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1 ***Ex vivo* approach supports both direct and indirect actions of melatonin on**
2 **immunity in pike-perch *Sander lucioperca***

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9 **ABSTRACT**

10 The melatonin hormone, which is a multifunctional molecule in vertebrates, has been shown to
11 exert complex actions on the immune system of mammals. In teleosts, the immunomodulatory
12 capacity of this hormone has seldom been investigated. In the present experiment, we exposed
13 *ex vivo* spleen and head kidney tissues of pike-perch to melatonin (Mel) and cortisol (Cort). We
14 applied three concentrations of both hormones, alone and in combination, namely (1) Mel (10,
15 100 or 1000 pg mL⁻¹) (2) Cort (50, 500 or 5000 ng mL⁻¹) (3) Mel+Cort (10+50, 100+500 or
16 1000 pg mL⁻¹+5000 ng mL⁻¹). Pure medium without Mel or Cort served as control. After 15 h
17 of incubation, we assessed the expression of a set of immunity-related genes, including genes
18 encoding for pro-inflammatory proteins (*il-1β*, *cxcl8* and *tnf-α*), acute-phase proteins (*fgl2*, *fth1*,
19 *hepc*, *hp* and *saal*) and key factors of the adaptive immune system (*fcbp4* and *tcrg*). Both Mel
20 and Cort, when used alone or combined at physiological concentrations, significantly
21 influenced immune gene expressions that may lead to a global immune stimulation. Our results
22 support both, an indirect action of the Mel hormone on the immune system through the
23 regulation of intermediates such as Cort, as well as a direct action on immune targets through
24 specific receptors.

25 **Keywords:** Melatonin; Immunity; Gene expression; Cortisol; Pike-perch

26 **1. INTRODUCTION**

27 In response to a growing market demand, aquaculture has strongly developed over the last few
28 decades. In order to improve productivity and profitability in fish culture, stocking density is
29 usually increased, with up to several hundred kg/m³ of fish. However, overcrowding in
30 production units tends to affect fish health, enhancing the susceptibility of fish to infections
31 which is a major bottleneck of aquaculture development (Conde-Sieira et al., 2014). Various
32 efforts have been undertaken to limit disease outbreaks by developing antibiotics and vaccines.
33 However, drug-resistant bacteria and limitations of vaccination have stimulated research on
34 alternatives based on the improvement of immunocompetence of cultured fish species through
35 the use of immunostimulants and the enhancement of fish welfare (Abarike et al., 2018).
36 Improving our knowledge on fish immunity is thus of great interest in order to optimize
37 management strategies and to limit disease outbreaks in fish farms.

38 Immune-neuroendocrine interactions in vertebrates have been at the center of interest for
39 decades and it has become evident that a bi-directional communication between the immune
40 and neuroendocrine systems is essential for the maintenance of homeostasis (Guerrero and
41 Reiter, 2002; Esteban et al., 2006; Mathieu et al., 2014). In fish, the effects of several hormones,
42 including cortisol (Cort), reproductive hormones (17 β -estradiol, testosterone, 11-
43 ketotestosterone, ...), growth hormone (GH) and prolactin (PRL), on immune functions have
44 been extensively documented (Harris and Bird, 2000; Cuesta et al, 2006; Yada, 2007; Paredes
45 et al., 2013; Nardocci et al., 2014; Chaves-Pozo et al., 2018). However, the immune modulation
46 by the melatonin hormone (Mel), a multifunctional molecule in vertebrates, is less understood
47 and merits more attention.

48 In vertebrates, the Mel hormone, a key hormone of the circadian axis, is mainly produced and
49 secreted by the pineal gland during the dark phase of the photoperiod (Vera et al. 2007;
50 Confente et al., 2010; Falcon et al., 2010). Through this daily rhythm, it relays information
51 about the time of the day and year to cells and organs (Kulczykowska et al., 2006; Migaud et
52 al., 2007; López-Patiño et al., 2014). This indoleamine is also known to regulate important
53 physiological functions like thermoregulation and reproduction in a wide range of vertebrates,
54 as well as immunity in mammals (Carrillo-Vico et al., 2005; Dumbell et al., 2016). In teleosts,
55 Mel also acts on important functions such as reproduction, smoltification, osmoregulation and
56 development (Downing et al., 2002; Falcon et al., 2007; 2010). Contrary to mammals, its
57 potential immunomodulatory capacity in teleosts has been rarely investigated (Cuesta et al.,

58 2008). The few available evidence suggest that Mel may act as an important fish immune
59 regulator. This action on immune cells and tissues could involve specific Mel receptors and/or
60 the regulation of the secretion of intermediates (glucocorticoids, GH, PRL) known to act on
61 immune functions (Esteban et al., 2006; Cuesta et al., 2008; Falcon et al., 2010; Esteban et al.,
62 2013).

63 As the main glucocorticoid in vertebrates, Cort is recognized also in teleosts to play a role in
64 stress responses and to be a crucial immunomodulator with complex actions (Esteban et al.,
65 2004; Cuesta et al., 2006; Oliveira et al., 2013). Depending on the type and intensity of stress,
66 Cort may act as an immune activator or suppressor, with acute stress generally resulting in
67 immune-enhancing processes and chronic stress generally leading to immunosuppression (Tort,
68 2011; Nardocci et al., 2014). Since a mutual inhibition has been characterized between stress
69 and circadian axes (López-Patiño et al., 2013; 2014; Conde-Sieira et al., 2014), Cort is a
70 potential intermediate of the indirect immunomodulation by Mel.

71 Over the last few years, pike-perch (*Sander lucioperca*) has become the most promising teleost
72 species of European inland aquaculture thanks to its fast growth and high-quality flesh
73 (Dalsgaard et al., 2013; Overton et al., 2015). However, percid fish seem to be more sensitive
74 to husbandry stressors than other species with a longer history of domestication (Jentoft et al.,
75 2005), which consequently may alter its immune functions (Mathieu et al., 2014). Efforts have
76 thus been deployed to improve pike-perch welfare to optimize its management in aquaculture.

77 Previous studies have proven that the light environment affects pike-perch stress status as well
78 as melatonin release by the pineal gland and that it further modulates its innate immune
79 functions (Baekelandt et al., 2019a,b; 2020). So far, no investigations exist on the mode of
80 action of the melatonin hormone on immune tissues of teleosts. We thus aimed to investigate
81 the direct action of Mel, with and without combination of Cort, on the expression of immune-
82 relevant genes in head kidney and spleen of pike-perch using an *ex vivo* approach. The selected
83 genes encode for pro-inflammatory proteins (*il-1 β* , *cxcl8* and *tnf- α*), acute-phase proteins (*fgl2*,
84 *fth1*, *hepc*, *hp* and *saal*) and key factors of the adaptive immune system (*fkbp4* and *tcrg*).

85 **2. Materials and methods**

86 **2.1. Animals and rearing conditions**

87 The present protocol (19 002 KE) has been carried out in agreement with the local Ethics
88 Committee for Animal Experiments. A stock of 200 pike-perch juveniles (120 ± 10 g) from the
89 Aquaculture Experimental Platform of the University of Lorraine, France, was transferred to
90 URBE facilities at the University of Namur, Belgium. They were maintained in 4 indoor 400-
91 L tanks of a recirculating aquaculture system for 4 weeks. Environmental conditions were kept
92 constant during that period. These were light intensity of 10 lux at water surface, photoperiod
93 of 12L(8:00 - 20:00)/12D, water temperature at 16°C, oxygen saturation of 90%, and a feeding
94 scheme twice a day with commercial pellets at 2% biomass.

95 **2.2. Sampling procedures and incubation**

96 On March 4th and 7th 2019, ten fish from each tank were randomly removed and euthanized
97 (overdose of anesthetic MS-222, 250 mg L⁻¹) before extracting the spleen and the head kidney.
98 Considering the two sampling days, 80 fish were collected in total. Organs were washed thrice
99 with Hanks' balanced salt solution (HBSS, Fisher Scientific, USA). They were then transferred
100 on 12-well culture plates, filled with HBSS supplemented with bovine serum albumin (BSA,
101 0.1%, Fisher Scientific, USA) and ascorbic acid (50 µM, Sigma-Aldrich, USA). The following
102 treatments were applied: (1) Mel (10, 100 or 1000 pg mL⁻¹) (2) Cort (50, 500 or 5000 ng mL⁻¹)
103 (3) Mel+Cort (10+50, 100+500 or 1000 pg mL⁻¹+5000 ng mL⁻¹). Medium without Mel or Cort
104 served as control. Thus, for one sampling day, each treatment was applied to 4 spleen and 4
105 head kidney tissues. The Mel and Cort doses were selected to according to existing literature,
106 considering 2 physiological and 1 pharmacological dose with a 10-fold factor between them.
107 The lowest concentrations of Mel (10 and 100 pg mL⁻¹) correspond to diurnal and nocturnal
108 levels of plasma melatonin for pike-perch maintained under steady conditions (Baekelandt et
109 al., 2019b). The lowest concentrations of Cort (50 and 500 ng mL⁻¹) consider plasma cortisol
110 levels under normal conditions and in response to acute stress (Baekelandt et al., 2019b). After
111 5 and 10 h of incubation, culture media were stored at -80°C and replaced with fresh media.
112 After 15h, culture media and organs were frozen in liquid nitrogen and transferred to -80°C
113 until further analysis.

114 2.3. Gene expression analysis

115 Total RNA isolation was performed using Extract-all® reagent (Eurobio, Paris, France)
116 following the manufacturer's instructions. Tissues were homogenized using a Bullet Blender
117 Storm 24 (NextAdvance, New York, USA) in tubes containing 0.5 mm zirconium oxide beads
118 (Dutscher, Brumath, France). Total RNA was resuspended in 100 µl of RNase-free water. Each
119 RNA sample was subjected to DNase treatment (DNase Ambion, Life Technologies) and
120 reverse-transcription (RevertAid™ H Minus First Strand cDNA Synthesis Kit, Thermo
121 Scientific) following the manufacturer's instructions. cDNA was then diluted 25 times for RT-
122 qPCR analysis and kept at -80°C.

123 The relative expression of targeted genes was investigated by RT-qPCR using specific primers
124 (Table 1, sequences published in Swirplies et al., 2019 and Baekelandt et al., 2019b). Primer
125 efficiencies were validated when ranging between 95 and 106 %. qPCR was performed using
126 SYBR® Green Supermix (Biorad, California, USA). A four-step experimental run protocol was
127 followed: denaturation (10 min at 95 °C), amplification (40 cycles, 10 s at 95 °C, 30 s at 60 °C),
128 melting curve (60 to 95 °C, heating rate 0.075°C s⁻¹) and a final cooling step (4°C) using a
129 QuandStudio™ 5 real-time PCR machine (Applied Biosystems).

130 Relative fold gene expression was calculated following the 2^{-ΔΔCt} method (Livak and
131 Schmittgen, 2001). Ct values were normalized with the geometric mean for *ma-18s*, *β-actin*
132 and *efl-α* whose expressions were stable under tested conditions. Values are expressed as fold
133 change, with the control equaling 1. Among the 10 immune genes targeted, 5 were detected in
134 both organs while the other 5 five were only detected in the head kidney (Table 1).

135 2.4. Lactate dehydrogenase activity

136 In order to evaluate the presence of damage and toxicity of tissues, lactate dehydrogenase
137 (LDH) activity was quantified using LDH Assay Kit (ab102526, Abcam, UK). Activity was
138 assayed according to the manufacturer's instructions. The analysis was performed twice in
139 media collected after 15 h of incubation.

140 2.5. Levels of hormones in culture medium

141 After 0, 5, 10 and 15 h of incubation, Mel and Cort concentrations were measured in the culture
142 medium using a Melatonin ELISA Kit (E-EL-M0788; Elabscience Biotechnology CO.) and a
143 Cortisol ELISA kit (DRG, EIA-1887, DRG International, USA).

144 **2.6. Statistical analysis**

145 Data are expressed as the mean \pm standard error of the mean (SEM). Kolmogorov and
 146 Smirnov's test was used to assess the normality of data sets ($p < .05$) and Bartlett's test was
 147 conducted to evaluate variance homogeneity ($p < .05$). Logarithmic transformations were made
 148 to achieve normality and homoscedasticity when necessary. No significant differences were
 149 detected between tanks (the fish were captured in 4 different tanks of the same RAS) or between
 150 sampling days (the experiment was conducted on two different dates). Results were then
 151 analyzed with a one-way ANOVA considering treatment as a fixed factor. When significant (p
 152 $< .05$), a Tukey's HSD post-hoc test was applied ($p < .05$). When the data, even after log-
 153 transformation, did not meet the assumptions for the parametric tests, a Kruskal-Wallis test for
 154 nonparametric analysis was applied, followed by a pairwise comparison using Dunn tests. The
 155 statistical tests and graphs were performed using JMP 12.1 Software (SAS Institute Inc., North
 156 Carolina, USA) and GraphPad Prism V5.04 (California, USA), respectively.

157 In addition, a redundancy analysis (RDA) and a hierarchical ascending classification (HAC),
 158 considering the Ward's distance, were performed with R software (package ade4) in order to
 159 characterize the samples' distribution regarding gene expression and treatment for both, kidney
 160 and spleen tissues. RDA and clustering analysis such as HAC have proven their value in omics'
 161 research (D'Haeseleer, 2005; Csala et al, 2017; Gold-Bouchot et al, 2017). The Ward's distance
 162 criteria achieved through the clustering enable to minimize the variance within each group
 163 formed by the clustering analysis (Shimodaira, 2002).

164

165 Table 1: Sequences of primers used for gene expression quantification in spleen and head
 166 kidney (HK), published in Swirplies et al. (2019) and Baekelandt et al. (2019b).

Gene	Sense primer (5' to 3')	Antisense primer (5' to 3')	Efficiency (%)
Reference genes			
<i>β-actin</i>	CGACATCCGTAAGGACCTGT	GCTGGAAGGTGGACAGAGAG	100
<i>efl-α</i>	TGATGACACCAACAGCCACT	AAGATTGACCGTCGTTCTGG	101
<i>rna-18s</i>	GCGGTAATTCAGCTCCAATAG	GCGGGACACTCAGTTAAGAGC	98
Target genes			
- in spleen & HK			
<i>cxcl8</i>	AACAGGGATGAGTCTGAGAAGC	GCTTGAAATGAAGTCTTACATGA	100
<i>fgl2</i>	ACTTTGAGGGTGTTCGGGAGTA	ACATATCGTTGTCGGGTCGG	105
<i>fth1</i>	ATTGAGACACACTACCTGGATGA	ACGGATTTAGCTGCTTTCTTTGC	106

<i>fkbp4</i>	ACTTGTAGGTGGAAGCTGTTGAAT	AAAAAGCTGTGTCTGGATGTGTTA	105
<i>il-1β</i>	TTCCCATCATCCACTGACA	ATTCACACACGCACACCATT	102
- in HK			
<i>hepc</i>	CCGTCGTGCTCACCTTTATT	GCCACGTTTGTGTCTGTTGT	97
<i>hp</i>	GCTGAAACTGGGGACATTTACG	GAGCGCAGAGCAGACGATTTTC	104
<i>saal</i>	CTGAAGGAGCTGGTGATATGTG	CTACTCTTTGCTTTTCACCTGATA	105
<i>tcrγ</i>	GTAATGTCTCTGTTGTGCCATATT	TCTCAGAGCAAATGCCATGGTC	99
<i>tnf-α</i>	CTGATTCGCCTCAACGTGTA	GGAGATGGGTCATGAGGAGA	99

168 **3. RESULTS**

169 **Levels of hormones in culture medium**

170 Every 5 h of incubation (before collection and renewal with fresh media), Mel and Cort
 171 concentrations were assessed in the culture media (Table 2), as well as in the stock solutions.
 172 No significant differences were detected between organs of the same treatment or between post-
 173 exposition time points.

174

175 Table 2: Melatonin (Mel, pg mL⁻¹) and cortisol (Cort, ng mL⁻¹) concentrations in culture media of stock solutions
 176 and in culture media after 5, 10 and 15 h of *ex vivo* exposition of spleen and head kidney (HK) from pike-perch.

	Stock solution	5 h	10 h	15 h
Mel10				
Spleen	12 ± 3	9 ± 2	8 ± 3	9 ± 1
HK		11 ± 2	7 ± 3	8 ± 2
Mel100				
Spleen	108 ± 8	92 ± 12	87 ± 7	91 ± 15
HK		102 ± 9	92 ± 13	89 ± 22
Mel1000				
Spleen	981 ± 27	934 ± 33	961 ± 42	943 ± 52
HK		969 ± 46	932 ± 75	961 ± 41
Cort50				
Spleen	68 ± 13	52 ± 24	61 ± 20	46 ± 17
HK		56 ± 17	48 ± 8	43 ± 16
Cort500				
Spleen	541 ± 62	480 ± 75	502 ± 39	482 ± 74
HK		512 ± 42	458 ± 21	462 ± 46
Cort5000				
Spleen	4780 ± 165	4530 ± 256	4620 ± 218	4490 ± 320
HK		4610 ± 184	4580 ± 312	4720 ± 162
Mel10/Cort50				
Spleen	14 ± 3/61 ± 9	11 ± 2/64 ± 11	9 ± 2/48 ± 9	8 ± 3/46 ± 10
HK		11 ± 2/58 ± 8	10 ± 3/47 ± 12	10 ± 2/52 ± 13
Mel100/Cort500				
Spleen	112 ± 10/512 ± 48	94 ± 15/481 ± 59	95 ± 18/473 ± 62	87 ± 8/504 ± 71
HK		92 ± 10/462 ± 68	106 ± 9/493 ± 36	93 ± 16/477 ± 35
Mel1000/Cort5000				
Spleen	1110 ± 59/5120 ± 306	1084 ± 78/4720 ± 256	960 ± 102/4840 ± 184	914 ± 96/4630 ± 317

177

178

179 **Lactate dehydrogenase activity**

180 After 15 h of incubation, no differences in lactate dehydrogenase activity were detected between
181 treatments and control condition, for both organs.

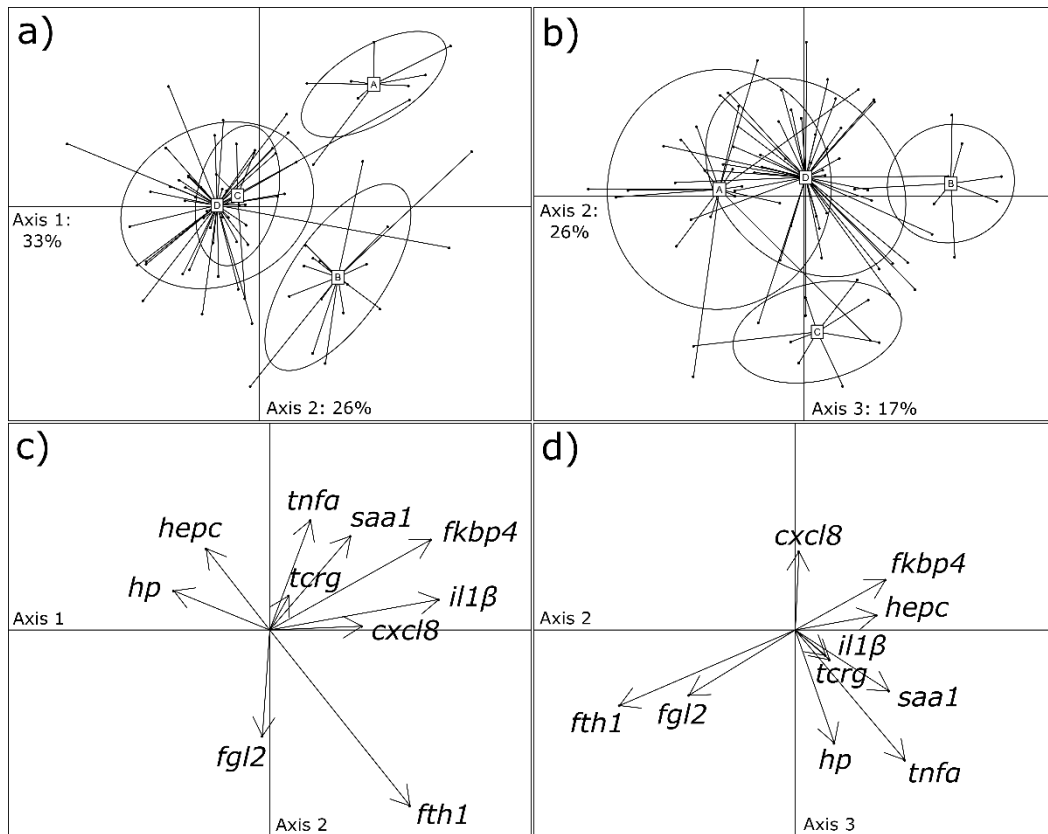
182 **Gene expression**

183 The RDA and clustering analysis of kidney tissues revealed four distinct groups (**Fig. 1**)
184 according to the following treatments: (A) Mel100; (B) Mel10 and Mel+Cort (10+50); (C)
185 Cort500; (D) Control, Mel1000, Cort50, Cort5000, Mel+Cort (100+500) and Mel+Cort
186 (1000+5000). The group “A” is mainly characterized by differentially expressed genes,
187 including *tnf-α*, *saal* and *fkbp4*. The “B” group is defined by *fth1*, *hepc* and *fkbp4*, and the “C”
188 group by *hp* and *tnf-α*. Concerning spleen tissue (**Fig. 2**), 3 groups can be distinguished: (A)
189 Mel10 and Cort50; (B) Mel+Cort (10+50; 100+500; 1000+5000); (C) Control, Cort500,
190 Cort5000, Mel100 and Mel1000. The dispersion, mainly defined on axis 1, is explained by *fgl2*,
191 and to a lesser extent by *il-1β*, *fth1* and *fkbp4* genes.

192 In the head kidney tissue, gene expression of *il-1β* and *fgl2* increased with the lowest Cort
193 concentration. A similar observation was made for *tnf-α*, *il-1β* and *hp* with Cort at 500 ng mL⁻¹
194 (**Fig. 3**). Mel treatments also significantly increased some gene expressions in comparison to
195 the control, including *fgl2*, *il-1β* and *fth1* at 10 pg mL⁻¹, *tnf-α*, *saal* and *il-1β* at 100 pg mL⁻¹
196 and *il-1β* at 1000 pg mL⁻¹. Finally, the mix Cort+Mel significantly increased *fth1* and *il-1β* at
197 the lowest concentration, as well as *fgl2* at pharmacological doses. On the contrary, the latter
198 concentration reduced *fth1* expression.

199 Concerning the spleen tissue (**Fig. 4**), several gene expressions increased significantly,
200 including *fth1*, *il-1β*, *fgl2* and *fkbp4* when exposed to Mel or Cort at the lowest concentration
201 (10 pg mL⁻¹ and 50 ng mL⁻¹, respectively). With the exception of *il-1β*, whose expression
202 decreased with Cort (5000 ng mL⁻¹), higher concentrations of Mel or Cort had no significant
203 effects compared to the control. Similarly, the mix Mel+Cort, whatever the concentration, did
204 not influence immune gene expressions in spleen. However, a significant difference was
205 detected for *il-1β* between the lowest and the highest concentrations.

206

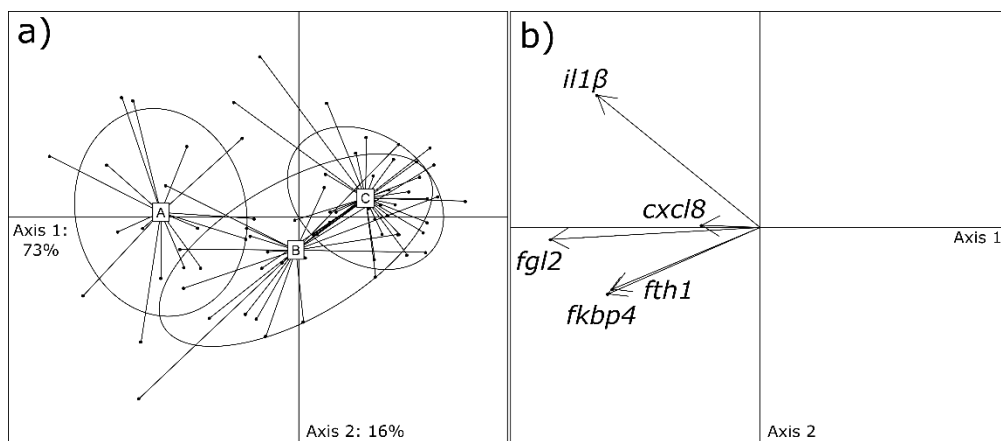


207

208 **Fig. 1:** Projection and clustering, on axes (a) 1 and 2 or (b) 2 and 3 of the redundancy analysis, of 80
 209 head kidney tissues according to their gene expression profiles after *ex vivo* hormonal treatments.
 210 Projection of gene expression outputs on axes (c) 1 and 2 or (d) 2 and 3 of the redundancy analysis. The
 211 cumulative projected inertia of axes 1, 2 and 3 reaches 76%. Clustering revealed four groups: [A]
 212 Mel100; [B] Mel10 and Mel+Cort (10+50); [C] Cort500; [D] Control, Mel1000, Cort50, Cort5000,
 213 Mel+Cort (100+500) and Mel+Cort (1000+5000). For each experimental condition, n=8.

214

215

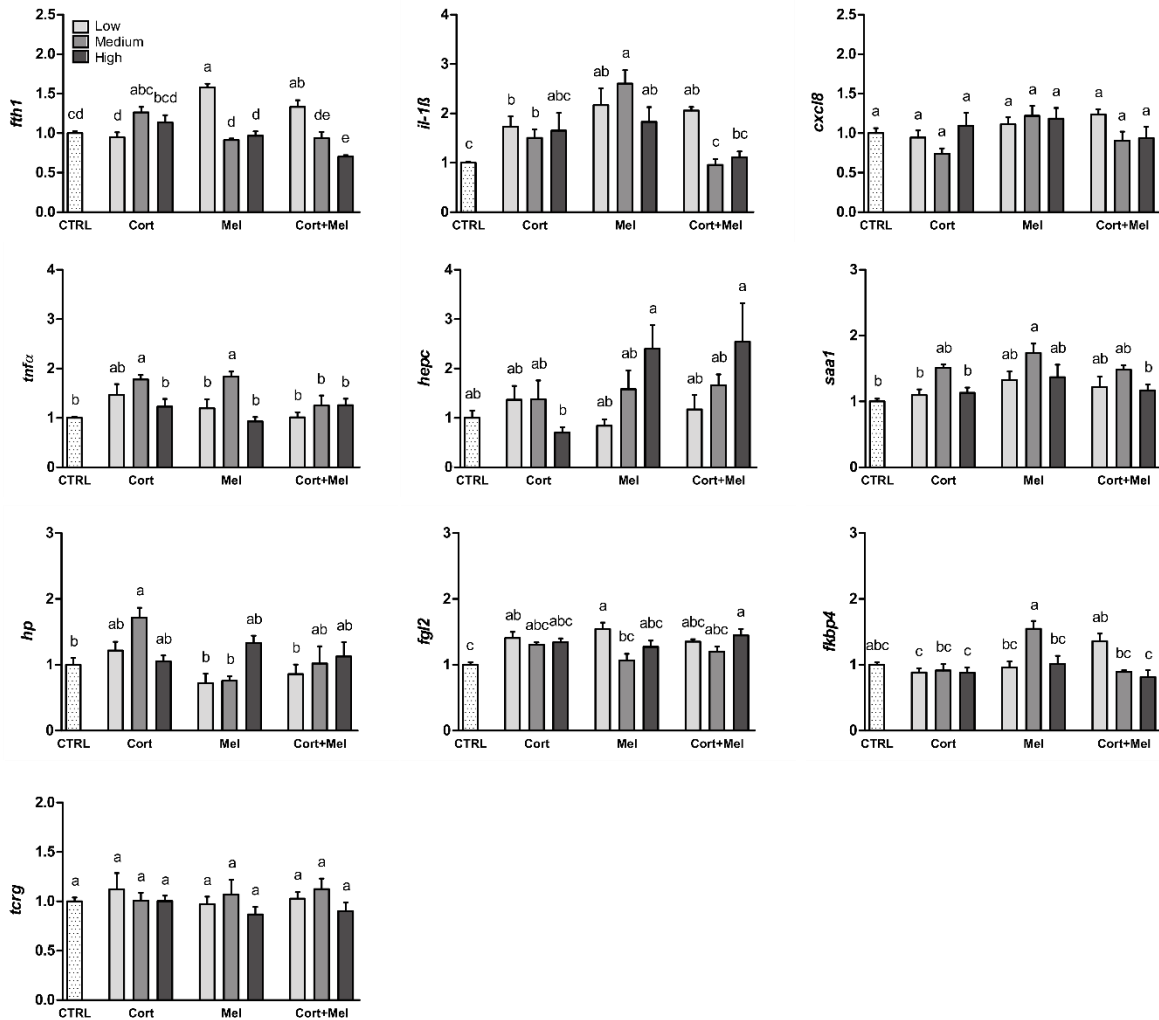


216

217 **Fig. 2:** Projection and clustering (a), on axes 1 and 2 of the redundancy analysis, of 80 spleen tissues
 218 according to their gene expression profiles after *ex vivo* hormonal treatments. Projection (b) of gene
 219 expression outputs on axes 1 and 2 of the redundancy analysis. The cumulative projected inertia of axes

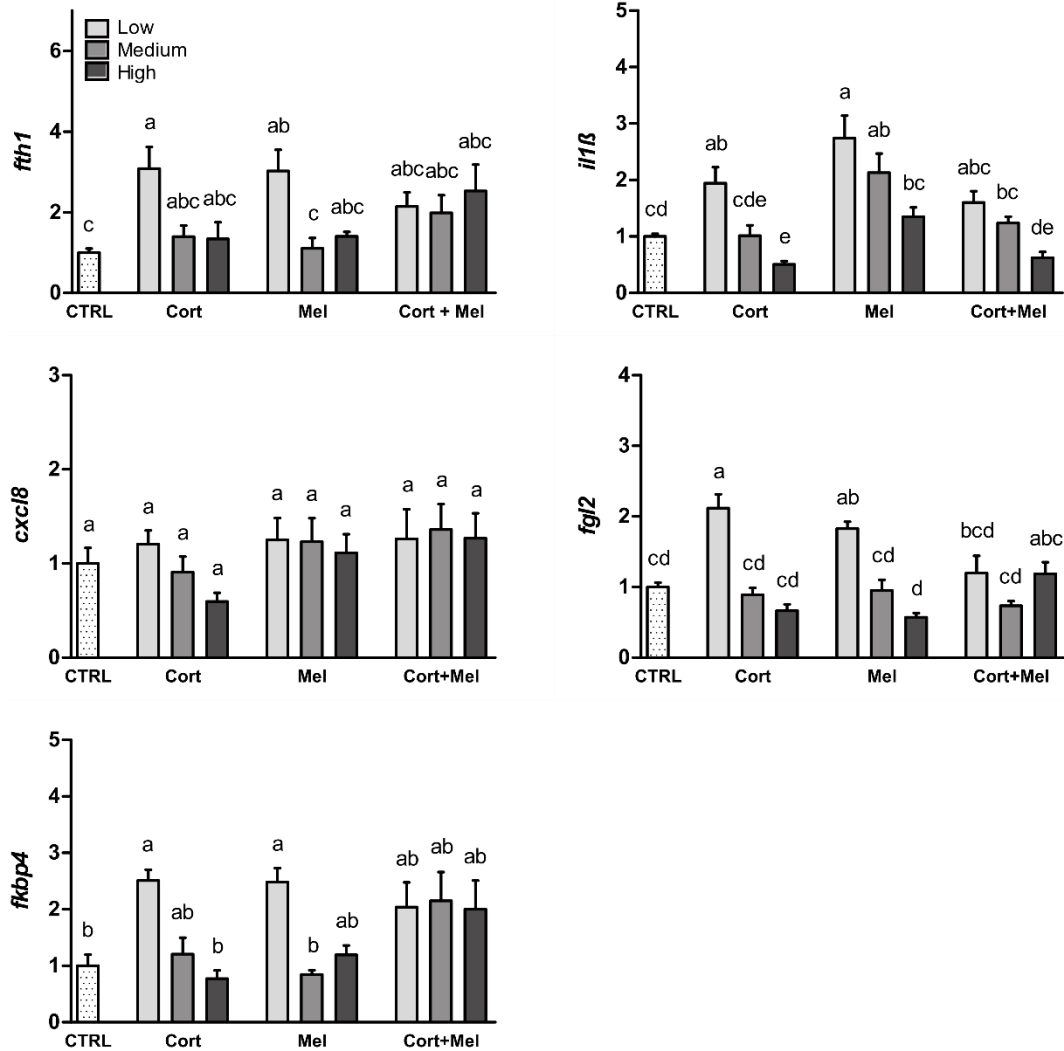
220 1 and 2 is 89%. Clustering revealed three groups: [A] Mel10 and Cort50; [B] Mel+Cort (10+50;
 221 100+500; 1000+5000); [C] Control, Cort500, Cort5000, Mel100 and Mel1000. For each experimental
 222 condition, n=8.

223
 224



225
 226 **Fig. 3: Relative expression of immune-relevant genes in head kidney tissue of pike-perch exposed**
 227 ***ex vivo* to melatonin and cortisol.** Treatments, tested in 3 concentrations (Low, Medium and High),
 228 included (1) Mel (10, 100 or 1000 pg mL⁻¹), (2) Cort (50, 500 or 5000 ng mL⁻¹) and (3) Mel+Cort
 229 (10+50, 100+500 or 1000 pg mL⁻¹+5000 ng mL⁻¹). Medium without Mel or Cort was used as a control.
 230 Data are expressed as means ± SEM (n = 8). Lower case letters indicate significant differences at p <
 231 .05.

232
 233



234

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Fig. 4: Relative expression of immune-relevant genes in spleen tissue of pike-perch exposed *ex vivo* to melatonin and cortisol. Treatments, tested in 3 concentrations (Low, Medium and High), included (1) Mel (10, 100 or 1000 pg mL⁻¹) (2) Cort (50, 500 or 5000 ng mL⁻¹) (3) Mel+Cort (10+50, 100+500 or 1000 pg mL⁻¹+5000 ng mL⁻¹). Medium without Mel or Cort was used as a control. Data are expressed as means ± SEM (n = 8). Lower case letters indicate significant differences at p < .05.

240 4. DISCUSSION

241 The actions of melatonin, with or without combination of cortisol, on immunity were
242 investigated by analyzing immune-related gene expressions in the main fish lymphoid organs,
243 the head kidney and the spleen. Both organs were exposed *ex vivo* to several concentrations of
244 Mel and/or Cort. The present protocol ensured constant concentrations of both hormones in
245 culture media throughout the experiment. Furthermore, the LDH activity revealed no damage
246 or toxicity of tissues following 15 h of hormonal treatments.

247 Globally, the most positively influenced gene expressions following Mel treatments included
248 two pro-inflammatory genes, namely *tnf- α* and *il-1 β* , three acute-phase protein (APP) genes,
249 *saal*, *fgl2* and *fh1*, as well as *fkbp4*, a gene involved in the regulation of immune gene
250 expression in B and T lymphocytes. Mel has been characterized as an immunostimulant
251 molecule under basal or immunosuppressive conditions, as demonstrated by enhanced immune
252 functions following its injection or ingestion in various vertebrates, such as fish (Cuesta et al.,
253 2008; Ren et al., 2015), birds (Brennan et al., 2002; Singh et al., 2010) and mammals (Liu et
254 al., 2001; Peña et al., 2007; Ahmad and Haldar, 2010). However, in the case of inflammatory
255 responses, Mel exerts anti-inflammatory properties to protect the organism from host tissue
256 damage (Carrillo-Vico et al., 2013; Tarocco et al., 2019). This anti-inflammatory function has
257 mainly been described in mammals (Lin et al., 2011; Xia et al., 2012) but only once in teleost.
258 In common carp (*Cyprinus carpio*), its administration during zymosan-induced peritonitis
259 reduced leukocyte migration to the peritoneum and induced a decrease of the respiratory burst
260 activity in peritoneal leukocytes (Kepka et al., 2015).

261 Considering pro-inflammatory and APP genes, the present results support the
262 immunoenhancing properties of the molecule under basal conditions (unstimulated immunity).
263 In pike-perch, it has been hypothesized that Mel would act on inflammatory cytokines since *in*
264 *vivo*, the daily cyclic release of Mel by the pineal gland were correlated with the day-night
265 variations of *tnf- α* and *il-1 β* gene expressions in the head kidney (Baekelandt et al., 2019).
266 Moreover, exogenous Mel has been described to increase *il-1 β* expression in the head kidney
267 of gilthead seabream (*Sparus aurata*) (Cuesta et al., 2008).

268 The acute-phase response is a series of non-specific and complex reactions occurring soon after
269 the onset of stress, injury, trauma, infection and inflammation, which aim to eliminate the
270 infectious agents and to restore homeostasis (Tothova et al., 2014; Yu et al., 2017). So far, the
271 only data showing an impact of Mel on APPs concern mammals. In castrated dogs, exogenous

272 Mel significantly reduced APPs and inflammatory cytokines, including SAA, CRP, IL-1 β and
273 TNF- α (Nazifi et al., 2020). In bovine mammary epithelial cells, Mel decreased LPS-induced
274 expression of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6), chemokines (chemokine CC
275 motif ligand (CCL)2, CCL5) and positive APPs (SAA, haptoglobin, C-reactive protein,
276 ceruloplasmin, α -1 antitrypsin) (Yu et al., 2017). While these studies defined a negative
277 regulation of acute-phase response during inflammatory processes, our results are consistent
278 with immunoenhancing properties of Mel under basal conditions.

279 In mammals, Mel influences the acquired immune response. T lymphocytes were shown to be
280 modulated by melatonin, from its development in thymus to its differentiation and even memory
281 (Garcia-Mauriño et al. 1999; Guerrero and Reiter, 2002; Glebezdina et al., 2019; Luo et al.,
282 2020). Several studies concluded that melatonin also plays a critical role in regulating the
283 activation of B cells (Yu et al., 2000; Cernysiov et al., 2009; Luo et al., 2020). The only study
284 considering potential Mel actions on T and B cells in teleosts revealed no effect on specific
285 markers at the transcript level (TCR α - T cell receptor alpha chain - and IgM, respectively),
286 suggesting a lack of effects on lymphocyte activation or proliferation (Cuesta et al., 2008).
287 However, the modulation of *fcbp4* following Mel treatment in this study may suggest an action
288 on fish specific immunity, as observed in other vertebrates like birds and mammals (Kharwar
289 et al., 2015; Li et al., 2015; Chen et al., 2016). Nevertheless, further investigations considering
290 the acquired immunity are needed.

291 No information was available about the potential direct and/or indirect actions of Mel on these
292 immune markers. In both organs, Mel activated a set of immune-related genes supporting the
293 hypothesis that Mel may act through specific receptors that are located on fish immune cells.
294 In vertebrates, several G protein-coupled membrane receptors with high affinity for Mel have
295 been identified, namely MT1 and MT2 (Dubocovich and Markowska., 2005). In addition, a
296 third melatonin receptor, Mel1c, was found exclusively in fish (*Xenopus* sp.) and birds
297 (Dufourny et al., 2008). The Mel receptors in mammals are expressed by numerous tissues such
298 as immune cells and tissues. In the human immune system, they are distributed in B and T
299 lymphocytes, monocytes, NK cells and mast cells (Carrillo-Vico et al., 2003; Pozo et al., 2004;
300 Lardone et al., 2009; Maldonado et al., 2010). They have further been detected in spleen,
301 thymus and lymphocytes of various vertebrates (rats, mice and birds, see Pozo et al., 1997;
302 Carrillo-Vico et al., 2003; Sanchez-Hidalgo et al., 2008; Wronka et al., 2008; Singh et al.,
303 2016). Concerning fish, Park et al. (2006) and Confente et al. (2010) described MT2 and MT1

304 in spleen of rabbitfish and Senegalese sole, respectively. However, their function in spleen
305 remains unclear and further characterization of Mel receptors in fish immune tissues are needed.

306 While the pineal gland is the main source of Mel, several extrapineal sources of this
307 indoleamine have been identified in several vertebrates like the retina, skin and gastrointestinal
308 tract (Wiechmann et al., 2013; Acuña-Castroviejo et al., 2014). Mel production has also been
309 detected in immune cells and tissues, including human lymphocytes, macrophages and Jurkat
310 cells (Carrillo-Vico et al., 2004; Lardone et al., 2006; Markus et al., 2017), murine thymus,
311 spleen, bone marrow cells and RAW264.7 macrophages (Gómez-Corvera et al., 2009; Muxel
312 et al., 2012) and rat mast cells and macrophages (Martins et al., 2004; Maldonado et al., 2010).
313 Considering that Mel receptors are found in immune cells and tissues, this immune-synthesized
314 Mel seems to play paracrine, autocrine and intracrine functions. In teleosts, the production of
315 Mel by immune cells and tissues has not yet been investigated, but following the results of our
316 gene expression experiment, its potential production and subsequent effects on immunity
317 cannot be excluded.

318 Cortisol is considered as the main hormone of stress responses. It is produced by interrenal cells
319 located in the head kidney of teleosts (Tort et al., 2011). The functions of cortisol during stress
320 reactions are numerous and include physiological, endocrine and immunological responses
321 (Tort et al., 2011; Cortés et al., 2013; Mathieu et al., 2014). Many studies have focused on the
322 regulation of immune defense through corticosteroids and both, activation and inhibition of
323 immune mediators have been described, depending on the stress event. While acute stress is
324 usually associated to immune activation, chronic stress is characterized by long-term exposure
325 to cortisol with subsequent immune depression or suppression (for further information see Tort
326 et al., 2011; Nardocci et al., 2014). In the present experiment, Cort influenced several immune
327 gene expressions in both, spleen and head kidney, depending on concentration. On the one
328 hand, in spleen, the lowest physiological Cort concentration led to an increase of pro-
329 inflammatory gene *il-1 β* , acute-phase genes *fgl2* and *fth1* as well as *fkbp4*. On the other hand,
330 *il-1 β* expression decreased with the pharmacological dose of Cort. Such action on pro-
331 inflammatory cytokine *il-1 β* has already been described *in vivo* in rainbow trout (*Oncorhynchus*
332 *mykiss*) following Cort application (Cortés et al., 2013). This effect may be explained by an
333 inhibition of NF- κ B signaling, leading to a decrease in the production of pro-inflammatory
334 cytokines such as IL-1 (Sternberg, 2006). In the head kidney tissue, only the high physiological
335 dose of Cort (500 ng mL⁻¹) led to a different expression profile, with increases in acute-phase
336 and pro-inflammatory genes, i.e. *fth1*, *il-1 β* , *tnf- α* and *hp*. While in spleen a stimulatory effect

337 was mainly observed at 50 ng mL⁻¹, which was lost at higher concentrations, in head kidney
338 tissues, stimulation was only observed at a concentration of 500 ng mL⁻¹. These different
339 sensitivities may be explained by different expressions or activities of glucocorticoids receptors
340 (GR). Both GR and mineralocorticoids receptors (MR) are capable of binding cortisol and, in
341 fish, four cortisol receptors (GR1a, GR1b, GR2 and MR) have been described whose activations
342 are concentration-dependent (Stolte et al., 2008; Nardocci et al., 2013). In addition, the dual
343 endocrine and hematopoietic functions of the head kidney tissue must be considered and
344 potential production and release of cortisol by the organ may have influenced the results.

345 In conclusion, both hormones at physiological concentrations significantly influenced the
346 immune-related genes in the present *ex vivo* experiment. In both organs, Mel treatment led to
347 an increase in immune-related genes, including genes involved in the inflammatory process,
348 acute-phase response and acquired immune response. These results confirm the
349 immunoenhancing properties of Mel under basal immune conditions in teleost. We further
350 demonstrated a direct action of Mel on immune organs. An indirect action of possibly even
351 greater importance, needs to be addressed in studies on melatonin-immunity interactions. In
352 addition, future investigations should consider the actions of the potential immune-derived
353 melatonin on the immunity of teleosts.

354

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