Supplementary File

Chronic inflammation modulates opioid receptor gene expression and triggers respiratory burst in a teleost model

Diogo Peixoto [1,2,3]. Marina Machado [2]. Rita Azeredo [2] and Benjamín Costas [2]*

[1] ICBAS - Instituto de Ciências Biomédicas Abel Salazar. Universidade do Porto. Porto. Portugal; dpeixoto@ciimar.up.pt (D.P.)
[2] CIIMAR - Centro Interdisciplinar de Investigação Marinha e Ambiental. Matosinhos. Portugal; mcasimiro@ciimar.up.pt (M.M.). mleme@ciimar.up.pt (R.A.) and bcostas@ciimar.up.pt (B.C.)
[3] Departamento de Biología. Facultad de Ciencias del Mar y Ambientales. Instituto Universitario de Investigación Marina (INMAR). CEIMAR-Universidad de Cádiz. Cádiz. Spain

* Correspondence: mleme@ciimar.up.pt (R.A.) and bcostas@ciimar.up.pt (B.C.)

Table S1 - Absolute values of total white, red and peritoneal cells, peripheral leucocytes (neutrophils, monocytes, lymphocytes and thrombocytes) and respiratory burst of European seabass after 24 hours, 7, 14 and 21 days after inflammation.

Parameters	Treatment	Time post-injection											
		24 hours			7 days			14 days			21 days		
WBC (x10⁴ µL⁻¹)	CTRL	4.33 ± 0.93			5.83 ± 1.55			4.77 ± 1.45			9.95 ± 2.51		
	FIA	5.5 ± 1.05			4.8 ± 0.71			4.53 ± 1.43			10.25 ± 1.75		
RBC (x10⁴ µL-1)	CTRL	16.15 ± 4.27			13.35 ± 2.5			15.13 ± 5.79			18.63 ± 6.31		
	FIA	14.53 ± 3.27			17.05 ± 2.62			11.08 ± 4.49			17.53 ± 2.58		
Hematocrit (%)	CTRL	26.33 ± 3.83			31.17 ± 2.48			25.33 ± 3.67			25.2 ± 4.82		
	FIA	30 ± 4.86			30.33 ± 2.81			26.83 ± 1.72			28.6 ± 4.04		
Neutrophils (x10⁴ µL⁻¹)	CTRL	0.11 ± 0.04	a#		0.04 ± 0.01	a#		0.07 ± 0.01	a		0.27 ± 0.09	b	
	FIA	0.25 ± 0.04	b*		0.2 ± 0.06	ab*		0.1 ± 0.04	a		0.18 ± 0.05	ab	
Monocytes (x10⁴ µL-1)	CTRL	0.08 ± 0.05	a		0.08 ± 0.01	a#		0.11 ± 0.02	ab		0.27 ± 0.09	b#	
	FIA	0.16 ± 0.08	a		0.33 ± 0.1	b*		0.6 ± 0.09	ab		0.57 ± 0.2	c*	
Lymphocytes (x10⁴ µL⁻¹)	CTRL	1.22 ± 0.16	a#		1.85 ± 0.24	a		1.45 ± 0.36	a		1.69 ± 0.24	b	
	FIA	1.96 ± 0.12	a*		1.54 ± 0.2	a		1.69 ± 0.24	a		4.29 ± 0.58	b	
Thrombocytes (x10⁴ µL⁻¹)	CTRL	2.95 ± 0.51			3.54 ± 0.69			3.59 ± 0.76			5.33 ± 0.76		
	FIA	3.19 ± 0.29			2.61 ± 0.38			2.6 ± 0.91			4.88 ± 0.27		
Total peritoneal cells (x10⁴ µL⁻¹)	CTRL	5.83 ± 3.44			9.88 ± 7.31			6.47 ± 6.66			7 ± 5.97		
	FIA	11.7 ± 3.58	a		42.15 ± 23.15	b*		46.92 ± 9.8	b*		35.23 ± 8.73	b*	
Respiratory burst (nmol O_2^-)	CTRL	1277.56 ± 1121.38			375.28 ± 172.56			486.33 ± 388.98			743.67 ± 903.51	#	
	FIA	2721.22 ± 1313.3	b		462.56 ± 175.24	a		800.39 ± 802.77	a		4134.39 ± 1294.93	b*	

Two-way ANOVA

Parameters	Time	Treatment	Time *vs* Treatment	Treatment		Time			
				CTRL	FIA	24 hours	7 days	14 days	21 days
WBC (x10⁴ μL⁻¹)	<0.01	ns	ns	-	-	a	a	a	b
RBC (x10⁴ μL⁻¹)	ns	ns	ns	-	-	-	-	-	-
Hematocrit (%)	<0.01	ns	ns	-	-	ab	b	a	ab
Neutrophils (x10⁴ μL⁻¹)	<0.01	<0.01	<0.01	*	#	a	ab	b	c
Monocytes (x10⁴ μL⁻¹)	<0.01	<0.01	0.005	*	#	a	b	ab	c
Lymphocytes (x10⁴ μL⁻¹)	<0.01	0.003	0.002	*	#	a	a	a	b
Thrombocytes (x10⁴ μL⁻¹)	<0.01	0.015	0.137	#	*	a	a	a	b
Total peritoneal cells (x10⁴ μL⁻¹)	0.008	<0.01	0.018	*	#	a	b	b	ab
Respiratory burst (nmol O^{-}_{2})	<0.01	<0.01	0.004	*	#	b	a	a	b

Values are presented as means ± SD (n = 6). Different low case letters stand for statistically significant differences attributed to sampling time. Symbols stand for significant differences between treatments. (Two-way ANOVA; Tukey post-hoc test; $p \leq 0.05$).

Table S2 - Plasma innate immune response parameters of European seabass after 24 hours, 7, 14 and 21 days after inflammation.

Parameters	Treatment	Time post-injection							
		24 hours		7 days		14 days		21 days	
IgMp (OD 450 nm)	CTRL	0.16 ± 0.111		0.132 ± 0.031		0.089 ± 0.028		0.219 ± 0.082	
	FIA	0.161 ± 0.056		0.109 ± 0.027		0.084 ± 0.036		0.255 ± 0.099	
Inihibited trypsin (%)	CTRL	81.193 ± 0.297		81.617 ± 0.992		83.682 ± 1.641		82.612 ± 2.78	
	FIA	81.267 ± 0.323		82.282 ± 1.782		81.877 ± 1.442		82.115 ± 1.83	
Total protease (%)	CTRL	7.755 ± 1.364		10.291 ± 0.808		9.08 ± 1.035		9.98 ± 0.92	
	FIA	9.608 ± 1.771		9.831 ± 1.443		10.98 ± 1.959		11.217 ± 0.952	
Peroxidase (U perox)	CTRL	38.693 ± 19.154		39.48 ± 20.309		60.283 ± 17.627		37.911 ± 21.77	
	FIA	32.361 ± 9.087		54.827 ± 13.395		42.333 ± 18.419		31.633 ± 23.743	
Lysozyme (mg mL^{-1})	CTRL	1.73 ± 1.213		4.328 ± 3.772		4.161 ± 1.356		2.713 ± 1.252	
	FIA	2.372 ± 1.493		9.721 ± 3.949		5.008 ± 1.204		4.459 ± 1.772	

Table S2 - Continuation.

Two-way ANOVA

Parameters	Time	Treatment	Time *vs* Treatment	Treatment		Time			
				CTRL	FIA	24 hours	7 days	14 days	21 days
IgMp (OD 450 nm)	<0.001	ns	ns	-	-	ab	a	a	b
Inihibited trypsin (%)	ns	ns	ns	-	-	-	-	-	-
Total protease (%)	0.003	0.006	ns	*	#	a	b	ab	b
Peroxidase (U perox)	ns	ns	ns	-	-	-	-	-	-
Lysozyme (mg mL^{-1})	<0.001	0.003	ns	*	#	a	b	ab	a

Values are presented as means ± SD (n = 6). Different low case letters stand for statistically significant differences attributed to sampling time. Symbols stand for significant differences between treatments. (Two-way ANOVA; Tukey post-hoc test; $p \leq 0.05$).

Table S3 - Quantitative expression of immune-related gene and opioids receptors of head-kidney of European seabass after 24 hours, 7, 14 and 21 days after inflammation.

Parameters		Treatment	Time post-injection														
			24 hours				7 days				14 days				21 days			
Normalized mRNA expression	*tgfβ*	CTRL	1.466	±	0.433		0.729	±	0.426		1.175	±	0.6		1.083	±	0.382	
		FIA	1.335	±	0.415		0.881	±	0.304		1.886	±	1.058		1.035	±	0.494	
	cxcr4	CTRL	1.093	±	0.591		1.046	±	0.339		1.82	±	1.357		1.148	±	0.240	
		FIA	0.65	±	0.222		1.272	±	0.382		2.389	±	0.353		1.788	±	0.548	
	il1β	CTRL	3.002	±	2.952		0.26	±	0.211		1.007	±	0.429		1.43	±	0.618	
		FIA	3.164	±	2.214		0.931	±	0.938		2.38	±	1.417		1.399	±	0.606	
	mmp9[1]	CTRL	2.501	±	0.85	b	0.528	±	0.31	a	0.928	±	0.43	a	1.439	±	0.69	ab
		FIA	2.39	±	0.67	b	0.852	±	0.5	a	2.431	±	3.07	ab	0.794	±	0.34	a
	casp1	CTRL	5.321	±	6.749		0.809	±	0.456		1.754	±	1.697		0.728	±	0.610	
		FIA	6.72	±	4.398		1.287	±	1.123		1.574	±	0.923		1.349	±	1.061	
	il10	CTRL	6.336	±	5.885		0.242	±	0.163		1.075	±	0.967		0.244	±	0.159	
		FIA	3.963	±	1.977		0.453	±	0.424		0.549	±	0.436		0.623	±	0.597	
	il34	CTRL	0.752	±	0.239		1.004	±	0.957		1.333	±	0.522		0.954	±	0.41	
		FIA	0.936	±	1.045		1.854	±	1.454		3.762	±	2.65		1.667	±	0.569	
	hep	CTRL	19.787	±	28.925		0.589	±	0.663		4.242	±	7.094		0.630	±	0.802	
		FIA	20.095	±	17.693		1.742	±	1.846		3.723	±	5.56		2.917	±	3.775	
	ifn-γ	CTRL	14.162	±	16.663		0.745	±	0.963		3.398	±	4.858		0.644	±	0.82	
		FIA	10.396	±	6.699		1.163	±	1.223		2.688	±	4.029		1.082	±	1.173	
	IgM[1]	CTRL	0.937	±	0.479		0.684	±	0.611		0.858	±	0.36		1.524	±	0.064	

	FIA	3.71	±	2.796	1.142	±	1.167	3.052	±	3.006	1.316	±	0.276

Table S3 - Continuation.

	ptx	**CTRL**	1.604	±	0.573	1.029	±	1.012	1.083	±	0.695	1.546	±	0.921
		FIA	1.397	±	0.203	1.092	±	0.463	1.073	±	0.705	1.376	±	0.469
	Opioids receptors													
Normalized mRNA expression	***nopr***	**CTRL**	3.551	±	3.116	0.291	±	0.313	2.001	±	2.01	0.539	±	0.309
		FIA	6.219	±	4.819	0.315	±	0.176	6.1	±	7.654	0.301	±	0.43
	muor	**CTRL**	11.026	±	9.459	0.583	±	0.741	1.452	±	1.575	1.625	±	1.557
		FIA	6.258	±	4.457	1.837	±	2.033	4.666	±	5.547	2.462	±	1.354
	kor1	**CTRL**	11.781	±	9.359	0.506	±	0.414	1.323	±	1.819	0.388	±	0.477
		FIA	7.621	±	4.403	1.275	±	1.239	1.296	±	1.4	1.244	±	1.005
	dor2	**CTRL**	9.674	±	10.706	0.708	±	0.79	2.98	±	4.781	0.576	±	0.596
		FIA	12.729	±	10.237	1.821	±	2.068	1.283	±	2.062	1.448	±	1.424
	ogfr1	**CTRL**	5.910	±	8.871	0.555	±	0.769	1.706	±	1.669	0.853	±	0.423
		FIA	5.839	±	5.666	1.067	±	1.191	1.4	±	2.012	0.864	±	0.779

[1] *non-parametric tests with Kruskal-Wallis pairwise comparisons*

Table S3 - Continuation.

Two-way ANOVA									
				Treatment		Time			
Parameters	**Time**	**Treatment**	**Time *vs* Treatment**	**CTRL**	**FIA**	**24 hours**	**7 days**	**14 days**	**21 days**
tgfβ	0.006	ns	ns	-	-	b	a	b	a
cxcr4	0.002	ns	ns	-	-	a	a	b	b
il1β	<0.001	0.05	ns	#	*	b	a	b	b
casp1	0.036	0.023	ns	#	*	b	a	a	a
il10	<0.001	ns	ns	-	-	a	b	b	b
il34	0.036	ns	ns	-	-	a	ab	b	ab
hep	0.02	ns	ns	-	-	b	a	ab	ab
ifn-y	ns	ns	ns	-	-	-	-	-	-
ptx	ns	ns	ns	-	-	-	-	-	-
Opioid receptors									
nopr	<0.001	ns	ns	-	-	b	a	b	a
muor	0.002	ns	ns	-	-	b	a	a	ab
kor1	<0.001	ns	ns	-	-	b	a	a	a

dor2	0.019	ns	ns	-	-	b	ab	ab	a
ogfr1	ns	ns	ns	-	-	-	-	-	-

Values are presented as means ± SD (n = 6). Different low case letters stand for statistically significant differences attributed to sampling time. Symbols stand for significant differences between treatments. (Two-way ANOVA; Tukey post-hoc test; $p \leq 0.05$).

Table S4 – Principal Component Analysis (PCA) eigenvalues.

	F1	F2	F3	F4	F5	F6	F7
Eigenvalue	10.316	4.064	2.400	1.691	1.241	0.885	0.403
Variability (%)	49.126	19.352	11.426	8.054	5.910	4.215	1.917
Cumulative %	49.126	68.478	79.904	87.958	93.868	98.083	100.000

Table S5 - Principal Component Analysis (PCA) eigenvectors.

	F1	F2	F3	F4	F5
Plasma IgM	0.002	-0.041	-0.542	-0.392	0.129
Anti-protease	0.016	0.122	-0.241	0.556	0.435
Protease	-0.201	0.294	-0.171	0.086	0.014
Peroxidase	-0.029	-0.223	0.441	-0.171	0.373
Lysozyme	-0.157	0.030	0.331	-0.146	0.464
Igs	-0.168	-0.347	-0.073	0.131	-0.324
tgfb	0.229	0.288	0.146	-0.012	-0.206
cxcr4	-0.140	0.383	0.213	-0.033	-0.032
il1b	0.253	0.272	-0.033	-0.121	0.063
mmp9	0.287	0.070	-0.017	-0.118	-0.149
casp1	0.276	-0.055	0.046	0.182	0.027
il10	0.291	-0.114	0.092	0.009	-0.056
igm	0.174	0.331	-0.212	0.096	0.063
ifn-y	0.280	-0.140	0.055	0.216	0.061
il34	-0.113	0.406	0.267	-0.015	0.038
ptx	0.230	0.067	-0.211	-0.398	0.211
nopr	0.253	0.105	0.167	0.262	-0.245
muor	0.277	0.112	0.081	-0.260	-0.073

kor1	0.267	-0.119	0.165	-0.102	-0.079
dor2	0.252	-0.255	0.017	0.019	0.180
ogfr1	0.276	-0.020	-0.071	0.196	0.322