~1000 µatm) relative to control fish (~450 µatm). Asterisks indicate a significant difference in expression between the two treatments (*p<0.1, **p<0.05, ***p<0.01).

Gene	RNAseq	qRT-PCR	Time
slc4a8	1.61**	1.94**	2 days
samhd1	6.14*	4.32*	2 days
klc8	2.0***	1.84	7 days
CACNA2D	-5.55*	-3.85***	7 days
tmp4	2.7*	2.05*	7 days

Table S13. Water chemistry parameters in the electrophysiology experimental setup.

Odorant	Treatment	pH	Temp °C	Salinity psu	TCO ₂ μmol kg ⁻¹ SW	pCO ₂ μatm	Alkalinity μmol kg ⁻¹ SW
L-serine	Control	8.13±0.01	21.7±0.1	35.5±0.2	2279±3	507±3	2588±13
	High CO ₂	7.81±0.01	21.5±0.1	35.2±0.2	2403±3	1144±5	2573±12
L-glutamate	Control	8.11±0.01	21.4±0.1	35.8±0.3	2324±13	537±33	2592±10
	High CO ₂	7.80±0.01	21.6±0.1	35.7±0.3	2448±16	1187±44	2568±13
L-cysteine	Control	8.11±0.01	21.5±0.2	35.8±0.3	2313±19	543±30	2572±13
	High CO ₂	7.86±0.01	21.7±0.2	35.8±0.3	2438±48	1175±48	2554±18
L-alanine	Control	8.11±0.01	21.5±0.1	35.7±0.2	2312±18	537±25	2580±11
	High CO ₂	7.81±0.01	21.7±0.1	35.7±0.2	2432±19	1177±41	2560±15
L-arginine	Control	8.09±0.01	21.4±0.1	35.5±0.2	2330±18	562±27	2580±13
	High CO ₂	7.80±0.01	21.6±0.1	35.5±0.2	2444±20	1211±47	2553±18
Cyprinol SO ₄	Control	8.17±0.01	21.2±0.2	36.2±0.1	2283±6	447±7	2583±9
	High CO ₂	7.82±0.01	21.3±0.2	36.2±0.1	2429±9	1113±22	2552±9
Scymnol SO ₄	Control	8.18±0.01	21.1±0.1	36.6±0.1	2551±13	426±6	2551±14
	High CO ₂	7.83±0.01	21.0±0.1	36.6±0.1	2395±15	1078±7	2521±16
Intestinal fluid	Control	8.17±0.01	21.2±0.2	36.2±0.2	2285±4	448±7	2584±7
	High CO ₂	7.82±0.01	21.2±0.2	36.2±0.2	2435±4	1117±28	2557±4
Bile	Control	8.18±0.01	21.3±0.3	36.2±0.2	2279±6	443±11	2581±7
	High CO ₂	7.83±0.01	21.3±0.3	36.2±0.2	2431±5	1120±28	2556±4
Alarm cue	Control	8.18±0.01	22.1±0.2	36.7±0.1	2222±11	428±4	2523±15
	High CO ₂	7.83±0.01	22.1±0.2	36.7±0.1	2366±15	1065±5	2494±18
Average	Control	8.15±0.01	21.3±0.2	36.2±0.2	2318±11	476±14	2554±11
	High CO₂	7.82±0.01	21.5±0.2	36.2±0.2	2422±15	1122±19	2522±13

Supplementary File 1 References:

71. Churcher AM, Hubbard PC, Marques JP, Canário AVM, Huertas M. Deep sequencing of the olfactory epithelium reveals specific chemosensory receptors are expressed at sexual maturity in the European eel *Anguilla anguilla*. *Molecular Ecology* 2015, **24**(4): 822-834.