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

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# ABSTRACT

The melatonin hormone, which is a multifunctional molecule in vertebrates, has been shown to exert complex actions on the immune system of mammals. In teleosts, the immunomodulatory capacity of this hormone has seldom been investigated. In the present experiment, we exposed *ex vivo* spleen and head kidney tissues of pike-perch to melatonin (Mel) and cortisol (Cort). We applied three concentrations of both hormones, alone and in combination, namely (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 $mL^{-1}$). Pure medium without Mel or Cort served as control. After 15 h of incubation, we assessed the expression of a set of immunity-related genes, including genes encoding for pro-inflammatory proteins (*il-1β*, *cxcl8* and *tnf-α*), acute-phase proteins (*fgl2*, *fth1*, *hepc*, *hp* and *saa1*) and key factors of the adaptive immune system (*fκbp4* and *tcrg*). Both Mel and Cort, when used alone or combined at physiological concentrations, significantly influenced immune gene expressions that may lead to a global immune stimulation. Our results support both, an indirect action of the Mel hormone on the immune system through the regulation of intermediates such as Cort, as well as a direct action on immune targets through specific receptors.



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

# 1. INTRODUCTION

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

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

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

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

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

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

Previous studies have proven that the light environment affects pike-perch stress status as well as melatonin release by the pineal gland and that it further modulates its innate immune functions (Baekelandt et al., 2019a,b; 2020). So far, no investigations exist on the mode of action of the melatonin hormone on immune tissues of teleosts. We thus aimed to investigate the direct action of Mel, with and without combination of Cort, on the expression of immune-relevant genes in head kidney and spleen of pike-perch using an *ex vivo* approach. The selected genes encode for pro-inflammatory proteins (*il-1β*, *cxcl8* and *tnf-α*), acute-phase proteins (*fgl2*, *fth1*, *hepc*, *hp* and *saa1*) and key factors of the adaptive immune system (*fκbp4* and *tcrg*).

# 2. Materials and methods

## 2.1. Animals and rearing conditions

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

## 2.2. Sampling procedures and incubation

On March 4$^{th}$ and 7$^{th}$ 2019, ten fish from each tank were randomly removed and euthanized (overdose of anesthetic MS-222, 250 mg $L^{-1}$) before extracting the spleen and the head kidney. Considering the two sampling days, 80 fish were collected in total. Organs were washed thrice with Hanks' balanced salt solution (HBSS, Fisher Scientific, USA). They were then transferred on 12-well culture plates, filled with HBSS supplemented with bovine serum albumin (BSA, 0.1%, Fisher Scientific, USA) and ascorbic acid (50 µM, Sigma-Aldrich, USA). The following treatments were applied: (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 served as control. Thus, for one sampling day, each treatment was applied to 4 spleen and 4 head kidney tissues. The Mel and Cort doses were selected to according to existing literature, considering 2 physiological and 1 pharmacological dose with a 10-fold factor between them. The lowest concentrations of Mel (10 and 100 pg $mL^{-1}$) correspond to diurnal and nocturnal levels of plasma melatonin for pike-perch maintained under steady conditions (Baekelandt et al., 2019b). The lowest concentrations of Cort (50 and 500 ng $mL^{-1}$) consider plasma cortisol levels under normal conditions and in response to acute stress (Baekelandt et al., 2019b). After 5 and 10 h of incubation, culture media were stored at -80°C and replaced with fresh media. After 15h, culture media and organs were frozen in liquid nitrogen and transferred to -80°C until further analysis.

## 2.3. Gene expression analysis

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

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

Relative fold gene expression was calculated following the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Ct values were normalized with the geometric mean for *rna-18s*, *β-actin* and *ef1-α* whose expressions were stable under tested conditions. Values are expressed as fold change, with the control equaling 1. Among the 10 immune genes targeted, 5 were detected in both organs while the other 5 five were only detected in the head kidney (Table 1).

## 2.4. Lactate dehydrogenase activity

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

## 2.5. Levels of hormones in culture medium

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

## 2.6. **Statistical analysis**

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

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

Table 1: Sequences of primers used for gene expression quantification in spleen and head 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 | | | |
| *β-actin* | CGACATCCGTAAGGACCTGT | GCTGGAAGGTGGACAGAGAG | 100 |
| *ef1-α* | TGATGACACCAACAGCCACT | AAGATTGACCGTCGTTCTGG | 101 |
| *rna-18s* | GCGGTAATTCCAGCTCCAATAG | GCGGGACACTCAGTTAAGAGC | 98 |
| Target genes | | | |
| - in spleen & HK | | | |
| *cxcl8* | AACAGGGATGAGTCTGAGAAGC | GCTTGGAAATGAAGTCTTACATGA | 100 |
| *fgl2* | ACTTTGAGGGTGTTCGGGAGTA | ACATATCGTTGTCGCGGTCGG | 105 |
| *fth1* | ATTGAGACACACTACCTGGATGA | ACGGATTTAGCTGCTTTCTTTGC | 106 |

| | | | | |
|---|---|---|---|---|
| | *fκbp4* | ACTTGTAGGTGGAACTGTTTGAAT | AAAAAGCTGTGTCTGGATGTGTTA | 105 |
| | *il-1β* | TTTCCCATCATCCACTGACA | ATTCACACACGCACACCATT | 102 |
| - | in HK | | | |
| | *hepc* | CCGTCGTGCTCACCTTTATT | GCCACGTTTGTGTCTGTTGT | 97 |
| | *hp* | GCTGAAACTGGGGACATTTACG | GAGCGCAGAGCAGACGATTTC | 104 |
| | *saa1* | CTGAAGGAGCTGGTGATATGTG | CTACTCTTTGCTTTTCACCTGATA | 105 |
| | *tcrg* | GTAATGTCTCTGTTGTGCCATATT | TCTCAGAGCAAATGCCATGGTC | 99 |
| | *tnf-α* | CTGATTCGCCTCAACGTGTA | GGAGATGGGTCATGAGGAGA | 99 |

167

# 3. RESULTS

## Levels of hormones in culture medium

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

Table 2: Melatonin (Mel, pg $mL^{-1}$) and cortisol (Cort, ng $mL^{-1}$) concentrations in culture media of stock solutions 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 |

| | | | |
|---|---|---|---|
| HK | 1060 ± 68/4950 ± 192 | 944 ± 138/4920 ± 281 | 983 ± 92/5030 ± 127 |

## Lactate dehydrogenase activity

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

## Gene expression

The RDA and clustering analysis of kidney tissues revealed four distinct groups (**Fig. 1**) according to the following treatments: (A) Mel100; (B) Mel10 and Mel+Cort (10+50); (C) Cort500; (D) Control, Mel1000, Cort50, Cort5000, Mel+Cort (100+500) and Mel+Cort (1000+5000). The group "A" is mainly characterized by differentially expressed genes, including *tnf-α*, *saa1* and *fκbp4*. The "B" group is defined by *fth1*, *hepc* and *fκbp4*, and the "C" group by *hp* and *tnf-α*. Concerning spleen tissue (**Fig. 2**), 3 groups can be distinguished: (A) Mel10 and Cort50; (B) Mel+Cort (10+50; 100+500; 1000+5000); (C) Control, Cort500, Cort5000, Mel100 and Mel1000. The dispersion, mainly defined on axis 1, is explained by *fgl2*, and to a lesser extent by *il-1β*, *fth1* and *fκbp4* genes.

In the head kidney tissue, gene expression of *il-1β* and *fgl2* increased with the lowest Cort concentration. A similar observation was made for *tnf-α*, *il-1β* and *hp* with Cort at 500 ng $mL^{-1}$ (**Fig. 3**). Mel treatments also significantly increased some gene expressions in comparison to the control, including *fgl2*, *il-1β* and *fth1* at 10 pg $mL^{-1}$, *tnf-α*, *saa1* and *il-1β* at 100 pg $mL^{-1}$ and *il-1β* at 1000 pg $mL^{-1}$. Finally, the mix Cort+Mel significantly increased *fth1* and *il-1β* at the lowest concentration, as well as *fgl2* at pharmacological doses. On the contrary, the latter concentration reduced *fth1* expression.

Concerning the spleen tissue (**Fig. 4**), several gene expressions increased significantly, including *fth1*, *il-1β*, *fgl2* and *fκbp4* when exposed to Mel or Cort at the lowest concentration (10 pg $mL^{-1}$ and 50 ng $mL^{-1}$, respectively). With the exception of *il-1β*, whose expression decreased with Cort (5000 ng $mL^{-1}$), higher concentrations of Mel or Cort had no significant effects compared to the control. Similarly, the mix Mel+Cort, whatever the concentration, did not influence immune gene expressions in spleen. However, a significant difference was detected for *il-1β* between the lowest and the highest concentrations.

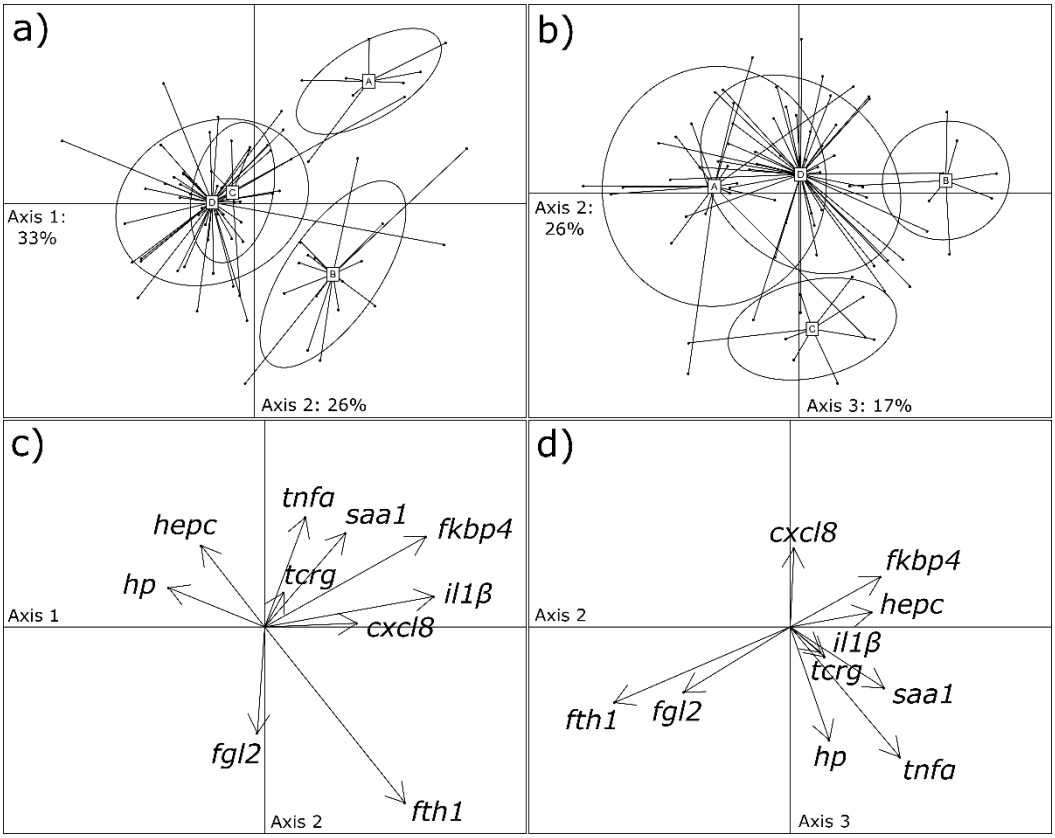


**Fig. 1**: Projection and clustering, on axes (a) 1 and 2 or (b) 2 and 3 of the redundancy analysis, of 80 head kidney tissues according to their gene expression profiles after *ex vivo* hormonal treatments. Projection of gene expression outputs on axes (c) 1 and 2 or (d) 2 and 3 of the redundancy analysis.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

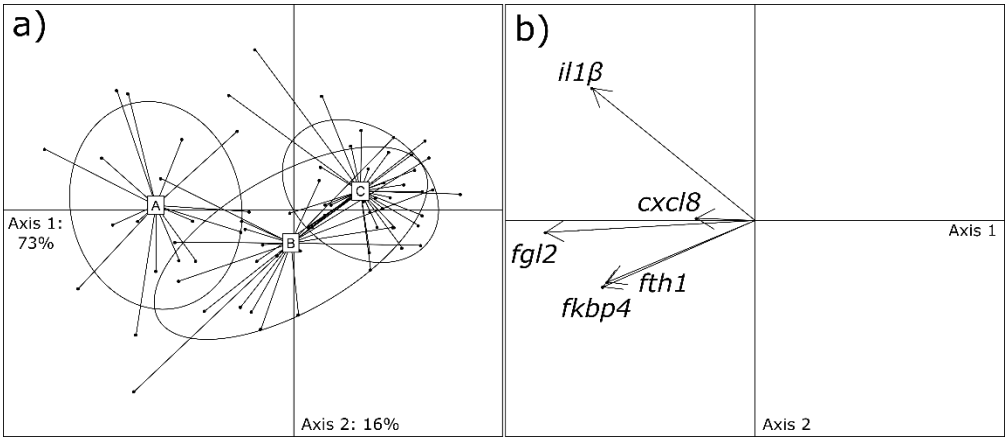


**Fig. 2**: Projection and clustering (a), on axes 1 and 2 of the redundancy analysis, of 80 spleen tissues according to their gene expression profiles after *ex vivo* hormonal treatments. Projection (b) of gene expression outputs on axes 1 and 2 of the redundancy analysis. The cumulative projected inertia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

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

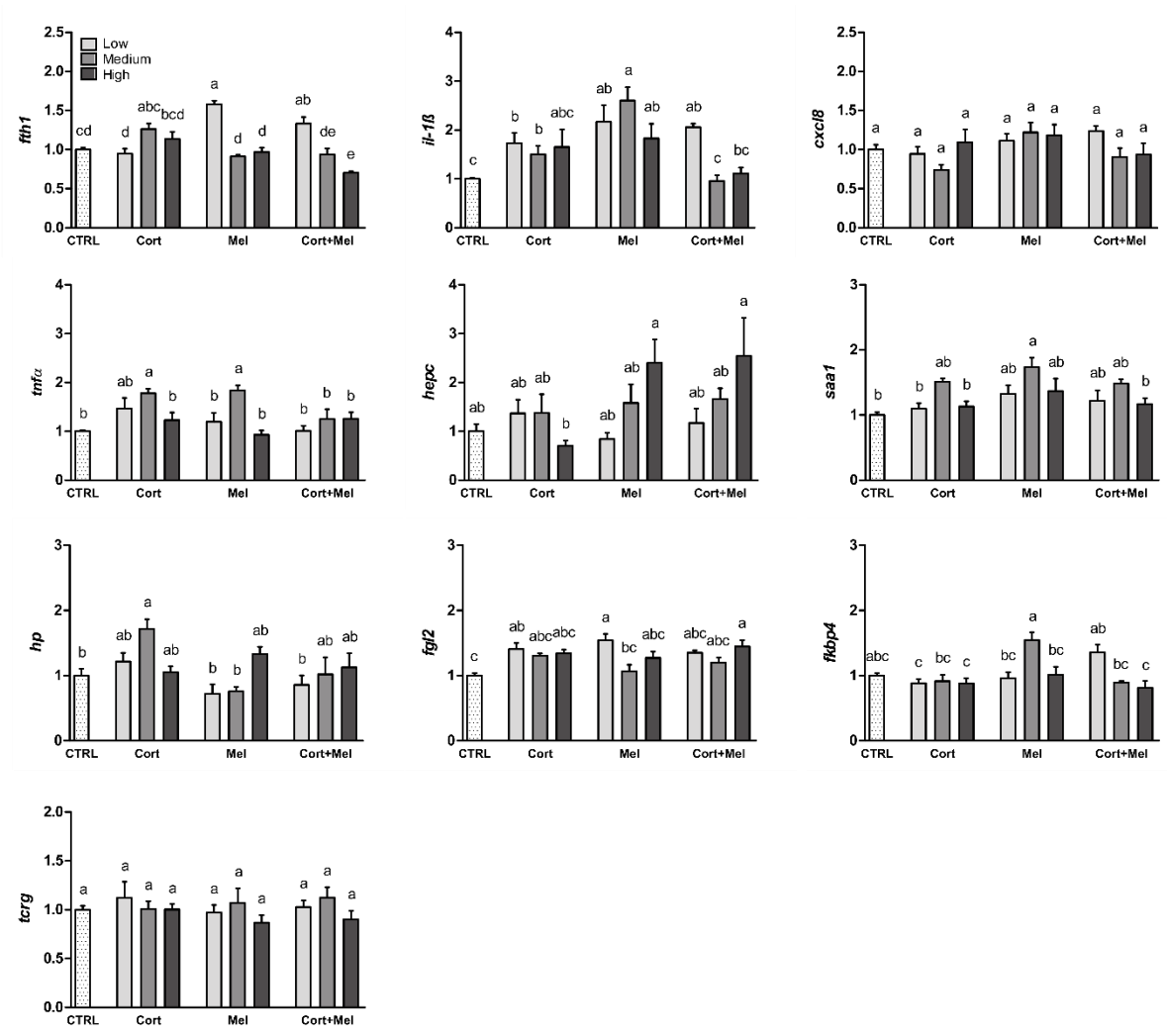


**Fig. 3: Relative expression of immune-relevant genes in head kidney tissue of pike-perch exposed *ex vivo* to melatonin and cortisol**. Treatments, tested in 3 concentrations (Low, Medium and High), included (1) Mel (10, 100 or 1000 pg $mL^{-1}$), (2) Cort (50, 500 or 5000 ng $mL^{-1}$) and (3) Mel+Cort (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 $p < .05$.

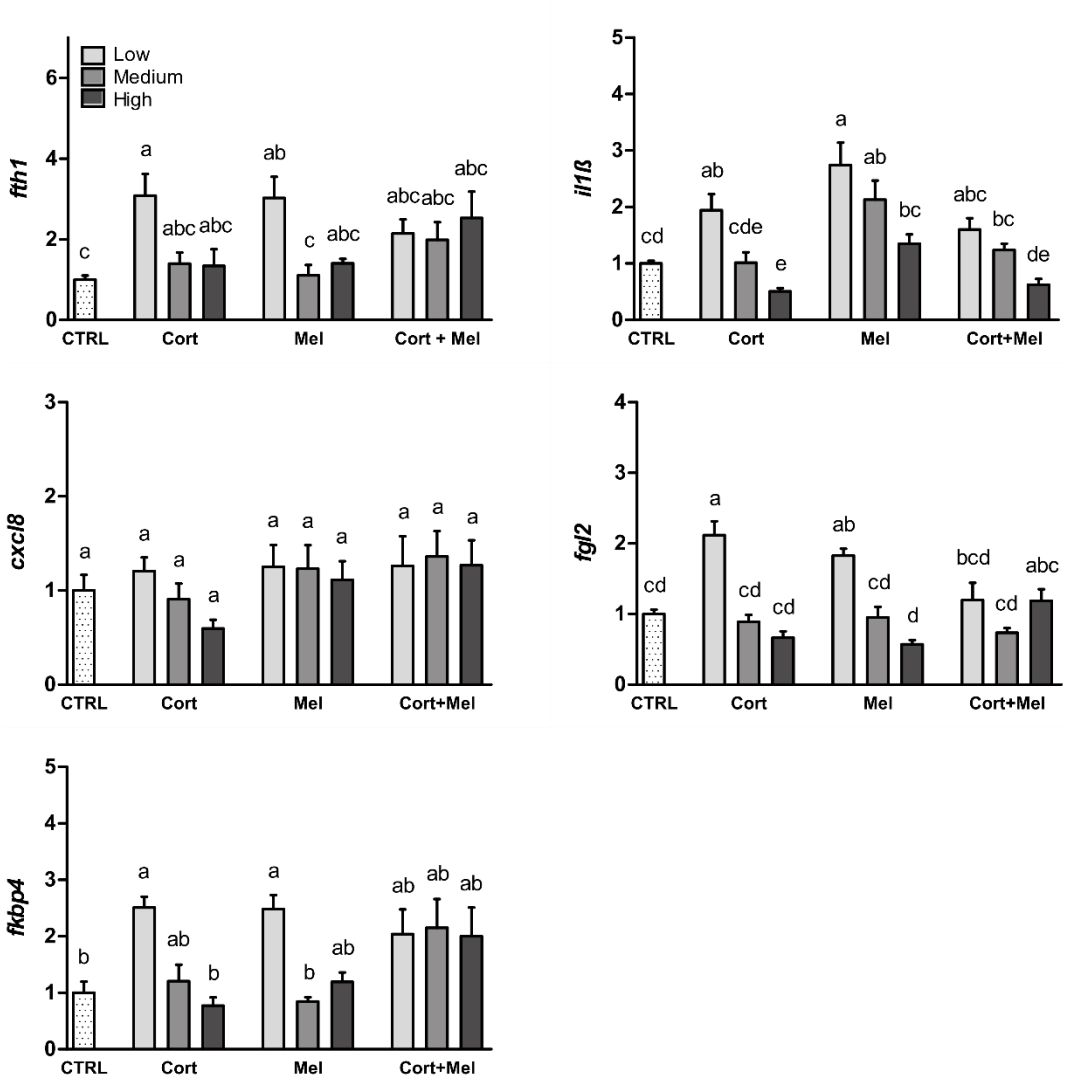


**Fig. 4: Relative expression of immune-relevant genes in spleen tissue of pike-perch exposed *ex vivo* to melatonin and cortisol**. Treatments, tested in 3 concentrations (Low, Medium and High), included (1) Mel (10, 100 or 1000 pg $mL^{-1}$) (2) Cort (50, 500 or 5000 ng $mL^{-1}$) (3) Mel+Cort (10+50, 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 $p < .05$.

# 4. DISCUSSION

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

Globally, the most positively influenced gene expressions following Mel treatments included two pro-inflammatory genes, namely *tnf-α* and *il-1β*, three acute-phase protein (APP) genes, *saa1*, *fgl2* and *fth1*, as well as *fκbp4*, a gene involved in the regulation of immune gene expression in B and T lymphocytes. Mel has been characterized as an immunostimulant molecule under basal or immunosuppressive conditions, as demonstrated by enhanced immune functions following its injection or ingestion in various vertebrates, such as fish (Cuesta et al., 2008; Ren et al., 2015), birds (Brennan et al., 2002; Singh et al., 2010) and mammals (Liu et al., 2001; Peña et al., 2007; Ahmad and Haldar, 2010). However, in the case of inflammatory responses, Mel exerts anti-inflammatory properties to protect the organism from host tissue damage (Carrillo-Vico et al., 2013; Tarocco et al., 2019). This anti-inflammatory function has mainly been described in mammals (Lin et al., 2011; Xia et al., 2012) but only once in teleost. In common carp (*Cyprinus carpio*), its administration during zymosan-induced peritonitis reduced leukocyte migration to the peritoneum and induced a decrease of the respiratory burst activity in peritoneal leukocytes (Kepka et al., 2015).

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

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

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

In mammals, Mel influences the acquired immune response. T lymphocytes were shown to be modulated by melatonin, from its development in thymus to its differentiation and even memory (Garcia-Mauriño et al. 1999; Guerrero and Reiter, 2002; Glebezdina et al., 2019; Luo et al., 2020). Several studies concluded that melatonin also plays a critical role in regulating the activation of B cells (Yu et al., 2000; Cernysiov et al., 2009; Luo et al., 2020). The only study considering potential Mel actions on T and B cells in teleosts revealed no effect on specific markers at the transcript level (TCRα - T cell receptor alpha chain - and IgM, respectively), suggesting a lack of effects on lymphocyte activation or proliferation (Cuesta et al., 2008). However, the modulation of *fκbp4* following Mel treatment in this study may suggest an action on fish specific immunity, as observed in other vertebrates like birds and mammals (Kharwar et al., 2015; Li et al., 2015; Chen et al., 2016). Nevertheless, further investigations considering the acquired immunity are needed.

No information was available about the potential direct and/or indirect actions of Mel on these immune markers. In both organs, Mel activated a set of immune-related genes supporting the hypothesis that Mel may act through specific receptors that are located on fish immune cells. In vertebrates, several G protein-coupled membrane receptors with high affinity for Mel have been identified, namely MT1 and MT2 (Dubocovich and Markowska., 2005). In addition, a third melatonin receptor, Mel1c, was found exclusively in fish (*Xenopus* sp.) and birds (Dufourny et al., 2008). The Mel receptors in mammals are expressed by numerous tissues such as immune cells and tissues. In the human immune system, they are distributed in B and T lymphocytes, monocytes, NK cells and mast cells (Carrillo-Vico et al., 2003; Pozo et al., 2004; Lardone et al., 2009; Maldonado et al., 2010). They have further been detected in spleen, thymus and lymphocytes of various vertebrates (rats, mice and birds, see Pozo et al., 1997; Carrillo-Vico et al., 2003; Sanchez-Hidalgo et al., 2008; Wronka et al., 2008; Singh et al., 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-synthetized
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-1β*, acute-phase genes *fgl2* and *fth1* as well as *fκbp4*. On the other hand,
330 *il-1β* expression decreased with the pharmacological dose of Cort. Such action on pro-
331 inflammatory cytokine *il-1β* has already been described *in vivo* in rainbow trout (*Oncorhyncus*
332 *mykiss*) following Cort application (Cortés et al., 2013). This effect may be explained by an
333 inhibition of NF-κB 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-1β, tnf-α* and *hp*. While in spleen a stimulatory effect

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

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

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