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Fish immunity

modulation by the light environment and the melatonin hormone in a percid fish, the pike-perch

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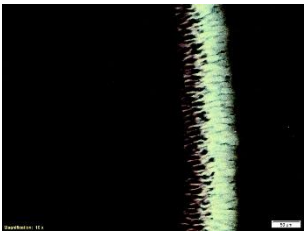

A dissertation submitted by

Sébastien BAEKELANDT

In fulfillment of the requirements

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ABSTRACT

Pike-perch (*Sander lucioperca*) is a valuable fish candidate for the expansion of the European aquaculture industry. However, several bottlenecks hamper its development including high mortalities that may reflect a low welfare related to a high stress responsiveness. The first objective of this thesis was thus to identify the main husbandry practices and environmental factors affecting pike-perch culture. From every tested modality, the light environment was identified as one of the most determining factor influencing its physiology.

Pike-perch possesses a *tapetum lucidum* that is a reflecting layer in the retina. It increases the eye sensitivity to the light, which enable that fish to hunt during dusk and night. However, inappropriate light environment for its culture may affect its physiology, including a higher stress level and a decrease in immunocompetence and growth. Light information is perceived by photoreceptors and converted by the pineal gland into a melatonin signal. This hormone conveys the information about the time of the day and the night to cells and organs and it is known to modulate numerous functions in vertebrates including behavior, reproduction and growth. However, while well described in mammals, the modulation of the immune system by melatonin has been poorly investigated in teleosts. We thus hypothesized that this hormone may play a central role by being a relay between the light, the stress axis and the immune system.

Several experiments were designed in order to better characterize the modulation of the immune system by the light environment, with special consideration for the melatonin hormone as a potential intermediate. We thus investigated the daily rhythmicity of innate immune markers and the impacts of several light intensities and spectrum on fish physiology. We then considered the modulation of the immune system by season-simulated photoperiods. All these experiments defined together that light modulates pike-perch immunity and that melatonin is involved. Some innate immune markers follow 24-h rhythmicity that is correlated to the day-night cycle. Natural photoperiod variations and associated decrease or increase in circulating melatonin led to a significant modulation of the immune system, with innate markers being stimulated under the fall-simulated photoperiod. Furthermore, unsuitable light environments affect fish immunity and growth and such impacts can be associated to both an increase in stress status and a decrease in circulating melatonin.

These experiments defined together that melatonin is involved in the modulation of the immune system in teleosts, but no information is available about the mode of action. It may include a direct action via specific melatonin receptors on immune targets or an indirect action through the regulation of hormonal secretions, including cortisol. An *ex vivo* experiment conducted on spleen and head kidney tissues of pike-perch aimed to better characterize the mode of action. Results suggest that both direct and indirect actions are involved.

All in all, the light environment can profoundly affect the fish immune system by inducing a higher stress status but also by modulating melatonin production and secretion. In addition, the action of melatonin on immune targets involves both direct actions through specific melatonin receptors and indirect actions through intermediates such as cortisol.

RÉSUMÉ

Le sandre (*Sander lucioperca*) est un poisson de haute valeur pour l'expansion de l'industrie aquacole européenne. Cependant, plusieurs freins entravent son développement, y compris des taux de mortalité élevés qui peuvent refléter un faible bien-être lié à une forte sensibilité au stress. Le premier objectif de cette thèse était donc d'identifier les principales pratiques d'élevage et les facteurs environnementaux affectant la culture du sandre. De chaque modalité testée, l'environnement lumineux a été identifié comme l'un des facteurs les plus déterminants influençant la physiologie de cette espèce.

Le sandre possède un *tapetum lucidum* qui est une couche réfléchissante localisée dans la rétine. Il augmente la sensibilité des yeux à la lumière, ce qui permet à ce poisson de chasser au crépuscule et pendant la nuit. Cependant, un environnement lumineux inapproprié pour sa culture peut affecter sa physiologie, avec un niveau de stress plus élevé et une diminution de l'immunocompétence et de la croissance. L'information lumineuse issue de l'environnement est perçue par des photorécepteurs au niveau de la rétine et est convertie par la glande pinéale en un signal hormonal, la mélatonine. Cette hormone transmet les informations sur le moment de la journée (jour/nuit) aux cellules et aux organes et elle est connue pour moduler de nombreuses fonctions chez les vertébrés, notamment le comportement, la reproduction et la croissance. Cependant, bien que bien décrite chez les mammifères, la modulation du système immunitaire par la mélatonine a été très peu considérée chez les téléostéens. Nous avons ainsi supposé que cette hormone pouvait jouer un rôle central en étant un relais entre la lumière, l'axe du stress et le système immunitaire.

Plusieurs expériences ont été conçues afin de mieux caractériser la modulation du système immunitaire par l'environnement lumineux, avec une attention particulière pour mélatonine. Nous avons donc étudié la rythmicité quotidienne des marqueurs immunitaires innés et les impacts de plusieurs intensités lumineuses et du spectre sur la physiologie du sandre. Nous avons ensuite considéré la modulation du système immunitaire par des photopériodes naturelles. Toutes ces expériences ont défini ensemble que la lumière module l'immunité du sandre et que la mélatonine est impliquée. Certains marqueurs immunitaires innés suivent une rythmicité de 24 h, ce qui est corrélé au cycle jour-nuit. Les variations naturelles de la photopériode et la diminution ou l'augmentation associée de la mélatonine dans le sang ont

conduit à une modulation significative du système immunitaire, les marqueurs innés étant stimulés sous photopériode décroissante. De plus, des environnements lumineux inadaptés affectent l'immunité et la croissance des poissons et ces impacts peuvent être associés à la fois à une augmentation de l'état de stress et à une diminution de la mélatonine dans le sang.

Dans l'ensemble, l'environnement lumineux peut affecter profondément le système immunitaire des poissons en induisant un état de stress plus élevé mais aussi en modulant la production et la sécrétion de mélatonine par la glande pinéale. De plus, l'action de la mélatonine sur les cibles immunitaires implique à la fois des actions directes via des récepteurs spécifiques à la mélatonine et indirectes via la modulation de la sécrétion d’autres hormones telles que le cortisol.

LIST OF ABBREVIATIONS

5-HIAA: hydroxyl-indol-acetic acid
5-HT: serotonin
AANAT: aryl-alkylamine N-acetyltransferase
ACH50: alternative complement activity
ACTH: Adrenocorticotropic hormone
ANOVA: analysis of variance
BSA: bovine serum albumin
C3: complement component 3
cAMP : cyclic adenosine monophosphate
Cort: cortisol
CV: final weight heterogeneity
DA: dopamine
DHBA: 2–3 dihydroxybenzoic acid
DOPAC: 3,4-dihydroxyphenylacetic acid
DP: dark phase
ef1- α: elongation factor-1α
ELISA: enzyme-linked immunosorbent assay
fgl2: fibrinogen like 2
fkbp4: FKBP prolyl isomerase 4
FIW: final individual weight
FSP: fall-simulated photoperiod
fth1: ferritin heavy chain 1
GC: glucocorticoid
GH: growth hormone
GPCR: G protein-coupled membrane receptors
GPR50: G protein-coupled receptor 50
GR1: glucocorticoid receptor 1
HAC: hierarchical ascending classification
hepc: hepcidin c
HIOMT: hydroxyindole-O-methyl transferase
HK: head kidney
hp: haptoglobin
HPI: hypothalamus-pituitary-interrenal cells
HPLC: high performance liquid chromatography
IGF-1: insulin-like growth factor 1
il-1: interleukine-1

irf-1: interferon-regulatory factor 1
LP: light phase
Lys: lysozyme
Mel: melatonin
MR: mortality rate
MS222: tricaine methanesulfonate
MT1: melatonin receptor 1
NF-kB: nuclear factor-kappa B
NK: natural killer
OD: optical density
OSA: octane sulfonic acid
PCA: principal component analysis
PRL: prolactine
QR2: quinone reductase 2
RAS: recirculating aquaculture system
RDA: redundancy analysis
ROS: reactive oxygen species
RZR/ROR: Retinoic Z receptor/Retinoic acid-related Orphan Receptor
saa1: serum amyloid A1
SEM: standard of the mean
SGR: specific growth rate
SSP: spring-simulated photoperiod
TCRα: T cell receptor alpha chain
tcrg: T cell receptor gamma locus
tnf- α: tumor necrosis factor α

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I. Context of the study

Pike-perch aquaculture

For a few decades, aquaculture has been developing, expanding and intensifying in almost all regions of the world in response to an increasing market demand and a decrease in wild fish stocks (Conte, 2004; Ashley, 2007; Teletchea, 2019; Reverter et al., 2020). However, in Europe, aquaculture has shown a weak increase in productivity which may be attributed to a low diversity of cultured fish species (FAO, 2017). In order to revitalize that sector, the European project DIVERSIFY was launched. Based on their biological and economical potential, 6 fish species were identified as new or emerging species for the expansion of European aquaculture industry. These species included the meagre (*Argyrosomus regius*), the greater amberjack (*Seriola dumerili*), the wreckfish (*Polyprion americanus*), the Atlantic halibut (*Hippoglossus hippoglossus*), the grey mullet (*Mugil cephalus*) and the pikeperch (*Sander lucioperca*). From all these fish species, pike-perch, which is the biological model of the present study, was the only freshwater one. Its natural distribution is limited to Europe and Asia and it inhabits large and turbid rivers as well as eutrophic lakes, brackish coastal lakes and estuaries (Lappalainen et al., 2003).

Due to its fast growth and high economical expectations, pike-perch became one of the most promising fish species to consider for inland aquaculture industry (Wang et al, 2009; Dalsgaard et al, 2013). In 2018, its production reached 1,557 t (Fig. 1) while nearly 20,000 t were captured from the wild (FAO, FishStat). However, the development of its culture is still quite limited and several bottlenecks have been identified, including a lack of knowledge of the genetic variability of broodstocks, a low larval survival, a high incidence of deformities and a high sensitivity to stressors leading to high and sudden mortalities. All those bottlenecks were considered in the DIVERSIFY project.

During both larval and juvenile stages of pike-perch, survival rate is estimated between 8 and 30% (Kestemont et al, 2007; Szkudlarek and Zakęś, 2007; Szczepkowski et al., 2011; Dalsgaard et al, 2013, Ljubobratović et al., 2015). In comparison, in Eurasian perch (*Perca fluviatilis*), survival rate is fluctuating between 60 and 70% during the first months of intensive culture in RAS (Mélard, 2008). That low survival rate for pike-perch is the result of multiple factors. The first mortality peak occurring during larval stage is

linked to the transition from endogenous to exogenous feeding and swim bladder inflation (Szkudlarek and Zakęś, 2007). A second peak corresponds to the occurrence of cannibalistic behavior (Ljubobratović et al., 2015). Then, considering that husbandry practices are not yet optimized, the high mortality during juvenile stage may also reflect a low welfare related to a high stress level. Thus, the University of Namur, in collaboration with the University of Lorraine (France), aimed to identify the best husbandry practices to consider in pike-perch culture with regards to growth, immune and physiological status of the fish.

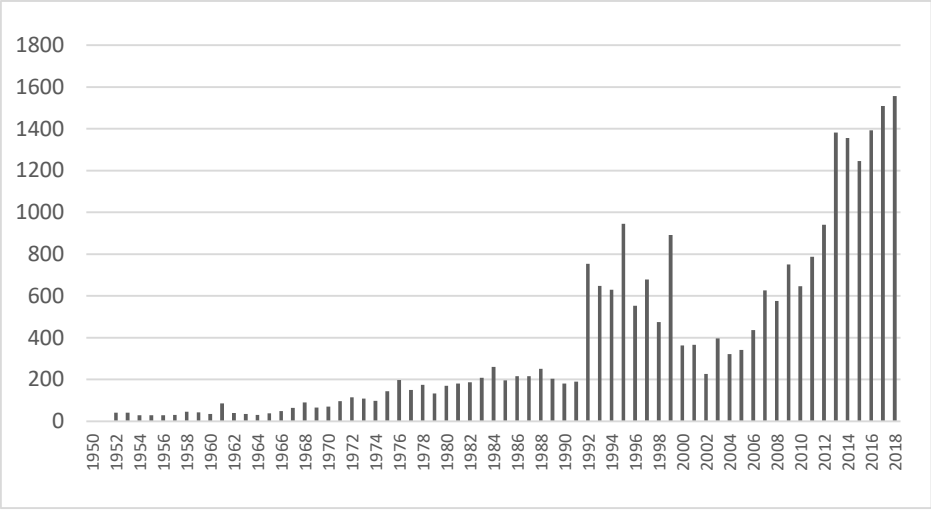

Figure 1: Global aquaculture production (in tonnes) of pikeperch, *Sander lucioperca* (FAO FishStat).

Pike-perch and light environment

Considering the light environment is crucial in aquaculture since it was shown to modulate numerous physiological functions in several fish species, including behavior, reproduction and growth (Downing, 2002; Falcón et al., 2007; Luchiari and Pirhonen, 2008; Falcón et al., 2010; Politis et al, 2014). Following preliminary observations and available data, it has been hypothesized that light is a determining factor for pike-perch welfare. The use of a red light was shown to improve its specific growth rate and feed

efficiency in cultured conditions. Moreover, a preference for low light intensities is consistent with the presence in the retina of a *tapetum lucidum* (Fig. 2) increasing the eye sensitivity to light (Luchiari et al., 2006; Feiner and Höök, 2015). This observation is consistent with the natural behavior of pike-perch since it is a crepuscular predator that is actively feeding during dusk and night (Luchiari et al, 2006; Zingel and Paaver, 2010; Dalsgaard et al, 2013). Such characteristics make the pike-perch a suitable animal model to focus on the impact of light on fish physiology.

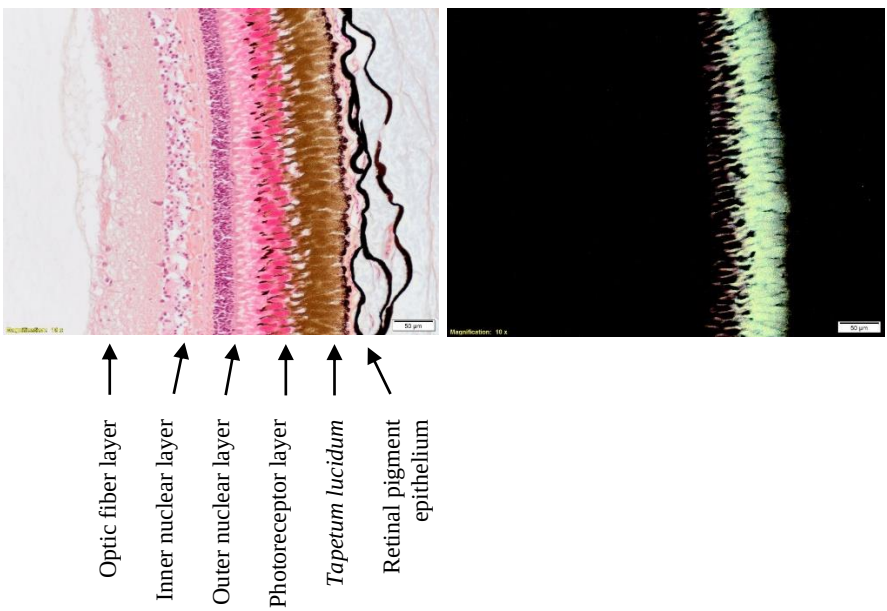

Figure 2: Histology of pikeperch retina (left) and its *tapetum lucidum* (right). Periodic acid-Schiff (PAS) stain.

Melatonin, stress and immunity

In addition to affect behavior, reproduction and growth of teleosts, the light environment has been shown to modulate stress status and stress-induced cortisol response (Volpato and Barreto, 2001; Karakatsouli et al, 2007; Eslamloo et al, 2015). Since cortisol is a strong immune-modulator of immune functions in fish, unsuitable light characteristics affect the immune

system (Sánchez-Vázquez et al., 2019). However, the effects of the light environment on fish immunity are poorly documented.

Considering that inappropriate light conditions may create a stressful environment for the fish and that the light is perceived by photoreceptors and converted into a melatonin signal by the pineal gland, it was hypothesized that this hormone may play a central role by being a relay between the light, the stress axis and the immune system. In parallel, the melatonin hormone is a multifunctional molecule that was shown in numerous vertebrates to modulate important physiological functions, including thermoregulation, reproduction, osmoregulation, development and migration (Carrillo-Vico et al., 2005; Falcón et al., 2010; Mehner et al., 2012; Dumbell et al., 2016).

A wide range of experimental evidence has shown a clear relationship between the neuroendocrine and immune systems in mammals. This link is illustrated by a two-way communication in which endogenous substances from the neuroendocrine system act on the immune system and vice versa (Carrillo-Vico et al., 2013). Numerous neuroendocrine responses follow circadian and circannual rhythmicity and, taking into account the close relationship between these systems, some immune parameters also exhibit both types of rhythmicity (Esteban et al., 2006; Carrillo-Vico et al., 2013; Esteban et al., 2013; Brüning et al., 2015). Thus, in many animals including ectotherms, the immune responses are known to vary seasonally with parameters being suppressed during winter and stimulated in summer in response to photoperiod and temperature variations (Bowden et al., 2007). The influence of melatonin on the immune system has been well established in higher vertebrates (Guerrero and Reiter, 2002; Carrillo-Vico et al., 2005; 2013). Generally, in mammals, melatonin stimulates basal immunity to ensure an optimal response to infection or exerts an anti-inflammatory action in the case of inflammatory responses to protect the organism from host tissue damage (Carrillo-Vico et al., 2013). Melatonin thus regulates cell dynamics, including the proliferative and maturational stages of all hematopoietic and immune cells lineages (NK cells, T and B lymphocytes, granulocytes, monocytes) involved in host defense, in both bone marrow and tissues (Miller et al., 2006).

Unlike mammals, the immunomodulatory capacity of melatonin is poorly documented in teleosts. Some studies, however, suggest that melatonin is a key hormone in the interaction between the endocrine and immune systems

(Esteban et al., 2013). The available data on the regulation of the immune system by melatonin mainly concern the daily rhythmicity of immune parameters such as lysozyme, complement or peroxidase. Although the activity profiles are variable between species, it has been shown that these different parameters follow a pattern based on the day-night cycle. In seabream (*Sparus aurata*), exogenous melatonin induces an increase in the expression of some immune genes (*il-1β*, *mhc* and *irf-1* - interferon-regulatory factor) in the anterior kidney (Esteban et al., 2006; Cuesta, 2008). Melatonin also appears to inhibit phagocytic activity in the snakehead *Channa punctatus* (Roy et al., 2008) and increase cytotoxic activity in seabream. In parallel, while melatonin is known to induce pro or antigonadal effects in response to an increasing or decreasing photoperiod (Maitra and Hasan, 2016), the effects of photoperiod on the immunomodulatory capacity of melatonin are not documented yet.

In teleosts, an additional interaction has been described between the melatonin hormone and the hypothalamo-pituitary interrenal cells (HPI) axis (López-Patiño et al., 2013; 2014; Conde-Sieira et al., 2014). Thus, several evidences support an anti-stress role of melatonin at both central and peripheral levels, including a suppressor effect on the HPI axis (López-Patiño et al., 2013; Gesto et al., 2016) while cortisol was shown to inhibit the production and secretion of melatonin by the pineal organ (López-Patiño et al., 2014). Considering the triangulation between the melatonin hormone, the HPI axis and the immune system is thus of a great interest.

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II. General methodology

This part presents the *in vivo* and *ex vivo* experiments done during this study, by describing the stocking conditions of the fish, the experimental facilities and the tested factors.

Fish origin and stocking conditions

The biological model used within the experiments is the pike-perch (*Sander lucioperca,* Czech origin) and a total of 4 *in vivo* and 1 *ex vivo* experiments were performed during this study. All the fish were provided by the Asialor farm (Dieuze, France) at a juvenile stage and transferred to the URBE facilities (UNamur, Belgium) or to the URAFPA facilities (ULorraine, France). The fish were then distributed into tanks for acclimation during 20 to 30 days or until they reached a specific weight. Conditions were always maintained stable during the acclimations: 21-23°C, 10-15 lux light intensity at water surface, 85-95% oxygen saturation, constant photoperiod 12:12, fed twice a day with commercial pellets at 1.5 or 2.0% biomass. These conditions are adapted to the needs of the species. Quality of the water was recorded regularly.

Experimental facilities and tested factors

The first and fourth experiments were conducted in URAFPA facilities (France). Each experimental unit (Fig. 1) was operating independently in a recirculating circuit (RAS) and was totally isolated from the others. While, for the first experiment, 8 environmental factors were considered and tested in two modalities (light intensity, light spectrum, photoperiod, oxygen saturation, density, temperature, manipulations and feed type) (see Experiment I "*Optimal aquaculture modalities for pike-perch*"), only the photoperiod was considered in the fourth experiment (see Experiment IV "*Photoperiod influences melatonin release and immunity*").

The second and third experiments took place at the University of Namur (Belgium). They were conducted in 24 indoor 100 L-tanks of two RAS (Fig. 2). For both of these experiments, only the light environment (light intensity and/or spectrum) was modified (see Experiment II "*Daily rhythms of immune markers*" and Experiment III "*Immune modulation by the light environment*").

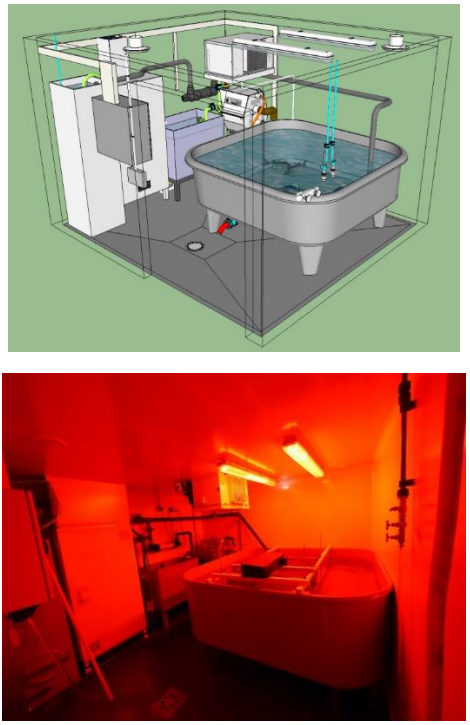

Fig. 1: Experimental unit, URAFPA facilities (Experiment I). Right picture: experimental tank with the high light intensity of red spectrum.

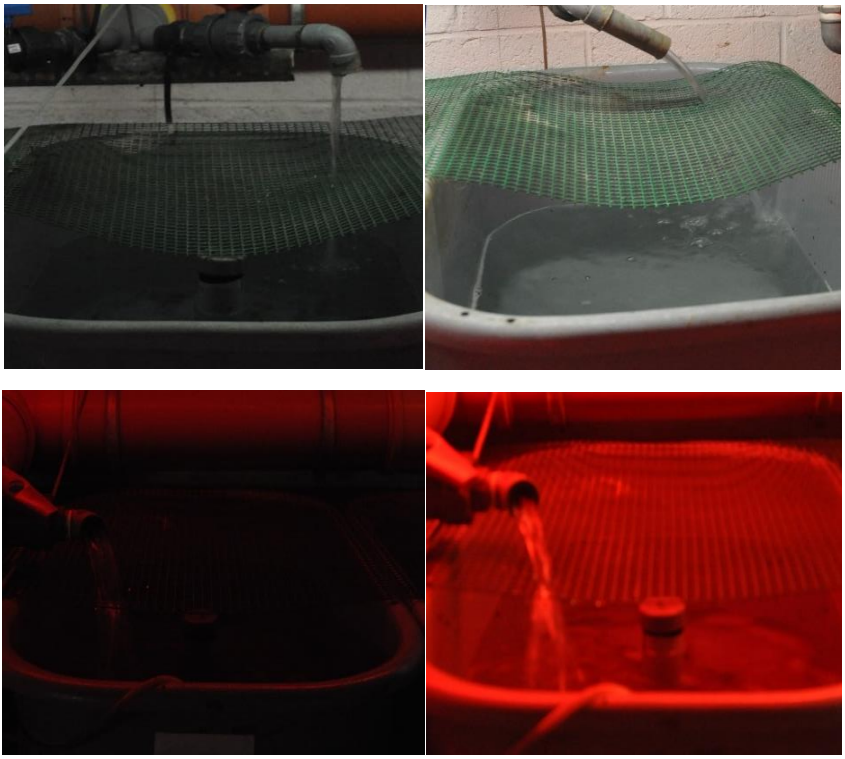

Fig. 2: Light environment of experimental tanks, considering 2 light spectra (white and red) and 2 light intensities (10 and 100 lux), at the University of Namur (see Experiment III).

Fig. 3: Four 400-L tanks of one recirculating aquaculture system, Unamur, Belgium.

Finally, the fish used for the *ex vivo* experiments were maintained in 4 indoor 400-L tanks of a same recirculating system at the University of Namur (Fig. 3). The fish were not exposed to any experimental conditions. Only specific organs were sampled for hormonal exposures (see Experiment V "*Ex vivo actions of melatonin and cortisol*").

Fish samplings

During samplings, several fish were randomly removed from an experimental tank and directly anesthetized in MS-222 (150 mg/l) in a bucket. As soon as they were anesthetized, fish head was covered with a tissue, and blood was quickly collected by caudal vein puncture with heparinized syringes within 5 min and centrifuged at 3,000 g during 10 min at 4 °C. Fish were then euthanized before collecting the specific organs. Organs and plasma were directly conditioned for further analyses. The same procedure was then applied for the next experimental tank. All the experiments were carried out in agreement with the European and local legislations on animal welfare.

Samplings occurred in France and in Belgium, during the day but also during the night for experiments II, III and IV. Despite different sampling locations and environments, efforts have been deployed to maintain similar sampling conditions between experiments. In addition, in order to reduce the total time of samplings of each experiment and to limit fish stress, at least 7 people were working together to sample all the fish collected from one experimental unit.

III. Objectives and structure of the study

A. GENERAL OBJECTIVES

There were two main objectives in the current work. Firstly, the thesis aimed to characterize the stress and immunological responses of cultured pike-perch (*Sander lucioperca*) to major aquaculture modalities and to identify best husbandry practices. Then, considering that the light environment can profoundly affect pike-perch physiology, we aimed to characterize the impacts of the light environment on fish immunocompetence and the role of the melatonin hormone as a potential relay between neuroendocrine and immune systems. For these purposes, 5 experiments were conducted.

B. THESIS OUTLINE

The manuscript is subdivided in several chapters and **Experiments I** to **V** are presented in a publication format:

- The following part is a ***literature review*** comparing the immunomodulatory capacity of the melatonin hormone in vertebrate taxa, including mammals, birds, fish, amphibians and reptiles.
- ***Experiment I "optimal aquaculture modalities for pike-perch"*** is dealing with the multifactorial experiment of the DIVERSIFY project. It aimed to identify the best husbandry practices for pike-perch culture.
- ***Experiment II "daily rhythms of immune markers"*** refers to the experiment aiming to describe daily rhythms of cortisol and humoral innate immunity.
- ***Experiment III "immune modulation by the light environment"*** concerns the experiment focusing on the effects of the day-night cycle, the light intensity and the light spectrum on fish physiology and makes a link between the stress status, the melatonin hormone and immune markers.
- ***Experiment IV "photoperiod influences melatonin release and immunity"*** is focused on the modulation of the immune system by season-simulated photoperiods and associated variations in circulating melatonin.
- ***Experiment V "ex vivo actions melatonin and cortisol"*** refers to the *ex vivo* experiment that aims to describe the actions of physiological and pharmacological doses of melatonin and cortisol on immune-related genes.

- Following those chapters, a ***general discussion*** combining all the results is proposed with a consideration of the potential bias and limitations that occurred during the thesis.
- Finally, some ***conclusions and perspectives*** are made.

IV. Literature review

Melatonin as an immune modulator: a similar pattern in all vertebrates?

A. INTRODUCTION

The melatonin hormone, which was first isolated in 1959 from the bovine pineal gland (Lerner et al., 1960), shows a high conservation within phylogenetically distant organisms. Melatonin is indeed present in bacteria, invertebrates, vertebrates, algae, plants and fungi (Hardeland et al., 2003; Carrillo-Vico et al., 2005). In vertebrates, it was initially described to be produced and secreted exclusively by the pineal gland. Later, other tissues such as skin, retina and gastro-intestinal tract were identified as non-endocrine extrapineal sources of melatonin in mammals (Bubenik, 2002; Acuna-Castroviejo et al., 2014), birds (Underwood et al., 1984), fish (Bayarri et al., 2004) and amphibians (Serino et al., 1993). Its synthesis always involves a four-enzymatic intracellular steps, including the formation of serotonin from the amino-acid tryptophan by tryptophan-5-hydroxylase and 5-hydroxytryptophan decarboxylase and then the conversion to melatonin by arylalkylamine N-acetyltransferase (AANAT) and hydroxyindole-O-methyl transferase (HIOMT) (Carrillo-Vico et al., 2005; Calvo et al., 2013).

Melatonin functions are numerous. In plants, melatonin plays important physiological functions by enhancing resistance to a range of biotic and abiotic stressors and regulating growth, morphogenesis and light-dark cycles (Arnao, 2014). In bacteria, melatonin has also been described to be involved in the response to abiotic stressors that have been reported to promote the generation of reactive oxygen species (ROS) (Arnao and Hernández-Ruiz, 2015; Gao et al., 2020). In vertebrates, the pineal gland is the main organ participating in the release of circulating melatonin. By being produced exclusively during the dark phase of the photoperiod in a wide range of vertebrates, melatonin relays the information about the time of the day and the year to cells and organs (Migaud et al., 2007; Vera et al. 2007; Confente et al., 2010; Falcon et al., 2010; López-Patiño et al., 2014). This time-keeping hormone thus regulates countless physiological functions in vertebrates, including the synchronization of circadian rhythms (sleep-wake timing, locomotor activity, thermal preferences, osmoregulation, metabolic activity)

as well as annual processes such as migration, growth and sexual maturation (Downing et al., 2002; Carrillo-Vico et al., 2005; Falcón et al., 2007; 2010; Dumbell et al., 2016).

In addition to act as an antioxidant, onco-static and antiaging compound, a large body of evidence, mainly accumulated in mammals, supports a complex action of melatonin on the immune system (Srinivasan et al., 2005; Carrillo-Vico et al., 2006; Calvo et al., 2013). In parallel, immune mediators have been shown to modulate melatonin production and release (Carrillo-Vico et al., 2013). Such interactions between neuroendocrine and immune systems for the maintenance of homeostasis are widely accepted in vertebrates. The communication between these systems occurs through neurotransmitters and hormones in the modulation of immune reactivity as well as immune-cells-derived soluble mediators, named cytokines, in the modulation of neuroendocrine functions. And, in this complex neuroendocrine-immunological network, the pineal-synthetized melatonin is considered as a crucial intermediate (Skwarlo-Sonta, 2002; Guerrero and Reiter, 2002; Esteban et al., 2006; Cuesta et al., 2008; Falcon et al., 2010; Esteban et al., 2013).

From the early 70's, an accumulation of anatomical, physiological and pharmacological evidences supporting the immunomodulatory effects of melatonin were collected. Surgical or functional pinealectomy was shown to highly impact the immune system affecting immune organs and immune responses. In chick embryo, the pineal ablation leads to a retarded development of the primary lymphoid organs, including thymus, spleen and burse of Fabricius, and a decreased immune response (Jankovic et al., 1994). In mice, the ablation of the pineal gland leads to an involution of the thymus, a depression of cell-mediated immune response and a decrease in antibody production, interleukin-2 (IL-2) production and natural killer (NK) cell activity (Vaughan and Reiter, 1971; Maestroni et al., 1986; Del Gobbo et al., 1989). In addition to pinealectomy, the action of melatonin on immunity is supported by a correlation between melatonin production and the circadian and seasonal variations of the immune system (Nelson et al., 1995; Nelson and Drazen, 2000). Some examples are the daily and seasonal rhythmicity of blood lymphocyte count (Blom et al., 1994; Haldar et al., 2001), cytokine production (Petrovsky and Harrison, 1997), humoral innate immune activities (Esteban et al., 2006) and cell proliferation in bone marrow and lymphoid system (Haus et al., 1983), with short photoperiods generally associated with

enhanced immune functions (Nelson, 2004). Melatonin was also shown to exert its action through specific membrane and nuclear receptors that are found on immune cells and tissues. Finally, several immunologically-relevant organs, tissues and cells such as leukocytes, bone marrow, thymus and spleen were identified as extrapineal sources of melatonin (Carrillo-Vico et al., 2004; Lardone et al., 2006; Maldonado et al., 2010; Muxel et al., 2012; Markus et al., 2018).

Numerous documents considering the functions of melatonin in mammal's immunity have been published so far, allowing scientists to draw well-defined schemes of action. However, its immune functions and modes of action in other vertebrates have been little or rarely investigated.

The aim of this review is to compare the specific actions of melatonin on immunity in different vertebrate taxa and to point out the lack of knowledge in non-mammalian vertebrates. It will not integrate all the information concerning mammals since specific reviews have been published so far (Carrillo-Vico et al., 2005; Calvo et al., 2013; Ren et al., 2017; Xia et al., 2018; Zhao et al., 2019).

B. MELATONIN RECEPTORS

The main effects of melatonin are primarily induced through melatonin receptors MT1 and MT2 that are high affinity G protein-coupled membrane receptors (GPCRs) (Dubocovich and Markowska., 2005; Emet et al., 2016). In a wide range of vertebrates, these receptors are both expressed in numerous tissues including immune cells and tissues (see Table 1). The main signaling pathways of activated MT1 and MT2 induce a decrease in cyclic adenosine monophosphate (cAMP) levels and/or the activation of PKC/ERK pathway (Hardeland et al., 2009; Oishi et al., 2018). G protein-coupled receptor 50 (GPR50) was described as the third receptor of the melatonin receptor subfamily in mammals. However, it does not bind to melatonin and is thus classified as orphan receptor (Oishi et al., 2018). Finally, another melatonin receptor, Mel1c, is found exclusively in fish, amphibians (*Xenopus* species) and birds (Dufourny et al., 2008; Emet et al., 2016). Since GPR50 might correspond to the mammalian ortholog of Mel1c, it is hypothesized that the receptor GPR50 lost its ability to bind melatonin during evolution (Oishi et al., 2018).

The melatonin receptors MT1 and MT2 are also described to form homo- and heteromers between each other or with other GPCRs (Oishi et al., 2018). MT1/MT2 heteromer was shown to regulate light sensitivity in the mouse retina, while MT1/GPR50 complex prevents melatonin binding to MT1 and subsequent inhibition of adenylyl cyclase (Levoye et al., 2006; Baba et al., 2013). However, such heteromerization of melatonin receptors has not been investigated yet in immune cells and tissues. Considering that functional properties of these complexes are different from monomers, they would make the understanding of the melatonin-immunity interactions more complex.

In addition to above-mentioned receptors, low-affinity binding sites of melatonin were detected. Several intracellular proteins, including quinone reductase 2 (QR2) and calmoduline have been suggested to interact with melatonin but only QR2, designated as the MT3 melatonin receptor, was confirmed as a melatonin target. However, no signaling pathway starting from this binding site is known (Nosjean et al., 2000; Oishi et al., 2018; Xia et al., 2018).

Finally, an interaction between melatonin and nuclear receptors belonging to RZR/ROR (Retinoic Z receptor/Retinoic acid-related Orphan Receptor) family, including RORα isoforms, has also been described (Carrillo-Vico et al., 2005). In several studies from the 90's performed on immune tissues of birds and mammals, the majority of specific melatonin binding was due to the nuclear fraction of tissue homogenates (Liu and Pang, 1993; Liu et al., 1995). The development of molecular biology as well as specific agonists and antagonists then led to the characterization, in various mammalian immune cells, of some nuclear receptors of RZR/ROR subfamily binding melatonin. They were identified in human and murine monocytes, macrophages, B lymphocytes, subsets of T lymphocytes and Jurkat cells (Carrillo-Vico et al., 2003b; Dzhagalov et al., 2004; Pozo et al., 2004; Moharrami et al., 2018) as well as in thymus and spleen of mouse and palm squirrel *Funambulus pennanti* (Carrillo-Vico et al., 2004; Gupta et al., 2015). More recent data confirmed that ROR subfamily isoforms are found in immune cells of mammals and that they play key roles in the development of several immune cells and in some immune responses (Cook et al., 2015). However, an absence of interactions of RORα receptors with melatonin was described (Slominski et al., 2016) suggesting an indirect action of melatonin on these receptors.

Table 1: Distribution of high-affinity melatonin receptors MT_1, MT_2 and Mel1c in immune cells and tissues of vertebrates.

Vertebrate	Organism	Distribution	Mel-receptor subtypes	References
Mammals	Human	B and T lymphocytes	MT_1	Carrillo-Vico et al., 2003 ; Pozo et al., 2004 ; Lardone et al., 2009
		Monocytes	MT_1	Pozo et al., 2004
		NK cells	MT_1	Pozo et al., 2004
		Mast cells	MT_1 & MT_2	Maldonado et al., 2010
		Jurkat cells	MT_1 & MT_2	Guerrero et al., 2000 Lardone et al., 2006
	Mouse	Thymus	MT_1	Carrillo-Vico et al., 2003
		Spleen	MT_1 & MT_2	Carrillo-Vico et al., 2003 Singh et al., 2016
	Rat	Thymus	MT_1 & MT_2	Pozo et al., 1997 Sanchez-Hidalgo et al., 2009
		Spleen	MT_1 & MT_2	Pozo et al., 1997 Sanchez-Hidalgo et al., 2009
		B and T lymphocytes	MT_1	Pozo et al., 1997
		Leukocytes	MT_2	Lotufo et al., 2001
	Palm squirrel	Thymus	MT_1 & MT_2	Ahmad and Haldar, 2010

		Spleen	MT_1 & MT_2	Ahmad and Haldar, 2010
	Golden hamster	Spleen	MT_1	Vishwas and Haldar, 2014; Verma and Haldar; 2018
		Thymus	MT_1	Vishwas and Haldar, 2014
		Bone marrow mononuclear cells	MT_1	Vishwas and Haldar, 2014
Birds	Duck	Spleen	Undefined	Yu et al., 1991
	Chicken	Thymus	MT_1 ; MT_2 & Mel1c	Wronka et al., 2008
		Spleen	MT_1 ; MT_2 & Mel1c	Wronka et al., 2008
		Bursa of Fabricius	MT_1 ; MT_2 & Mel1c	Wronka et al., 2008
	Pigeon	Spleen	Undefined	Poon et al., 1993
	Jungle bush quail	Spleen	MT_1 ; MT_2	Yadav et al., 2011
		Splenocytes	MT_1 ; MT_2 & Mel1c	Yadav et al., 2014
		Lymphocytes in BALT	MT_2	Kharwar and Haldar, 2011
Fish	Common carp	Leukocytes	MT_1	Kepka et al., 2015
	Senegalese sole	Spleen	MT_1	Confente et al., 2010
	Rabbitfish	Spleen	MT_2	Park et al., 2006
	European sea bass	Blood cells	MT_2	Sauzet et al., 2008
Amphibians & Reptiles	MT1 and MT2 receptors were characterized in brain but no study so far has investigated their peripheral distribution.			

C. IMMUNE SYSTEM-SYNTHESIZED MELATONIN

While the pineal gland was initially described as the exclusive source of melatonin in the 60s and 70s, the production of melatonin by trout retina was reported by Gern and Ralph in 1979. Several extrapineal sources of melatonin were then identified in vertebrates, including gastrointestinal tract, skin, Harderian gland and, to a greater interest for this review, the immune system cells (Wiechmann et al., 2013; Acuña-Castroviejo et al., 2014; Markus et al., 2018). This production was observed in human lymphocytes, macrophages, bone marrow cells and Jurkat cells (Conti et al., 2000; Carrillo-Vico et al., 2004; Lardone et al., 2006; Markus et al., 2018), murine thymus, spleen, bone marrow cells and RAW264.7 macrophages (Gómez-Corvera et al., 2009; Muxel et al., 2012) and rat mast cells and macrophages (Martins et al., 2004; Maldonado et al., 2010). In addition, melatonin and/or its biosynthetic machinery was described in thymus of human and rat and in human peripheral blood cells (Carrillo-Vico et al., 2004; Naranjo et al., 2007). Since all these immune cells and tissues express melatonin receptors (Table 1), it became quite evident that melatonin synthetized by the immune system plays paracrine, autocrine and intracrine functions in the regulation of innate and acquired immune responses (Markus et al., 2018, Xia et al., 2018; Zhao et al., 2019). The biological significance of this immune-cell derived melatonin has also been explored, but to a lesser extent. It has been demonstrated that melatonin regulates the IL-2/Il-2 receptor system in human lymphocytes as well as phagocytosis of human colostral mononuclear cells (Carrillo-Vico et al., 2005; Pires-Lapa et al., 2013). For additional information, readers are invited to consult the paper of Markus et al. (2018) reviewing the shift of melatonin production from the pineal gland to the immune cells during acute inflammatory responses.

Although specific melatonin receptors have been detected on immune cells and tissues of birds and fish (Table 1), no study, to our knowledge, has investigated yet the presence of such immune biosynthesis in immune-derived cells of non-mammalian vertebrates or its biological significance.

D. MELATONIN ACTIONS ON INNATE IMMUNITY

The innate immune responses are the early line of defense against invading pathogens. It is composed of a set of different cells, including macrophages, dendritic cells, neutrophils, eosinophils, basophils, mast cells and NK cells, in addition to various humoral components. These humoral markers include lytic enzymes, components of the complement pathways, cytokines, chemokines and antibacterial peptides (Magnadóttir, 2006).

In mammals, the actions of melatonin on cellular innate immunity have been extensively analyzed and reviewed elsewhere (Calvo et al., 2013; Xia et al., 2018). In addition to influence leukocyte populations (Currier et al., 2000; Srinivasan et al., 2005), melatonin was shown to modulate the main functions of these cells, with the role on macrophage biology being the most documented. It concerns, in macrophages, the expression of cell surface molecules, including major histocompatibility complex class I and II, the production and secretion of free radicals and cytokines, including IL-12, TNF-α, IL-6 and IL-1β, as well as their phenotype polarization (Calvo et al., 2013; Xia et al., 2018; Xu et al., 2018). Melatonin also modulates phagocytosis and ROS production of neutrophils as well as cytokine production and cytotoxic activity of NK cells (Pieri et al., 1998; Recchioni et al., 1998; Hriscu, 2005; Miller et al., 2006). In addition, it favors the recruitment of eosinophils and neutrophils to inflammatory sites (Calvo et al., 2013).

Despite the differences in the organization of some immune relays and a huge gap of knowledge in the melatonin-immunity interactions in non-mammalian vertebrates, the available data suggest a similar key role of melatonin on their innate immune system. In reptiles, the only two available studies described in a snake model an action of melatonin on nitric oxide and superoxide productions by macrophages and on phagocytic activity of neutrophils (Tripathi and Singh, 2014; Singh et al., 2016). Furthermore, monocyte, eosinophil and basophils counts increased in response to melatonin (Tripathi and Singh, 2014). In birds, melatonin stimulates heterophil phagocytic activity and total leukocyte counts and modulates splenocyte activity measured through blastogenic response, apoptosis and Il-2 production (Rodriguez et al., 2001; Brennan et al., 2002; Terrón et al., 2005). In fish, melatonin was shown *in vivo* to enhance several innate immune responses,

including phagocytic, peroxidase, ROS and cell-mediated cytotoxic activities as well as neutrophil migration to an injury site (Cuesta et al., 2008; Ren et al., 2015).

Melatonin is mainly seen as an immunostimulant molecule, as demonstrated by the enhancement of immune functions following its injection or ingestion in various vertebrates (Table 2). And these immunoenhancing properties are best demonstrated in cases in which the immunity is depressed as a consequence of aging, stress or treatment (Carrillo-Vico et al., 2005). However, anti-inflammatory properties of melatonin in mammals were described, leading scientists to describe it as an immune buffer compound. Using *in vitro* approaches, Xia et al. (2012) described an inhibition of LPS-stimulated production of TNF-a, Il-1b, IL-6 and IL-8 in RAW264.7 cells following melatonin treatment. During multiple organ dysfunction syndrome following heatstroke in rat, melatonin reduced plasma systemic inflammation response molecules, including IL-1b and IL-6, and promoted plasma levels of an anti-inflammatory cytokine, namely IL-10 (Lin et al., 2011). Melatonin was also shown to lower pro-inflammatory cytokines levels in different cases of inflammation, including neuroinflammation in streptozotocin-induced diabetic neuropathy, cerulean-induced pancreatitis and periodontitis in rat, as well as duchenne muscular dystrophy in human (Chahbouni et al., 2010; Jung et al., 2010; Negi et al., 2011; Kara et al., 2013). This anti-inflammatory property of melatonin was shown to be mediated by the inhibitions of both inflammasome and NF-κB activations (Markus et al., 2013; Tarocco et al., 2019). Melatonin thus preserves the subtle pro- and anti-inflammatory balance by acting as an immune stimulant under basal or immunosuppressive conditions to ensure an optimal response to infection or as an anti-inflammatory compound in the case of inflammatory responses to protect the organism from host tissue damage (Carrillo-Vico et al., 2013; Tarocco et al., 2019).

Such buffering role of immune responses was explored only once in non-mammalian vertebrates. In common carp *Cyprinus carpio*, during zymosan-induced peritonitis, the administration of melatonin reduced leukocyte migration to the peritoneum and induced a decrease of the respiratory burst activity in peritoneal leukocytes (Kepka et al., 2015). However, more investigations are needed to hypothesize a similar melatonin dual function as observed in mammals.

Table 2: Immunomodulatory effects of exogenous melatonin.

Vertebrate taxa	Organism	Protocol	Effect on the immune system	References
Mammals	Human	injection	+ neutrophil chemotactic response + expression of intracellular chemokines in neutrophils	Peña et al., 2007
	Mouse	i.p. injection	+ gene expression of M-CSF, TNF-α, TGFβ and SCF in peritoneal macrophages + gene expression of IL-1β, IFNγ, M-CSF, TNFα and SCF in splenocytes	Liu et al., 2001
		Oral administration	+ survival of precursor B cells	Yu et al., 2000
	Rat	In vitro (1.2 nM); neutrophils and PBMCs	+ chemotaxis response	Peña et al., 2007
		i.p. injection	+ peritoneal leukocytes	Peña et al., 2007
	Northern palm squirrel	Intratesticular injection	+ lymphatic tissue weight + lymphocyte count +lymphocyte proliferation in spleen and thymus + Il-2 + leukocyte	Ahmad and Haldar, 2010
		s.c. injection	+ leukocyte count + blastogenic response of splenocytes	Rai et al., 2009
		s.c. injection	+ lymphocyte count of blood and bone marrow + blastogenic stimulation ratio of spleen and thymus	Rai and Haldar, 2003

	Sheep	Implant or injection	+ CD4+ T lymphocytes + antibody titer	Ramos et al., 2018
Birds	Jungle bush quail	*In vitro (430 nM – 2 µM)* ; lymphocytes	+ proliferation	Kharwar et al., 2015
		s.c. injection	+ spleen weight + leukocyte count - Apoptosis + blastogenic response of splenocytes	Singh et al., 2010
		In vitro (2.15 pM) ; splenocytes	+ Il-2 production + blastogenic response	Singh et al., 2010
	Japanese quail	Added in drinking water	+ primary antibody titers + cutaneous basophil hypersensitivity reaction to phytohemagglutinin	Moore and Siopes, 2003
	Chicken	s.c. injection (40 mg/kg)	+ leukocyte count + T and B lymphocytes proliferation	Brennan et al., 2002
	Ring dove	Oral administration	+ phagocytosis - Superoxide radical levels	Terrón et al., 2005
Fish	Gilthead seabream	i.p. injection	+ cytotoxic, phagocytic and respiratory burst activities of head-kidney leukocytes + peroxidase activity in serum	Cuesta et al., 2008
		In vitro (20 – 400 µM) ; head kidney leukocytes	+ respiratory burst activity - peroxidase activity	Cuesta et al., 2007
	Common carp	i.p. injection (during zymosan-induced peritonitis)	- neutrophil and lymphocytes counts - respiratory burst in inflammatory leukocytes - leukocyte apoptosis	Kepka et al., 2015

		In vitro (10^{-6} – 10^{-10} M)	- leukocyte migration	Kepka et al., 2015
	Zebrafish	Pretreatment of larvae	+ neutrophil migration	Ren et al., 2015
	Sea bass	*In vitro (400 µM)* ; head kidney leukocytes	- peroxidase activity	Cuesta et al., 2007
Reptiles	Asiatic water snake	i.p. injection	+ thymus weight + nitrite release and superoxide production by splenic macrophages + mitogen-induced splenic lymphocyte proliferation + monocyte, eosinophil and basophil counts + nitrite release and superoxide production by leukocytes - Leukocyte phagocytosis	Tripathi and Singh, 2014 Singh et al., 2016
Amphibians	No study has investigated the effects of exogenous melatonin on immunity of amphibians			

E. MELATONIN ACTIONS ON SPECIFIC IMMUNITY

The main components of the acquired immune response, which is antigen-specific, are T and B lymphocytes in addition to circulating proteins including antibodies and cytokines. Seen as one of the most important cell types of the immunity, T lymphocytes are the most considered cells in the study of the melatonin immunomodulation and several recent reviews have been published so far in mammals (Ren et al., 2017; Zhao et al., 2019). In addition to express receptors for melatonin and to possess its biosynthetic machinery, T-cells were shown to be modulated by melatonin in mammals, 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). After exposure of the rat thymus to microwaves, melatonin stimulated the proliferation rate of thymocytes and decreased apoptosis (Sokolovic et al., 2013). Melatonin also activated proliferation markers of CD4+ and naïve CD4 T lymphocytes in murine spleen (Yoo et al., 2016) and modulated the differentiation of activated CD4+ T cell into specific T subsets (Ren et al., 2017). Finally, it promoted the survival of human and mouse T cells that may affect the generation of T memory cells (Yu et al., 2000; Pedrosa et al., 2010; Ren et al., 2017).

Several studies concluded that melatonin also plays a critical role in regulating the activation of B cells in mammals (Luo et al., 2020). In mouse, when orally administered, it promotes the survival of precursor B-cells in bone marrow (Yu et al., 2000). Melatonin also modulates both T-cell-dependent and T-cell-independent antibody production by B cells (Cernysiov et al., 2009). If used as an adjuvant in sheep vaccination, melatonin induced higher antibody titer and an increase in IgG+ B lymphocytes which both seem to be the result of an enhancement of T CD4 cell activation cooperating with B lymphocytes for adequate response to vaccine antigen (Ramos et al., 2018).

In birds, Chen et al. (2016) showed in pinealectomized chicken a partial restoration of T-cell proliferative activity with exogenous melatonin. Exogenous melatonin also increased T and B lymphocyte populations in chicken and in Jungle bush quail (Brennan et al., 2002; Singh et al., 2010) and *in vitro* proliferation of lymphocytes isolated from lung tissue of Jungle bush quail (Kharwar et al., 2015). The suppressive effects of dexamethasone and testosterone on lymphocyte counts were antagonized by melatonin

supplementation (Singh and Haldar, 2005; Singh et al., 2010). Finally, melatonin enhanced B lymphocyte proliferation isolated from the bursa of Fabricius of broilers (Li et al., 2015).

The only study focusing on T and B lymphocyte in teleosts observed no effects of exogenous melatonin 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). And no data were found concerning amphibians and reptiles.

F. INDIRECT ACTION ON IMMUNE TARGETS

Considering that several hormones are well-known immune modulators and that melatonin influences the secretion of these hormones, it became evident that melatonin acts on immune targets, not only through specific receptors, but also via intermediates. One of the main considered groups of molecules is glucocorticoids (GC) whose production is regulated by the hypothalamic-pituitary (HPA) axis in mammals and birds and by the hypothalamic-pituitary-interrenal cells (HPI) axis in fishes, amphibians and reptiles (Sopinka et al., 2015).

Melatonin was shown to modulate the secretion of the main GCs in vertebrates, namely cortisol in teleosts and corticosterone in birds, non-human mammals, reptiles and amphibians. And both cortisol and corticosterone are strong immune modulators (Tort, 2011; López-Patiño et al., 2013; Conde-Sieira et al., 2014; Morey et al., 2015; Sopinka et al., 2015; Taves et al., 2016, Berechshencko et al., 2018). However, the mechanisms of such action on the stress axis are not fully understood (Gesto et al., 2016). In mammals, melatonin was shown to inhibit the ACTH-mediated cortisol production in the adrenal gland (Torres-Farfan et al., 2003; Gesto et al., 2016). In the study of Gesto et al. (2016), adding melatonin in fish tank (100 nM) was effective in reducing in Solea *senegalensis* the intensity of the stress response and in attenuating the post-stress increase in *crh* gene expression in the hypothalamus. Such observation, taking into account that specific melatonin receptors have been detected on fish hypothalamus (Gaildrat and Falcón, 2000; Falcón et al., 2003; 2007; Maitra and Hasan, 2016), suggests that effects of melatonin on the HPI axis are mediated through an interaction

with the hypothalamic control of pituitary function. In addition, the detection of melatonin receptors on pituitary gland provides evidence that melatonin might also modulate directly the neuroendocrine functions of this tissue (Maitra and Hasan, 2016). Melatonin was thus shown to modulate GH and PRL secretions by the pituitary gland (Falcón et al., 2003), but no information was found regarding ACTH secretion.

The neuroendocrine system was also shown to modulate immune functions through the release of prolactin (PRL), growth hormone (GH) and insulin-like growth factor-1 (IGF-1) (Kelley et al., 2007) whose secretions are all influenced by melatonin (Falcón et al., 2003; Oner et al., 2009; Molik et al., 2010). Finally, sex hormones influence both innate and adaptive immunity, with androgens showing mainly anti-inflammatory properties and estrogens being both pro- and anti-inflammatory (Gilliver, 2010; Berechshencko et al., 2018). And in mammals, melatonin is a modulator of androgen and estrogen production in gonadal cells, and a modulator of the levels of associated receptors (Menéndez-Menéndez and Martinez-Campa, 2018).

G. MODULATION OF PINEAL ACTIVITY

In fish, several environmental factors have been described to influence production and release of melatonin by the pineal organ. Is most fish species, the melatonin rhythmic profile is thus under the control of internal factors named circadian clocks which are untrained by light (photoperiod) and temperature, the two main synchronizing signals of the environment (Masuda et al., 2003; Sánchez-Vázquez et al., 2019). Those factors are thus crucial pineal modulator and an increase in melatonin levels were reported in fish reared in shorter photoperiod or higher temperature (Esteban et al., 2006; 2013). Inhibition of melatonin production and secretion was also described in several fish species following a stress event. Such inhibition is mediated by cortisol (Benyassi et al., 2001; Falcón et al., 2010; López-Patiño et al., 2014). In rainbow trout (*Oncorhynchus mykiss*), increasing external salinity promotes melatonin synthesis by the pineal organ through an activation of AANAT protein synthesis (López-Patiño et al., 2011). Age has also to be considered since a progressive decline in melatonin production occurs in zebrafish (*Danio rerio*) from year 1 to year 4 (Zhdanova et al., 2008).

H. A SIMILAR ACTION IN VERTEBRATES

The high conservation of melatonin structure and its associated biosynthetic machinery have been demonstrated in phylogenetically distant organisms. Some authors also suggest that the neuroendocrine-immune interactions through melatonin is evolutionary conserved, at least in vertebrates (Kepka et al., 2015). While the literature is abundant for mammals, only little attention has been paid on birds and fish. In addition, very few studies have considered this melatonin action in reptiles and none was found about amphibians. In the present document, we defined that, in all vertebrate taxa to the exception of amphibians due to inexistent information, the available data demonstrated a common action of the melatonin hormone on the immune system.

Plenty of protocols have been followed since 80's, from *in vitro* to *in vivo* approaches. They considered different animal models, environments and procedures, leading sometimes to contradictory results. However, all these studies have participated in the better understanding of one of the countless significant actions of melatonin. Even if a general observation can be drawn considering non-mammalian vertebrates supporting a common action shared between vertebrate taxa, additional investigations are needed to better clarify the immune-modulatory actions of melatonin, considering both direct and indirect actions.

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V. Optimal aquaculture modalities for pike-perch

Foreword: This first experiment on pike-perch was conducted at the University of Lorraine, France. It aimed to characterize the effects of major husbandry practices and environmental factors on pike-perch physiology and to identify optimal rearing conditions ensuring low stress level and thereby good growth and survival. The hypothesis was that several factors, considered alone or in combinations, may create stressful conditions for the fish, leading to negative impacts on immunocompetence, growth and survival.

Multifactorial analyses revealed optimal aquaculture modalities improving husbandry fitness without clear effect on stress and immune status of pike-perch Sander lucioperca

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A. ABSTRACT

High mortality and impairment in growth rate during pike-perch (*Sander lucioperca*) ongrowing are among the major bottlenecks for its development

in aquaculture. These failures may be related to high stress responsiveness since the rearing conditions are not yet optimized for this species. The objectives were to characterize the stress and immunological responses of pike-perch to major aquaculture modalities, and to identify the optimal aquaculture conditions for improving its welfare status. In a screening experiment, eight factors considered as relevant for the welfare of pike-perch were compared in two modalities using a fractional multifactorial design (2^{8-4}). Each experimental unit represented a combination of 8 factors in two modalities including grading, stocking density (15 *vs* 30 $kg.m^{-3}$), feed type (sinking *vs* mid-floating), light intensity (10 *vs* 100 lux), light spectrum (red *vs* white), photoperiod (long *vs* short), dissolved oxygen (60 *vs* 90 %) and temperature (21 *vs* 26 °C). Fish sampling occurred on days 36 and 63. Stress markers (glucose, cortisol and brain serotonergic activity), innate immune parameters (plasma lysozyme and complement activities) and expression of some immune genes were assessed. Light intensity and the type of feed clearly appeared as directive factors for pike-perch culture. A strong effect of the feed type was observed on growth parameters while survival was impacted by high light intensity. Light characteristics (intensity, spectrum and photoperiod) and temperature were identified as determining factors for physiological and immune markers. No obvious relation was established between stress status and growth parameters and further investigations are needed to improve management strategies of pike-perch culture and knowledge on the relations between environmental conditions, stress and immunity in percid fish.

B. INTRODUCTION

Over the last decade, European inland aquaculture has shown weak increase in productivity despite the increase in the demand for fish products throughout the world (FAO, 2014). This low performance in fish production might be attributed to the low number of fish species that are cultivated in Europe. Indeed, European aquaculture is mainly focused on 5 fish species: Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*). Therefore, it is essential to diversify the fish species pool in order to increase fish productivity throughout Europe and to tap into new niche markets. Pike-perch (*Sander lucioperca)* is one of the most promising freshwater fish species for diversification and an attractive alternative for inland aquaculture species according to its relatively fast growth compared to other, its high quality flesh and a favorable market acceptance leading to high economical expectations species (Hilge and Steffens, 1996; Barry and Malison, 2004; Wang et al, 2009; Dalsgaard et al, 2013).

However, the culture of pike-perch is still limited by an unpredictable high mortality rate and impairment in growth rate during both larval and juvenile stages with survival rate estimated between 8 and 30 % (Kestemont et al, 2007; Szkudlarek and Zakes, 2007; Dalsgaard et al, 2013). A low welfare related to high stress level may be one of the major causes of high mortality rate observed for young pike-perch. While intensive aquaculture is well mastered for salmonid species such as Atlantic salmon and rainbow trout, aquaculture technology for percid fishes is not yet optimal. It has also been demonstrated that percid fish such as Eurasian perch (*Perca fluviatilis*) are more sensitive to some aquaculture stressors such as emersion and handling (Jentoft et al, 2005). For the latter species, it was also demonstrated that moderate hypoxia may considerably impair the behavior but also the physiological status such as the immune functions (Strand et al, 2007; Douxfils et al, 2014). Stress responsiveness has received little attention in pike-perch whatever its developmental stage. It has been reported that light characteristics such as light intensity and light spectrum have a huge impact on the pike-perch physiology and behavior due to some retinal histo-anatomical features (presence of *tapetum lucidum* and macroreceptors) which improve their vision in dim environment (Kozlowski et al, 2010; Luchiari et al, 2009; Sarameh et al, 2012). It was especially observed that pike-perch

exhibited higher growth rate and food conversion rate under high light intensity (100 lux) of red spectrum than under white spectrum (Luchiari et al, 2009). But there is no clear information about the optimal intensity or light spectrum. Luchiari et al (2006) also showed a clear preference of pike-perch for low light intensities. These findings are in agreement with the behaviour of juvenile and adult pike-perch in natural environments since this species is a crepuscular predator that is actively feeding during dusk and night (Luchiari et al, 2006; Zingel and Paaver, 2010; Dalsgaard et al, 2013). The effects of temperature conditions on pike-perch stress physiology are also reported inconsistently. It was reported that the optimal temperature for pike-perch is in the range from 10 to 27 °C (Frisk et al, 2012), but fish size should be considered when optimizing temperature level in aquaculture production. According to some authors, better growth and feed utilization for ongrowing pike-perch are achieved under high temperature conditions ranging between 25-28 °C (Rónyai and Csengeri, 2008; Wang et al, 2009; Dalsgaard et al, 2013). Nonetheless, Frisk et al. (2013) reported that a smaller fraction of metabolic scope was utilized for digestion at 19 °C compared to 25 °C, indicating that low temperature conditions may be more suitable for pike-perch reared under intensive culture conditions. Extended and continuous photoperiods have been proposed to improve the growth performance in some fish species by increasing food intake (Biswas et al, 2016), but no attempt has been done for pike-perch. Photoperiod manipulations should be appropriate to the feeding behaviour of targeted fish species to avoid a possible stress side effect.

The relationships between population parameters such as stocking density and physiological stress status or immune competence have been described in various fish species (Barton, 2002; Pankhurst, 2011) whereas limited information is available for pike-perch. Preliminary observations reported that high stocking density has no marked effects on growth and food utilization of young pike-perch, and that pike-perch juveniles can be maintained at high densities comprised between 30 and 60 $kg.m^{-3}$ without any increase in physiological stress response (Molnar et al, 2004; Steenfeldt et al, 2010; Dalsgaard et al, 2013). On the contrary, another study reported that high density may increase the susceptibility to diseases for pike-perch juveniles (Jensen et al, 2011). According to our observations, juveniles of pike-perch in intensive culture conditions are found to feed both on the ground and in the water column. Mid-floating feed has lower sinking velocity

and better dispersion in the water which can improve feeding behavior in some fish species. No clear data are available on juvenile pike-perch and current practices are not yet standardized.

Numerous other stress factors can be encountered by cultured fish from water temperature to grading size and, as in any biological system, the overall performances are the result of interactions from multiple factors (Trabelsi et al, 2011). Therefore, fractional factorial approach has been proposed to consider simultaneously the impact of a large number of interrelated aquaculture factors using a few number of experimental units (Kobilinsky, 2000; Hamre et al, 2004; Gardeur et al, 2007; Teletchea et al, 2009).

It has been reported that a bi-directional communication between corticotropic axis and immune system is essential to maintain homeostasis in mammals and teleosts (Tort, 2011; Mathieu et al, 2014; Nardocci et al, 2014). Many pieces of evidence clearly support this close interaction since some of the immune and endocrine messengers belong to the same family of molecules and that the head kidney plays a central role in stress response regulation (Tort, 2011). In Eurasian perch, it has been shown that some corticosteroid hormones namely cortisol and 11-deoxycorticosterone are active mediators of the immune activity (Mathieu et al., 2014). However, the relationships between stress and immunity in pike-perch have received little attention.

In aquaculture, prolonged, repeated and/or unavoidable other stressors are largely associated to maladaptive physiological effects including failures in immune functions and disease resistance (Fast et al, 2008; Douxfils et al, 2011; Tort, 2011).The objectives of the present study were thus *(a)* to characterize the effects of major husbandry and environmental factors on growth related parameters and physiological status of cultured pike-perch and *(b)* to identify the optimal husbandry and environmental conditions which may induce mild physiological stress response, and thereby improve the growth and welfare of pike-perch in intensive culture conditions. To achieve these objectives, we selected 8 major husbandry and environmental factors according to the current pike-perch aquaculture practices and available information on potential stress impact of those factors on percid fish. These factors were compared in two modalities using a fractional factorial design.

C. MATERIALS AND METHODS

1. Experimental design

Taking into account current practices in major European pike-perch intensive farms and available data in the literature (Luchiari et al, 2006; Teletchea et al, 2009; Wang et al, 2009; Sarameh et al, 2012; Dalsgaard et al, 2013), eight environmental factors at two levels (Table 1) were selected and tested in a multifactorial experiment based on a $2^{(8-4)}$ reduced factorial design (Kobilinsky, 2000; Torstensen et al, 2001; Hamre et al, 2004; Gardeur et al, 2007; Mairesse et al, 2007; Blanchard et al, 2008). With a full factorial design study of 8 factors at 2 levels, 256 (2^8) treatments would be tested resulting in the calculation of the main effects and all the interactions. With a fractional factorial design study, the number of combinations (treatments) is reduced from 256 (2^8) to 16 (2^4). To generate such a design, an alias structure was selected (Table 2), determining which effects are confounded with others (Hamre et al, 2004, Trabelsi et al, 2011). Thus, it is possible to calculate main effects separated from each other and from the effects of two-factor interactions (Kobilinsky, 2000; Hamre et al, 2004; Gardeur et al, 2007). The main advantage of this approach is that it considers simultaneously the impact of a large number of interrelated aquaculture factors using a limited number of experimental units. With a $2^{(8-4)}$ reduced factorial design, each of the 16 combinations was tested once but each level of every factor was repeated eight times (Table 3).

Table 1
Selected modalities for the 8 environmental factors.

Factor - *abbreviation*	Level	
Light intensity (lux)	100	10
Light spectrum	Industrial white	Red (610 nm)
Photoperiod (L:D)	24:00	10:14
Density (kg m^{-3})	30	15
Temperature (°C)	26	21
Oxygen saturation (%)	90	60
Feed type	Mid-floating	Sinking
Fish grading	Yes	No

Table 2
Aliasing structure considering the 8 environmental factors: light intensity (Int.), oxygen saturation (Oxy.); density (Dens.), light spectrum (Spec.), photoperiod (Photo.), water temperature (Temp.), grading and feed type.

Group	Aliased effects
1	Int.*Oxy. ; Dens.*Grading ; Spec.*Temp. ; Photo.*Feed
2	Int.*Temp. ; Dens.*Photo. ; Spec.*Oxy. ; Feed*Grading
3	Int.*Dens. ; Spec.*Feed ; Photo.*Temp. ; Grading*Oxy.
4	Int.*Spec. ; Dens.*Feed ; Photo.*Grading ; Temp.*Oxy.
5	Int.*Grading ; Dens.*Oxy. ; Spec.*Photo. ; Temp.*Feed
6	Int.*Photo. ; Dens.*Temp. ; Spec.*Grading ; Feed*Grading
7	Int.*Feed ; Dens.*Spec. ; Photo.*Oxy. ; Temp.*Grading

Table 3

Combinations (c1 - c16) of the factors following a $2^{(8-4)}$ reduced factorial design and results. Final weight heterogeneity (CV); Light spectrum: W = White, R = Red; Feed: S = Sinking, F = mid-Floating; Grading: Y = with manipulations mimicking grading, N = without grading. Global score: note of interest for each combination, based on husbandry output variables. The grey lines correspond to the five best combinations according to the global score of interest.

	Variables tested								Variables studied																			
Combination of the factors	Light intensity (lux)	Density (kg.m^{-3})	Light spectra	Photoperiod (h)	Temperature (°C)	Feed	Grading	Oxygen saturation (%)	Final individual weight (g)	Mortality Rate (%)	CV (%)	Specific growth rate (%d-1)	Plasma Cortisol (D36, ng.mL^{-1})	Plasma Cortisol (D63, ng.mL^{-1})	Plasma Glucose (D36, µg.mL^{-1})	Plasma Glucose (D63, µg.mL^{-1})	Serotonergic activity (D36)	Serotonergic activity (D63)	ACH50 (D36)	ACH50 (D63)	Lysozyme activity (D36, U)	Lysozyme activity (D63, U)	Relative *C3* gene expression to ef1+βactin (D36)	Relative *lysozyme* gene expression to ef1+βactin (D36)	Relative *C3* gene expression to ef1+βactin (D63)	Relative *lysozyme* gene expression to ef1+βactin (D63)	Global score of interest	Rank of the global score
c1	10	30	W	24	21	S	Y	90	168	4	37	0.9	84	31	457	318	0.84	0.61	98	202	17	19	0.011	0.018	0.0001	0.079	3.9	2
c2	100	15	R	10	26	F	N	60	146	3	50	0.7	17	13	378	427	0.71	0.77	145	142	16	19	0.027	0.016	0.0003	0.027	1.3	6
c3	100	15	W	24	21	S	N	60	172	13	40	1.0	20	15	355	371	0.94	0.74	162	215	16	18	0.014	0.045	0.0001	0.114	3.1	5
c4	100	30	R	10	21	S	N	90	143	31	29	0.7	19	14	264	340	0.79	0.72	153	216	17	19	0.027	0.024	0.0001	0.042	0.4	8
c5	10	15	R	10	21	S	Y	60	131	7	53	0.5	15	14	424	379	0.96	0.81	157	181	16	19	0.002	0.029	0.0002	0.025	-0.5	10
c6	10	15	W	10	21	F	N	90	88	7	67	0	16	14	342	365	0.53	0.82	76	264	15	15	0.004	0.061	0.0005	0.090	-5.4	16
c7	100	15	R	24	21	F	Y	90	113	24	61	0.3	17	13	273	343	0.69	0.78	140	210	14	19	0.021	0.024	0.0008	0.065	-4.0	15
c8	10	15	W	24	26	F	Y	60	146	10	53	0.7	60	13	322	359	0.60	0.72	125	244	16	21	0.004	0.022	0.0004	0.030	0.4	7
c9	100	15	W	10	26	S	Y	90	158	13	39	1.1	26	13	371	411	0.87	0.75	132	225	17	18	0.015	0.055	0.0024	0.088	3.1	4
c10	100	30	W	10	21	F	Y	60	122	41	52	0.7	17	14	339	317	1.06	0.76	173	261	17	20	0.008	0.028	0.0000	0.011	-3.0	13

c11	100	30	W	24	26	F	N	90	148	18	61	0.8	89	29	328	405	0.81	0.72	101	257	15	20	0.012	0.045	0.0010	0.015	-0.6	11
c12	10	30	R	10	26	F	Y	90	114	24	57	0.3	23	13	304	325	1.19	0.68	96	250	11	17	0.006	0.061	0.0002	0.014	-3.6	14
c13	100	30	R	24	26	S	Y	60	151	32	37	0.8	47	15	466	412	0.71	0.77	100	233	18	20	0.019	0.067	0.0005	0.021	0.3	9
c14	10	30	R	24	21	F	N	60	117	4	72	0.4	17	13	271	337	0.94	0.75	132	298	15	16	0.012	0.033	0.0013	0.019	-2.8	12
c15	10	30	W	10	26	S	N	60	167	3	36	0.9	27	15	420	373	0.67	0.73	124	249	16	16	0.027	0.049	0.0005	0.017	4.0	1
c16	10	15	R	24	26	S	N	90	169	7	40	0.9	18	15	375	369	0.89	0.64	67	267	14	18	0.003	0.019	0.0001	0.010	3.4	3
Mean									140	15	49	0.7	32	16	356	366	0.82	0.73	124	232	16	18	0.014	0.037	0.0005	0.042		
SD									24	12	13	0.3	24	6	63	35	0.17	0.06	31	38	2	2	0.008	0.017	$\hat{0}$.0006	0.034		

Table 4
List of the husbandry variables and formulas. CV: Final weight heterogeneity.

Variables	Calculation
Final individual weight (g)	= mean of the final individual weight
Mortality rate (%)	= (number of dead individuals / initial number of individuals) *100
CV (%)	= standard deviation of final individual weight *100 / mean of the final individual weight
Specific growth rate (% day^{-1})	= (Ln(final individual weight) – Ln (initial individual weight)) *100 / duration of the experiment

Table 5
Sequences and melting temperatures (Tm) of primers used for gene expression quantification.

Gene	GenBank accession #	Sens	Sequence (5' to 3')	Tm (°C)	Efficiency
Complement component 3 (C3)	MF472630	Forward	TGGTGATGTGAGAGGAGCAG	56.5	92 %
		Reverse	GACGTCATGGCAACAGCATA	55.5	
Lysozyme	MF472629	Forward	AGCCAGTGGGAGTCGAGTTA	57.8	100 %
		Reverse	CATTGTCGGTCAGGAGCTCA	57.0	
β-actin	MF472627	Forward	CGACATCCGTAAGGACCTGT	56.5	98 %
		Reverse	GCTGGAAGGTGGACAGAGAG	57.3	
EF1alpha	MF472628	Forward	TGATGACACCAACAGCCACT	56.8	100 %
		Reverse	AAGATTGACCGTCGTTCTGG	54.9	

2. Animals and rearing conditions

The present experiment was carried out in agreement with the European and French national legislations on animal welfare (protocol number: C54-547-1).

A stock of 3200 mixed-sex juveniles pike-perch (20-30 g) was provided by Asialor farm (Dieuze, France) and transferred to the URAFPA facilities at the University of Lorraine, France. Fish were first reared in 6 large tanks for acclimation and ongrowing until they reached 91 ± 5 g body weight. Working with fish around 90 g was relevant since high mortality rate is still observed at this developmental stage. During acclimation, they were maintained in constant conditions (temperature: 23 °C; light intensity: 10 lux; photoperiod: 12D:12L) and fed twice daily at 1.5 % biomass with a sinking food. They were then distributed into 16 indoor 200 L-tanks and stocked at two densities (15 or 30 kg m^{-3}) according to Table 3. Each of the 16 experimental units was operating independently in a recirculating circuit (RAS). Rearing conditions, including light intensity, light spectrum, photoperiod, temperature, feed type, grading manipulations and oxygen saturation were then applied as indicated in Table 3. Temperature, light intensity and oxygen saturation were checked daily. Fish diets consisted in mid-floating or sinking feed (D-D Optibream 2P or 2P-Optobream, 4 mm, Skretting, France) containing the same contents of crude proteins (46%) and lipids (16%). Food fixed at 1.5% biomass was distributed during a photophase of either 24 h or 10 h depending on the tank photophase. Manipulations mimicking grading were applied every two weeks. For this purpose, fish were starved one day before handling. All fish were harvested from each tank by a handling-net, and put in one or two basins with water. Then, they were transferred to a fish size grader to simulate the sorting process. Duration of grading manipulations varied between 15-20 min per tank.

3. Sampling procedure

Fish were sampled on days 36 and 63 and were starved one day before. Six fish were removed randomly from each tank and anesthetized with MS-222 (150 mg l^{-1}). Blood was quickly collected by caudal vein puncture with heparinized syringes within 5 min and centrifuged at 7,500 g during 10 min at 4 °C. Plasma was stored at -80 °C until assayed. Fish were then rapidly killed by cervical dislocation to collect the whole brain and the anterior

kidney. These organs were directly frozen in liquid nitrogen and stored at -80 °C.

4. Husbandry variables

Final individual weight (FIW), mortality rate (MR) and weight heterogeneity (CV) were all determined on day 63 for each experimental condition (Table 4). Specific Growth Rate (SGR) was also estimated at days 24 and 63.

5. Stress indicators

Cortisol and glucose assays

Plasma cortisol was assayed in triplicate using a cortisol ELISA kit (DRG, EIA-1887), following the manufacturer's instructions (BioSource, Belgium). The intra-assay coefficient of variation was 3.6 %. The assay dynamic range was between 0 and 800 ng ml^{-1} and the analytical sensitivity was 2.5 ng ml^{-1}. Plasma glucose was determined calorimetrically based on a glucose oxidase/peroxidase method described by Trinder (1969).

Brain neurotransmitters

High Performance Liquid Chromatography (HPLC) was performed according the methods of Lepage et al (2000), with some modifications, to assess in whole brain serotonergic activity, expressed as serotonin (5-HT) / hydroxyl-indol-acetic acid ratio (5-HIAA) ratio.

For each fish, the whole brain tissue was weighed out and homogenized for 6 min in perchloric acid 4% (250 µL per 50 mg of tissue) containing 4 µM 2-3 dihydroxybenzoic acid (DHBA) as internal standard. The homogenate was sonicated for 20 s and then centrifuged at 21.000 g for 20 min at 4 °C. The supernatant was transferred to a new tube, mixed with HPLC mobile phase (v/v; 75 mM NaH_2PO_4, 7 mM octane sulfonic acid (OSA, Sigma-Aldrich) and 10 % MeOH adjusted to pH 3) and centrifuged at 21.000 g for 20 min at 4 °C. The whole procedure was carried out on ice.

HPLC analysis was performed using a GP50 gradient pump (Dionex, Sunnyvale, USA) equipped with an autosampler FAMOS (LC packings). Neurohormones were monitored using a DC amperometry detector (Dionex, Sunnyvale, USA) with Glassy Carbon Working Electrode (0.80V, Ag/AgCl – P/N 061677). The mobile phases were all degassed with helium. Chromeleon™ software (6.8) (Dionex, Sunnyvale, USA) was used for data

acquisition and processing. The samples were individually applied (50 µl) on a 2.6 µm particle size (150 x 4.6 mm, I.D.) C_{18} analytical Kinetex column at 1 ml/min. The mobile phase consisted of 75 mM NaH2PO4, 7 mM octane sulfonic acid (OSA, Sigma-Aldrich) and 10 % MeOH adjusted to pH 3.0. The column was reconditioned by washing with 95 % MeOH during 10 min and then re-equilibrated with buffer during 20 min. The column was kept at 25 °C.

Purified 5-HT and 5-HIAA were obtained from Sigma-Aldrich. Standard solutions were treated similarly to samples. Concentrations of the compounds were calculated by interpolation of their respective standard curves. The intra-assay coefficients of variation were 5.9 % and 7.1 % respectively.

6. Immune variables

Plasma lysozyme activity

Lysozyme activity was evaluated in plasma samples by the turbimetric method (Siwiki and Studnicka, 1987; Douxfils et al, 2012). Plasma samples (10 µl) were mixed with 10 µl of Na_2HPO_4 0.05 M pH 6.2 and 130 µl *Micrococcus lysodeikticus* (Sigma-Aldrich) solution (0.6 g/L). This assay was performed in triplicate. Absorbance was measured at 450 nm every 5 min during 30 min at room temperature. Lysozyme activity (units) is defined as the amount of enzyme decreasing the turbidity of 0.001 OD per min.

Plasma haemolytic activity of the alternative complement pathway

Plasma complement (ACH50) was assayed by measuring the haemolytic activity in plasma samples using rabbit erythrocytes as targets (Sunyer and Tort, 1995). In brief, serial dilutions (from 15 to 160 times) of plasma samples were performed in veronal buffer (Biomerieux, Marcy-l'Etoile, France). Then 60 µl of each dilution were mixed with 10 µl of 3 % rabbit erythrocytes suspended in veronal buffer. After incubation at 37 °C for 100 min and centrifugation at 2000 g for 10 min at 4 °C, supernatants were collected and read at 405 nm. The spontaneous hemolysis was obtained by adding veronal buffer to 10 µl of rabbit erythrocytes and total lysis was obtained by mixing 10 µl of rabbit erythrocytes to distilled water (total volume = 70 µl). ACH50 was calculated by linear regression and corresponds to the lysis of 50 % of the rabbit erythrocytes.

7. Expression levels of some immune genes in anterior kidney

For further immune markers, the expression level of two immune-related genes was determined in anterior kidney tissues, namely C-type lysozyme and complement component 3 (C3). Primer sequences are presented in Table 5. Total RNA from anterior kidney was extracted using TRIzol Reagant (ThermoFisher Scientific) according to manufacturer's instructions. Tissue samples were homogenized using a SpeedMill PLUS homogenizer (AnalytikJena, Germany) in tubes containing ceramic beads and TRIzol Reagant. Total RNA was resuspended in 50 µl of DPEC-treated water. RNA integrity and concentration was checked by denaturating gel electrophoresis (1.2% agarose) and OD_{260}/OD_{280} and OD_{260}/OD_{230} nm absorption ratio using Nanodrop-1000 (ThermoFisher Scientific). Twelve µg of each RNA sample were treated with FreeDNA kit (Ambion, Austin, TX, USA) to remove genomic DNA. mRNA was then retrotranscribed with Reverse Transcription System kit (Promega, Wisconsin, USA) according to manufacturer's instructions. The cDNA was then 20 times diluted and aliquoted. qPCR was performed using Power SYBR® Green PCR Master Mix (Applied Biosystem, Warrington, UK), 2.5 µl of both right and left primers (5 µM) and 5 µl of the diluted cDNA. A four steps experimental run protocol was followed: denaturation (10 min at 95 °C), amplification (40 cycles, 15 s at 95 °C, 1 min at 60 °C), melting curve (40 to 95 °C, heating rate 0.10 °C s^{-1}) and a final cooling step (4°C) using a StepOne plus real time PCR machine (Applied Biosystem). Relative quantification of the target gene transcript was calculated following the Pfaffl method (Pfaffl, 2001), considering the Ct value and the primer efficiency. The relative expression levels of C-type lysozyme and complement C3 in each sample were normalized with the geometric mean of ef1-α and β-actin calculated by the relative standard curve method.

8. Statistical analyses

To determine the best combinations of factors-modalities, each experimental unit was assigned to a global score of interest. This global score was calculated using results of husbandry output variables and was based on the transformation of each output in centered reduced output (Gardeur et al, 2007). Principal component analyses (PCA) were also performed to analyze the global effect of combinations on husbandry output variables.

Main effects and two factor-interactions were then analyzed using Analys software (Kobilinsky, 2000). This method is first based on the detection of potentially active effects using Daniel graphics (Daniel, 1959). It is followed by ANOVA to test these potentially active effects. Significant results ($p < 0.05$) were finally confirmed by ANOVA ($p < 0.05$) using Statistica software version 10 (StatSoft Inc., France, 2011). Significant correlations were then calculated between growth parameters and stress and immune markers.

D. RESULTS

1. Husbandry parameters

Global score results showed that the combination 15 (c15) resulted in the best husbandry performances (Table 3). High global scores of interest (> 3) were also obtained for c1, c16, c9 and c3. All these experimental treatments resulted in the highest FIW (158 to 172 g), the lowest MR (3 to 13 %) and the highest SGR (0.9 to 1.1 % d^{-1}). The lowest weight heterogeneity was observed for c1, c4, c9, c13 and c15 and averaged 36 %. The five best combinations were commonly influenced by the type of feed (sinking) as input variable. The worst husbandry outputs were observed with combinations c6, c7, c10 and c12 and they had in common the use of mid-floating feed. Further ANOVA revealed no effect of feed type on SGR between D0 and D24 while sinking feed significantly improved SGR between D24 and D63 ($p < 0.05$).

Further comparison by PCA approach confirmed that only the feed type was the major influencing factor husbandry parameters but also with the light intensity (Fig. 1). Indeed, on the axis 1, c1, c3, c9, c15 and c16 were characterized by a high SGR, a high FIW and a low CV. The use of sinking feed mainly defined these combinations confirming that the use of such feed type led to significant higher SGR (Fig. 2a, $p < 0.01$) and FIW (Fig. 2b, $p < 0.001$) with values reaching respectively 0.86 ± 0.19 % d^{-1} and 157 ± 15 g. This type of feed also decreased the CV from 59 ± 8 % to 39 ± 7 % (Fig. 2c, $p < 0.01$). The opposite results were observed for c6, c7, c12 and c14. On the second axis, combinations c4, c10 and c13 displayed a high MR and they were mainly defined by the high light intensity vector. The opposite results were observed with the low light intensity for c2, c5 and c8. Lowest MR was observed under low light intensity (8 ± 7 %) compared to high light intensity (22 ± 12 %) ($p < 0.05$) (Fig. 2d).

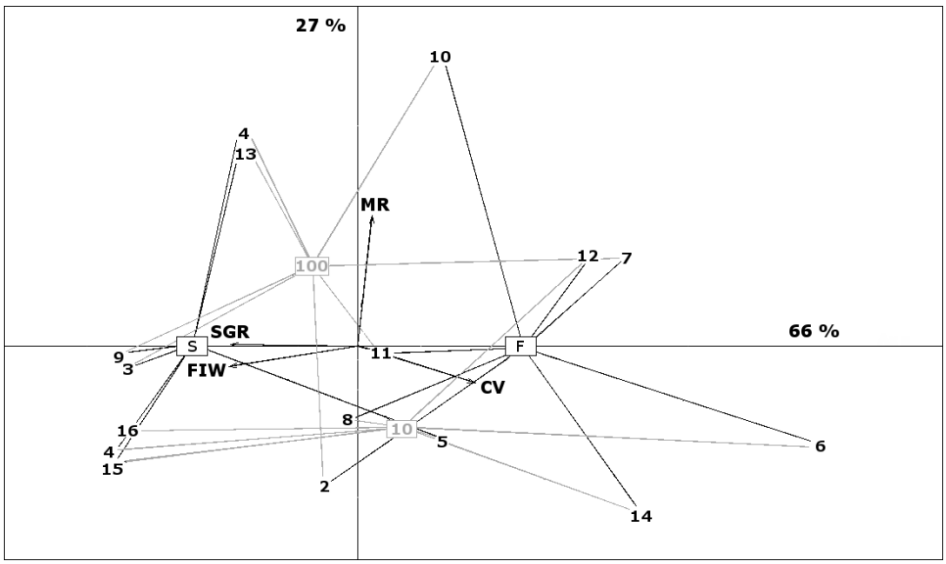


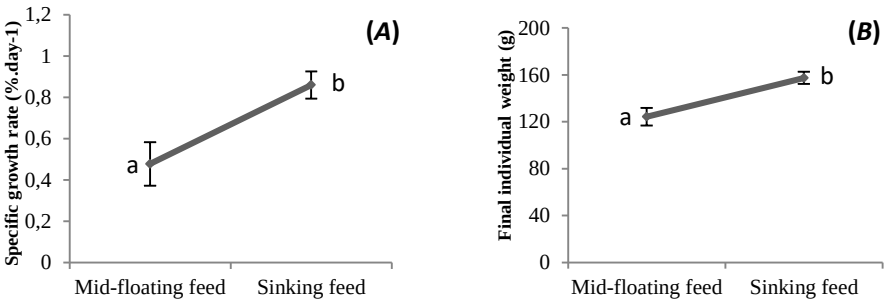
Fig. 1: Projections of husbandry outputs and combinations (1 to 16) on the two first axis of the principal component analysis. The plans 1 and 2 of the PCA explain 93 % of the projected inertia. Axis 1 (inertia 66 %) represents Specific growth rate (SGR), Final individual weight (FIW), final weight heterogeneity (CV) and the type of feed (Sinking *S vs* mid-floating *F*). Axis 2 (inertia 27 %) represents mortality rate (MR) and light intensity (10 *vs* 100 lux).

2. Stress and immune parameters

The experimental combinations (c1, c3, c4, c5, c9, c13, c15 and c16) that included the sinking feed induced higher glucose level (392 ± 66 µg.ml^{-1}) on day 36 (Fig. 3a, $p < 0.05$) but not on day 63. High temperature level (26 °C; combinations c2; c8; c9; c11; c12; c13; c15; c16) also increased glycaemia on day 63 (Fig. 3b, $p < 0.05$). No statistical influencing factor was observed for plasma cortisol level both on D36 and D63. Plasma cortisol values were nearly to basal level both on D36 and D63, except for C1 and C8 on D36; and a significant decrease was observed between D36 and D63 ($p < 0.05$). Therefore, non-significant coefficients of correlation could be calculated between cortisol or glucose values and those growth parameters on D36 and D63.

While no single factor influenced serotonergic activity in the brain, light spectrum and light intensity appeared clearly to act synergistically since the serotonergic activity increased when the red light was used with low light intensity (combinations c5, c12, c14 and c16) or when white light was combined with high light intensity (combinations c3, c9, c10 and c11) (Fig. 3c, $p < 0.001$). It is interesting to notice that the best three ranked combinations (c15, c1 and c16) in terms of husbandry performances were characterized by low values of serotonergic activity. Moreover, significant negative coefficient of correlation ($R^2 = 0.31$; $p < 0.05$) was calculated between values of serotonergic activity on D63 and those of final body weight. So, red light spectrum at low intensity or white light spectrum at high intensity may induce both a higher stress status in pike-perch.

Low oxygen saturation significantly increased complement activity in plasma on D36 ($p < 0.05$, Fig. 4a) but not on D63, and no significant factor interaction was detected. Values for complement activity increased between D36 and D63 ($p < 0.001$). Light intensity and photoperiod acted in synergy on complement gene expression level in kidney on day 36 and such effect is not detected on D63. Significantly lower values were observed when a 10 lux intensity was combined with a short photoperiod ($p < 0.05$) (Fig. 4b). No effect on lysozyme activity and C-type lysozyme expression was observed.



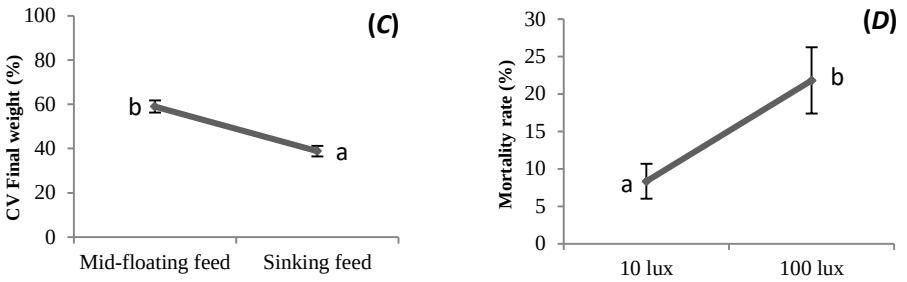


Fig. 2: Effects of tested factors on (*A*) specific growth rate, (*B*) final individual weight, (*C*) final weight heterogeneity, and (*D*) mortality rate. Day 63. Data are presented as mean ± SEM (n = 8). Lowercase letters indicate a significant difference at $p < 0.05$.

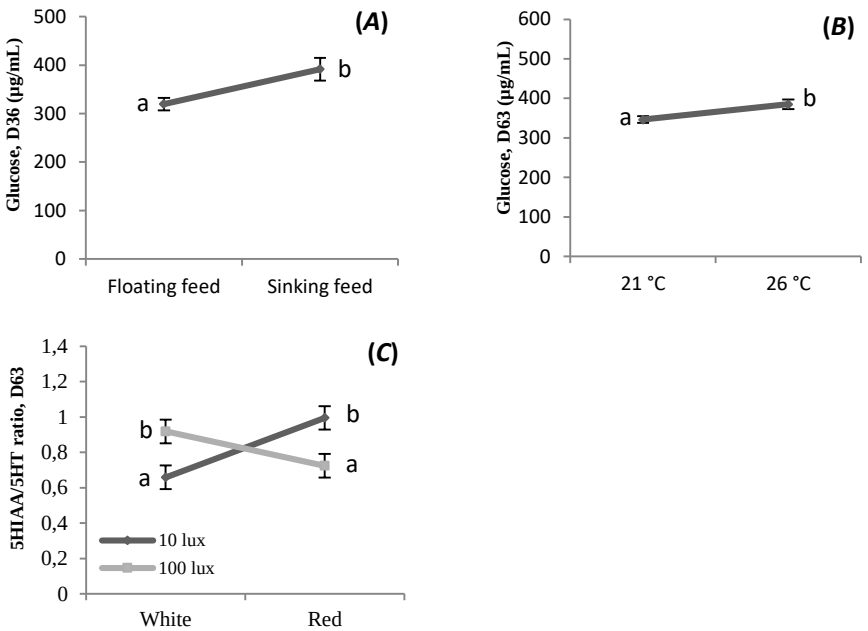


Fig. 3: Effects of tested factors on (*A*) plasma glucose D36, (*B*) plasma glucose D63, and (*C*) serotonergic activity in brain. Data are presented as mean ± SEM (n = 8 *(A)*; 8 *(B)*; 4*(C)*). Lowercase letters indicate a significant difference at $p < 0.05$.

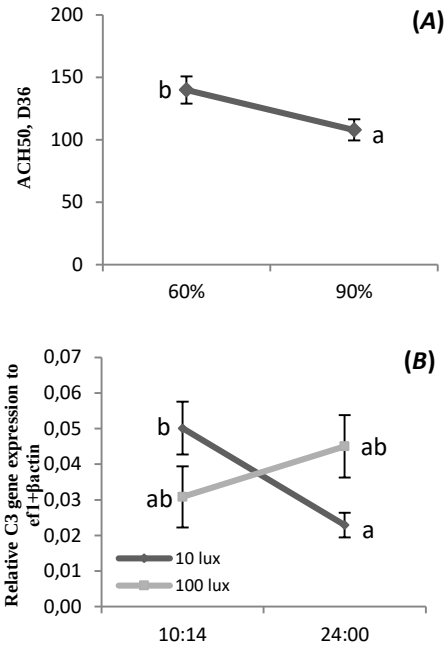


Fig. 4: Effects of tested factors on (*A*) plasma complement activity, and (*B*) relative C3 gene expression. Data are presented as mean ± SEM (n = 8 *(A)*; 4 *(B)*). Lowercase letters indicate a significant difference at $p < 0.05$.

E. DISCUSSION

The present study aimed to define the main directive factor-modalities for pike-perch aquaculture as well as the best combination of husbandry factors inducing the lowest stress status together with high husbandry performances and better welfare status. To our knowledge, very few studies have examined the optimization of aquaculture conditions for percid fish by taking into account a wide range of environmental and husbandry factors. Despite the loss of resolution compared to a full factorial design, fractional factorial approach has the main advantage of considering simultaneously the impact of a large number of inter-related aquaculture factors while traditional full factorial design rarely exceeds 3 factors (Hamre et al, 2004; Gardeur et al, 2007; Trabelsi et al, 2011).

In the present study, we have considered a 2 month-experiment to better assess the effects of the 8 environmental factors on growth related parameters, stress markers and immune status. Only considering mortality, 3 of the 16 tanks exceeded 30 % of mortality while 4 other tanks did not reach 5 %. Such difference was not expected within 2 months but allowed us to consider more especially some factors as crucial for welfare and survival of pike-perch in intensive culture conditions.

Directive factors improving husbandry performances

The classification of the combinations and the PCA have revealed the type of feed and the light intensity as the two main directive factors for husbandry variables, which are regarded as valuable indicators for estimating the fitness in aquaculture conditions (Moberg and Mench, 2001). The use of sinking feed improved growth performances and decreased weight heterogeneity. Fish were fed with sinking feed before the experiment and no habituation to mid-floating feed was done. However, the change of feeding strategy and the lack of habituation do not have impacted feeding behavior during the first days since no effect of feed type was detected on SGR between D0 and D24 in contrast to differences observed on D63 at the end of the experiment. The use of mid-floating feed has thus impacted, independently of the lack of habituation, food intake resulting in decreased growth performances. It has been demonstrated that the use of sinking feed has a better effect on food intake and thereby on husbandry performances comparing to mid-floating feed in other fish species such as the Atlantic halibut *Hippoglossus hippoglossus* (Kristiansen and Ferno, 2007).

The present study revealed that light intensity is a determining factor for pike-perch with better survival observed under low light intensities. Light intensity affects many behavioral and biological processes in fish, such as foraging and growth (Fraser and Metcalfe, 1997; Trippel and Neil, 2003; Luchiari et al, 2006). The present study confirmed that pike-perch reared under conditions of low light intensity set at 10 lux displayed better husbandry performances than those submitted to 100 lux. Such preference of pike-perch for low light intensities has already been described by Luchiari et al (2006) since this species is a nocturnal and crepuscular predator. *Sander* species also possess a *tapetum lucidum* that is a specific anatomo-histological tissue of the retina which greatly amplifies the eye sensitivity to light (Feiner and Höök, 2015). This preference for low light intensities can be related to an innate behavior

to avoid possible harmful effects of light on light-sensitive eyes (Sandström, 1999; Luchiari et al, 2006).

It is surprising that other tested factors such as grading were not demonstrated as directive influencing factors on husbandry variables in the context of the present study. Indeed, it has been reported that frequent manipulations markedly affect growth rate of young Eurasian perch (Jentoft et al, 2005; Strand et al, 2007), a species biologically close to pike-perch. Perhaps, the frequency of grading every two weeks, and the relative manipulations were not so detrimental at the developmental stage used in the present experiment. Temperature level was not also found as main directive factor for husbandry performances, and the positive interactions with sinking feed were observed with the treatments which included the lowest temperature. So, our finding did not corroborate previous reports that high temperature promotes growth rate in pike-perch juveniles (Rónyai and Csengeri, 2008; Wang et al, 2009; Dalsgaard et al, 2013), but may support the hypothesis that more energy is spent for increased metabolic rates when pike-perch are reared at 25°C (Frisk et al, 2013). And considering that high temperature may weaken the immunocompetence and thereby increase pathogen outbreaks in intensive culture conditions (Raida and Buckmann, 2007; Martins et al., 2011), we therefore conclude that temperature around 21°C is more favourable for pike-perch culture.

Our results also support observations reporting that high stocking density has no marked effects on growth and food utilization of young pike-perch, and that small juveniles can be kept at high densities ranging between 15 and 30 $kg.m^{-3}$ without any increase in physiological stress response (Molnar et al., 2004; Steenfeldt et al., 2010; Dalsgaard et al., 2013). It was also reported that higher densities (30 to 60 $kg.m^{-3}$) for larger pike-perch up to 2 kg are associated to good growth without any increase in crowding stress parameters (Dalsgaard et al., 2013).

Percid fish display sexual growth dimorphism as females grow faster than males (Craig, 2000). Therefore, rearing all-female populations would improve production of these species and this would be achieved by hormonal sex reversal treatment (Rougeot, 2015). The difference of growth performances between mixed-sex families and all-female families reaches 30 % in juvenile Eurasian perch after 360 days of rearing (Rougeot and Mélard, 2008). However, the methods for all-female production have not been yet

optimized for pike-perch, and since sex-determination in not possible with juvenile pike-perch, a mixed-sex population was used in the present experiment.

Directive modality interactions for pike-perch physiological and immune status

In the present experiment, no effect of the different culture conditions on plasma cortisol level was observed. Cortisol is well known to be part of the stress response and to be a major stress indicator promoting the metabolic pathways that increase plasma glucose levels in response to energy expenditure (Laiz-Carrion, 2003; Milla et al, 2010; Oliveira et al, 2013). A decrease in cortisol release along exposure to long-lasting stressors is a mechanism that minimizes the deleterious effects of sustained cortisol elevation on biological functions (Milla et al, 2015). While the assessment of cortisol is used as a valuable indicator of first stress response, it has been debated whether such indicator is a reliable indicator of welfare status since itself is not predictive of the fish's ability to cope with a stress situation (Ellis, 2012). A previous experiment on pike-perch (unpublished data) showed that plasma cortisol level returned to basal level as soon as 1 h post-stress while glucose peak was sustained for more than 3 h. A similar rapid decrease in the amplitude of stress response was characterized in juvenile Eurasian perch following a single or multiple emersion stressor (Douxfils et al, 2014). These observations may suggest a habituation to stress and/or a rapid metabolism of cortisol indicating the interest for using various stress indicators to account for the stress responsiveness in pike-perch. While assaying cortisol from plasma samples has already been used in several fish species with good results when collected within 5 min to avoid handling-induced cortisol (Wang et al, 2004; Douxfils et al, 2011), non-invasive methods have also been developed including direct fecal corticoid metabolites measurement and cortisol release measurement in water (Ellis et al, 2004; Cao et al, 2017). These methods should be better considered for offering various advantages including the absence of fish disturbance and the reduction of the number of animals required (Fanouraki et al, 2008).

The significant impact of high temperature on plasma glucose observed in the present study may be related to a better feeding behaviour since the feed type influenced glycaemia. Higher temperature conditions ranging between 25-28 °C have been described to promote growth and feed utilization in pike-perch

juveniles (Rónyai and Csengeri, 2008; Wang et al, 2009, Dalsgaard et al, 2013). However, if this is convenient for juveniles, it cannot be extrapolated to larger pike-perch without investigation since several studies have shown that optimal temperature seems to decrease with fish weight as observed with the African catfish (*Clarias gariepinus*) or the Atlantic cod (*Gadus morhua)* (Hogendoorn et al, 1983; Björnsson et al, 2007; Rónyai and Csengeri, 2008; Wang et al, 2009). In the present experiment, while the feeding behavior may have been improved by high temperature, this factor did not significantly affect growth performances. Frisk et al. (2013) reported that a smaller fraction of metabolic scope was utilized for digestion at 19 °C compared to 25 °C, indicating that low temperature conditions are more favorable for pike-perch reared under intensive culture conditions. Even if this was not evidenced in the present study showing low cortisol levels on both D36 and D63, high temperature condition may induce a long-term stress that leads to energy reallocation resulting in less energy available for some biological functions such as growth, reproduction or disease resistance (Schreck et al, 2001; Tort, 2011; Segner et al, 2012, Milla et al, 2015).

Serotonergic activity has been described several times as a good indicator of acute and chronic stress in various fish species including the Senegalese sole *Solea senegalensis* and several salmonids (Winberg and Nilsson, 1993; Gesto et al, 2013; 2016; Conde-Sieira et al, 2014). When considering serotonergic activity level in the presented study, red light spectrum at low intensity or white light spectrum at high intensity induced both a higher stress status. Environmental colors affect the vision of fishes, influencing for example food intake, signals for hierarchical status, reproduction, growth and even survival (Downing, 2002; Politis et al, 2004; Luchiari and Pirhonen, 2008). Species-dependent preferences are a reflection of the photic environment the populations have evolved in (Migaud et al, 2007). Luchiari et al (2009) showed an improved specific growth rate and feed efficiency under red light, only considering high light intensity. In parallel, Luchiari et al (2006) described a preference for low light intensities. Among the three stress indicators tested in the present study, only brain serotonergic activity showed plausible correlation with husbandry performances, indicating that high stress level is one of the main causes of low husbandry performances in pike-perch culture. By elsewhere, brain serotonergic activity may be considered as a more reliable stress indicator in percid fish than plasma cortisol. It has been suggested that a low degree of responsiveness to stressors in cultured fish

may result in better performances (Pottinger and Pickering, 1997). The converse may also be true considering the greater advantage of fish with a high degree of stress responsiveness, through a faster acclimation to environmental and social changes (Pottinger and Carrick, 1999). While the latter authors have described no correlation between cortisol response and growth performances in rainbow trout, better growth was obtained in low cortisol responders (Fevolden et al, 2002). And, as well as plasma cortisol level, it was reported that brain serotonergic, noradrenergic and dopaminergic activities are influenced by dominant-subordinate relationships in several fish species, including the rainbow trout (Overli et al, 1999). Social dominance was not followed in the present experiment but could have influenced the lack of relationship between growth and cortisol level since it has been shown in other fish species that socially defeated animals exhibit increased glucocorticoid secretion, sustained sympathetic activation and other physiological stress responses (Winberg and Lepage, 1998; Overli et al, 1999).

In terms of cortisol level, it is also surprising that grading manipulations did not induce higher stress status of pike-perch since grading practice has already been reported to markedly affect fish welfare (Jentoft et al, 2005; Strand et al, 2007). Before starting the experiment, the fish were size graded several times according to common practices of intensive pike-perch culture. Grading manipulations were then applied every two weeks according to the protocol. To explain the absence of the expected impact on cortisol level, it could be hypothesized that pike-perch submitted to such sequential stressors can exhibit a habituation on its cortisol response and an attenuation of the response as shown in several fish species (Schreck, 2000; Koakoski et al, 2013). However, the results on habituation and accumulation are highly dependent on the type of stressor, the length of time between discrete stressors and the number of repeated stressor (Koakoski et al, 2013). In the present experiment, the purpose was not to characterize the stress response after fish manipulations but to characterize chronical effects of several husbandry practices and, therefore, the long-term impact on stress markers. It is worth noting that serotonergic activity appeared as more sensitive marker than cortisol since plausible correlation was established with growth rate.

Concerning immune status, humoral immune activities were slightly impacted by some tested factors during the 2-month period. The effects of low oxygen saturation on complement activity on day 36 were no longer

observed on day 63, suggesting only a short-term effect. This decrease in the impact of low oxygen saturation may be account for the increase in ACH50 values between D36 and D63. On the other hand, the effect of low oxygen level on humoral immunity may be explained by the fact that pike-perch requires high oxygen saturation and recommendations are to maintain the oxygen level above 50 % saturation to sustain growth and biofilter performance (Dalsgaard et al, 2013). In contrast to what was observed for growth rate, long photoperiod combined with high light intensity, which was characterized as a stressful factor, led to an increase in the immune expression level of both tested genes. Long photoperiod increased the exposure time to stressful light conditions that may have resulted in a short-term immune stimulation. However, since no single effect of photoperiod on stress and immune status was detected, the impact of such factor should be investigated in further experiments. The other factors tested in the present experiment were not revealed as directive variables for stress or immune status.

The lack of significant impact for high stocking density (30 $kg.m^{-3}$) corroborates previous reports indicating that pike-perch juveniles from 10 to 50 g should be maintained at densities below 15 - 30 $kg.m^{-3}$ while fish up to 2 kg can be kept around 30 - 60 $kg.m^{-3}$ without any increase in crowding stress parameters, suppressed growth, or increase in feed conversion ratio (Steenfeldt et al, 2010). The lack of enough information on the imposing stress events for pike-perch in intensive aquaculture did not allow further discussion concerning the impact of the tested factors-modalities on immune status. So, further investigations are needed to explore potential effects of high stress responsiveness on immune status of pike-perch.

F. CONCLUSION

This experiment was based on a factorial fractional design study which allowed simultaneously to study the effects of 8 common husbandry practices in two modalities on pike-perch growth and welfare. Several husbandry practices, including light intensity, feed type and temperature were revealed to be directive factors for husbandry and stress parameters in pike-perch aquaculture while immune status was mainly influenced by photoperiod and oxygen saturation level. Best husbandry performances were thus obtained with sinking feed and low light intensity, and no obvious relation was

established between stress status and growth parameters. Further investigations focusing on light characteristics, temperature and feed type are needed to improve management strategies of pike-perch culture, and knowledge on the interrelations between stress and immunity in percid fish.

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VI. Daily rhythms of immune markers

Foreword: Since the light environment profoundly affects pike-perch physiology, we considered the modulation of the immune system by the light environment whose characteristics are spectrum, intensity and photoperiod. Available data confirmed that several fish species are sensitive to the light in terms of behavior, growth and reproduction. However, few authors have considered the immune system. In this experiment, we thus aimed to identify the 24-h profiles for rhythmicity in several stress and innate immune markers. Furthermore, the potential effects of two light spectra on those parameters were compared.

Influence of the light spectrum on the daily rhythms of stress and humoral innate immune markers in pike-perch *Sander lucioperca*

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A. ABSTRACT

This study investigated the daily variations of stress markers namely plasma cortisol and glucose and some humoral innate immune markers, including peroxidase, lysozyme and complement activities, of pike-perch (*Sander lucioperca*) and the effect of light spectrum on these variations. Fish were reared under a white or red light spectrum at a constant photoperiod (12D:12L). Samples were collected at 22:00, 04:00, 10:00 and 16:00 at days

1 and 42 of the experiment. After 42 days, the use of a red light spectrum led to a significant increase in final bodyweight. Specific growth rate reached 2.1 ± 0.18 and 1.8 ± 0.17 % d^{-1} under red and white spectra respectively. The profiles of plasma cortisol followed a cyclic activity with a surge during photophase at 10:00 without any effect of the light spectrum at day 42. Both lysozyme and peroxidase activities in blood followed a day-night variation with a peak at 4:00 corresponding to low cortisol values. No rhythmicity was detected for the complement activity but higher values were observed at 16:00 when cortisol values were lowest. Light spectra also influenced humoral immune markers with an increase in lysozyme activity and a decrease in peroxidase activity in a red light environment. The present results indicate a strong effect of the light environment, including the light-dark cycle and the light spectrum, on pike-perch physiology. Especially, some innate immune status seemed stimulated during the dark phase in relation to a decrease in the stress level markers. Such parallelism in the relationship between the immune status and stress markers may be affected positively or negatively by the light characteristics. Humoral immune markers were also modulated according to the light spectrum without no clear trend (stimulation or inhibition) for the immunocompetence status.

B. INTRODUCTION

Due to its fast growth, high quality flesh and high economical expectation, pike-perch *Sander lucioperca* is one of the most promising freshwater fish species for the diversification of European inland aquaculture (Wang et al, 2009; Dalsgaard et al, 2013; Overton et al, 2015). However, its culture is still limited by impairment in growth rate and high mortality rate during the young developmental stages. These failures may be related to inadequate rearing conditions inducing high stress level since the pike-perch aquaculture management has not been optimized yet. It has been shown that percid fish are more sensitive to aquaculture stressors than other species with a longer history of domestication (Jentoft et al, 2005). And since decreased welfare may lead to increased stress level and to disease outbreaks, it is essential to improve its management strategy. In previous studies (Luchiari et al, 2006; 2009; Baekelandt et al, 2018), light was defined as a determining factor affecting physiology and, by the way, culture of pike-perch. However, the effects of the light environment, including light-darkness cycle and light spectrum, on physiology and immunity of pike-perch, are poorly documented and would merit more attention.

The aquatic environment is critical for the maintenance of fish homeostasis. It is well established that a perturbation of the pathogen-host-environment balance favors disease outbreaks that can severely limit aquaculture success (Esteban et al, 2006). From environmental cues, photoperiod is one of the major factors regulating a wide range of biological processes. The light-darkness cycle is perceived by photoreceptors and integrated into a melatonin rhythmic signal. It has been described several times to play in almost all vertebrates, a central role in driving circadian rhythms, including locomotor activity, thermal preferences, rest, osmoregulation and metabolic activity, as well as annual processes such as growth and sexual maturation (Falcón et al, 2007; 2010). Few studies also support a circadian and circannual activity of the immune system (Esteban et al, 2006; Morgan et al, 2008). Esteban et al (2006) pointed out a variation of some humoral immune markers in seabream and sea bass based on the light-darkness cycle. In addition to vary seasonally, immunity was shown to be influenced by artificial photoperiod (Leonardi and Klempau, 2003). While only little is known on this matter, a better understanding of the immune regulation by the light-darkness cycle would improve management strategies of cultured species. And as already used to control timing of broodstock spawning, smoltification and early maturation

in several fish species (Falcón et al, 2010), photoperiod manipulation could be an interesting tool for improving their immunocompetence.

Environmental colors affect the vision of teleosts, influencing for example food intake, signals for hierarchical status, reproduction, growth and even survival (Downing, 2002; Politis et al, 2014; Karakatsouli et al, 2007; Luchiari and Pirhonen, 2008). The use of optimal light colors was described to decrease stress status and stress-induced cortisol response in several fish species (Volpato and Barreto, 2001; Karakatsouli et al, 2007; Eslamloo et al, 2015). Unnatural light spectra also negatively affect important aspects of larval development and performance (Villamizar et al, 2009; Blanco-Vives, 2010). In pike-perch, it was shown that the use of red light improved specific growth rate and feed efficiency, with no consideration on the stress status or the immune system (Luchiari et al, 2009). These species-dependent preferences are explained by their specific natural habitat characteristics in relation to the adaptations of their visual system (Karakatsouli et al, 2007; Migaud et al, 2007). In the case of pike-perch, natural habitats are typically eutrophic and only light above 600 nm, including red wavelengths, penetrates below 1 m (Luchiari et al, 2009). Considering current practices in pike-perch culture and previous data (Baekelandt et al, 2018), red and industrial white light spectra were chosen to better evaluate the potential effects of the light spectrum on humoral innate immune markers.

As a potent immunosuppressive agent in vertebrates with complex actions on immune cells, cortisol has to be taken into account when investigating the potential effects of the light environment on the immune system (Tort et al, 2011). This glucocorticoid is well known to be part of the stress response and to be a major stress indicator promoting the metabolic pathways that increase plasma glucose levels in response to energy expenditure (Laiz-Carrion, 2003; Milla et al, 2010; Oliveira et al, 2013).

Globally, involvement of light characteristics in the regulation of the immune system in fish is poorly documented. Therefore, as a first step in describing the immunomodulation by the light environment, we tested in pike-perch the 24-h profiles for rhythmicity in cortisol and glucose release and in humoral innate immune markers, including peroxidase, lysozyme and complement activities. Furthermore, the effects of two light spectra (red and white) on stress status and on the latter immune markers were assessed.

C. MATERIALS AND METHODS

1. Animals and rearing conditions

A stock of 960 pike-perch (*S. lucioperca*) juveniles from Asialor farm (Dieuze, France) were transferred to URBE facilities at the University of Namur, Belgium. Animals were randomly distributed in 24 indoor 100 L-tanks. They were acclimated for 20 days under constant white lightning conditions (industrial white spectrum, 10 lux, 12 h of night duration from 8 pm to 8 am) and 22 ± 0.5 °C water temperature until they reached 10 ± 1 g bodyweight. At day 1 of the experiment, the white spectrum was replaced by a red spectrum (610 nm) for half of the tanks. Intensity was maintained at 10 lux at water surface. Fish were reared under these conditions (red or white spectrum) with a 12 L:12 D daily cycle for 42 days. They were fed twice a day at 10:30 and 18:00 with a commercial pellet diet (44 % proteins and 26 % lipids; Coppens, Netherlands) at 2.0 % biomass during all the experimental period. These rearing conditions were adapted according to a previous multifactorial experiment comparing major husbandry practices in pike-perch culture (Baekelandt et al, 2018).

The present protocol (16 276 KE) has been carried out in agreement with the local Ethics Committee for Animal Experiments.

2. Sampling procedures

Samplings occurred at 22:00, 4:00, 10:00 and 16:00 h (5 fish per time and per tank) at both days 1 and 42. To avoid repetitive stressful events on fish and potential artefacts on results, 6 tanks (3 per condition) were assigned at each time of sampling. Each treatment group had thus 3 replicates. Fish were starved one day before samplings. Five fish were removed randomly from each tank and anesthetized with MS-222 (150 mg L^{-1}) in a bucket covered with a tissue. As soon as they were anesthetized, fish head was covered with a tissue and blood was quickly collected by caudal vein puncture with heparinized syringes and centrifuged at 3,000 g during 10 min at 4 °C. The time between fish capture from their rearing tank and the blood sampling was optimized within 4 min. Plasma was aliquoted and stored at -80 °C until assayed.

3. Husbandry variables

Final individual weight (FIW) and Specific Growth Rate (SGR) were determined on day 42 for each experimental condition. SGR was estimated according to the formula: ((Ln(final individual weight) – Ln (initial individual weight)) *100 / duration of the experiment).

4. Cortisol and glucose assays

Cortisol was assayed in triplicate using a cortisol ELISA kit (DRG, EIA-1887), following the manufacturer's instructions (BioSource, Belgium). The intra-assay coefficient of variation was 3.6 %, the assay dynamic range was between 0-800 ng mL^{-1} and the analytical sensitivity was 2.5 ng ml^{-1}. Plasma glucose, also assayed in triplicate, was determined calorimetrically based on a glucose oxidase/peroxidase method described by Trinder (1969).

5. Plasma lysozyme activity

Lysozyme activity was evaluated in plasma samples by the turbimetric method (Siwiki and Studnicka, 1987; Douxfils et al, 2012). Plasma samples (10 µl) were mixed with 10 µl of Na_2HPO_4 0.05 M pH 6.2 and 140 µl *Micrococcus lysodeikticus* (Sigma-Aldrich) solution (0.6 g/L). This assay was performed in triplicate. Absorbance was measured at 450 nm every 2 min during 20 min at room temperature. Lysozyme activity (units) is defined as the amount of enzyme decreasing the turbidity of 0.001 OD per min.

6. Peroxidase activity

The total peroxidase activity in plasma was assessed following the method described in Quade and Roth (1997). Briefly, 15 µl of plasma was diluted in 140 µl of HBSS without Ca+2 or Mg+2 and mixed with 50 µl of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (Sigma) and 5 mM H2O2. The reaction was stopped after 2 min by adding 50 µl of 4 M sulphuric acid and absorbance was measured at 450 nm. The peroxidase activity was determined defining as one unit the peroxidase that produces an absorbance change of 1 OD.

7. Plasma alternative complement pathway

The alternative complement pathway (ACH50) was assayed by measuring the haemolytic activity in plasma samples using rabbit erythrocytes as targets

(Sunyer and Tort, 1995). Briefly, 10 µl of rabbit red blood cells suspension suspended at 3% in veronal buffer (Biomerieux, Marcy-l'Etoile, France) were mixed with serial dilutions of plasma (from 40 to 800 times). Plates were then read at 405 nm after incubation at 28 °C for 120 min. The spontaneous hemolysis was obtained by adding veronal buffer to 10 µl of rabbit erythrocytes and total lysis was obtained by mixing 10 µl of rabbit erythrocytes to distilled water (total volume = 70 µl). ACH50 was calculated using 4-parameter logistic regression and corresponds to the lysis of 50 % of the rabbit erythrocytes.

8. Statistical analysis

Data are expressed as the mean ± standard error (SEM). Kolmogorov and Smirnov's test was used to assess the normality of data sets ($p < 0.05$) and Bartlett's test was conducted to evaluate variance homogeneity ($p < 0.05$). Results from day 1 and day 42 were separately analyzed with a two-way ANOVA ($p < 0.05$) taking the light spectrum (white or red) and the time of the day (4:00; 10:00; 16:00 and 22:00) as the two-modality factors. When significant, mean values were compared according to Tuckey's HSD post-hoc test ($p < 0.05$). The results were analyzed with JMP 12.1 software (SAS Institute Inc., North Carolina, USA). Moreover, a nonlinear regression with a cosine function (Refinetti et al, 2007) was performed to test 24-h profiles for rhythmicity under white and red spectra.

D. RESULTS

Rearing pike-perch juveniles in a red light environment resulted in a significant increase in husbandry performances (Fig. 1) with a higher final body weight and Specific Growth Rate (SGR) ($p < 0.001$). SGR reached 2.1 ± 0.18 and 1.8 ± 0.17 % d^{-1} under red and white spectra respectively.

Plasma cortisol level was influenced by the day-night variation at both days 1 and 42 (Fig. 2). Values reached 82 ± 17 ng ml^{-1} during the photophase at 10:00 and significantly ($p < 0.01$) dropped to 28 ± 12 ng ml^{-1} during the scotophase, regardless of the day of sampling and light spectrum. Moreover, at day 1, the red light spectrum led to a significant decrease in plasma cortisol ($p < 0.01$) compared to the white spectrum. Concerning plasma glucose level, no clear rhythmicity was detected (Fig. 3) whatever the light spectrum.

However, the red spectrum induced a higher plasma glucose at days 1 and 42 ($p < 0.01$) compared to the white one.

Lysozyme and peroxidase activities both followed a daily rhythm (Fig. 4 and 5) ($p < 0.001$). They peaked at 4:00 and then dropped by a 25 to 40 % during the photophase at 10:00 and 16:00. Moreover, at day 42, the lysozyme activity was found to be stimulated by the red light while the peroxidase activated by the white one. As regards to ACH50 activity, the maximum activity was observed during the photophase around 16:00 corresponding to a high level of plasma cortisol level, but no significant rhythmicity was detected (Fig. 6).

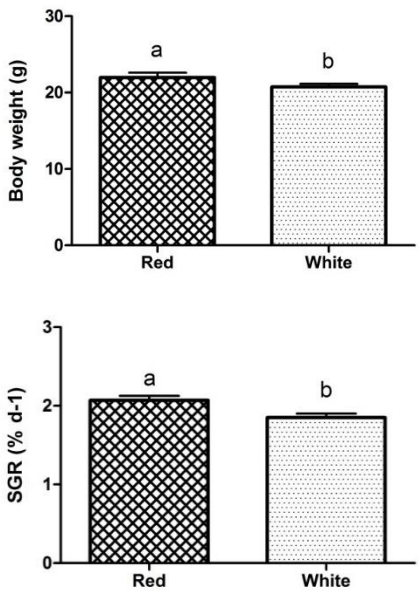

Fig. 1: Effects of the light spectrum on final body weight and Specific Growth Rate (SGR) of pike-perch juveniles reared under a red or a white light spectrum. Data are expressed as means ± SEM (n = 12). Lowercase letters indicate significant differences at $p < 0.05$.

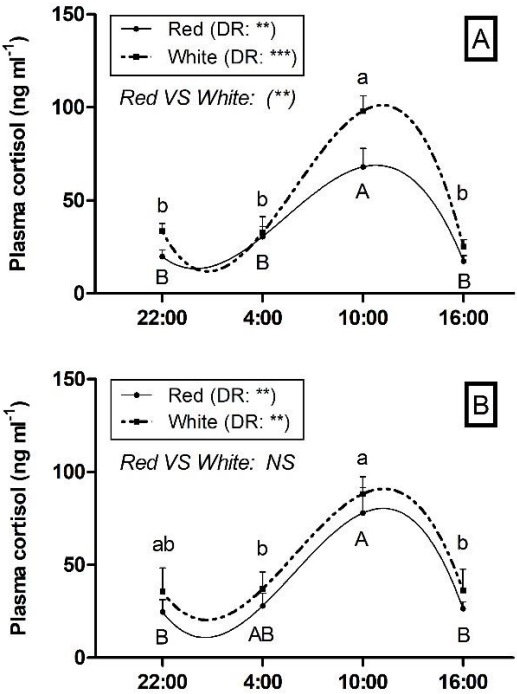

Fig. 2: Daily variations of plasma cortisol in pike-perch juveniles at days 1 (A) and 42 (B). Data are expressed as means ± SEM (n = 12). Lowercase and capital letters indicate significant differences at $p < 0.05$ between sampling time points, for red and white spectrum respectively. In boxes, (*), (**) and (***) indicate significant daily rhythmicity (DR) at $p < 0.05$, 0.01 and 0.001 respectively. Effect of the light spectra was also tested (NS: not significant).

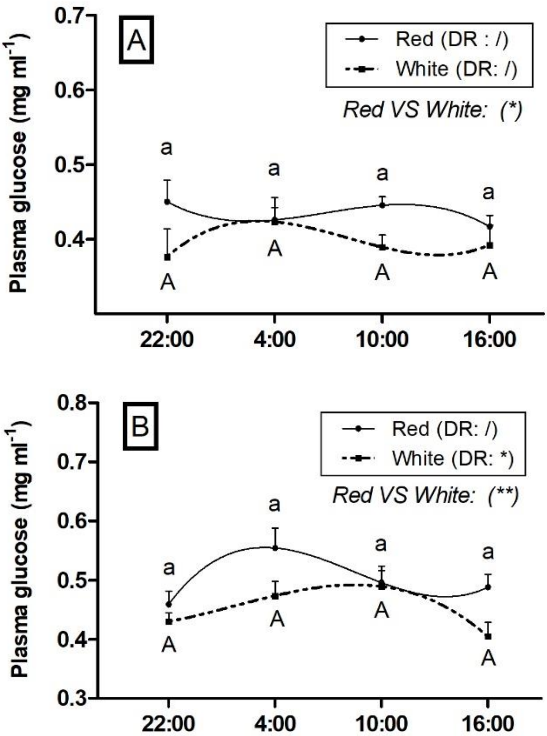

Fig. 3: Daily variations of plasma glucose in pike-perch juveniles at days 1 (A) and 42 (B). Data are expressed as means ± SEM (n = 12). Lowercase and capital letters indicate significant differences at $p < 0.05$ between sampling time points, for red and white spectrum respectively. In boxes, (*), (**) and (***) indicate significant daily rhythmicity (DR) at $p < 0.05$, 0.01 and 0.001 respectively. Effect of the light spectra was also tested.

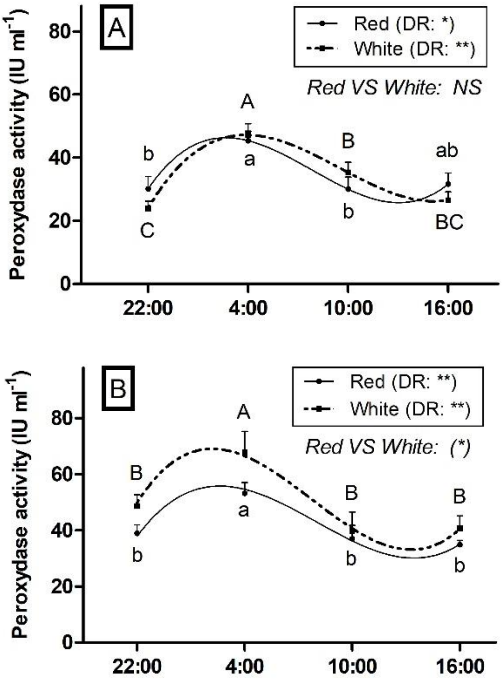

Fig. 4: Daily variations of plasma peroxidase activity in pike-perch juveniles at days 1 (A) and 42 (B). Data are expressed as means ± SEM (n = 12). Lowercase and capital letters indicate significant differences at $p < 0.05$ between sampling time points, for red and white spectrum respectively. In boxes, (*), (**) and (***) indicate significant daily rhythmicity (DR) at $p < 0.05$, 0.01 and 0.001 respectively. Effect of the light spectra was also tested (NS: not significant).

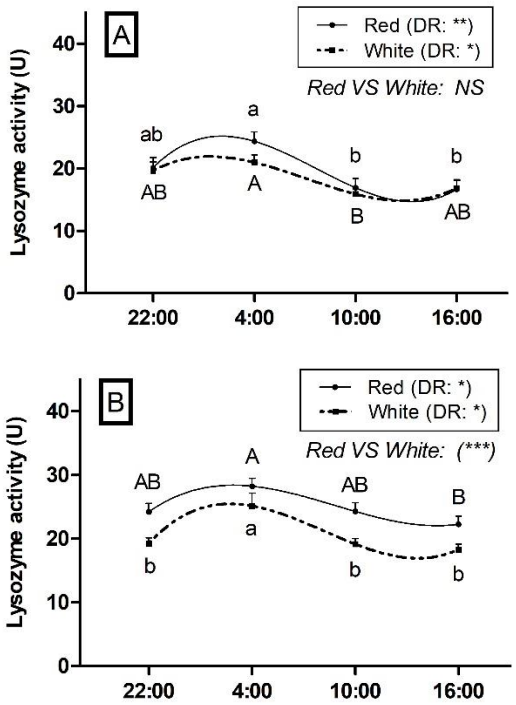

Fig. 5: Daily variations of plasma lysozyme activity in pike-perch juveniles at days 1 (A) and 42 (B). Data are expressed as means ± SEM (n = 12). Lowercase and capital letters indicate significant differences at $p < 0.05$ between sampling time points, for red and white spectrum respectively. In boxes, (*), (**) and (***) indicate significant daily rhythmicity (DR) at $p < 0.05$, 0.01 and 0.001 respectively. Effect of the light spectra was also tested (NS: not significant).

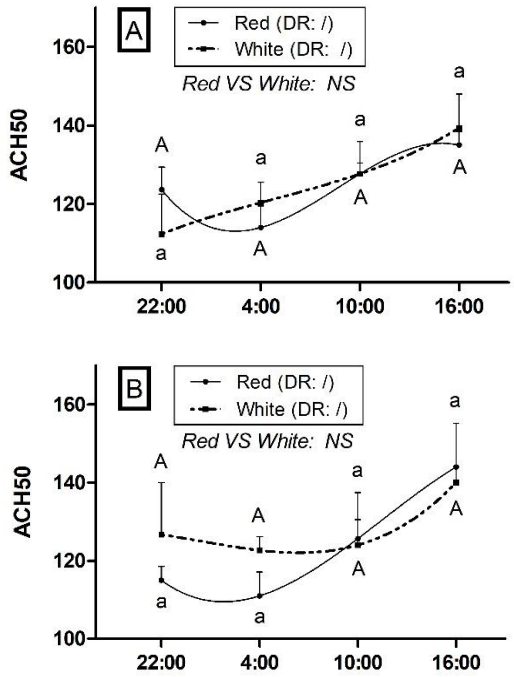

Fig. 6: Daily variations of plasma hemolytic complement activity in pike-perch juveniles at days 1 (A) and 42 (B). Data are expressed as means ± SEM (n = 12). Lowercase and capital letters indicate significant differences at $p < 0.05$ between sampling time points, for red and white spectrum respectively. In boxes, (*), (**) and (***) indicate significant daily rhythmicity (DR) at $p < 0.05$, 0.01 and 0.001 respectively. Effect of the light spectra was also tested (NS: not significant).

E. DISCUSSION

Using immuno-stimulants and vaccines on fish to prevent disease is of a growing interest in aquaculture (Mastan, 2015). Additionally, acting on fish environment as a first step may be an easier way to prevent disease outbreak. Photoperiod is known to regulate energetically expensive functions such as breeding and birthing but also to entrain circadian rhythms including osmoregulation, locomotor activity and metabolic activity (Falcón et al, 2007; 2010). In mammals, the effects of photoperiod on the immune system

are well documented while little is known in teleosts. And improving immunocompetence of commercially important species through photoperiod manipulations could be a very interesting tool.

In the present study, a daily cyclic activity was observed for humoral innate immune markers, namely lysozyme and peroxidase activities. Such daily variations in similar immune markers were already observed, including ACH50 activity in gilthead seabream (*Sparus aurata*) and seabass (*Dicentrarchus labrax*) and peroxidase and lysozyme activities in seabass and Nile tilapia (*Oreochromis niloticus*) (Esteban et al, 2006; Lazado et al, 2016). However, cyclic activity of these immune markers seems to be species-specific since their peaks were observed during the light-phase or the dark-phase depending on the species (Esteban et al, 2006), suggesting that the responsiveness of humoral factors to a pathogen may differ according to the time of the day and to the species considered. In the study of Esteban et al (2006), these daily variations were correlated to the daily cyclic activity of the pineal gland which releases the melatonin hormone during the dark phase of the photoperiod. Complementary experiments to the current study conducted in pike-perch reared under the same lighting conditions (constant photoperiod, industrial white light spectrum, 10 lux, 12 h of night duration from 8 pm to 8 am) revealed basal values around 15 pg ml^{-1} during the day and up to 100 pg ml^{-1} during the dark period, at 04:00 (unpublished data). Little is known on the regulatory mechanisms and different models of action have been proposed, including a direct action of melatonin on immune cells and tissues through specific receptors, an indirect action via various hormones (glucocorticoids, growth hormone, prolactin) or a combination of both models (Cuesta, 2008; Falcón et al, 2010; Esteban et al, 2013).

Cortisol is the main glucocorticoid taking part in the stress response and it is well known to act on immune cells and tissues through specific receptor (Tort et al, 2011; Mathieu et al, 2013). In the present study, we described in pike-perch the existence of a daily rhythmicity in plasma cortisol levels with a surge during photophase at 10:00. However, daily variations in cortisol are species-dependent. For instance, a peak of plasma cortisol occurs during the night in salmonids while it happens during photophase at 15:00 in Senegalese sole and at light onset in goldfish and Eurasian perch (Noeske and Spieler, 1983; Laidley and Leatherland, 1988; Oliveira et al, 2013; Brüning et al, 2015). Such differences between species in terms of cortisol profile can be explained, at least partially, by the meal time that could be perceived as an

entraining signal of cortisol rhythm (Kulczykowska and Sánchez Vázquez, 2010, Oliveira et al, 2013). In the current study, the decrease in peroxidase and lysozyme activities during photophase is correlated to the peak of plasma cortisol. Considering as a potent immunosuppressive agent, it is suggested that cortisol may be involved in the morning decrease in these humoral immune activities. A wide range of data describes an enhancement of humoral components after an acute stress (*i.e.* short release of cortisol in plasma) while a chronic stress is more suppressive (Demers and Bayne, 1997; Tort et al, 2011; Cortés et al, 2013).

In contrast to plasma cortisol profile, no clear daily cycle was detected concerning plasma glucose profile. However, it had been described to follow a cyclic rhythm in several fish species including rainbow trout and tench (*Tinca tinca*) (De Pedro et al, 2005; Polakof et al, 2007). Glucose level in plasma does not only depend on cortisol through the regulation of neoglucogenesis during a stress event, but also on diet composition and feeding habits (Oliveira et al, 2013). Fish used in this experiment were not fed during the days of sampling which could have resulted in this absence of glucose rhythmicity. Montoya et al (2010) described in gilthead seabream an absence of glucose rhythmicity when the fish are fed every day at the same time, indicating a lack of a clear daily rhythm in blood glucose or a daily rhythm related to the species or experimental conditions.

Spectral composition in water greatly varies between environments and photopigments of fish show adaptations to available wavelengths (Bayarri et al, 2002). Luchiari et al (2009) described in pike-perch the advantageous effects of long-wavelengths light, especially red light compared to white or short-wavelength lights, including a better feeding behavior and feed efficiency. This preference for long-wavelength environments is related to cones containing photopigments absorbing at 603 and 535 nm in the retina (Luchiari et al, 2009). In the present experiment, the use of a red light spectrum improved growth performances of pike-perch, confirming the results from Luchiari et al (2009). Moreover, and for the first time in pike-perch, we described an effect of the light spectrum on the innate immune system after a long-term exposure (42 days). Since opposite responses for lysozyme and peroxidase activities were observed when fish were reared under a red light spectrum, we are not able at this stage to define a clear trend (stimulation or inhibition) of the immune system. The only study found on immune regulation by the light spectrum in teleosts focused on goldfish

(Eslamloo et al, 2015). This study revealed that a red or a blue environmental lights are chronically stressful and immunosuppressive with increase in plasma cortisol and decreases in lysozyme and plasma antiprotease. In our study, low cortisol values almost corresponded to a higher stimulation in all the tested immune markers, confirming the previous studies that variations in immune markers are negatively affected by high cortisol release following a stress event (Tort et al, 2011; Cortés et al, 2013). But, other hormones may also have acted on these immune markers including melatonin, growth hormone, prolactin or other glucocorticoids (Falcón et al, 2010, Mathieu et al, 2013). However, more investigations on innate and acquired immune markers are needed to better define and describe the immunoregulation by the environmental light colors in teleosts.

To conclude, light environment is a crucial factor to be considered in pike-perch culture. The day-night cycle regulated both endocrine and some immune functions, suggesting that photoperiod manipulation may be a valuable tool to improve immunocompetence of cultured fish species. Additionally, while the use of a red light improved husbandry performances, light spectrum also modulated innate humoral immune activities through a putative action on HPI axis. However, no clear trend direction of the light spectrum on the immune system was defined and more investigations focusing on the immune modulation by the light environment and the underlying mechanisms are needed.

F. ACKNOWLEDGMENT

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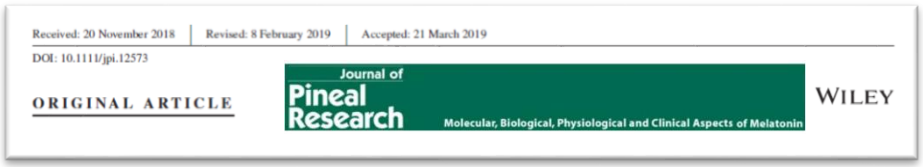
VII. Immune modulation by the light environment

Foreword: Both day-night cycle and light spectrum influenced innate immune activities in pike-perch. Considering that the light information in vertebrates is converted into a melatonin signal by the pineal gland, we focused on the hypothesis that melatonin, a multifunctional molecule, may play a central role by being a relay between the light, the stress axis and the immune system. In the following experiment, we considered the three light characteristics and we measured stress levels, immune status and plasma melatonin content in pike-perch exposed to various light environments.

Are cortisol and melatonin involved in the immune modulation by the light environment in pike-perch *Sander lucioperca?*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A. ABSTRACT

The pineal gland is the main organ involved in the transduction process converting environmental light information into a melatonin response. Since light environment was described as an important factor that could affect physiology of teleosts, and because melatonin is a crucial hormone regulating numerous physiological processes, we hypothesized that environmental light may act on both stress and circadian axes which in turn could influence the immune status of pike-perch. Therefore, we investigated the effects of two light spectra (red and white) and two light intensities (10 and 100 lx) with a constant photoperiod $12L_{(8:00\text{-}20:00)}$/12D on pike-perch physiological and

immune responses. Samples were collected at 04:00 and 16:00 at days 1 and 30 of the experiment. Stress markers, plasma melatonin levels, humoral innate immune markers and expression of key immune genes in the head-kidney were assessed. Light intensity clearly affected pike-perch physiology. This included negative growth performances, increase in stress status, decrease in plasma melatonin levels, and immune depression. Light spectrum had only little influences. These results demonstrate that high stress status may have impacted melatonin production and secretion by the pineal organ. The drop in circulating melatonin and the increase in stress status may both be involved in the immune suppression.

B. INTRODUCTION

Pike-perch (*Sander lucioperca)* is one of the most promising freshwater fish species for diversification of European aquaculture and an attractive inland aquaculture species [1-3]. However, its culture is still limited by impairment of growth and survival rates during the young developmental stages. It has been shown that percid fish are more sensitive to aquaculture stressors than other species with a longer history of domestication, such as rainbow trout [5], and that frequent manipulations or emersion can decrease growth rate or induce an immunodepression in percid fish juveniles [5, 6]. Stress is thus one of the possible explanations for these production deficiencies since pike-perch aquaculture management has not been optimized yet. In previous studies [7-10], light was defined as a determining factor affecting physiology and, by the way, culture of pike-perch. However, the effects of the light environment, including the light intensity and the light spectrum, on the physiology and immunity of pike-perch, and more generally of teleosts, are poorly documented and would merit more attention.

Visual systems of fishes are adapted to their specific natural habitat characteristics. It is essential to maintain fish in optimal light environment since light intensity and environmental colors were both shown to affect the vision of the fish. On the one hand, spectral content influences food intake, reproduction, growth and even survival of various fish species [11-16]. In pike-perch, it was described that long-wavelength light, especially red light compared to white or short-wavelength lights, improved feeding behavior, feed efficiency and growth [8, 10]. Considering these information, and because current practices for pike-perch rearing are not yet optimized [9], red and industrial white light spectra were chosen to better evaluate the potential effects of the light spectrum on pike-perch physiology. On the other hand, light intensity is described to affect many behavioral and biological processes in fish, such as foraging and growth [7, 17, 18]. In a previous study [9], we demonstrated that pike-perch reared under conditions of low light intensity set at 10 lx displayed better husbandry performances than those submitted to 100 lx. Such preference of pike-perch for low light intensities was also described by Luchiari et al [7] since this species is a crepuscular predator actively feeding during dusk and night [2, 19].

As the key hormone of the circadian axis, melatonin is produced and secreted by the pineal gland during the dark phase of the photoperiod [20, 21].

Through the secretion of this hormone, the pineal gland processes light information and provides information such as time of the day and season for cells and organs [22, 23]. In mammals, melatonin is largely described to act on important physiological functions, including thermoregulation, reproduction and immune functions [24, 25]. In various fish species, its involvement in daily rhythms including rest, skin pigmentation, osmoregulation, thermoregulation and locomotor activity and annual processes such as reproduction, development and migration is well documented [21, 26-28]. Nevertheless, its potential role on immune functions is not well described. Few evidences suggest that melatonin may act as an important immune regulator [21, 29-31]. In pike-perch, we have already described daily cyclic activities of several humoral innate immune markers correlated to the light-dark cycle [10], but the relation with melatonin secretion was not reported.

Considering cortisol is crucial when studying the potential impact of the light environment on circadian axis and immune system. In vertebrates, cortisol is known as a potent immunosuppressive agent with complex actions on immune cells and tissues [32-34]. Furthermore, a bi-directional communication between the HPI axis and the melatonin axis was also revealed. On the one hand, an anti-stress role for melatonin was described, including reduced post-stress plasma cortisol levels in Senegalese sole (*Solea senegalensis*) and rainbow trout [35, 36]. On the other hand, stress has been shown, with cortisol as a mediator, to negatively affect melatonin release by the pineal organ of rainbow trout, with highest reduction observed in fish exposed to long-term stress [37].

Only few studies have focused on immune regulation by spectral content or light intensity and none has considered the potential involvement of both the HPI and the circadian axis. Eslamloo et al. [16] revealed that red or blue environments are chronically stressful and immunosuppressive with increase in plasma cortisol and decrease in lysozyme and plasma antiprotease. In rainbow trout, light color affects both serotonergic and dopaminergic activities which have been described several times as good indicators of acute and chronic stress in fish species with no consideration of the immune system [13, 36, 38, 39]. A preliminary study in pike-perch showed that light spectrum influences humoral immune markers with an increase in lysozyme activity and a decrease in peroxidase activity in a red light environment (when

compared with white light environment) without clear effect on cortisol release [9].

Since light environment is described to affect physiology of teleosts, and because melatonin is a crucial hormone regulating many physiological processes including the stress response and potentially the immune system, we hypothesized that environmental light may act on melatonin and cortisol secretions which in turn could influence the immune status of pike-perch. Therefore, we investigated during scotophase (04:00) and photophase (16:00) the effects of two light spectra (red and white) and two light intensities (10 and 100 lx) of a constant photoperiod $12L_{(8:00\text{-}20:00)}/12D$ on melatonin secretion, stress level (glucose, cortisol, brain dopaminergic and serotonergic activities and the Glucocorticoid Receptor 1 gene expression), humoral innate immune markers (plasma lysozyme and peroxidase activities), expression of key immune genes in the head-kidney and growth performances of pike-perch during a 30-day experiment.

C. MATERIALS AND METHODS

1. Animals and rearing conditions

A stock of 1,000 pike-perch (*S. lucioperca*) juveniles from Asialor farm (Dieuze, France) was transferred to URBE facilities at the University of Namur, Belgium. Animals were randomly distributed in 24 indoor 100 l-tanks of a recirculating aquaculture system (RAS). They were acclimated for 30 days under constant lighting conditions (spectrum: white; light intensity at water surface: 10lx; photoperiod: $12L_{(8:00\text{-}20:00)}/12D$) and 22 ± 0.5 °C water temperature until they reached 19 ± 4 g body weight. Water was continuously aerated (90% of oxygen saturation) and water inflow into the tank was regulated to ensure a complete water exchange once per hour. They were fed manually twice a day at 8:00 and 14:00 with a commercial pellet diet (Skretting, France) at 2.0 % biomass during all the experimental period. At day 0, new light conditions were applied, with 6 tanks per experimental condition: 10 lx-white; 10 lx-red; 100 lx-white and 100 lx-red. Light intensity was measured at water surface and spectra included a white (industrial white - Osram, cool white 840 Lumilux, Germany) and a red color (red filter, 610 nm, Lumis, Belgium). The group exposed to the 10-lux light of white spectrum was considered as the control group since fish were acclimated in

these light conditions. These light settings are also the main conditions encountered by this species in its natural environment. The photoperiod was the same as used for acclimation. Water analysis (ammonia, nitrite and nitrate) were done twice a week and concentrations did not exceed 0.02, 0.1 and 2 mg/l respectively. The present protocol (16 276 KE) has been carried out in agreement with the local Ethics Committee for Animal Experiments.

2. Sampling procedures

Samplings occurred during scotophase at 04:00 and photophase at 16:00, at both days 1 and 30. To avoid repetitive stressful events on fish and potential artefacts on results, 12 tanks (3 per condition) were assigned at each time of sampling. Each treatment group had thus 3 replicates. Fish were starved one day before samplings. Five fish were removed randomly from each tank and anesthetized with MS-222 (150 mg/l) in a bucket covered with a tissue. As soon as they were anesthetized, fish head was covered with a tissue and blood was quickly collected by caudal vein puncture with heparinized syringes within 5 min and centrifuged at 3,000 g during 10 min at 4 °C. Plasma was aliquoted and stored at -80 °C until assayed. Fish were then euthanized before collecting the whole brain and the anterior kidney. These organs were directly frozen in liquid nitrogen and stored at -80 °C until assayed.

3. Husbandry performances

Final individual weight (FIW) and Specific Growth Rate (SGR) were determined on day 30 for each experimental condition.

4. Stress indicators

Cortisol was assayed in triplicate using a cortisol ELISA kit (DRG, EIA-1887), following the manufacturer's instructions (BioSource, Belgium). Plasma glucose, also assayed in triplicate, was determined calorimetrically according to Trinder [40].

High Performance Liquid Chromatography (HPLC) was performed according to the methods of Lepage et al. [41], with some modifications, to assess in whole brain the serotonergic and dopaminergic activities expressed as hydroxyl-indol-acetic acid (5-HIAA) / serotonin (5-HT) and 3,4-dihydroxyphenylacetic acid (DOPAC) / dopamine (DA) ratios, respectively. Tissues were weighed out and homogenized using a Bullet Blender Storm 24 (NextAdvance, New York, USA) in tubes containing 0.5 mm zirconium

oxide beads (Dutscher, Brumath, France) and mobile phase (1.5 ml per g of tissue) used in the chromatography. They were then submitted to ultrasonic disruption. Homogenates were centrifuged (20,000 x g, 10 min, 4 °C) and filtered through 0.5 µm filters (Phenomenex, California, USA). An aliquot (35 µl) of the filtrate was injected into the HPLC system. The whole procedure was carried out on ice.

HPLC analysis was performed using a GP50 gradient pump (Dionex, Sunnyvale, USA) equipped with an autosampler FAMOS (LC packings). Neurohormones were monitored using a DC amperometry detector (Dionex, Sunnyvale, USA) with Glassy Carbon Working Electrode (0,700 V, Ag/AgCl – P/N 061677). Chromeleon™ software (6.8) (Dionex, Sunnyvale, USA) was used for data acquisition and processing. The samples were individually applied on a 2.6 µm particle size (150 x 4.6 mm, I.D.) C_{18} analytical Kinetex column at 1 $ml.min^{-1}$. The mobile phase consisted of 65 mM NaH2PO4, 1.63 mM octane sulfonic acid (OSA, Sigma-Aldrich), 0.1 mM EDTA-Na2 and 13 % MeOH adjusted to pH 2.79 with orthophosphoric acid. The column was kept at 25 °C.

Purified hormones were obtained from Sigma-Aldrich (Saint-Louis, USA). Standard solutions were treated similarly to samples. Concentrations of the compounds were calculated by interpolation of their respective standard curves. The intra- and inter-assay coefficients of variation for tested hormones were respectively under 5.9 % and 7.4 %.

5. Humoral immune variables

Lysozyme activity was evaluated in plasma samples by the turbimetric method [42, 43]. Activity (units) is defined as the amount of enzyme decreasing the turbidity of 0.001 OD per min.

The total peroxidase activity in plasma was assessed following the method described in Quade and Roth [44]. The activity was determined defining as one unit the peroxidase that produces an absorbance change of 1 OD.

6. Gene expression analysis

Total RNA isolation was performed using Extract-all® reagent (Eurobio, Paris, France) following manufacturer's instructions. 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.

The relative expression of several immune-related genes were investigated by RT-qPCR in anterior kidney tissues, including genes involved in bactericidal defense, namely C-type lysozyme (*lys*), Hepcidin c (*hepc*) and complement C3 (*c3*), and in pro-inflammatory action, namely interleukine-1 (*il-1*) and Tumor Necrosis Factor alpha (*tnf-α*). In addition, expression of glucocorticoid receptor 1 (*gr1*) and reference genes *β-actin* and elongation factor alpha (*ef1- α*) were assessed. Efficiencies of primers (Table 1) were validated when ranged between 90 and 105 %. The relative mRNA levels of *c3*, *lys*, *il-1*, *gr1*, *hepc* and *tnf-α* in each sample were normalized with the geometric mean of *ef1-α* and *β-actin* calculated by the relative standard curve method [45].

Table 1: Sequences and melting temperatures (Tm) of primers used for gene expression quantification.

Gene	GenBank accession #	Sens	Sequence (5' to 3')
β-actin	MF472627	Forward	CGACATCCGTAAGGACCTGT
		Reverse	GCTGGAAGGTGGACAGAGAG
ef1- α	MF472628	Forward	TGATGACACCAACAGCCACT
		Reverse	AAGATTGACCGTCGTTCTGG
tnf-α	MK167462	Forward	CTGATTCGCCTCAACGTGTA
		Reverse	GGAGATGGGTCATGAGGAGA
hepc	MK036790	Forward	CCGTCGTGCTCACCTTTATT
		Reverse	GCCACGTTTGTGTCTGTTGT
il-1	MK036791	Forward	TTTCCCATCATCCACTGACA
		Reverse	ATTCACACACGCACACCATT
Complement component 3 (*c3*)	MF472630	Forward	TGGTGATGTGAGAGGAGCAG
		Reverse	GACGTCATGGCAACAGCATA
lys	MF472629	Forward	AGCCAGTGGGAGTCGAGTTA
		Reverse	CATTGTCGGTCAGGAGCTCA
gr1	MK036792	Forward	GGACAGGGTGAAACCAAAGA
		Reverse	TGAGGTGCTGATGACAGAGG

7. Melatonin content in plasma

Plasma melatonin was assayed in triplicate using a Melatonin ELISA kit (E-EL-M0788, Elabscience Biotechnology Co., USA), following the manufacturer's instructions. Parallelism to the standard curve was confirmed using serially diluted plasma sample. Recovery rate was estimated around 90 to 95 % for melatonin values ranging from 5 to 100 pg ml^{-1}. Intra and inter-assays of coefficients were 5.8 % and 7.4 % respectively (n = 4). Nocturnal plasma samples were diluted to get values between 5 and 100 pg ml^{-1}.

8. Statistical analyses

Data are expressed as the mean ± standard 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 ($p < .05$). Results were analyzed with a four-way ANOVA ($p < .05$) taking the light spectrum (white or red), the light intensity (10 or 100 lx), the time of the day (04:00 or 16:00) and the day (1 or 30) as two-modality factors. Statistics were performed using the fish as the experimental unit with the exception of growth parameters (final body weight and SGR). Tank effect was previously tested not significant. When interactions were tested significant, values were compared according to Tuckey's HSD post-hoc test ($p < .05$). Correlations between plasma melatonin values and other examined parameters were tested for significance. The results were analyzed with JMP 12.1 software (SAS Institute Inc., North Carolina, USA) and graphs were performed with GraphPad Prism V5.04 (California, USA).

D. RESULTS

Rearing pike-perch juveniles during 30 days in a 10 lx-light environment resulted in a significant increase in husbandry performances (Fig. 1) with a higher final body weight and Specific Growth Rate (SGR) ($p < .01$) compared to 100 lux-light experimental conditions. SGR reached 1.60 ± 0.25 and 1.16 ± 0.41 % d^{-1} under low and high light intensity respectively. Moreover, a trend of increase in growth performances by the red spectrum was observed at high light intensity. Mortality rate averaged 4 % and no difference was detected between the experimental conditions.

Plasma cortisol level showed a significant day-night variation with the highest values at night, regardless the light conditions (intensity and spectra) and the day of sampling (Fig. 2). However, at day 1, the use of a 100-lx light resulted in a significant increase in plasma cortisol during light-phase with values reaching 52 ± 12 ng ml^{-1}. Such daily rhythmicity was not detected for plasma glucose (Fig. 3).

The application of a 100-lx light of white or red spectra resulted in a significant increase in the serotonergic activity at day 1 during the light-phase compared to the dark-phase of the photoperiod (Fig. 4). Additionaly, a long-term exposure of pike-perch juveniles to high light intensity led to a significant increase in the 5HIAA/5HT ratio regardless the time of the day and the light spectrum. Moreover, the white light spectrum at 100 lx led to a significant increase in the dopaminergic activity at day 30 during the night (Fig. 5).

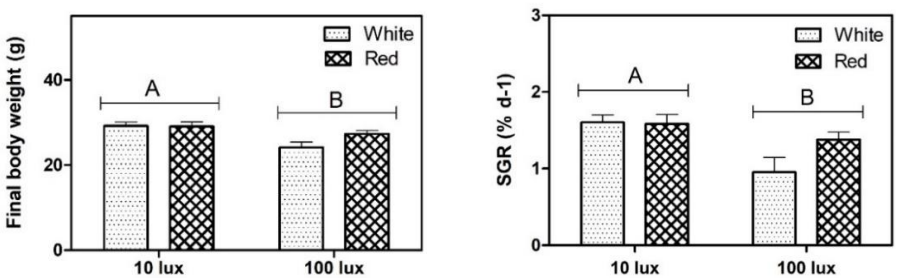


Fig. 1: Effects of the light spectrum and the light intensity on (left) final body weight (g) and (right) Specific Growth Rate (SGR, % d^{-1}) of pikeperch juveniles reared under 4 light conditions, combining 2 light intensities (10 and 100 lx) and 2 light spectra (cool white and red at 610 nm). Data are expressed as means ± SEM (n = 6 tanks). Capital letters indicate significant differences at $p < .05$.

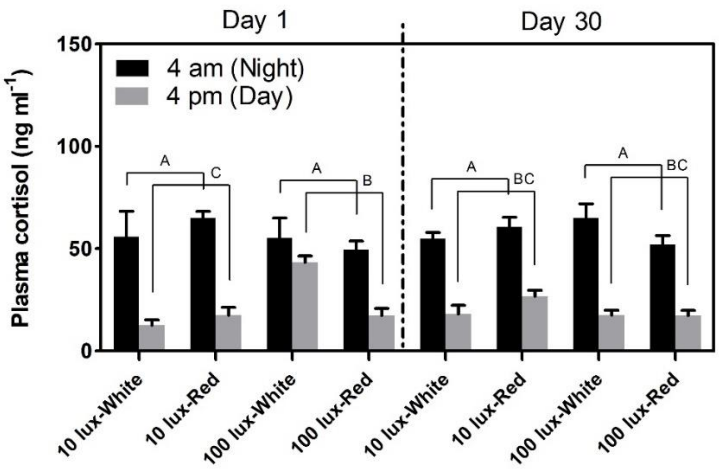


Fig. 2: Day-night variation (16:00 *vs* 4:00) of plasma cortisol at days 1 and 30 in pikeperch juveniles reared under 4 light conditions, combining 2 light intensities (10 and 100 lx) and 2 light spectra (cool white and red at 610 nm). Data are expressed as means ± SEM (n = 9). Capital letters indicate significant differences at $p < .05$.

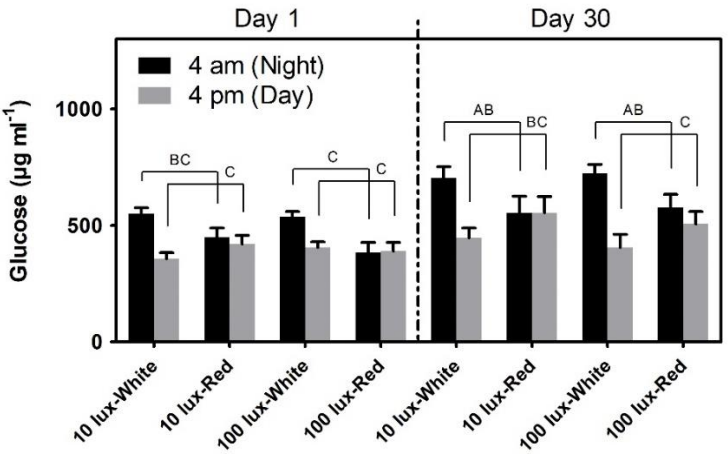


Fig. 3: Day-night variation (16:00 *vs* 4:00) of plasma glucose at days 1 and 30 in pikeperch juveniles reared under 4 light conditions, combining 2 light intensities (10 and 100 lx) and 2 light spectra (cool white and red at 610 nm). Data are expressed as means ± SEM (n = 9). Capital letters indicate significant differences at $p < .05$.

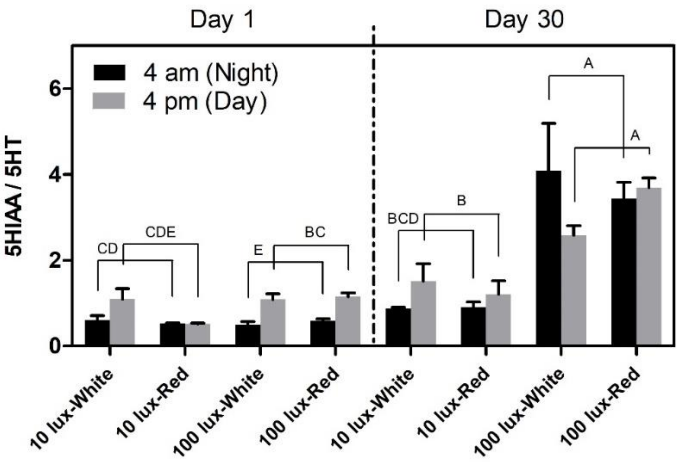


Fig. 4: Day-night variation (16:00 *vs* 4:00) of serotonergic activity in the whole brain (5-HIAA / 5-HT ratio) at days 1 and 30 in pikeperch juveniles reared under 4 light conditions, combining 2 light intensities (10 and 100 lx) and 2 light spectra (cool white and red at 610 nm). Data are expressed as means ± SEM (n = 9). Capital letters indicate significant differences at $p < .05$.

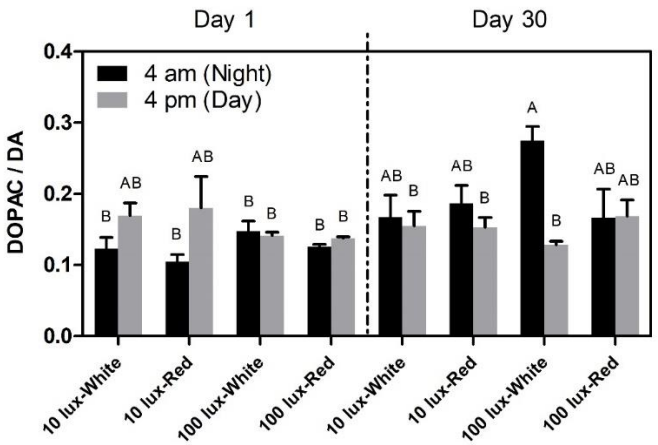


Fig. 5: Day-night variation (16:00 *vs* 4:00) of dopaminergic activity in the whole brain (DOPAC / DA ratio) at days 1 and 30 in pikeperch juveniles reared under 4 light conditions, combining 2 light intensities (10 and 100 lx) and 2 light spectra (cool white and red at 610 nm). Data are expressed as means ± SEM (n = 9). Capital letters indicate significant differences at $p < .05$.

Concerning plasma melatonin, values reached 100 pg ml^{-1} at 04:00 (night) and significantly dropped to 11 ± 4 pg ml^{-1} at 16:00 (day) whatever light spectrum or light intensity or the sampling day (Fig. 6). Increasing the white not red light intensity from 10 to 100 lx was followed by a significant decrease of the night plasma melatonin at day 1. Moreover, after 30 days, a significant drop in the night plasma melatonin level was detected under a high light intensity for both red and white light spectra at 04:00 (Fig. 6). In addition, significant correlations ($p < .01$) were observed between melatonin and several markers including plasma cortisol (0.79), glucose (0.37), lysozyme (0.54) and peroxidase (0.51) and *il-1* (-0.61), *tnf-α* (-0.54) and *gr1* (-0.45) gene expressions in the head kidney.

Concerning lysozyme and peroxydase activities, they both showed day-night variations (Fig. 7, Fig. 8) with activity peaks at night. Moreover, the lysozyme activity was significantly decreased after 30 days when fish were exposed to a high light intensity.

The relative *gr1* expression was mainly influenced by the light intensity since increases were observed during the light-phase under the 100-lx light at both days 1 and 30 (Fig. 9a). While no differences was detected for the *lys* expression (Fig. 9f), the high light intensity also influenced *c3* expression with a significant increase during the light-phase, compared to the dark phase of the photoperiod, at day 30 whatever the light spectrum (Fig. 9b). Similar observation was made for *hepc* expression at both days 1 and 30 (Fig. 9c). *tnf-α* and *il-1* expressions both followed a daily cyclic activity with increases at 16:00 (Fig. 9d,e). Furthermore, these two gene expressions were also significantly decreased after a long-term exposure of pike-perch juveniles to the 100-lx light during the day compared to the 10-lx light.

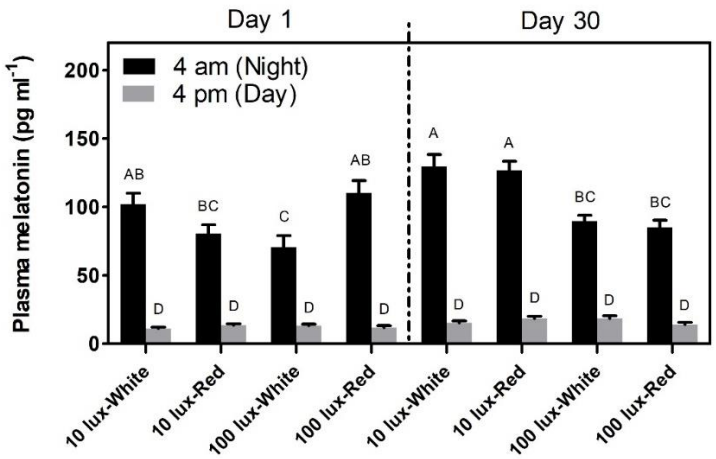


Fig. 6: Day-night variation (16:00 *vs* 4:00) of plasma melatonin at days 1 and 30 in pikeperch juveniles reared under 4 light conditions, combining 2 light intensities (10 and 100 lx) and 2 light spectra (cool white and red at 610 nm). Data are expressed as means ± SEM (n = 12). Capital letters indicate significant differences at $p < .05$.

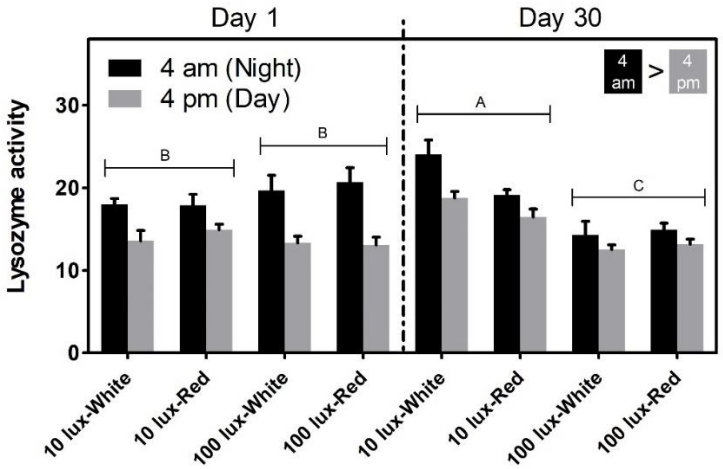


Fig. 7: Day-night variation (16:00 *vs* 4:00) of plasma lysozyme activity at days 1 and 30 in pikeperch juveniles reared under 4 light conditions, combining 2 light intensities (10 and 100 lx) and 2 light spectra (cool white and red at 610 nm). Data are expressed as means ± SEM (n = 12). Capital letters indicate significant differences at $p < .05$.

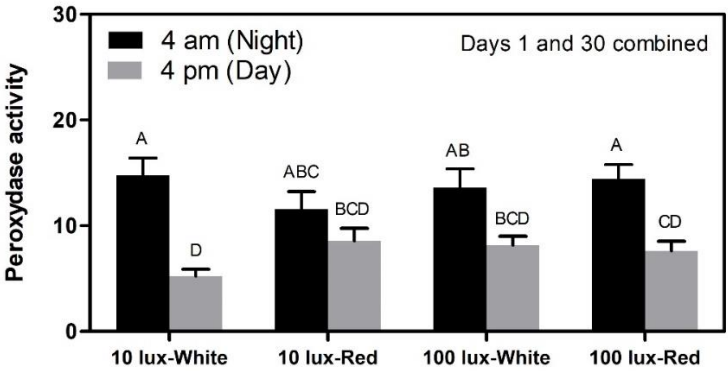
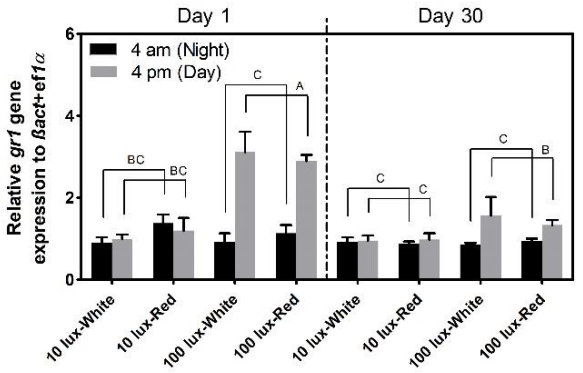
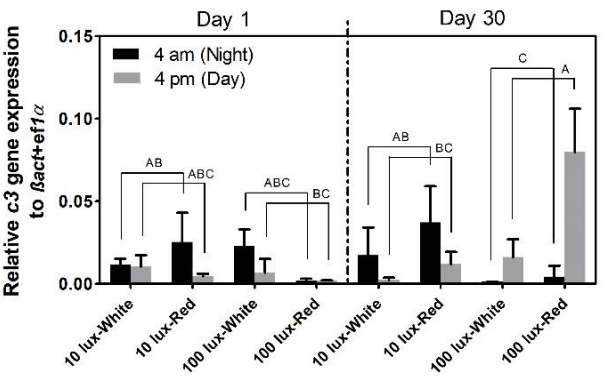


Fig. 8: Day-night variation (16:00 *vs* 4:00) of plasma peroxidase activity at days 1 and 30 in pikeperch juveniles reared under 4 light conditions, combining 2 light intensities (10 and 100 lx) and 2 light spectra (cool white and red at 610 nm). Data are expressed as means ± SEM (n = 12). Capital letters indicate significant differences at $p < .05$. Data from days 1 and 30 were combined since no statistical difference was detected.

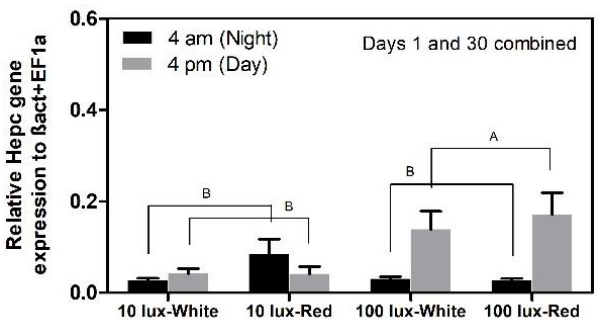
a)


Day 1
Day 30
4 am (Night)
4 pm (Day)
Relative gr1 gene expression to ßact+ef1α
6
4
2
0
BC
BC
C
A
C
C
C
B
10 lux-White
10 lux-Red
100 lux-White
100 lux-Red
10 lux-White
10 lux-Red
100 lux-White
100 lux-Red

b)


Day 1
Day 30
4 am (Night)
4 pm (Day)
Relative c3 gene expression to ßact+ef1α
0.15
0.10
0.05
0.00
AB
ABC
ABC
BC
AB
BC
C
A
10 lux-White
10 lux-Red
100 lux-White
100 lux-Red
10 lux-White
10 lux-Red
100 lux-White
100 lux-Red

c)


4 am (Night)
4 pm (Day)
Days 1 and 30 combined
Relative Hepc gene expression to ßact+EF1a
0.6
0.4
0.2
0.0
B
B
B
A
10 lux-White
10 lux-Red
100 lux-White
100 lux-Red

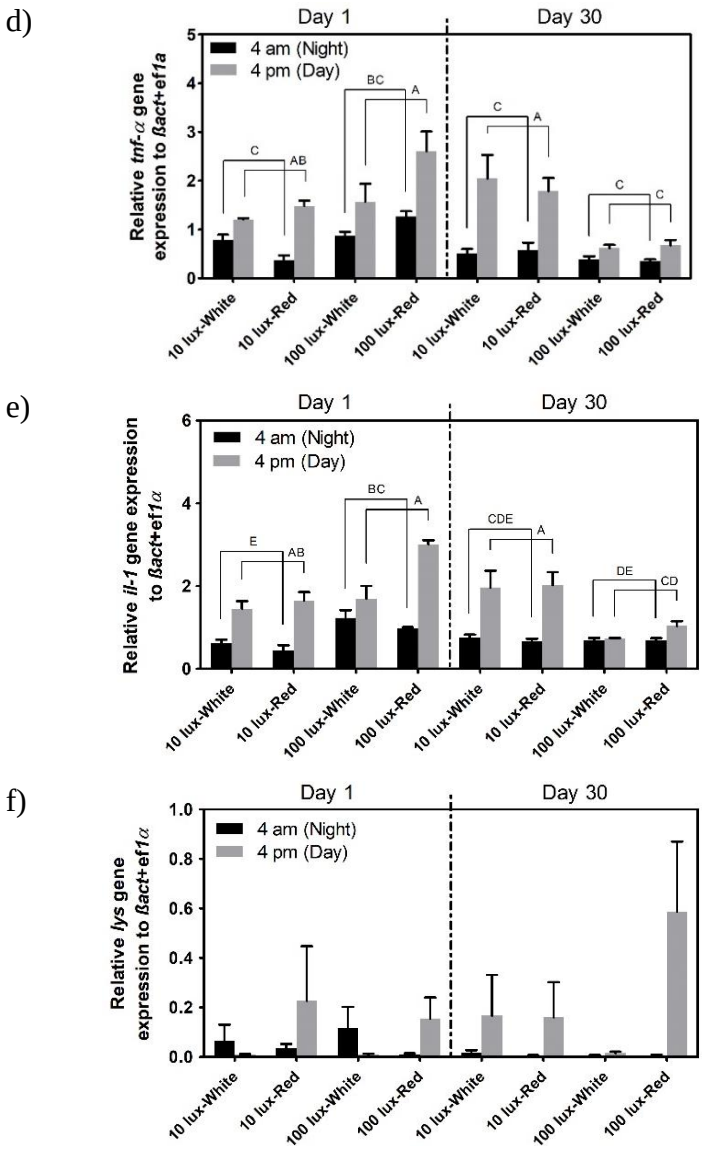


Fig. 9: Day-night variation (16:00 *vs* 4:00) of relative (a) *gr1*, (b) *c3*, (c) *hepc*, (d) *tnf-α*, (e) *il-1* and (f) *lys* expressions at days 1 and 30 in the head kidney of pikeperch juveniles reared under 4 light conditions, combining 2 light intensities (10 and 100 lx) and 2 light spectra (cool white and red at 610 nm). Data are expressed as means ± SEM (n = 9). Capital letters indicate significant differences at $p < .05$. Modalities from a tested factor were combined when no statistical difference was detected.

E. DISCUSSION

Visual systems of fish are adapted to the specific environment they inhabit. A preference of pike-perch for low light intensities and red color are explained by specific adaptations of its retina. It includes a *tapetum lucidum*, that is a specific anatomo-histological tissue of the retina which greatly amplifies the eye sensitivity to light, and specific cones absorbing red wavelengths [7, 8, 10, 46]. In the present experiment, the best husbandry performances were obtained with a 10-lx light supporting previous results [9] while light spectra did not significantly affect growth. In Baekelandt et al. [10], we described better growth performances of pike-perch reared for 42 days under a red light environment. Although not significant, the results obtained in the present experiment followed the same trend at high light intensity.

Cortisol, considered as a major stress marker in fish, plays a crucial role in several biological functions including stress response, growth, immune functions and energy metabolism [33, 34, 47]. In the present experiment, the increase in plasma cortisol at day 1 under a 100-lx light confirmed that the exposure to high light intensities is stressful for pike-perch. The absence of cortisol peak at day 30 may result of a mechanism that minimizes the deleterious effects of sustained cortisol elevation on biological functions when organism is exposed to long-lasting stressors [48]. However, the lack of such cortisol increase at day 30 also raises the question about the relevance of this latter stress marker in percid fish. A previous experiment on pike-perch (unpublished data) showed that plasma cortisol level returned to basal level within 1 h post-stress. A rapid decrease in the amplitude of stress response was also described in juvenile Eurasian perch submitted to single or multiple emersion stressor [6]. Such observations may suggest a habituation to stress and/or a rapid metabolism and clearance of cortisol indicating the interest for using various stress indicators to account for the stress responsiveness in percids, such as brain neurotransmitters [9]. Additionally, plasma level of glucose strongly depends on cortisol (through the regulation of neoglucogenesis during a stress event), but also on feeding (diet composition, feeding habits) [34]. Glucose responses did not follow the same pattern as plasma cortisol (no day-night variation and no increase at day 1 under a 100-lx white spectrum) pointing the importance of the feeding habits. For instance, it was shown in gilthead seabream (*Sparus aurata*) that feeding the fish every day at the same time lead to an absence of glucose rhythmicity

[49]. Brain serotonergic and dopaminergic activities have been described several times as reliable indicators of acute and chronic stress in various fish species since these brain monoamine neurotransmitters are involved in primary responses to stress [35, 36, 38, 39, 50]. In some fish species, the responses of the dopaminergic activity have been shown to be highly dependent on the nature of the stressor and the brain region while increase in the serotonergic activity is consistently observed after a stress event [38, 39, 51]. In the present experiment, the only increase in DOPAC/DA ratio was detected at day 30 during the night under a high, white light intensity while the 100-lx light induced a higher serotonergic activity at both days 1 and 30. Therefore in percid fish, the dopaminergic system seems less sensitive to light-induced stress, suggesting that the serotonergic activity would be a more key stress indicator in that case, since plasma cortisol level was not indicative for a stressful status. In a previous study, we also observed an increase in serotonergic activity after a sixty-day exposure of pike-perch juveniles to high light intensity of white spectra, while plasma cortisol level was almost at a basal threshold [9], confirming altogether the high sensitivity of this brain monoamine activity to high light intensities in pike-perch.

The exposure of pike-perch to the 100-lx light was followed by a decrease in day-night variations of circulating melatonin with drops observed during the night at both days 1 and 30. Similar results were already described in López-Patiño et al. [37] with stress (chasing or high stocking density) affecting production and release of the melatonin hormone by the pineal organ of rainbow trout. Melatonin synthesis was reported to be modulated by glucocorticoid hormones [52]. Exogenous cortisol in rainbow trout led to a decrease in the aryl-alkylamine N-acetyltransferase 2 (*AANAT2*) enzyme activity – considered as the rate-limiting enzyme of melatonin synthesis – and to its mRNA abundance in the pineal organ at night [21, 37]. Such observations suggest that high cortisol levels following stress events result in a decrease in melatonin synthesis and release and that such effect might be mediated by specific glucocorticoid receptors on the pineal organ.

Furthermore, in fish, several evidences support an anti-stress role of the melatonin hormone at both central and peripheral levels, including a suppressor effect on the HPI axis [35, 39, 53]. Exogenous melatonin minimized the stress response in Senegalese sole and rainbow trout with an inhibition of the HPI axis and a decrease in both dopaminergic and serotonergic activities in the hypothalamus [35, 36]. Cortisol secretion was

also affected by subchronic melatonin treatment in goldfish [53]. However, the underlying mechanism of this anti-stress action is not fully understood. Gesto et al. [39] suggested a possible action on hypothalamus with a reduction of the post-stress activation of the neuronal pathways regulating stress at this tissue. Moreover, melatonin receptors were detected in the pituitary gland of some fish species and may regulate the release of several hormones including adrenocorticotropin (ACTH), growth hormone and prolactin [20, 54, 55]. In these studies, the anti-stress role of melatonin was only detected by using exogenous melatonin treatment and no evidence suggested that physiological concentrations in fish plasma could significantly inhibit the stress response. Thus, our results suggest that the stressful light environment has significantly impacted melatonin synthesis and release by the pineal organ through the activation of the HPI axis. However, the absence of plasma cortisol rise at day 30 suggests that another underlying mechanism may be involved, such as an increase in glucocorticoid receptor expression in the pineal gland as we observed in the head kidney at day 30. Further research is needed to clarify this hypothesis.

Coping with a stressor involves a metabolic reorganization that can affect food intake, reproduction, growth and immune functions [35, 47]. Long-term exposure to a stressor is characterized by an immune depression or suppression including decreases in innate immune activities, leucocyte populations and antibodies levels and increase in disease susceptibility in various fish species (6, 47, 56, 57). In the present study, rearing pike-perch during 30 days in stressful light conditions led to a significant depression in some innate immune functions, including a decrease in plasma lysozyme activity and in *tnf-α* and *il-1* expressions in the head-kidney. Lysozyme, considered as a key element of the humoral innate immunity, is one of the main anti-bacterial molecules in teleosts. Mainly produced by neutrophils [58], this protein was positively or negatively modulated according to stress duration and cortisol release [59 – 62]. *Tnf*-α is a pro-inflammatory cytokine and its expression is known to be inhibited following a stress event [32, 62]. Expression of *tnf-α*, as other cytokines including *il-1*, seems to be down-regulated by glucocorticoids through the inhibition of *NF-kB* as observed in mammals [47, 64, 65]. As for maintaining the bactericidal defense, hepc expression showed the opposite trend compared to values of *tnf-α* and *il-1* expressions with a stimulation under a high light intensity. Hepcidin mRNA was downregulated in spleen of Eurasian perch (*Perca fluviatilis*) after

handling stress [33] while no effect was observed in rainbow trout's liver following handling and confinement stress [66]. The up-regulation of hepc expression at day 1 and day 30 in the current study may be the result of a process counteracting a possible decrease in protein abundance and/or activity. However, while no subsequent elevation of plasma cortisol was reported at day 30 which could jeopardize the hypothesis of the possible effect of the HPI axis on the immune system, an increase in *gr1* gene expression was detected in the head-kidney suggesting that basal plasma cortisol levels (as observed at day 1 under a 10-lx white spectrum) could have a greater impact on targeted cells and tissues.

The 100-lx light environment has thus clearly impacted pike-perch immunity in the current study, including lysozyme and expression of pro-inflammatory genes, but the underlying mechanisms are still unclear. A direct effect of glucocorticoids on immune cells and tissues seems consistent according to our results. However, a possible effect of the decreasing plasma melatonin levels on immunity cannot be discarded. Several studies support the melatonin as a regulator of fish immune defense. Exogenous melatonin in gilthead seabream increased several innate immune responses including peroxidase, phagocytic ability and cytotoxic activity of head-kidney leucocytes and expression of immune-relevant genes in head-kidney such as *il-1β*, major histocompatibility complex and interferon-regulatory factor-1 [67]. In the present study, day-night variations of activity were observed for humoral innate immune markers including peroxidase and lysozyme activities and immune genes involved in inflammatory activity, namely *tnf-α* and *il-1*. Such day-night rhythmicity of humoral innate immune variables was already described in several teleosts including gilthead seabream and seabass (*Dicentrarchus labrax*) [30, 68]. In a previous study on pike-perch, both lysozyme and peroxidase activities were already shown to follow a daily rhythmicity with peaks at 04:00 and drops by a 25 to 40 % during the photophase at 10:00 and 16:00 [10]. However, we show for the first time day-night variations in immune mRNA gene expressions in association to the cyclic release of melatonin in blood, supposing a potential effect of this hormone on the immune system. Different models of action have been proposed, including a direct action on immune cells and tissues through specific receptors and/or an indirect action via several candidate hormones (glucocorticoids, growth hormone, prolactin) [21, 29, 31]. In various fish species, three high affinity melatonin receptor subtypes with different

distributions were identified: *MT1*, *MT2* and *Mel1c* [21, 69]. *MT1* and *MT2* are both found in the retina, the brain and some peripheral tissues while Mel1c is detected in the skin and the retina [20, 21, 55, 70]. To our knowledge, no study has examined their presence in immune cells or tissues in teleosts. However, they are found in the kidney of rainbow trout, flounder (*Platichthys flesus*), sea bream, golden rabbitfish (*Siganus guttatus*) and Senegalese sole and in the spleen of the golden rabbitfish and seabream [20, 70, 71].

Daily rhythms of immune variables were also correlated to the daily cyclic release of cortisol in plasma, which could support an indirect action of melatonin on immune markers in the present study. Such night increase in plasma cortisol was already described in salmonids [72]. However, cortisol peaks happen during photophase at 15:00 in Senegalese sole and at light onset in goldfish and Eurasian perch [23, 34, 73]. A previous experiment in pike-perch also showed a cortisol peak at 10:00 during photophase [10] while humoral immune activity peaks also occurred during the night as in the present experiment. Such differences between species and experiments could be mainly explained by the meal time that can be perceived as an entraining signal of cortisol rhythm [34, 74]. In the present experiment, fish were fed daily at 8:00 and 14:00 and it not clear whether the observed cortisol peak at 04:00 was related to anticipative behavior of morning feeding.

Both the regulation of the HPI axis and the circadian axis may have resulted in the decrease in some bactericidal and pro-inflammatory functions. However, the present results cannot define the importance of the effects of each axis on immune variables neither if the melatonin hormone acted directly on immune variables nor if it acted through other hormonal regulation.

In conclusion, environmental light characteristics can profoundly affect fish physiological and immune responses. Increasing the light intensity in the rearing conditions of pike-perch led to an increase in stress status, a slight decrease in plasma melatonin levels, an immune depression and a negative effect on growth performances. Since melatonin is thought to be a crucial immune modulator, both the decrease in circulating melatonin and the increase stress status may have led to the immune depression with a decrease in lysozyme activity and pro-inflammatory gene expressions. However, little is known on the underlying mechanisms and more investigations are needed

to clarify the direct and/or indirect actions of the melatonin hormone on immunity.

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VIII. Photoperiod influences melatonin release and immunity

Foreword: The previous experiment supports an action of melatonin on pike-perch innate immunity. In order to better characterize *in vivo* the immune modulation by the melatonin hormone, we considered a fourth experiment to characterize some immune activities when fish are exposed to seasonal-simulated photoperiods. We hypothesized that natural variations of photoperiod and subsequent changes in melatonin release profile may act on immune status of pike-perch with limited effect on the stress axis.

Seasonal simulated photoperiods influence melatonin release and immune markers of pike perch *Sander lucioperca*

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A. ABSTRACT

Melatonin is considered as the time-keeping hormone acting on important physiological functions of teleosts. While the influence of melatonin on reproduction and development is well described, its potential role on immune

functions has little been considered. In order to better define an immune modulation by the melatonin hormone, we hypothesized that natural variations of photoperiod and subsequent changes in melatonin release profile may act on immune status of pike-perch. Therefore, we investigated during 70 days the effects of two photoperiod regimes simulating the fall and spring in western Europe, on pike-perch physiological and immune responses. Samples were collected at 04:00 and 15:00 at days 1, 37 and 70. Growth, plasma melatonin levels, innate immune markers and expression of immune-relevant genes in head kidney tissue were assessed. While growth and stress level were not affected by the seasonal simulated photoperiods, nocturnal levels of plasma melatonin were photoperiod-dependent. Innate immune markers, including lysozyme, complement, peroxidase and phagocytic activities, were stimulated by the fall-simulated photoperiod and a significant correlation was made with plasma melatonin. In addition to bring the first evidence of changes in fish immunocompetence related to photoperiod, our results provide an additional indication supporting the immunomodulatory action of melatonin in teleosts.

B. INTRODUCTION

As photoperiod transducer, the melatonin hormone is mainly produced and secreted by the pineal gland during the night [1-5]. Through this activity, the pineal gland converts light information into a melatonin signal and thus relays information such as the time of the day and year for cells [6,7]. The melatonin hormone is indeed seen as the main actor for anticipating changes in season since its peak of production and release by the pineal organ is directly proportional to the length of the night and thus provides a direct transduction of night length [8]. In mammals as well as in teleosts, melatonin is described to act on important physiological functions, including development and reproduction [3,9-13]. In mammals, it is also known to interact with the immune system [4,12,14-18]. However, such immunomodulatory effects of melatonin have little been investigated in fish. Nevertheless, the available information supports an immune regulation by the melatonin hormone in teleosts [3,19-22]. In pike perch, a potential dual action of cortisol and melatonin hormones on immune defenses was described [23,24]. In addition, these experiments defined a correlation between daily cyclic activities of humoral innate immune markers and the nocturnal peak of plasma melatonin.

The life of the organisms is strongly conditioned by seasons, and this influence is more and more marked further from the equator. Seasonality is known to modulate reproductive activity and to influence food intake, locomotor activity, growth performance and immune responses of teleosts [8,25,26]. In temperate latitudes, the main factors characterizing the seasonal cycle are photoperiod and temperature. From these two factors, the annual cycle of changing photoperiod is the most precise temporal cue for determining the time of year and it has already been well established to influence growth, feeding, smoltification and reproduction [8,25,27,28]. Photoperiod manipulation is also used in aquaculture to modulate sexual maturation and growth of various fish species [29], including the Eurasian perch (*Perca fluviatilis*) and the pike perch (*Sander lucioperca)* [30]. However, the available information of such influence on fish immune system is very scarce. Considering the potential immunomodulatory action of the melatonin hormone and the annual rhythmicity of melatonin secretion by the pineal gland, it is feasible that changing photoperiod co-ordinates fish immunity through the modulation of melatonin secretion. Since bi-directional communications are described in teleosts between HPI axis and both melatonin axis and immune system, several stress markers were considered

in the present experiment. While cortisol is known as a potent immunosuppressive agent in vertebrates [31,32], a mutual inhibition was described between HPI and melatonin axes [33-35]. In addition, brain serotonergic and dopaminergic activities, which are both indicators of acute and chronic stress in teleosts, were affected by light color and intensity but no consideration of the photoperiod was made [24,35-38]. Pike perch is the most promising freshwater fish species for the diversification of inland aquaculture industry in Europe. The eyes of this species possess a *tapetum lucidum* that is a specific tissue of the retina which greatly amplify the eye sensitivity to light [24,39]. This is in agreement with behavior of pike perch since it is a crepuscular predator actively feeding during dusk and night [40,41]. Previous experiments have defined a high sensitivity of this species to the light environment. Both light intensity and light spectrum were defined as determining factors affecting its physiology, including endocrine and immune functions [23,40,42,43]. However, as the third light characteristic, photoperiod and its potential effects on fish immunity have still not been considered.

In order to better define in fish the effects of photoperiodic changes on the immunocompetence and the potential key role of the melatonin hormone in this regulation, this study investigated in pike perch the effects of two photoperiod regimes simulating the fall and the spring in western Europe.

C. MATERIAL AND METHODS

1. Animals and rearing conditions

The experiment was carried out at the Aquaculture Experimental Platform (AEP, registration number for animal experimentation C54-547-18) belonging to the URAFPA lab and located at the Faculty of Sciences of the University of Lorraine (France). All experimental manipulations were carried out in agreement with the European and French national legislations on animal welfare after evaluation and approval of the experimental project (protocol number: APAFIS10285-201706201445413) by the local ethic committee in France (Name: CELMEA; French code: 066). A stock of 1,500 mixed-sex pike perch juveniles was provided by Asialor farm (Dieuze, France) and transferred to the facilities. Animals were randomly distributed into 12 indoor 2000-L tanks. Each of these 12 experimental units was

operating independently in a recirculating system (RAS). Fish were acclimated for 29 days in constant conditions (temperature: 21°C; light intensity: 15 lx; photoperiod: LD 12:12) and fed once daily at 2% biomass. They reached 149 ± 21 g at the first day of the experiment. In order to simulate the fall and the spring light conditions in Western Europe, gradual changes in LD from $12_{(8:00\text{-}20:00)}$:12 to 8(10:21-18:16):16 or 12(8:00-20:00):12 to 16(7:25-23:30):8, respectively, were set up for 70 days according to natural photoperiods in Paris, France. Additionally, a dusk and a dawn of 30 min were programmed. Every other rearing condition was maintained constant during all the 70-day experiment. In order to limit high size heterogeneity due to a high social dominance observed in pike perch, fish were fed once a day during the light phase, 2.5 h after sunrise.

2. Sampling procedures

Samplings at days 1, 37 and 70 occurred during scotophase at 04:00 h and photophase at 15:00 h. To avoid stress artefacts of nocturnal fishing on diurnal samplings, the number of tanks was doubled. Each of the 4 treatment groups, considering the two simulated photoperiods and the two sampling times, had thus 3 replicates. Five fish were removed randomly from each tank and anesthetized with MS-222 (150 mg L^{-1}). Blood was quickly collected by caudal vein puncture with heparinized syringes within 4 min and centrifuged at 3,000 g during 10 min at 4°C. Fish were then euthanized before collecting the spleen, the whole brain and the anterior kidney. Plasma, brain and anterior kidney were directly frozen in liquid nitrogen and stored at -80°C until assayed. Spleen was stored on ice in L-15 media.

Final individual weight and specific growth rate were determined on day 70 for each experimental condition. Specific growth rate was estimated according to the formula: ((Ln (final individual weight) – Ln (initial individual weight)) *100 / duration of the experiment). Mortality was recorded along the whole experiment.

Since a slight gonadal development was observed for both males and females at D70, the gonadosomatic index (%) was estimated according to the formula: (gonad weight * 100 / body weight).

3. Stress indicators

Plasma cortisol and glucose

Cortisol was assayed in triplicate using a cortisol ELISA kit (DRG, EIA-1887), following the manufacturer's instructions (BioSource, Belgium). The intra-assay coefficient of variation was 3.6%, the assay dynamic range was between 0-800 ng mL^{-1} and the analytical sensitivity was 2.5 ng mL^{-1} [23]. Plasma glucose, also assayed in triplicate, was determined calorimetrically based on a glucose oxidase/peroxidase method described by Trinder [23, 44].

Brain neurotransmitters

High Performance Liquid Chromatography (HPLC) was performed according to the methods of Lepage et al. [45], with some modifications [24], to assess in whole brain the serotonergic and dopaminergic activities expressed as hydroxyl-indol-acetic acid (5-HIAA)/serotonin (5-HT) and 3,4-dihydroxyphenylacetic acid (DOPAC)/dopamine (DA) ratios, respectively.

4. Melatonin content in plasma

As described in Baekelandt et al. [24], plasma melatonin was assayed in triplicate using a Melatonin ELISA kit (E-EL-M0788, Elabscience Biotechnology Co., USA), following the manufacturer's instructions. Recovery rate was estimated around 90 to 95% for melatonin values ranging from 5 to 100 pg mL^{-1}. Intra and inter-assays of coefficients were 5.8 and 7.4%, respectively (n = 4). Nocturnal plasma samples were diluted to get values between 5 and 100 pg mL^{-1}.

5. Humoral immune variables

The total peroxidase activity in plasma was assessed following the method described in Quade and Roth [46]. One unit of peroxidase activity corresponds to an absorbance change of 1 OD.

The alternative complement pathway (ACH50), as described in Baekelandt et al. [23], was assayed by measuring the haemolytic activity in plasma samples using rabbit erythrocytes as targets [47]. Briefly, 10 µL of rabbit red blood cells suspension suspended at 3% in veronal buffer (Biomerieux, Marcy-l'Etoile, France) were mixed with serial dilutions of plasma (from 40 to 800 times). Plates were then read at 615 nm after incubation at 28°C for 120 min. The spontaneous hemolysis was obtained by adding veronal buffer

to 10 µL of rabbit erythrocytes and total lysis was obtained by mixing 10 µL of rabbit erythrocytes to distilled water (total volume = 70 µL). ACH50 corresponds to the lysis of 50% of the rabbit erythrocytes.

Lysozyme activity was evaluated in plasma samples by the turbimetric method [48,49]. Lysozyme activity (units) is defined as the amount of enzyme decreasing the turbidity of 0.001 OD per min.

6. Phagocytic activity

Spleen tissues were washed once with L-15 medium. They were then gently mashed with 9 mL of L15 medium supplemented with bovine serum albumin (10%, Sigma-Aldrich) and Penicillin– streptomycin (P/S) (1%, Sigma-Aldrich) through a 100 µm nylon mesh grid. Cell suspensions were kept at 4°C for 16h and then centrifuged and washed twice with L15 medium. Cells were suspended in 1 mL of L-15 medium containing 1% of P/S.

Cell mortality was assessed by flow cytometry with a FACSVerse (BD Biosciences, USA) using propidium iodide (PI) probe (1 µg mL^{-1}). Samples were considered for analysis when survival exceeded 90%. For the phagocytosis assay, $1x10^6$ cells were incubated with yellow-green fluorescent latex beads (Fluoresbrite®, Polyscience; 2 µm diameter) for 18h at 21°C with a 1/100 ratio cell-beads ratio. Non-ingested beads were eliminated following a centrifugation step (400 x g, 10 min, 4°C). Cells were fixed with a 0.5% formaldehyde and 0.2% sodium azide PBS fixating solution. The phagocytic activity (percentage of cells that have ingested three or more beads) was measured through cytometric analysis [50].

7. Gene expression analysis

Total RNA isolation from anterior kidney tissue, which is important in hematopoiesis and immunity in fish, was performed using Extract-all® Reagent (Eurobio) following manufacturer's instructions and description in Baekelandt et al. [24]. 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. The relative expression of several immune-related genes was investigated by RT-qPCR, including genes involved in bactericidal defense, namely C-type lysozyme (*lys*), hepcidin c (*hepc*), and complement C3 (*c3*), and in pro-inflammatory action, namely interleukin-1

(*il-1*) and tumor necrosis factor alpha (*tnf-α*). In addition, expression of reference genes *β-actin* and elongation factor alpha (*ef1-α*), whose expressions were tested stable in experimental conditions, were assessed. Efficiencies of primers (Table 1; [24]) were validated when ranged between 90 and 105%. The relative mRNA levels of *c3*, *lys*, *il-1*, *hepc*, and *tnf-α* in each sample were normalized with the geometric mean of *ef1-α* and *β-actin* calculated by the relative standard curve method [51].

Table 1: Sequences of primers used for gene expression quantification [24].

Gene	GenBank accession #	Sens	Sequence (5' to 3')
β-actin	MF472627	Forward	CGACATCCGTAAGGACCTGT
		Reverse	GCTGGAAGGTGGACAGAGAG
ef1- α	MF472628	Forward	TGATGACACCAACAGCCACT
		Reverse	AAGATTGACCGTCGTTCTGG
tnf-α	MK167462	Forward	CTGATTCGCCTCAACGTGTA
		Reverse	GGAGATGGGTCATGAGGAGA
hepc	MK036790	Forward	CCGTCGTGCTCACCTTTATT
		Reverse	GCCACGTTTGTGTCTGTTGT
il-1	MK036791	Forward	TTTCCCATCATCCACTGACA
		Reverse	ATTCACACACGCACACCATT
c3	MF472630	Forward	TGGTGATGTGAGAGGAGCAG
		Reverse	GACGTCATGGCAACAGCATA
lys	MF472629	Forward	AGCCAGTGGGAGTCGAGTTA
		Reverse	CATTGTCGGTCAGGAGCTCA

8. Statistical analysis

Data are expressed as the mean ± standard error (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. Results were analyzed with a three-way ANOVA ($p < .05$) taking the photoperiod regime (fall and spring), the day of sampling (D1, D37 and D70) and the time of the day (4:00 and 15:00) as factors. Statistics were performed using the fish as the experimental unit with the exception of growth parameters (final individual weight and specific

growth rate). Tank effect was previously tested not significant. When interactions were tested significant, values were compared according to Tukey's HSD post-hoc test ($p < .05$). In addition, correlations between immune markers and gonadosomatic index as well as between immune activities and night variations of plasma melatonin were tested for significance. The results were analyzed with JMP 12.1 software (SAS Institute Inc., North Carolina, USA) and graphs were performed with GraphPad Prism V5.04 (California, USA).

D. RESULTS

Final body weight (282.8 ± 33.5; 276.5 ± 36.3 g) and specific growth rate (1.04 ± 0.28; 1.12 ± 0.37% d^{-1} for the fall and spring-simulated photoperiod, respectively) did not show any difference between experimental groups. At D70, while the tested photoperiods did not influence the gonadosomatic index for females (0.30 ± 0.04%), a significant difference for males was detected with increased gonadal development with the fall-simulated photoperiod (FSP) (0.41 ± 0.17%) compared to the spring-simulated photoperiod (SSP) (0.22 ± 0.09%). No correlation between immune markers and sexes or gonadosomatic index was tested significant.

About the tested stress markers, a significant day-night variation was observed for plasma cortisol ($p < .001$), with values reaching 28 ± 4 and 14 ± 2 ng mL^{-1} during the dark and the light phases respectively (Fig. 1). For plasma glucose (Fig. 1), the highest values were observed for the SSP at D70 (4:00) while the lowest were detected for the FSP at D1 (15:00) ($p < .05$). While no effects were observed concerning the dopaminergic activity (Fig. 2), the serotonergic activity was influenced by the time of the day with increases during the light phase ($p < .05$). The SSP also led to an increase in 5HIAA / 5HT ratio from D1 to D37 ($p < .05$) (Fig. 2).

While constant during the light phase, plasma melatonin values were significantly influenced by the seasonal simulated photoperiods during the night. It showed a progressive increase for the FSP from 89 ± 14 at D1 to 125 ± 18 pg mL^{-1} at D70 and a decrease for the SSP from 88 ± 17 to 68 ± 14 pg mL^{1} (Fig. 3). No difference was detected between sexes.

Peroxidase and lysozyme activities in plasma and phagocytic activity in spleen all followed a day-night variation with increased activities at 4:00 whatever the simulated photoperiod ($p < .001$) (Fig. 4a,b,d). The opposite

scheme was observed for plasma complement activity (Fig. 4c) with the highest values detected at 15:00 ($p < .001$). In addition, lysozyme, peroxidase and complement activities were significantly influenced by the simulated photoperiod with increases observed from D1 to D70 for the FSP. Furthermore, the lysozyme activity significantly decreased from D1 (15:00) to D70 (15:00) for the SSP ($p < .05$). An increase in phagocytic activity was also observed at D37 ($p < .05$) for both photoperiods (Fig. 4d). Significant positive correlations between night variations of plasma melatonin and immune markers were tested significant, including for lysozyme (correlation: 0.41; $p < .001$), peroxidase (0.28; $p < .01$) and complement (0.38; $p < .01$).

Considering expression of immune-related genes, no effects were detected on *il-1* and *hepc* (Fig. 5a,b). However, *c3* gene expression increased at D70 for the FSP ($P < .001$) (Fig. 5c). In addition, an increase in *tnf-α* gene expression was detected at D70 whatever the experimental group while a decrease in *lys* gene expression was observed at D70 compared to D37 (Fig. 5d,e).

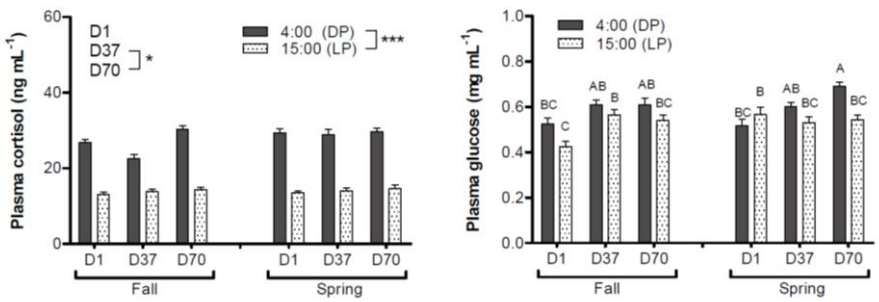

Fig. 1: Day-night (DP: dark phase; LP: light phase) variations of plasma cortisol (left) and glucose (right) in pike-perch juveniles exposed during 70 days to photoperiods simulating the fall or the spring in western Europe. Data are expressed as means ± SEM (n = 12). Capital letters indicate significant differences at $p < .05$. Stars (*) and (***) indicate main effects (day of sampling or time of the day) significant at $p < .05$ and .001, respectively.

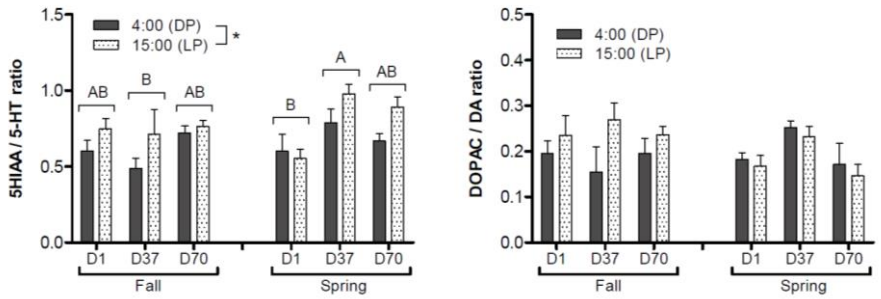

Fig. 2: Day-night (DP: dark phase; LP: light phase) variations of brain serotonergic (left) and dopaminergic (right) activities in pike-perch juveniles exposed during 70 days to photoperiods simulating the fall or the spring in western Europe. Data are expressed as means ± SEM (n = 12). Capital letters indicate significant differences at $p < .05$. Stars (*) indicate main effect (time of the day) significant at $p < .05$.

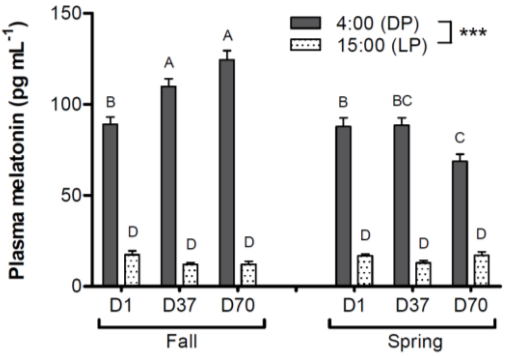

Fig. 3: Day-night (DP: dark phase; LP: light phase) variations of plasma melatonin in pike-perch juveniles exposed during 70 days to photoperiods simulating the fall or the spring in western Europe. Data are expressed as means ± SEM (n = 9). Capital letters indicate significant differences at $p < .05$.

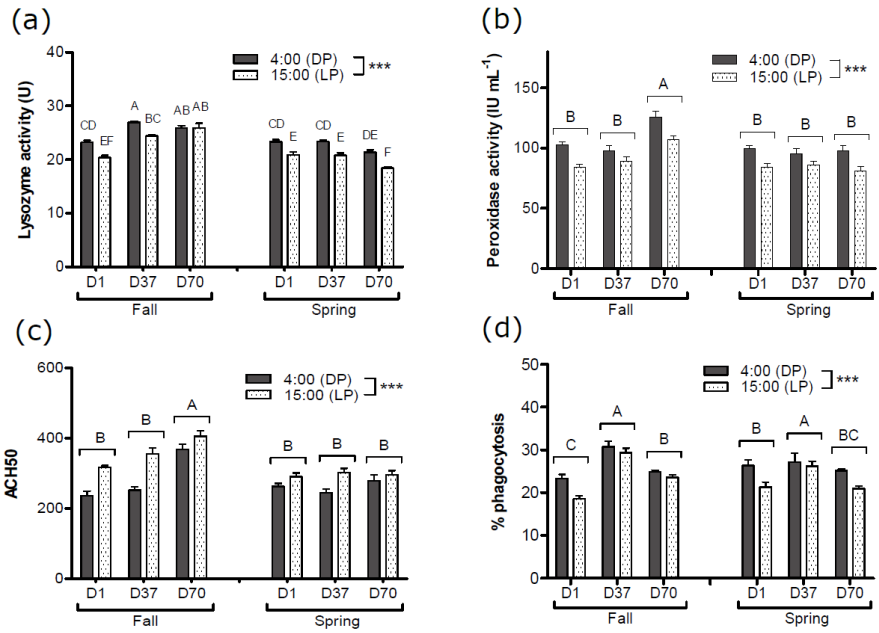

Fig. 4: Day-night (DP: dark phase; LP: light phase) variations of (a) lysozyme, (b) peroxidase and (c) hemolytic complement activities in plasma and (d) phagocytic activity in spleen of pike-perch juveniles exposed during 70 days to photoperiods simulating the fall or the spring in western Europe. Data are expressed as means ± SEM (n = 15). Capital letters indicate significant differences at $p < .05$. Stars (***) indicate main effect (time of the day) significant at $p < .001$.

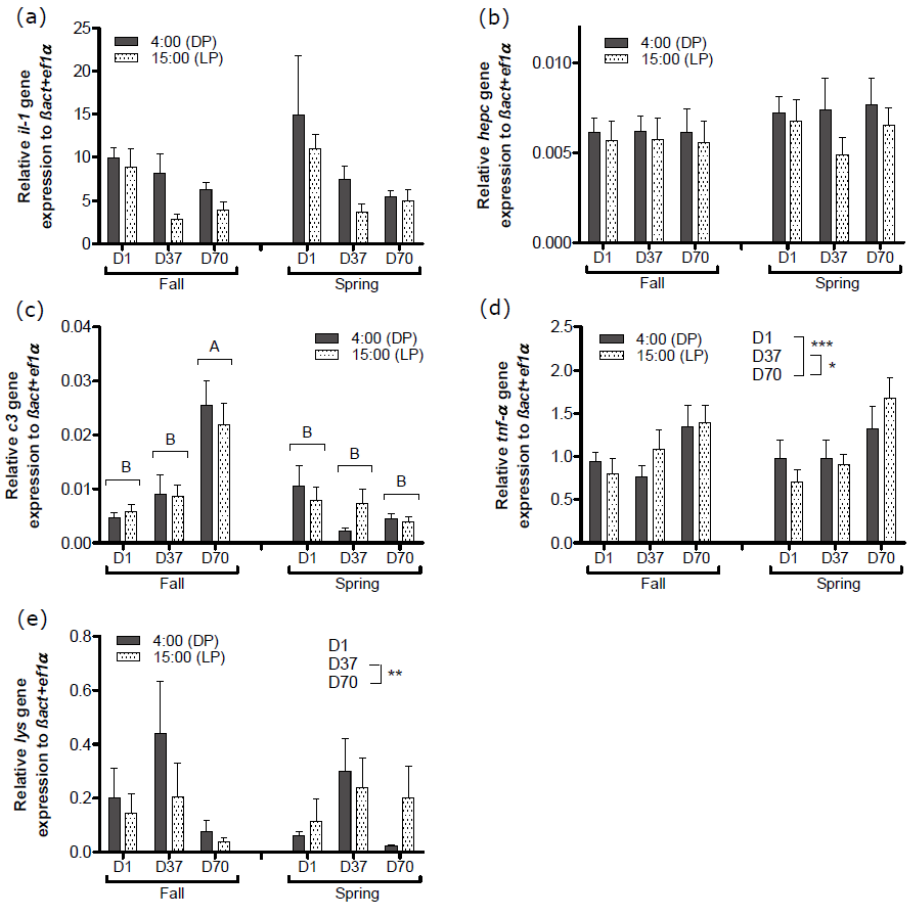

Fig. 5: Day-night (DP: dark phase; LP: light phase) variations of (a) *il-1*, (b) *hepc*, (c) *c3*, (d) *tnf-α* and (e) *lys* gene expression in head kidney of pike-perch juveniles exposed during 70 days to photoperiods simulating the fall or the spring in western Europe. Data are expressed as means ± SEM (n = 12). Capital letters indicate significant differences at $p < .05$. Stars (*), (**) and (***) indicate main effects (day of sampling or time of the day) significant at $p < .05$, .01 and .001, respectively.

E. DISCUSSION

Seasonality, that is mainly characterized by variations of both temperature and day length, is described to affect immune responses of vertebrates, including fish, reptiles, birds and mammals [25,26,52-54]. Thus seasonal variations of several innate immune markers, including complement,

peroxidase and lysozyme activities, have been observed in sea bass (*Dicentrarchus labrax*), dab (*Limanda limanda*), halibut (*Hippoglossus hippoglossus*) and Asian catfish (*Clarias batrachus*) [55-58]. Temperature was described several times to be the main seasonal factor operating on fish immune system since lower temperatures lead to a shutdown or slowing of immune mechanisms leading to an increase in fish susceptibility to disease [58-60]. However, the study of Valero et al. [58] has reported that seasonal variations of some immune markers are not related with the temperature and that they could be explained by daylight changes, supporting that both temperature and photoperiod variations act significantly on fish immune system throughout the year. Nevertheless, to our knowledge, no studies conducted specifically on photoperiod in a seasonal context has been published until now. Several studies have focused on photoperiod manipulation (extreme light regimes including constant light or darkness). This practice, that has become common in the aquaculture industry to obtain out-of-season reproductions [25], affects the levels of stress hormones in rainbow trout (*Oncorhynchus mykiss*), which consequently alter immune functions [29]. It was also shown to affect larval development and survival of European sea bass and Senegalese sole [61,62].

In the present experiment, the season-simulated photoperiod regimes did not influence growth parameters. Since seasonality was shown to highly influence growth of various fish species [25,57], our results suggest that such effect is mainly driven by temperature and/or food availability. In addition, values of stress markers suggest that natural gradual changes in L:D are not stressful for pike perch and that it should still be considered in management strategies of fish culture. Since tryptophan is a precursor of both melatonin and serotonin hormones [35], the daily rhythm in 5HIAA/5-HT ratio may be the result of a decrease in tryptophan availability during the dark phase of the photoperiod due to melatonin synthesis.

An early gonadogenesis was observed at day 70 for both sexes, with the highest development for males maintained under the FSP. The decrease in day length is involved in the initiation of gametogenesis in many fish species including the walleye (*Sander vitreus*) [63] and the Eurasian perch [64]. Our results are also consistent with results from Ben Ammar et al. [65] showing that the initiation of gonadogenesis seems to be mainly driven by photoperiodic changes. However, both temperature and photoperiod

variations must be considered in the control of pike-perch reproductive cycle [65,66].

A potential effect of such gonadal development on the immune system cannot be discarded since interactions between neuroendocrine and immune systems are widely accepted [67,68]. Moreover, season-dependent changes in fish immune system were shown to be correlated with changes in the levels of circulating sex hormones [69]. For example, phagocytosis and lysozyme activities, respiratory burst activity in blood leukocytes as well as ROS and NOS production are all influenced by estrogens in various fish species. However, in the present experiment, the correlation study does not highlight any relation between immune markers and gonadal development, supporting that immune status of young pike perch may be only slightly influenced by the initiation of gonadogenesis.

We showed for the first time the variations of immune markers according to the season-simulated photoperiod. Lysozyme, complement and peroxidase activities, as well as *c3* gene expression in head kidney, all increased following the decrease in day length during 70 days. In contrast, only lysozyme showed a decrease under the spring-simulated photoperiod. In addition, the correlation study support that variations of innate immune markers are related to the nocturnal variations in plasma melatonin. The progressive exposure to shorter photoperiods leads to an increase in melatonin production and release by the pineal gland since its activity is directly proportional to the length of the night [8]. Few evidences have suggested that the melatonin hormone may act as an important immune regulator in teleosts [3,20]. For instance, several immune markers including peroxidase, phagocytic ability and cytotoxic activity of head kidney leucocytes as well as the expression of immune genes in head kidney (*il-1β*, major histocompatibility complex, and interferon-regulatory factor-1) are increased in gilthead seabream (*Sparus aurata*) following intraperitoneal injection of melatonin [20]. And, in zebrafish, endogenous melatonin enhances neutrophil migration following increase in cytokine expressions such as Il-8 and Il-1β [22].

Our results defined also day-night variations of lysozyme, peroxidase, complement and phagocytic activities. Such cyclic activity has already been described in several fish species including pike perch, Nile tilapia (*Oreochromis niloticus*), gilthead seabream and sea bass [19,23,24,70). The

day-night variations of immune variables, as observed in the present experiment, are comparable to the variations of both plasma melatonin and cortisol [23,24]. Considering that cortisol is a potent immunosuppressive agent with complex actions on immune cells and tissues [31,32] and that an anti-stress role for melatonin has been defined [33,35], the present results may support that melatonin acts on immune activities through the modulation of cortisol release in plasma [3,71]. However, cortisol peak was described to occur at different times of the day according to the experiment and the fish species, supposing that it is, at least partially, light- and melatonin-independent. Innate immune markers are also differently stimulated according to the time of the day. Even if the underlying mechanisms are still not well understood and that differences of cyclic pattern are observed among fish species [19,23,24], there are always one or several innate immune activities enhanced whatever the time of the day, ensuring a constant immune protection against pathogens.

All in all, the daily rhythms of innate immune markers and the up- and down-regulations according to the photoperiod support a significant effect of the melatonin hormone on the innate immune system of fish. However, the model of action still needs investigations. Melatonin could act on immune cells and tissues indirectly through the regulation of several hormones (glucocorticoids, growth hormone, prolactin…) or through specific melatonin receptors [3]. In several teleost species, three high-affinity receptor subtypes were identified, including MT1, MT2 and Mel1c [72]. While several studies focused on melatonin receptor distribution [73-76], few has examined their presence in immune cells or tissues. They are found in the kidney and the spleen of several fish species [74,76,77] but no study has investigated the exact location of these receptors in the immune tissues neither their functional significance.

In addition, the present study did not examine the potential role of an internal clock machinery in immune modulation according to seasonal simulated photoperiods. Organisms, including fish, show circadian rhythms (repeating roughly every 24 h) of activity, food intake, body change color, oxygen consumption and some physiological parameters [3,78]. These rhythms are under the control of different environmental cues, with the light being the strongest of these synchronizers. Other environmental parameters changing daily or annually, including temperature and food availability, are also critical [78]. The main characteristic of these circadian rhythms is the persistence of

their oscillations for a certain period of time, even in the absence of these environmental cues, by being driven by a circadian clock [3]. In zebrafish, a complex network of coexisting central and peripheral clocks was described and peripheral tissue pacemakers, that have been identified in several extra-retinal/extra-pineal tissues, were shown to be directly responsive to light [79]. However, while several studies described daily variations of immune activities [19,23], none has investigated in fish, and to our knowledge, circadian activity of the immune system or peripheral clock in immune tissues. The potential presence of such pacemaker in immune tissue should be investigated in order to better describe the relationship between the photoperiod, the clock machinery and the immune system.

In conclusion, this study showed for the first time in a teleost fish an innate immune modulation according to the seasonal and daily variations of photoperiod. As the time-keeping hormone, melatonin is seen as one of the main mediator acting on fish immune system. Such regulation may involve both direct and indirect action of melatonin on immune targets. Better consideration of the light environment is suggested to improve immunocompetence of cultured fish species and to limit disease outbreaks.

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G. REFERENCES

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IX. *Ex vivo* actions of melatonin and cortisol

Foreword: In the previous *in vivo* experiments, the results suggest an action of melatonin on pike-perch immunity. However, no data clearly characterized the mode of action (direct and/or indirect modulation) in teleosts. Melatonin could act on immune targets through specific receptors and/or via intermediates such as cortisol. We thus investigated with an *ex vivo* approach the direct actions of melatonin, combined or not with cortisol, on the expression of immune-relevant genes in the two main hematopoietic organs of teleosts, namely the head kidney and the spleen.

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

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A. ABSTRACT

The melatonin hormone, which is a multifunctional molecule in vertebrates, was shown to exert complex actions on the immune system of mammals. However, the immunomodulatory capacity of this hormone has only been little investigated in teleosts. In the present experiment, we exposed *ex vivo* the spleen and the head kidney tissues of pike-perch to melatonin (Mel) and cortisol (Cort). Three concentrations of both hormones, alone or in combination, were used, 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}). Medium without Mel or Cort was used as a 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. The results support both an indirect action of the Mel hormone on the immune system through the regulation of intermediates as Cort, as well as a direct action on immune targets through specific receptors.

B. INTRODUCTION

A great development in aquaculture has been observed for the last few decades in response to an increasing market demand. 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 outbreak by developing antibiotics and vaccines. However, the development of drug-resistant bacteria and the 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 a center of interest for decades and it became 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 are 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), which is a multifunctional molecule in vertebrates, is less understood and would merit 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 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 in a wide range of vertebrates, including thermoregulation, reproduction and immunity in mammals (Carrillo-Vico et

al., 2005; Dumbell et al., 2016). In teleosts, Mel also acts on important functions including reproduction, smoltification, osmoregulation and development (Downing et al., 2002; Falcon et al., 2007; 2010). However, and contrary to mammals, its potential immunomodulatory capacity has been rarely investigated in teleosts (Cuesta et al., 2008). The few available evidences 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 known in teleosts to be part of the stress response 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 an 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, it has been shown that percid fish are 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 whose aquaculture management has not been optimized yet.

The light environment was shown in previous studies to affect pike-perch stress status and melatonin release by the pineal gland and to modulate its innate immune functions (Baekelandt et al., 2019a,b; 2020). However, no investigations have been performed so far about the mode of action of the melatonin hormone on immune tissues of teleost. We thus aimed to investigate by an *ex vivo* approach the direct action of Mel, combined or not with Cort, on the expression of immune-relevant genes in head kidney and spleen of pike-perch. The set of selected genes encodes 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*).

C. MATERIAL AND METHODS

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 during 4 weeks in 4 indoor 400-L tanks of a recirculating aquaculture system. Environmental conditions were kept constant during that period. It included light intensity at water surface (10 lux), photoperiod ($12L_{(8:00\ -\ 20:00)}$/12D), water temperature (16°C), oxygen saturation (90%) and feeding (twice a day with commercial pellets at 2% biomass).

2. Sampling procedures and incubation

The experiment was performed twice on March 4th and 7th, 2019. For one day of sampling, ten fish from each tank were randomly removed and euthanized (overdose of anesthetic MS-222, 250 mg L^{-1}) before collecting the spleen and the head kidney. Thus, the total number of collected fish, considering the two sampling days, was 80. Organs were washed thrice with Hanks' balanced salt solution (HBSS, Fisher Scientific, USA). They were then transferred, in 12-well culture plates, in HBSS supplemented with bovine serum albumin (BSA, 0.1%, Fisher Scientific, USA) and ascorbic acid (50 µM, Sigma-Aldrich, USA) and submitted to one of the following treatments: (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. Thus, for 1 day of sampling, each treatment was applied to 4 spleen and 4 head kidney tissues. The Mel and Cort doses were selected to be consistent with the literature, considering 2 physiological and 1 pharmacological doses and a 10-fold factor between them. The lowest concentrations of Mel (10 and 100 pg mL^{-1}) corresponds to diurnal and nocturnal levels of plasma melatonin for pike-perch maintained in steady conditions (Baekelandt et al., 2019b). The lowest concentrations of Cort (50

and 500 ng mL^{-1}) consider plasma cortisol levels in normal conditions and in response to an acute stress (Baekelandt et al., 2019b). After 5 and 10 h of incubation, culture media were collected for storage at -80°C and replaced with fresh media. After 15h, the culture media and the organs were frozen in liquid nitrogen and then transferred at -80°C until further analysis.

3. Gene expression analysis

Total RNA isolation was performed using Extract-all® reagent (Eurobio, Paris, France) following 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 ranged between 95 and 106 %. qPCR was performed using SYBR® Green Supermix (Biorad, California, USA). A four steps 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™ 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 of *rna-18s*, *β-actin* and *ef1-α* whose expressions were stable in tested conditions. Values are expressed as fold change considering the control condition set at the value 1. Among the 10 immune genes targeted, 5 were detected in both organs while 5 others were detected only in the head kidney (Table 1).

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 manufacturer's instructions. The analysis was performed in duplicate in media collected after 15 h of incubation.

5. Levels of hormones in culture medium

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

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 a same RAS) or between sampling days (the experiment was conducted in two sets). Results were then analyzed with a one-way ANOVA considering the treatment as a fixed factor. When significant ($p < .05$), a Tukey's HSD post-hoc test was applied ($p < .05$). When data, even transformed, 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 test. 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 on R software (package ade4) in order to characterize the samples distribution considering the gene expression data and the treatments 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 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')	Eff. (%)
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
fkbp4	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

D. RESULTS

1. Levels of hormones in culture medium

Mel and Cort concentrations were assessed in culture media, every 5 h of incubation (before being collected and renewed with fresh media) as well as in stock solutions. No significant differences were detected between organs of a same treatment or between post-exposition time points.

2. Lactate dehydrogenase activity

After 15 h of incubation, no differences in lactate dehydrogenase activity were detected between treatments and control condition, for both head kidney and spleen culture media.

3. Gene expression

The RDA and clustering analysis on kidney tissues revealed four distinct groups (Fig. 1) constituted with 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, "C" group, by *hp* and *tnf-α*. Concerning spleen tissue (Fig. 2), 3 groups were revealed: (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 *il-1β*, *fth1* and *fκbp4* genes.

In the head kidney tissue, an increase in *il-1β* and *fgl2* was detected with the lowest Cort concentration and 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 control condition, 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. However, this latter concentration was shown to reduce *fth1* expression.

Concerning the spleen tissue (Fig. 4), the analysis revealed significant increases of several gene expressions, 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). To the exception of *il-1β* whose expression decreased with Cort (5000 ng mL^{-1}), higher concentrations of Mel or Cort has no significant effects in comparison to control condition. 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.

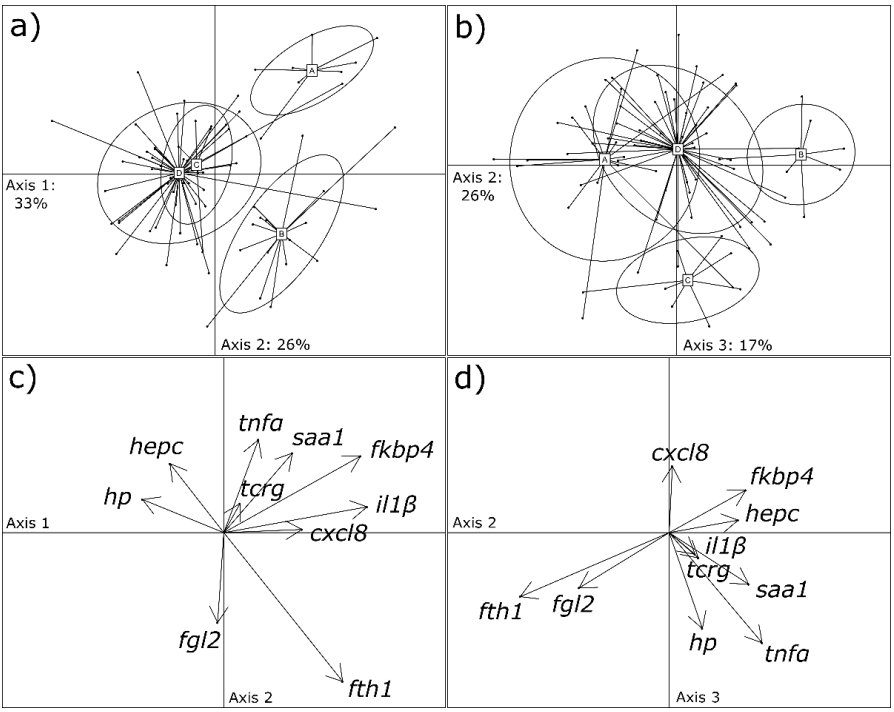

Fig. 1: Projection and clustering, on axes (a) 1 and 2 or (b) 2 and 3 of the redundancy analysis, of 80 head kidney tissues according to their gene expression profiles after *ex vivo* hormonal treatments. Projection of gene expression outputs on axes (c) 1 and 2 or (d) 2 and 3 of the redundancy analysis. 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.

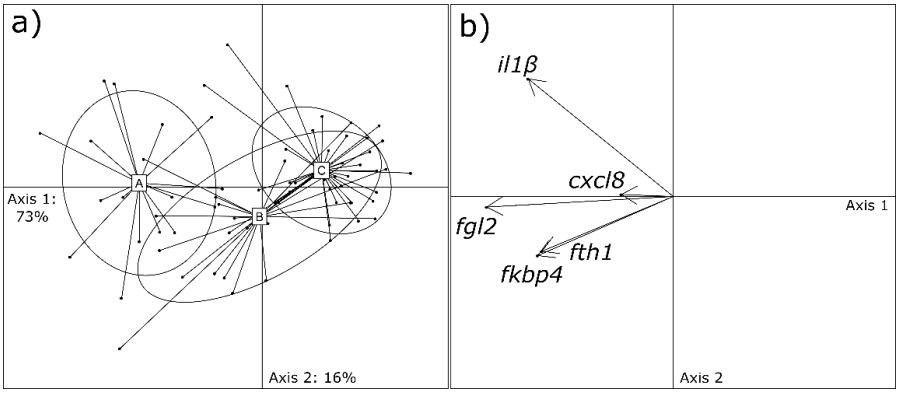

Fig. 2: Projection and clustering (a), on axes 1 and 2 of the redundancy analysis, of 80 spleen tissues according to their gene expression profiles after *ex vivo* hormonal treatments. Projection (b) of gene expression outputs on axes 1 and 2 of the redundancy analysis. The cumulative projected inertia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

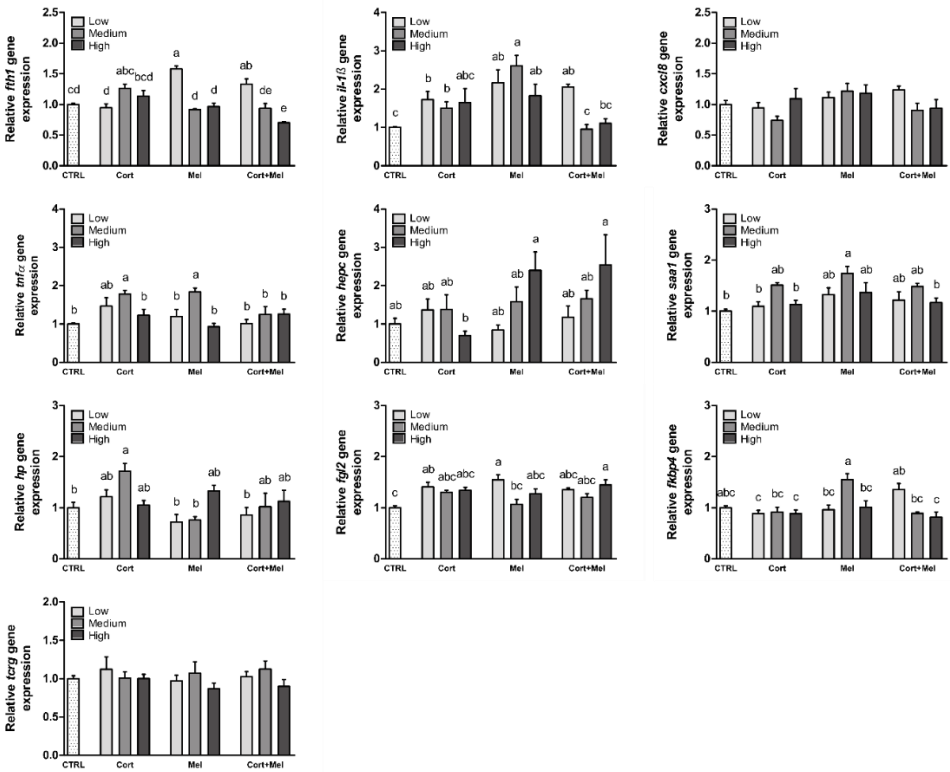

Fig. 3: Relative expression of immune-relevant genes in head kidney tissue of pike-perch exposed *ex vivo* to melatonin and cortisol. Treatments, tested in 3 concentrations (Low, Medium and High), included (1) Mel (10, 100 or 1000 pg mL^{-1}), (2) Cort (50, 500 or 5000 ng mL^{-1}) and (3) Mel+Cort (10+50,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

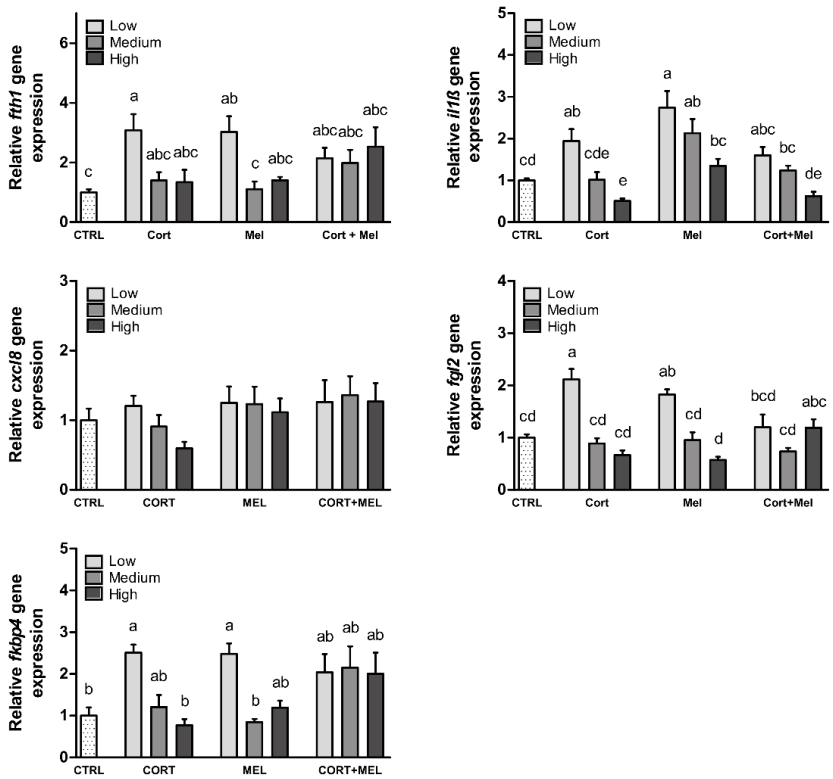

Fig. 4: Relative expression of immune-relevant genes in spleen tissue of pike-perch exposed *ex vivo* to melatonin and cortisol. Treatments, tested in 3 concentrations (Low, Medium and High), included (1) Mel (10, 100 or 1000 pg mL^{-1}) (2) Cort (50, 500 or 5000 ng mL^{-1}) (3) Mel+Cort (10+50, 100+500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

E. DISCUSSION

The actions of melatonin, combined or not with cortisol, on immunity were investigated through the analysis of immune-related gene expressions in the main fish lymphoid organs, namely the head kidney and the spleen. Both organs were thus 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*, and *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 the enhancement of immune functions following its injection or ingestion in various vertebrates, including 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 been largely described in mammals (Lin et al., 2011; Xia et al., 2012) but was explored 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).

The present results, considering pro-inflammatory and APP genes, thus support the immunoenhancing properties of the molecule under basal condition (unstimulated immunity). In pike-perch, an action of Mel on inflammatory cytokines was hypothesized since a correlation was established *in vivo* between the daily cyclic release of Mel by the pineal gland and the day-night variations of *tnf-α* and *il-1β* gene expressions in the head kidney (Baekelandt et al., 2019). Moreover, exogenous Mel was also 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). The only data showing an action 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 negative regulation of acute-phase response during inflammatory process, our results are consistent with immunoenhancing properties of Mel in 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 *fkbp4* following Mel treatment may suggest an action on fish specific immunity, as observed in other vertebrates, including birds and mammals (Kharwar et al., 2015; Li et al., 2015; Chen et al., 2016). However, 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 were identified, including MT1 and MT2 (Dubocovich and Markowska., 2005). In addition, a third melatonin receptor, Mel1c, is found exclusively in fish, amphibians (*Xenopus* species) and birds (Dufourny et al., 2008). The Mel receptors in mammals are expressed by numerous tissues including immune cells and tissues. Their distribution in human's immune system includes 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). Moreover, they were detected in spleen, thymus and lymphocytes of various vertebrates including rats, mice and birds (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 in spleen of rabbitfish and Senegalese sole respectively. However, no information about their function in spleen is available and further characterization of Mel receptors in fish immune tissues are needed.

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

Seen as the main hormone of the stress response, cortisol is produced by interrenal cells located in the head kidney of teleosts (Tort et al., 2011). The functions of cortisol in the stress response are numerous and include physiological, endocrine and immunological responses (Tort et al., 2011; Cortés et al., 2013; Mathieu et al., 2014). Many studies have focused on the regulation of immune defenses by corticosteroids and both activation or inhibition of immune mediators were described depending on the stress event. While an acute stress is usually associated to an immune activation, a chronic stress is characterized by long-term exposure to cortisol leading to an immune depression or suppression (for further information, reader is invited to consult Tort et al., 2011; Nardocci et al., 2014). In the present experiment, Cort, depending on the concentration, influenced several immune gene expressions in both spleen and head kidney. On the one hand, in spleen, the lowest physiological Cort concentration led to an increase of pro-inflammatory gene *il-1β,* acute-phase genes *fgl2* and *fth1* as well as *fκbp4.* In opposition, *il-1β* expression decreased with the pharmacological dose of Cort. Such action on pro-inflammatory cytokine *il-1β* was already described *in vivo* in rainbow trout (*Oncorhyncus mykiss*) following Cort implantation (Cortés et al., 2013). This effect may be explained by an inhibition of NF-κB signaling, leading to

a decrease in the production of pro-inflammatory cytokines such as IL-1 (Sternberg, 2006). On the other hand, in the head kidney tissue, only the high physiological dose of Cort (500 ng mL^{-1}) led to a different expression profile, with increases in acute-phase and pro-inflammatory genes, including *fth1*, *il-1β, tnf-α* and *hp*. While stimulatory effect in spleen was mainly observed at 50 ng mL^{-1} and was lost at higher concentration, it was mainly observed around 500 ng mL^{-1} in the head kidney tissue. 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 have been described, including GR1a, GR1b, GR2 and MR, 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.

Considering both Cort and Mel actions on immune tissues was relevant since a mutual inhibiton was characterized in teleosts. Thus, increase in plasma Mel through injection or oral administration was shown to reduce the activation of the HPI axis under prolonged stress conditions in various fish species, including in European sea bass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*) (Herrero et al., 2007; Conde-Sieira et al., 2014). In parallel, Cort was shown to lower melatonin synthesis in the pineal organ (Nikaido et al., 2010; López-Patiño et al., 2014). Moreover, a combined action of Mel and Cort may be hypothesized. In the present results, an increase in *il-1β* and *tnf-α* expressions in the head kidney is observed with Mel or Cort (high physiological dose). However, such increase is lost when exposing the organ to both hormones. An interaction between those systems (receptor level) can be hypothesized. In mammals, exogenous Mel was shown to upregulate the expression of MT1 and MT2, while the same expression is decrease following dexamethasone (synthetic glucocorticoid) treatment (Gupta et al., 2013). In parallel, GR expression is reduced following Mel treatment, suggesting that the modulation occurring between those systems is also mediated through the expression of their receptors (Gupta et al., 2013).

Nuclear factor-kappa B (NF-κB) is involved in cellular responses to stimuli such as stress and in regulation of the immune response to infection. The anti-inflammatory property of Mel was shown to be mediated by the inhibitions of both inflammasome and NF-κB activations (Markus et al., 2013; Favero et

al., 2017; Tarocco et al., 2019). In rat, Mel treatment alters NF-κB expression, thus taking part in the protection against cyclophosphamide-induced urotoxicity (Tripathi and Jena, 2010). Concerning fish, in *Labeo rohita* hepatocytes, Mel improved H2O2-induced oxidative stress through modulation of Erk/Akt/NF-κB (Moniruzzaman et al., 2018). Since NF-κB activation is also modulated by Cort (Dong et al., 2018), it would be of a great interest to better consider this nuclear factor in the modulation of the immune system by Mel and Cort.

In conclusion, both hormones at physiological concentrations influenced significantly the immune-related genes of the present *ex vivo* experiment. In both organs, Mel treatment led to an increase in immune-related genes, including genes involved in inflammatory process, acute-phase response and acquired immune response. These results confirm, in teleost, the immunoenhancing properties of Mel in basal immune conditions. Furthermore, we showed a direct action of Mel on immune organs but the indirect action, that could even be of a greater importance, must be considered in melatonin-immunity interactions studies. In addition, future investigations may consider the actions of the potential immune-derived melatonin on immunity of teleosts.

F. ACKNOWLEDGMENT

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X. Supplementary data

From a transcriptomic analysis (Jennifer Rocher, ULorraine), sequences of MT1 and MT2 melatonin receptors were collected in order to design specific primers. At the end of the PhD thesis, we were able to specifically amplify these genes (Table 1).

Primer efficiencies were validated when ranged between 95 and 105 %. qPCR was performed using SYBR® Green Supermix (Biorad, California, USA). A four steps 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™ 5 real-time PCR machine (Applied Biosystems). Specificity was checked (melting curve and gel electrophoresis). Furthermore, amplified sequences were sent for DNA sequencing (Genewiz, Sanger sequencing). Blast analysis revealed high identities with published sequences for pike-perch (>99%).

Table 1: Sequences of primers used for gene expression quantification [24].

Gene	Sens	Sequence (5' to 3')
mt1	Forward	GGATCCAGATGCGAAGGTAA
	Reverse	CCACAGCCTCAAGTACGACA
mt2	Forward	GCTCGGGAAGTCTCTGTCAC
	Reverse	CCTGAGTGGCTTTTTGTGGT

Amplification was successfully performed for *mt1* and *mt2* genes in brain, liver, head kidney and spleen of pike-perch. It thus confirms the expression of these receptors in various organs, including in the main organs of the fish immune system.

XI. General Discussion

While aquaculture has been developing in almost all regions of the world, the European aquaculture industry has shown limited increase in productivity. This can be attributed to a low diversity of cultured species since the European fish production is dominated by only five species, including rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), Atlantic salmon (*Salmo salar*), seabass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) (FAO). In order to boost this major economic activity, the FP7 - European Commission funded project 'DIVERSIFY' was launched to overcome the documented bottlenecks in the production of six selected fish species with high aquaculture potential, including the pike-perch (*Sander lucioperca*). Research was carried out in various scientific disciplines including reproduction and genetics, nutrition, larval husbandry, grow out husbandry, fish health and socioeconomics.

Pike-perch is a valuable candidate for the diversification of European inland aquaculture industry. However, one major bottleneck identified in its culture is a high sensitivity to stressors leading to high and sudden mortalities, with survival rate estimated between 8 and 30% during larval and juvenile stages (Kestemont et al, 2007; Szkudlarek and Zakes, 2007; Dalsgaard et al, 2013). Considering that *(i)* newly aquaculture species are more sensitive to aquaculture stressors than some highly domesticated species such as salmonids (Jentoft et al., 2005) and that *(ii)* husbandry practices are not optimized and standardized for pike-perch, we aimed to identify the best husbandry practices to consider in pike-perch culture with regards to growth, immune and physiological status of the fish. Indeed, in aquaculture, prolonged, repeated and/or unavoidable stressors are largely associated to maladaptive physiological effects including failures in immune functions and disease resistance (Fast et al, 2008; Douxfils et al, 2011; Tort, 2011). A multifactorial experiment considering eight environmental factors was designed taking into account current practices in major European pike-perch intensive farms and available data in the literature.

Experiment I: objectives, results and limitations

Experiment I: ***Optimal aquaculture modalities for pike-perch***

Objectives (O) and hypothesis (H): reminder

O_1: characterize the effects of major husbandry practices and environmental factors on pike-perch physiology.
O_2: identify optimal rearing conditions ensuring low stress level and thereby good growth and survival.

H: several husbandry practices and environmental factors, considered alone or in combination, reduce stress in pike-perch, leading to improved immunocompetence, growth and survival.

As hypothesized, the experiment highlighted several factors impacting pike-perch stress status and grow out, including the feed type and the light environment. While the light intensity was the only factor explaining the mortality rate, the type of feed influenced all husbandry parameters. In parallel, stress and immune markers were influenced by temperature and oxygen saturation, respectively (Baekelandt et al., 2018). However, only a few output variables were tested, bringing limited conclusions.

By considering a fractional factorial approach, the first experiment allowed integrating simultaneously a large number of experimental factors with a limited number of experimental units. In comparison, a traditional full factorial design rarely exceeds 3 factors while we considered 8 factors. Despite these advantages, this kind of protocol has some limitations. First, each factor is tested in two modalities only, and the modalities of one factor must be quite different (*e.g.* 10/14 vs 24/0 for the photoperiod). This method thus allows pointing out the important factors to consider, but not the optimal conditions within each factor. And if extreme modalities have marked effects on the output results, it may mask the effects of other factors. Moreover, some factors are susceptible to induce non monotonic responses, and this is impossible to determine by using two modalities. Second, another main limitation is about the results since interactions between 3 or more factors are considered not relevant. Indeed, the impacts of fish grading according to combined factors such as light intensity and light spectrum can be

hypothesized but cannot be tested. Third, to create such an experimental design, an alias structure is generated to define which effects are confounded with others (Baekelandt et al., 2018). It requires, based on literature and practices in industries, to define before the experiment which factors and interactions are more relevant for the model since all the two-way interactions cannot be tested. Fourth, factors and modalities were defined according to constraints observed in Asialor farm (France). Those limitations, which may be limited to that company, raise the question about the possible transfer of the results to other fish companies. Fifth, rearing conditions of the multifactorial experiment were imposed (tank colour, size, shape…) as defined in Asialor farm. Considering that these factors may highly impact fish behaviour and physiology, we may hypothesize limitations about the transfer of the results to other fish facilities.

Because of those limitations, this kind of protocol has to be seen as a screening study. In the DIVERSIFY project, in order to confirm the results of the multifactorial protocol, an additional experiment was conducted in industrial conditions (Fish2Be). Growth and physiological status were compared between standard husbandry conditions usually applied in routine by the farmer and the best rearing conditions identified in the multifactorial experiment. Good growth results were obtained during 83 days for both conditions, with no statistical differences. However, the transfer of the fish to bigger tanks from another fish farm (Inagro) resulted in poor growth results and high mortalities. Results were thus not exploitable but it pointed out, as previously defined, that pike-perch is a stress-sensitive species as other percids.

The two objectives of the study were thus partially achieved. Several factors, including the type of feed and the light intensity, are important for the ongrowing of pike-perch juveniles. It may be associated to a higher stress level and lowered immunocompetence but the limited output variables and the high variability of the results do not allow drawing conclusions. Then, based on husbandry outputs, we identified some optimal combinations (Baekelandt et al., 2018). However, since we only tested two modalities per factor, we were unable to define the optimal conditions (best temperature or stocking density) neither their potential evolution (optimal density according to the fish weight). For instance, it is reported that pike-perch juveniles can be kept at high densities ranging between 15 and 30 kg m^{-3} without any increase in physiological stress response, and larger pike-perch up to 2 kg can

be maintained between 30 and 60 kg m^{-3} (Steenfeldt et al., 2010; Dalsgaard et al., 2013). Concerning optimal temperature, we showed best growth performances under both 21 and 26°C showing that pike-perch has a broad thermal optimum, already defined between 10 and 27°C for adults (Frisk et al., 2012). Several growth studies on juvenile pike-perch demonstrated that the optimal temperature is 25-30°C (Kestemont et al., 2003; Wang et al., 2009). Such difference between juveniles and adults may be explained by a preference of bigger fish for cooler water (Morita et al., 2010). However, the good growth observed at 21°C (combinations 1 and 3, see Experiment I) may support the hypothesis that more energy is spent for increased metabolic rates when pike-perch are reared at 25°C (Frisk et al, 2013). And taking into account that higher temperatures may increase pathogen outbreaks in fish farms, the use of a temperature around 21°C may be more consistent. While we bring interesting and important results for the improvement of pike-perch culture during the juvenile stage, including about the light environment and the temperature, additional investigations are needed to identify optimal conditions and their interactions with other parameters such as fish weight.

Experiment II: objectives, results and limitations

Rearing conditions in production units tend to affect fish health, enhancing the susceptibility of fish to infections. Since it is a major bottleneck of aquaculture development, various efforts have been undertaken to limit disease outbreaks by developing antibiotics and vaccines or by acting on fish immunocompetence through the use of immunostimulants and the enhancement of fish welfare (Abarike et al., 2018). From this point of the study, we focused on the immune system for which improving our knowledge is of a great interest to optimize management strategies and to limit disease outbreaks in fish farms.

Since the light environment profoundly affects pike-perch physiology, we considered the modulation of the immune system by the light environment whose characteristics are spectrum, intensity and photoperiod. Available data confirmed that several fish species are sensitive to the light in terms of behavior, growth and reproduction (Falcón et al., 2007; Luchiari and Pirhonen, 2008; Falcón et al., 2010; Politis et al, 2014; Baekelandt et al., 2018). However, few authors have considered the immune system.

Experiment II: ***Daily rhythms of immune markers***

Objective and hypotheses: reminder

O: identify the 24-h profiles for rhythmicity in several stress and innate immune markers and the potential influence of the light spectrum.

H_1: innate immune markers follow daily rhythmicity.

H_2: red light environment is beneficial for pike-perch.

For this second experiment, the first hypothesis was validated. Both lysozyme and peroxidase followed significant day-night variations. And the daily decrease in their activities was correlated to the cortisol peak that occurred during photophase, around 10:00. Because cortisol is mainly described as an immunomodulator agent (Tort, 2011), cortisol was suggested to be involved in the morning decrease of these humoral immune activities. However, this correlation was not validated in similar experiments. Considering literature data, immune activities mainly follow day-night cycle while cortisol rhythmicity is dependent on the fish species and the experiment. And it has been described that the meal time that can be perceived as an entraining signal of cortisol rhythm (Kulczykowska and Sánchez Vázquez, 2010, Oliveira et al, 2013). In the study of Esteban et al. (2006) conducted on seabream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*), daily variations in humoral immune activities are correlated to the daily cyclic activity of the pineal gland which releases the melatonin hormone during the dark phase of the photoperiod. Such correlation was hypothesized but could not be tested in the Experiment II.

The results have also validated the second hypothesis, when considering the fish growth. Growth parameters were improved under the red light, as already described by Luchiari et al. (2009). They showed in pike-perch the advantageous effects of long-wavelengths light, especially red light compared to white or short-wavelength lights, including a better feeding behavior and feed efficiency. An effect of the light spectrum was also observed on humoral innate immune markers. However, we obtained opposite responses for lysozyme and peroxidase after 42 days and no clear

trend (stimulation or inhibition of the immune system) could be defined (Baekelandt et al., 2019a).

This second experiment brought evidences that the light environment modulates pike-perch physiology, including growth and immunity. Since the light information is perceived by photoreceptors and converted into a melatonin signal by the pineal gland, this neurohormone was a good candidate to explain day-night variations in immune activities. However, the analysis could not be performed, which is the main limitation of this experiment. Among several tested protocols, the only one we validated on pike-perch plasma is an ELISA kit that necessitates 50 µL of plasma. Due to fish size, such quantity was not available after immune assays. However, the analysis was performed in the third experiment.

Experiment III: objectives, results and limitations

In mammals, the immunomodulatory actions of the melatonin hormone are well documented (Calvo et al., 2013; Ren et al., 2017; Xia et al., 2018; Zhao et al., 2019). However, in teleosts, such role on immune functions has been little investigated. A better understanding of the immune system and the relation with the neuroendocrine system and the environment is of a great interest to improve fish immunocompetence.

Experiment III: ***Immune modulation by the light environment***

Objective and hypothesis: reminder

O: identify if melatonin plays a central role by being a relay between the light, the stress axis and the immune system.

H: the light environment influences melatonin and cortisol secretions, leading to change in fish immunocompetence.

In this experiment, we took into consideration the triangulation between the melatonin hormone, the HPI axis and the immune system (Fig. 1). These relations are based on data collected in various fish species (Laiz-Carrión et

al., 2003; Tort, 2011; López-Patiño et al., 2013; 2014; Conde-Sieira et al., 2014). A mutual inhibition occurs between the HPI and the melatonin axes, and cortisol is a major immune modulator. A direct action of melatonin on immunity has not been investigated in fish, but an indirect action through the modulation of cortisol release has been suggested.

The hypothesis of the third experiment was verified. The high light intensity, as suggested in the first experiment, is a stressful environment for pike-perch. After 42 days of exposition, we noticed a decrease in fish immunocompetence, as well as a decrease in plasma melatonin (Baekelandt et al., 2019b). Such observation is consistent with a physiological response to chronic stress, consecutively acting on pineal gland and immune tissues.

Since melatonin is mainly seen as an immune activator in a wide range of vertebrates, including in mammals, birds and reptiles (Peña et al., 2007; Ahmad and Haldar, 2010; Singh et al., 2010; 2016; Ramos et al., 2018), the reduction in circulating melatonin may be involved in the decrease in immune activities and pro-inflammatory gene expressions. However, the experimental design did not allow drawing conclusions about the mode of action of the hormone on pike-perch immunity.

In this experiment, we considered that the pineal activity was inhibited by the HPI axis, following exposure to high light intensities. However, the pineal gland may contain itself light-sensitive photoreceptor cells (Li et al., 2012). Migaud et al. (2007) have shown that the photic regulation of pineal melatonin production has evolved within teleosts. In salmonids, the pineal organ responds directly to light independently of the eyes. In Nile tilapia (*Oreochromis niloticus niloticus*) and African catfish (*Clarius gariepinus*), it is not or slightly light sensitive, requiring eyes to perceive light and inhibit melatonin synthesis. In pike-perch, no information is available about the presence of such pineal photoreceptors. However, if detected, it could be hypothesized that the light has, at least partially, modulated pineal activity through these pineal photoreceptors. For now, no data can confirm or refute such hypothesis.

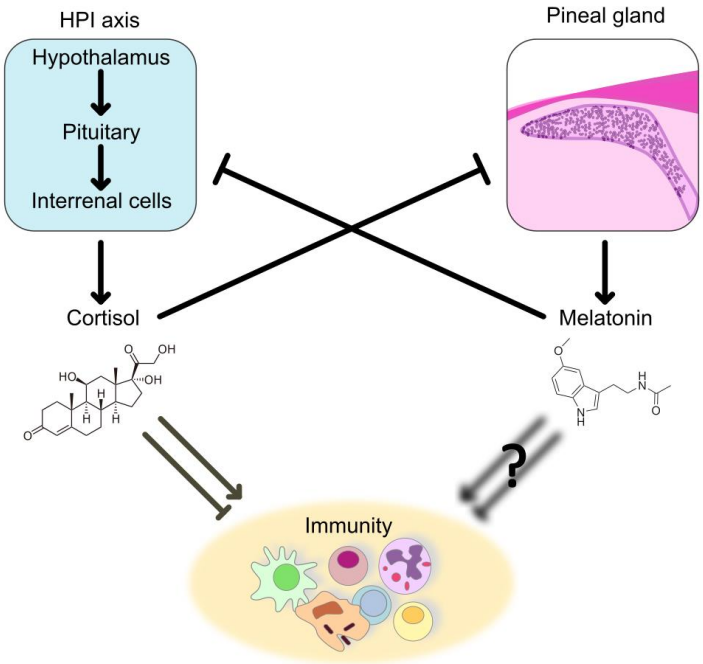

Figure 1: Triangulation between the HPI axis releasing cortisol, the pineal gland releasing melatonin and the immune system in teleosts.

Experiment IV: objectives, results and limitations

Seasonality is characterized by variations of temperature and photoperiod influencing fish immune system throughout the year. On one hand, lower temperatures lead to a shutdown or slowing of immune mechanisms leading to an increase in fish susceptibility to disease. On the other hand, it was reported that seasonal variations of some immune markers are photoperiod-dependent (Valero et al. 2014).

In order to better define the immunomodulatory capacity of melatonin, we designed this fourth experiment in order to isolate its potential direct action. We thus investigated the effects of two photoperiod regimes simulating the fall and the spring in Western Europe, on pike-perch physiological and immune responses.

Experiment IV: *Photoperiod influences melatonin release and immunity*

Objective and hypothesis: reminder

O: Characterize a potential direct action of melatonin on pike-perch immunity.

H: Natural variations of photoperiod and subsequent changes in melatonin release profile influence pike-perch immune status with limited effect on the stress axis.

The hypothesis of the study was verified. The season-simulated photoperiods influenced innate immune markers, with increased activities under the fall-simulated photoperiod. The progressive exposure to shorter photoperiods leads to an increase in melatonin production and release by the pineal gland since its activity is directly proportional to the length of the night (Baekelandt et al., 2020). Furthermore, the natural gradual changes in photoperiod was tested not stressful for pike-perch, and no change in cortisol release was detected.

The significant correlation between melatonin content and immune markers reinforces the idea of an immunomodulatory role of the hormone. Moreover, the results support an action of melatonin independently of the HPI axis. It may suggest a potential direct action but other candidates may have influenced immune markers such as growth hormone, prolactine or reproductive hormones (Oner et al., 2009; Falcón et al., 2010; Molik et al., 2010). A better consideration of these hormone in the experiment would have been of a great interest.

All the fish were collected and sampled at 04:00 and 15:00, whatever the photoperiod regime and the day of samplings. This may have created an experimental bias since the time between samplings and sunset/sunrise varied throughout the experiment – which is unavoidable in this kind of experiment. Indeed, in teleosts, different melatonin profiles were recorded (Falcón et al., 2010). In salmonids and Atlantic halibut (*Hippoglossus hippoglossus*), the amount of melatonin is produced in an on/off manner. In Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*), there is a discrete peak

in late dark phase and, in Nile tilapia (*Oreochromis niloticus*), there is a discrete peak in mid dark phase with an anticipation of sunrise (Falcón et al., 2010). The melatonin profile was not tested for pike-perch and we have no evidence that all our samplings occurred at the maximum of the melatonin peak. At 04:00 during the dark phase, an anticipation of sunrise may have occurred, mainly for the spring-simulated photoperiod, leading to variations in tested markers. However, in order to limit possible effects, the samplings were finished at least 1h before the twilight.

For this experiment, it would have been of a great interest to focus on melatonin receptors. The three receptors MT1, MT2 and Mel1c have been identified in several teleosts. A few studies have detected MT1 or MT2 in immune organs but they did not investigate their location within tissue neither their functional significance (Park et al., 2006; Sauzet et al., 2008; Confente et al., 2010). In addition to confirm their presence in the pike-perch hematopoietic organs, it would have been interesting to follow their expression according to the photoperiod regime and the time of the day. In the Senegalese sole (*Solea senegalensis*), both MT1 and MT2 exhibit daily and seasonal variations in several organs, including retina, optic tectum and pituitary (Confente et al., 2010).

Experiment V: objectives, results and limitations

An *ex vivo* experiment was designed in order to focus on the suggested direct action of melatonin on the main immune organs of teleosts, namely the head kidney and the spleen. These organs were exposed to several concentrations of cortisol and/or melatonin.

Experiment V: ***Ex vivo actions of melatonin and cortisol***

Objective and hypothesis: reminder

O: Investigate by an *ex vivo* approach the suggested direct action of melatonin, combined or not with cortisol, on the expression of immune-relevant genes in head kidney and spleen of pike-perch.

H: Both melatonin and cortisol modulate immune gene expressions.

The hypothesis for this last experiment was validated. Both hormones significantly influenced immune gene expressions, leading to a global immune stimulation. Considering the triangulation (Fig. 1), the results support both an indirect action of melatonin on the immune system through the regulation of intermediates such as cortisol, as well as a direct action on immune targets through specific receptors.

However, we cannot define the importance of both ways of action (direct vs indirect). Furthermore, results may have been influenced by endogenous cortisol secreted by interrenal cells (head kidney) as well by melatonin whose production may occur in immune cells (head kidney and spleen). Immune-synthetized melatonin has been described in immune cells and tissues of several mammals and it plays paracrine, autocrine and intracrine functions (Lardone et al., 2006; Maldonado et al., 2010; Muxel et al., 2012; Markus et al., 2017). Even if it has not been investigated in teleosts yet, it has to be considered in future experiments.

For this *ex vivo* experiment, we decided to submit the immune organs of pike-perch to different concentrations of melatonin and/or cortisol. Those concentrations included 2 physiological and 1 pharmacological doses, with a 10-fold factor between them. The lowest concentrations of melatonin (10 and 100 pg mL^{-1}) corresponds to diurnal and nocturnal levels of plasma melatonin for pike-perch maintained in steady conditions. The lowest concentrations of cortisol (50 and 500 ng mL^{-1}) consider plasma cortisol levels in normal conditions and in response to an acute stress. The pharmacological doses of melatonin (1 ng mL^{-1}) and cortisol (5 µg mL^{-1}) may be considered as toxic for cells. However, no cell death was revealed in preliminary tests. Furthermore, concerning melatonin, several studies in teleosts have already used high pharmacological doses of melatonin (IP injections), including 5 mg kg^{-1} in tilapia *Oreochromis niloticus* (Kim et al., 2017) and 10 mg kg^{-1} in goldfish *Carassius auratus* (Jung et al., 2016) without observing any mortality or deleterious effects. Even if the authors did not measure melatonin in plasma, such injection is supposed to induce higher melatonin concentrations than used in our *ex vivo* experiment. About cortisol, in rainbow trout (*Oncorhynchus mykiss*), plasma values exceeded 2 µg mL^{-1} after injection of an implant associated with a heat stress (Basu et al. 2001). However, even if several authors already used pharmacological doses of both hormones, the absence of more negative effects on immune organs with such hormonal cocktail raises the question about the existence of an experimental

bias. In our *ex vivo* experiment, full organs were directly plunged in culture media supplemented with hormones. It may be hypothesized that such procedure can limit the contact of internal parts of the organ with hormones, thus suggesting that hormonal concentrations in the organs is much lower than in media. Measuring hormonal contents in organs would have been performed to confirm that hypothesis. In addition, in order to better characterize the triangulation between the melatonin hormone, the HPI axis and the immune system, it would be interesting to test the effects of melatonin and cortisol at concentrations measured in pike-perch exposed to chronic stress.

The *ex vivo* expositions were conducted at 16°C instead of 21°C as applied in the other experiments. Since fish were reared at 16°C (before arriving at the University of Namur) and because we wanted to limit possible negative impact on pike-perch behavior, the acclimation in our facilities occurred under the same environmental conditions. However, knowing that the temperature is a crucial factor that can modulate pineal activity (Esteban et al., 2013) and, more generally, metabolism of ectotherm species, such difference would ultimately limit comparisons between experiments, and conclusions.

Further investigations with such *ex vivo* protocol would consider the use of specific antagonists to melatonin receptors, including luzindole and 4-phenyl-2-propionamidotetralin (4P-PDOT) (Liu et al., 2016). Luzindole has thus already been used in several fish studies, including in zebrafish *Danio rerio* (Ren et al., 2015) and in common goldfish *Carassius auratus* (Ribelayga et al., 2004). However, to our knowledge, there is no specific antagonist or agonist to MT1 or MT2, limiting the understanding of the physiological functions of these receptors.

Melatonin and fish immunity

The influence of melatonin on the immune system has been well established in higher vertebrates (Guerrero and Reiter, 2002; Carrillo-Vico et al., 2005; 2013). Generally, in mammals, melatonin stimulates basal immunity to ensure an optimal response to infection or exerts an anti-inflammatory action in the case of inflammatory responses to protect the organism from host tissue damage (Carrillo-Vico et al., 2013). Melatonin thus regulates cell dynamics,

including the proliferative and maturational stages of all hematopoietic and immune cells lineages (NK cells, T and B lymphocytes, granulocytes, monocytes) involved in host defense, in both bone marrow and tissues (Miller et al., 2006).

Since light is mainly perceived by the retina and transferred into a melatonin signal by the pineal gland, melatonin was considered as a potential actor in the immune modulation by the light environment in fish. Such hypothesis was supported by numerous data available in mammals, birds and reptiles and by a high conservation of the hormone within phylogenetically distant organisms (Calvo et al., 2013; Ren et al., 2017; Xia et al., 2018; Zhao et al., 2019). The objective was thus to characterize the role of the melatonin hormone as a potential relay between neuroendocrine and immune systems. We have defined that all three light characteristics can modulate pike-perch immunity and a significant correlation was established with plasma melatonin. Several humoral innate immune markers follow daily cyclic activity and variations of both light intensity and spectrum affect melatonin release, stress status and immune markers.

Considering that cortisol modulates immune functions in vertebrates through specific receptors and that a mutual inhibition was described between HPI and melatonin axes (López-Patiño et al., 2013; 2014; Conde-Sieira et al., 2014), it was considered that melatonin modulates immunity through, at least, an indirect action. Furthermore, we hypothesized a direct action suggesting specific melatonin receptors on immune cells and tissues, as widely described in mammals (Lardone et al., 2009; Maldonado et al., 2010; Singh et al., 2016). Such direct action was primarily supported by the results of the *ex vivo* experiment (see Experiment V) and secondly by the detection of MT1 and MT2 mRNA in spleen and head kidney tissues (unpublished data). We thus confirmed a direct action of melatonin on fish immunity, as previously described in birds and mammals. The experiments also defined the immunoenhancing properties of the hormone in pike-perch with increases in humoral activities and immune-genes expression. However, some anti-inflammatory properties of the hormone cannot be excluded when considering that melatonin may buffer the immune system as observed in mammals (Xia et al., 2012; Carrillo-Vico et al., 2013) and in common carp (*Cyprinus carpio*) (Kepka et al., 2015). More investigations are needed about a potential dual function of melatonin in teleosts. However, considering the

results of the present study and the data available in other vertebrates, a similar immune function of melatonin can be hypothesized.

The present study did not consider any potential role of an internal clock machinery in the modulation of fish immunity. All the organisms show circadian rhythms (repeating roughly every 24h) whose main characteristic is the persistence of their oscillations for a certain period of time, even in the absence of the environmental cues (the light being the main of these cues, but also the temperature, the food availability…) (Zhdanova and Reebs, 2006). The persistence of such oscillations is induced by an internal clock machinery. In zebrafish (*Danio rerio*), a complex network of coexisting central and peripheral clocks was described and peripheral tissue pacemakers, that have been identified in several extra-retinal/extra-pineal tissues, were shown to be directly responsive to light (Whitmore et al., 2000; Prokkola and Nikinmaa, 2018). However, a high intra- and interspecies variability in the design and function of the fish circadian systems was described (Prokkola and Nikinmaa, 2018). For now, no investigations have been carried out on the existence of such clock machinery in immune organs and tissues of teleosts but we can hypothesize that immune functions are also under the control of internal clocks. In our experiments, we described day-night variations of several immune markers but we did not test the persistence of their oscillation in a constant environment (24h of light, no feeding). Future experiments may consider such circadian rhythms and the potential action of pacemakers.

Fish model

Studying pike-perch was initially of a great interest since it is one of the most valuable candidate for the development of fish industry in Europe. Additionally, its high sensitivity for the light environment make this species a good model to study the effects of the light on fish physiology, and more particularly on melatonin release and immunity. This sensitivity is mainly due to the *tapetum lucidum* that is a reflecting layer in the retina. It thus increases eye sensitivity to the light. This is consistent with the natural behavior of pike-perch since it is a crepuscular and nocturnal predator mainly found in turbid rivers and eutrophic lakes. However, in cultured conditions, the use of clear water associated with high light intensities can impact pike-perch physiology.

However, for no apparent reason, difficulties can be encountered in the maintenance of this species. While all the fish were reared in the same conditions (water quality, feed, light environment, temperature…), some batches of juveniles have slightly acclimatized after transfer to new facilities, leading to poor growth results and high mortality rates. Moreover, as said before, in the DIVERSIFY experiment that aimed to confirm the results of the multifactorial design (unpublished results), an excellent growth was observed during the two first months but growth stagnation and high mortalities occurred after transfer by car, suggesting a strong impact of this stress event. The hypothesis of unsuitable conditions for bigger juveniles may be suggested, but is not consistent with results of the other experiments. Those observations rise the question about the stress sensitivity and the potential relation with the domestication level and the geographic origin of the fish. Unpublished data defined that pike-perch is a stress-sensitive species as reported for other percid fish such as the Eurasian perch (*Perca fluviatilis*) (Milla et al, 2010; Douxfils et al, 2014). Furthermore, it was shown that newly aquaculture species, in comparison to some highly domesticated species such as salmonids, are more sensitive to aquaculture stressors (Jentoft et al, 2005). Our study thus defined important environmental factors to consider, but the optimization of pike-perch culture will necessitate to consider the genetic of the fish as well.

High stress responsiveness and suboptimal/inadequate environmental rearing conditions are suggested to be the main cause of high mortalities in pike-perch juveniles. However, other factors to consider in such species culture are the social dominance and the cannibalistic behaviour. In some pike-perch batches maintained in our facilities, social dominance was higher and cannibalistic behaviour was observed (even with fish around 30 g) while the same environmental conditions were applied for all batches (light, temperature, oxygen, density…). Such observation was thus suggested to be dependent on the life history of the fish (before arriving to our facilities) as well as on its domestication level.

In pike-perch culture, various domesticated populations, including strains from different geographic origins and wild populations, are actually used (Teletchea and Fontaine, 2014). For all our experiments, the fish were provided by Asialor farm (Dieuze, France), ensuring the same geographical origin (Czech origin). However, no information was collected about the domestication level, defined as the adaptation of an animal to the human

environment and its constraint (Milla et al., 2020). And domestication process can highly influence fish physiology. During the domestication process, a set of biological responses occurs, leading to genetic, genomic and phenotypic modifications and allowing the fish to better adapt to humans and captive environments (Teletchea, 2015; Milla et al., 2020). In Eurasian perch (*Perca fluviatilis*), domestication process modifies digestion ability in larvae, with modification of digestion ability occurring at the very beginning of ontogeny (Palińska-Żarska et al., 2020). It has also been shown that the stress response to acute or chronic stressors is reduced with domestication (Lepage et al., 2000; Milla et al., 2020). In *Melanoteania duboulayi*, F15 fish show lower increase in plasma cortisol than F0, when confined in beaker for 30 min (Zuberi et al., 2014). In the Eurasian perch, the effect of domestication level on stress response varies depending on the type of aquaculture stressor. In parallel, an improvement of immune competence through the domestication process of Eurasian perch was also described since there is a decrease in both innate and specific immune response in F1 fish exposed to various aquaculture stressors while a stimulation of immunity occurred in F4 ones (Douxfils et al, 2011; 2012; 2014). The interest of a selection of broodstock source followed by a domestication program thus seems obvious in the success of the aquaculture industry (Ikhwanuddin and Abol-Munafi, 2016).

While we tried to standardize the experiments by considering the same rearing and samplings conditions, we were dependent on the fish and facilities availability. It thus explains discrepancies in fish size/age at the beginning of the experiments. While results are consistent between experiments, such difference has to be considered when studying the neuroendocrine and immune systems. In mammals, secretion of melatonin by the pineal gland decreases with age. Such progressive decline in melatonin was also observed in zebrafish (*Danio rerio*) from 1 to 4 years of age (Zhdanova et al., 2008). In our experiments, potential decrease in melatonin release and associated impacts on results seem limited since we only considered pike-perch juveniles. Barcellos et al (2012) have also shown that the age of the fish is a determining factor for the functioning of the HPI axis. It could be explained by differences in the timing of maturation of the HPI axis and by variations in the perception and response to stressors (Barcellos et al., 2012; Koakoski et al., 2012).

As previously explained, optimization of pike-perch culture is required, and the current study highlighted key elements to consider. The environment is

crucial for animals, and behaviour, reproduction, immunity, growth and even survival of fish will be improved if optimal environmental conditions are considered. Pike-perch was shown to be light-sensitive, which involves the use of low light intensities (Baekelandt et al., 2018; 2019a; 2019b). Data also suggest the use of a red spectrum (Luchiari et al., 2009; Baekelandt et al., 2019a). For different reasons previously explained, the results of the current study do not allow to better define optimal rearing conditions such as temperature or density. However, the thesis pointed out the importance of the genetic of the species. Considering the high variability between batches (in terms of feeding behaviour and stress sensitivity, leading to low immune defences and high mortalities), efforts should focus on a selective breeding program of pike-perch.

Sensitivity to the light environment

In this study and according to available data in fish literature, we defined through several experiments that pike-perch is a light-sensitive species that prefers dark environments. However, fish were initially acclimated in low light conditions and were never exposed to high light environments. Changing suddenly the light environment modifies inevitably fish behaviour and induces a stress response, as observed in the experiments. A long-term exposure to high light intensities was considered as a chronic stress (Baekelandt et al., 2019b), suggesting that the fish was unable to acclimatize to these new environmental conditions within several weeks.

However, among several pike-perch farms we visited, one of them uses higher light intensities (> 70 lux) than suggested in our study (10 lux). No stress parameters were assessed on those fish, but their behaviour did not seem impacted by the light. Since that farmer has been rearing pike-perch in those conditions for several years, and maybe for several fish generations, it supposes a selection of fish showing best growth (i.e. less impacted by potential stressor) or a fish adaptation. A better resistance to high light intensities may occur at the eye level. Indeed, the reflecting *tapetum lucidum* can be darkened by a screening pigment. In the light, pigment granules move distally toward the retina, preventing light from reflecting back to the retina (Douglas and Djamgoz, 1990; Bone and Moore, 2008). In the dark, the mechanism is reverse, uncovering the *tapetum lucidum* and allowing light to reflect. A greater production and migration of those granules may thus

involve a better resistance to some light environments. A histological survey of such pigment granules migration would be considered in future experiments.

Immune markers

Several immune markers were assessed during this study, including humoral activities, splenic phagocytosis and related-gene expression. To the exception of some genes, all these markers are related to the innate system that has been considered in teleosts as an essential component in combating pathogens due to limitations of the adaptive immune system, their poikilothermic nature, their limited repertoire of antibodies and the slow proliferation, maturation and memory of their lymphocytes (Whyte, 2007; Uribe et al., 2011). These immune markers have also been selected since they are widely assessed in immune-related fish studies, allowing comparison between studies and species. In future experiments, considering additional innate immune markers should be of a great interest to better understand the relation between melatonin and fish immunity. Suggested markers are protease inhibitors, cytokines (including pro and anti-inflammatory interleukins) and antimicrobial peptides that are polypeptides targeting bacterial walls and being found in fish mucus, liver and gills (Uribe et al., 2011).

The present study has little considered the specific immunity that provides a specific response to antigens, antibodies and effector cells with high specificity. In mammals, the actions of melatonin on T and B lymphocytes have been widely demonstrated (Ren et al., 2017; Zhao et al., 2019; Luo et al., 2020). In birds, lymphocyte populations are also influenced by melatonin (Singh et al., 2010; Kharwar et al., 2015; Chen et al., 2016). However, in teleosts, the only similar study found in literature observed no effects of exogenous melatonin 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, such studies focusing, at the transcript level, only on a few genes, as performed during the *ex vivo* experiment (see Experiment V), bring limited conclusions since variations in gene expression does not specifically reflect variations in protein abundance or activity. Additional studies with a special consideration for the specific immunity should be driven to better define the immunomodulation by the melatonin hormone in teleosts. Several markers

including antibodies populations, T and B cell populations, immunological memory and specific cytokines should be considered.

Future research about melatonin in fish

Our study is a first approach concluding that melatonin is an immunomodulatory hormone in pike-perch. This modulation occurs through an indirect action via intermediates and through a direct action via specific receptors, including MT1 and MT2. Considering the available data in other fish species as well as in birds and mammals, a similar function between teleosts and other vertebrates is hypothesized but cannot be confirmed without additional investigations. These experiments should focus on the anti-inflammatory properties of melatonin, on the immune-synthesized melatonin and associated functions and on the indirect actions of melatonin considering hormones such as PRL, GH and IGF-1. Furthermore, investigations should be performed on fish specific immunity since we mainly focused on the innate system.

The present study also described some specific melatonin receptors (MT1 and MT2) on the main hematopoietic organs of teleosts, namely the head kidney and the spleen, thus confirming the direct action of the hormone. Additional investigations should be performed on these receptors, considering their expression as well as their day-night and seasonal variations, as shown in retina, optic tectum and pituitary of Senegalese sole (*Solea senegalensis*) (Confente et al., 2010). The functional significance of such variations remains to be determined but it suggests that both melatonin and melatonin receptors rhythms must be considered when studying melatonin activities. Combining different experimental and analytical approaches, including transcriptomic, proteomic and immunohistochemistry, is of a great interest to follow the expression and to localize the receptors within immune organs and tissues.

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XII. Conclusion and perspectives

Due to some retinal adaptations, pike-perch is a light-sensitive species. Long-term exposure to high light intensities profoundly affects its physiology due to a high-stress status leading to poor growth status, high mortalities and decreased immunocompetence. In addition, both light spectrum and photoperiod influences pike-perch innate immunity. Considering that the melatonin hormone is a multifunctional molecule conveying the light information to cells and organs, we hypothesized that melatonin is a relay between the neuroendocrine and the immune systems. While well described in mammals, this function has been only little investigated in teleosts. The *in vivo* and *in vitro* experiments described in this study defined together that melatonin has a key role in the modulation of immune functions, not only via intermediates such as cortisol, but also through specific receptors that are found on main hematopoietic organs of pike-perch. This modulation mainly involves a stimulation of the innate system. However, with regard to research data on other vertebrates, it is assumed that melatonin, depending on the immune status of the organism, could act as a buffer with immunostimulatory or immunosuppressive actions. Further experiments should thus focus on this buffering role as well as on the specific immune system of teleosts.

XIII. List of publications

Article published or submitted

Baekelandt S, Cornet V, Mandiki SNM, Lambert J, Dubois M, Kestemont P (2020). Ex vivo approach supports both direct and indirect actions of melatonin on immunity in pike-perch *Sander lucioperca*. Submitted.

Baekelandt S, Milla S, Cornet V, Flamion E, Ledoré Y, Redivo B, Antipine S, Mandiki SNM, Houndji A, El Kertaoui N, Kestemont P (2020). Seasonal simulated photoperiods influence melatonin release and immune markers of pike perch *Sander lucioperca*. *Sci Rep*., *10*: 2650.

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Oral communications

Baekelandt S, Milla S, Cornet V, Flamion E, Ledoré Y, Redivo B, Mandiki SNM, El Kertaoui N, Kestemont P (2019). Evidences for the immunomodulation by the melatonin hormone in pikeperch *Sander lucioperca*. International society of fish and shellfish immunology.

Baekelandt S, Mandiki SNM, Kestemont P (2017). Light environment affects endocrine and immune rhythms in pikeperch (*Sander lucioperca*). European Aquaculture Society.

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Mandiki SNM, Redivo B, **Baekelandt S**, Douxfils J, Lund I, Höglund E, Kestemont P (2016). Long-term tryptophan supplementation decreased the welfare and innate immune status of pikeperch juveniles. *Fish & Shellfish Immunology*, *53*:113-114.

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Other activities during the PhD

Supervision of 12 students from high schools and universities (bachelor and master degrees).

Master in management at the University of Namur (evening classes, 2016-18).