New species for EU aquaculture

Deliverable Report

Deliverable No:	D22.2		**Delivery Month:**	55
Deliverable Title:	Validation of optimal rearing variables under commercial farm conditions			
WP No:	22		**WP Lead beneficiary:**	P16. FUNDP
WP Title:	Growth and husbandry - pikeperch			
Task No:	22.2		**Task Lead beneficiary:**	P29. Fish2Be
Task Title:	Characterization of pikeperch growth, immune and physiological status in farm conditions			
Other beneficiaries:	P9. UL	P21. DTU		
Status:	Submitted		**Expected month:**	42
…………				

Lead Scientist preparing the Deliverable: Kestemont Patrick (FUNDP)

Other Scientists participating: Mandiki Robert, Baekelandt Sebastien (FUNDP), Bossuyt Jiri (Fish2Be)

Objective: Characterization of pikeperch (*Sander lucioperca*) growth, immune and physiological status in farm conditions

Description: Based on the results from multifactorial experiments (see Task 22.1), growth and physio-immunological status of different batches of pikeperch (*Sander lucioperca*) at different developmental stages (from 10 g to 1.5 kg) were compared, in farm conditions, between standard husbandry conditions usually applied in routine by the SMEs and the best rearing conditions identified in Task 22.1. From this task, the expected results will help to recommend the best conditions applicable in pikeperch farming for reducing stress level and supporting maximal growth performances.

Table of contents

SUMMARY 3
1. **OBJECTIVES** 4
2. **BACKGROUND** 4
3. **VALIDATION FARM EXPERIMENT:** 5
   A. MATERIALS AND METHODS 5
      Experimental design and rearing conditions 5
      Samplings and output variables 7
      Statistical analyses 8
   B. RESULTS 8
      Husbandry performances 8
   C. DISCUSSION 10
4. **SUPPLEMENTARY-LAB CONFIRMATION EXPERIMENT:** 12
   A. MATERIALS AND METHODS 12
      Experimental design and rearing conditions 12
      Samplings and output variables 12
      Stress indicators 12
      Humoral immune variables 13
      Gene expression analysis 13
   B. RESULTS 14
      Growth performances 14
      Physiological stress response 14
      Immune status 15
   C. DISCUSSION 17
5. **GENERAL CONCLUSIONS** 18
6. **REFERENCES** 18

SUMMARY

This report is targeted on the study of the husbandry and environmental requirements of pikeperch (*Sander lucioperca*) during on-growing, with emphasis on the effects of (a) multifactorial stress factors, (b) farm conditions and (c) the domestication level and geographical origin, on growth, immune and physiological status.

The specific objectives of the performed work were (i) to test at a commercial farm level the identified optimal rearing conditions for pikeperch by determining their effects on growth related parameters, immune and physiological status at different developmental stages (from 10 g to 500 g) and (ii) to determine the stress sensitivity to grading manipulations when fish are reared under red or white light spectrum. For this experiment, a stock of pikeperch juveniles was produced by Fish2Be farm (Belgium) and reared in 2,000 l-tanks according to rearing methods as usually followed in commercial farms. Each tank was exposed to a white or a red light spectrum. The experiment lasted 83 days and final body weight ranged between 90 and 100 g. Grading process and samplings occurred on days 49 and 83. Light spectrum did not affect husbandry performances or stress response. However, results on the stress response during the grading process show more clearly than those from the multifactorial study in Task 22.1 that pikeperch juveniles are highly sensitive to emersion stress. In aquaculture, prolonged, repeated and/or unavoidable other stressors are largely associated to maladaptive physiological effects including failures in immune functions and disease resistance. The stress responsiveness to frequent manipulations in pikeperch juveniles reared in farm intensive conditions may be the major factor affecting their immune-competence since a relationship between stress response and immune status has been established in other percid fish.

A complementary *in vivo* experiment was performed in FUNDP facilities to deepen the understanding of the mechanisms by which light intensity affects the stress and immune response in pikeperch juveniles, according to previous results (Deliverable 22.1). We investigated the effects of the light intensity (10 vs 100 lux) and two light spectra (white and red) on stress status, humoral innate immune response and expression profile of immune-relevant genes in pikeperch for 30 days. While light spectrum had little influence on tested variables, the use of a high light intensity was followed by long-term stress associated to an immune suppression. Cortisol and several immune variables also followed a day-night variation. Since the secretion of the melatonin hormone by the pineal gland follows a circadian rhythm by being produced only during the dark phase of the photoperiod, it is thought to be a crucial regulator of stress-induced hormones and immune functions. However, this hypothesis needs further investigations.

1. Objectives

(1) To validate, in a commercial farm, conditions identified as optimal for pikeperch rearing (see multifactorial experiment, Deliverable 22.1) by assessing growth related parameters and physio-immunological status of pikeperch at different developmental stages (from 10 g to 500 g) (Validation *farm experiment*).

The results from this experiment are expected to serve as recommendations for best conditions applicable in pikeperch farming for reducing stress level and supporting maximal growth performances.

(2) To better understand the combining effects of light intensity (10 *vs* 120 lux) and the light spectra (white *vs* red) on stress status, humoral innate immune response and expression profile of immune-relevant genes in pikeperch (*additional lab confirmation experiment*).

2. Background

High mortality and impairment in growth rate during pikeperch 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. Unsuitable light characteristics may induce high stress intensity, which may negatively affect growth processes in fish. It was reported that pikeperch exhibited higher growth and food conversion rates under high light intensity of red spectrum than fish under white spectrum (Luchiari et al., 2009). But there is no consensus about intensity or light spectrum because better growth rates were obtained with low light intensity in the case of white spectrum in agreement with the behavior of juvenile and adult pikeperch in natural environments, where this species is considered a crepuscular predator that is actively feeding during dusk and night (Luchiari et al., 2006; Zingel and Paaver, 2010; Dalsgaard et al., 2013).

During their young developmental stages, pikeperch are submitted to frequent grading manipulations, about every two weeks, to avoid high size heterogeneity. Grading manipulations may be stressful for the overall juvenile populations (Jentoft et al., 2005; Strand et al., 2007; Douxfils et al., 2014) since fish are submitted to netting and partial air exposure. The response related to aquaculture stressors has not yet been described for pikeperch but it has been reported that exposure of other percid fish to grading and emersion stressors may impair significantly various physiological pathways (Milla et al., 2010, Douxfils et al., 2012).

Although it is suggested that stress can impact negatively the immune defense in fish, stress response is a beneficial physiological adjustment to maintain homeostasis. So, the trade-off between stress and immune functions depends on the stress intensity and may be species related in fish. Characterization of stress sensitivity is an important factor for optimizing the commercial production of a new species in aquaculture. Indeed, it has been reported that reduction of stress responsiveness may be an important part of domestication, because of the positive selection of stress-resistant fish with an improvement of fitness along generations (Douxfils et al., 2011, 2012). In salmonids, this improvement was associated with low cortisol response, which was shown to be highly heritable through generations (Pottinger and Carrick, 1999, Felvoden et al, 2002). However, Volckaert et al. (2012) demonstrated that heritability of cortisol response to stress was low in European sea bass indicating that the reported decrease in stress responsiveness with selection may be species related.

Since the stress responsiveness in pikeperch has received little attention, characterization of the relationship between stress due to aquaculture management and the immune competence is a relevant step for improving the performance of this species in intensive production systems. Such characterization was firstly

investigated by a multifactorial experiment including 16 factors-modalities (Deliverable 22.1), and the results indicated that light characteristics were among the most directive factors affecting stress response and husbandry parameters in pikeperch. Therefore, from the combination of results on husbandry performances and on stress and immune status, two rearing factor-modalities representing two rearing systems were selected as optimal rearing for on growing of pikeperch in intensive culture. These selected two rearing systems were mainly characterized by low light intensity of red or white light spectrum, and then were recommended for validation investigation in farm commercial conditions (Deliverable 22.2). We also tested to what extent fish reared under red or white light conditions are sensitive to grading manipulations since this practice is unavoidable in pikeperch culture production.

3. Validation farm experiment:

The specific objectives were:

(a) To test at a pilot commercial farm level, the identified optimal rearing conditions for pikeperch by determining their effects on growth related parameters, immune and physiological status at different developmental stages of pikeperch (from 10 g to 500 g).

(b) To determine the stress sensitivity of fish reared under red or white light spectrum to grading manipulations

A. Materials and methods

Experimental design and rearing conditions

For this experiment, a stock of 8100 pikeperch juveniles was produced by Fish2Be farm (Belgium). Once they reached 11 g, fish were randomly distributed into 6 indoor 2,000 l-tanks. After a 2-week acclimation, the industrial white light spectrum was replaced by a red spectrum (610 nm) for half of the tanks. Since the objective was to validate the effects of the light spectrum in farm conditions, the same rearing methods as usually followed in commercial farms were used. Light intensity (10 lux at water surface), photoperiod (12 L:12 D daily cycle) and temperature (21 °C) were maintained constant. Grading process was applied on days 49, 83, 133, 186 and 291 in order to reduce size heterogeneity, then dividing the experiment in 5 periods (see **Table 3.1**). With the increase in individual body weight, the stocking density is usually reduced in the commercial production system of pikeperch. Therefore, on day 83 (beginning of the third period), 500 fish from each tank were transferred into 1,500 l- tanks, at Inagro's facilities (Belgium) and the same rearing conditions were applied.

Table. 3.1. Husbandry performances (CV: weight heterogeneity; SGR: specific growth rate and mortality rate) of pikeperch reared under a white (W) or a red (R) spectrum for periods 1 (27 Mar. to 15 May), 2 (16 May to 19 Jun.), 3 (20 Jun. to 9 Aug.), 4 (10 Aug. to 2 Oct.) and 5 (3 Oct. to 16 Jan.). Each light condition was applied in 3 tanks. Lowercase letters indicate a significant difference at $p < 0.05$.

Period 1 (27 Mar. - 15 May)

		27-Mar			15-May				
		Body weight (g)	CV (%)	Biomass (kg)	Body weight (g)	CV (%)	Biomass (kg)	Mortality rate (%)	SGR (%d-1)
W	1	11.24	22.5	15.9	36.79	36.1	50.8	2.26	2.76
	2	12.84	21.4	16.4	47.83	25.6	60.2	1.56	3.06
	3	11.03	23.9	15.3	35.92	29.2	48.7	2.16	2.75
R	4	9.95	21.6	13.5	37.15	29.4	49.8	1.54	3.06
	5	11.60	24.3	15.2	44.04	34.9	56.3	2.07	3.10
	6	11.01	21.8	15.0	41.68	30.0	55.6	2.13	3.10

Period 2 (16 May - 19 June)

		16-May			19-Jun				
		Body weight (g)	CV (%)	Biomass (kg)	Body weight (g)	CV (%)	Biomass (kg)	Mortality rate (%)	SGR (%d-1)
W	1	38.79	11.3	46.8	92.65	25.8	108.6	2.16	2.64
	2	49.38	12.7	58.5	103.01	28.9	119.5	1.61	2.23
	3	38.95	12.5	44.4	89.12	26.6	98.7	2.28	2.51
R	4	40.22	10.6	44.1	98.45	26.6	105.0	2.19	2.71
	5	45.34	10.7	54.4	93.20	28.2	109.5	1.67	2.18
	6	44.00	15.7	51.2	99.76	25.4	113.2	1.98	2.48

Period 3 (20 Jun. May - 9 Aug.)

		20-Jun			9-Aug				
		Body weight (g)	CV (%)	Biomass (kg)	Body weight (g)	CV (%)	Biomass (kg)	Mortality rate (%)	SGR (%d-1)
W	1	106	17.2	53.0	122	40.3	59.7	2.20	0.28
	2	115	19.7	53.0	141	38.6	69.1	1.60	0.40
	3	106	15.7	57.5	121	39.8	58.7	2.80	0.26
R	4	106	16.2	54.0	127	37.7	62.6	1.80	0.37
	5	108	18.6	57.5	119	37.6	57.9	2.40	0.19
	6	115	15.6	53.0	120	39.6	58.3	2.60	0.08

		Period 4 (10 Aug. - 2 Oct.)							
		10-Aug			2-Oct				
		Body weight (g)	CV (%)	Biomass (kg)	Body weight (g)	CV (%)	Biomass (kg)	Mortality rate (%)	SGR (%d-1)
W	1	133	32.8	46.1	173	63.6	59.7	0.62	0.49
	2	151	30.0	53.5	202	56.6	70.0	1.66	0.55
	3	131	32.8	48.1	178	65.1	63.0	2.29	0.58
R	4	145	28.8	45.1	183	57.3	53.7	3.74	0.44
	5	124	28.5	44.7	141	63.0	49.3	2.49	0.25
	6	130	32.1	46.0	173	61.5	60.2	1.66	0.54

		Period 5 (2 Oct. - 16 Jan.)							
		3-Oct			16 Jan.				
		Body weight (g)	CV (%)	Biomass (kg)	Body weight (g)	CV (%)	Biomass (kg)	Mortality rate (%)	SGR (%d-1)
W	1	257	20.8	22.9	335	22.1	28.8	3.37	0.25
	2	243	25.7	27.0	319	25.6	31.3	14.61	0.26
	3	247	19.9	25.5	398	27.0	39.8	3.37	0.45
R	4	235	29.2	22.4	376	30.6	31.2	2.25	0.45
	5	232	26.7	16.9	358	30.5	24.3	5.62	0.41
	6	236	56.2	26.9	349	32.8	35.0	5.62	0.37

Samplings and output variables

In order to assess the effects of grading procedures on stress and immune status, we sampled fish 2 h before and 30 min after the grading manipulations on days 49, 83, 133, 186 and 291. The grading process consisted in chasing, emersion, and manipulations in order to reduce size heterogeneity by discarding smallest and biggest fish. Since high size heterogeneity observed in pikeperch culture may highly influence the physiological stress response, each sampling consisted in capturing 4 of the smallest and 4 of the biggest fish. These fish were then 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 4,000 g for 5 min at 4 °C. Plasma was aliquoted and stored at -80 °C until assayed.

Due to poor growth parameters (strong heterogeneity and low gains of weight and biomass) after the tank transfer (periods 3 to 5), it was decided to focus on the 2 first samplings (days 49 and 83) to evaluate the physio-immunological status of pikeperch.

Plasma cortisol was assayed in duplicate 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).

Lysozyme activity was evaluated in plasma samples by the turbimetric method (Siwicki and Studnicka, 1987; Douxfils et al, 2012). Plasma samples (10 µl) were mixed with 140 µl *Micrococcus lysodeikticus* (Sigma-Aldrich) solution (0.6 g l^{-1}). This assay was performed in duplicate. Absorbance was measured at 450 nm every minute for 15 min at room temperature. Lysozyme activity (units) is defined as the amount of enzyme decreasing the turbidity of 0.001 OD min^{-1}.

The total peroxidase activity in plasma was assessed following the method described in Quade and Roth (1997). Briefly, 10 µl of plasma were diluted in 140 µl of HBSS without Ca2+ or Mg2+ 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.

Statistical analyses

Data for growth performances were compared using one-way ANOVA ($p < 0.05$) taking the light spectrum as a two-modality factor ($n = 3$). Stress markers and immune data are expressed as the mean ± SEM. Replicate tank was considered as the statistical unit ($n = 3$). Kolmogorov and Smirnov's test was used to assess the normality of data sets ($p < 0.05$) and Levene's test was conducted to evaluate variance homogeneity ($p < 0.05$). Results were then analyzed with a linear model taking the light spectrum (red or white), the day of sampling (0, 49 or 83) and the body weight as factors. The results were analyzed with JMP 12.1 software (SAS Institute Inc., North Carolina, USA).

B. Results

Husbandry performances

Specific Growth Rate (SGR, % day^{-1}) for the periods 1 and 2 varied between 2.2 and 3.1 (**Table 3.1**). Individual body weight values on day 83 ranged between 90 and 100 g. Mortality did not exceed 3% and size heterogeneity (CV) reached 29% at the end of the second period. No difference was detected in any husbandry parameter between the two light conditions (**Table 3.1, Figure 3.1**).

For the periods 3 to 5, SGR values did not exceed 0.58 % day^{-1} (**Table 3.1**). Moreover, high CV values were observed during period 4 (up to 65%), and resulted in decreased total biomass since more fish were discarded during the grading process. For the fifth period, the red light rearing conditions resulted in higher CV than the white light rearing conditions (**Table 3.1**) but no effect was detected on SGR or mortality.

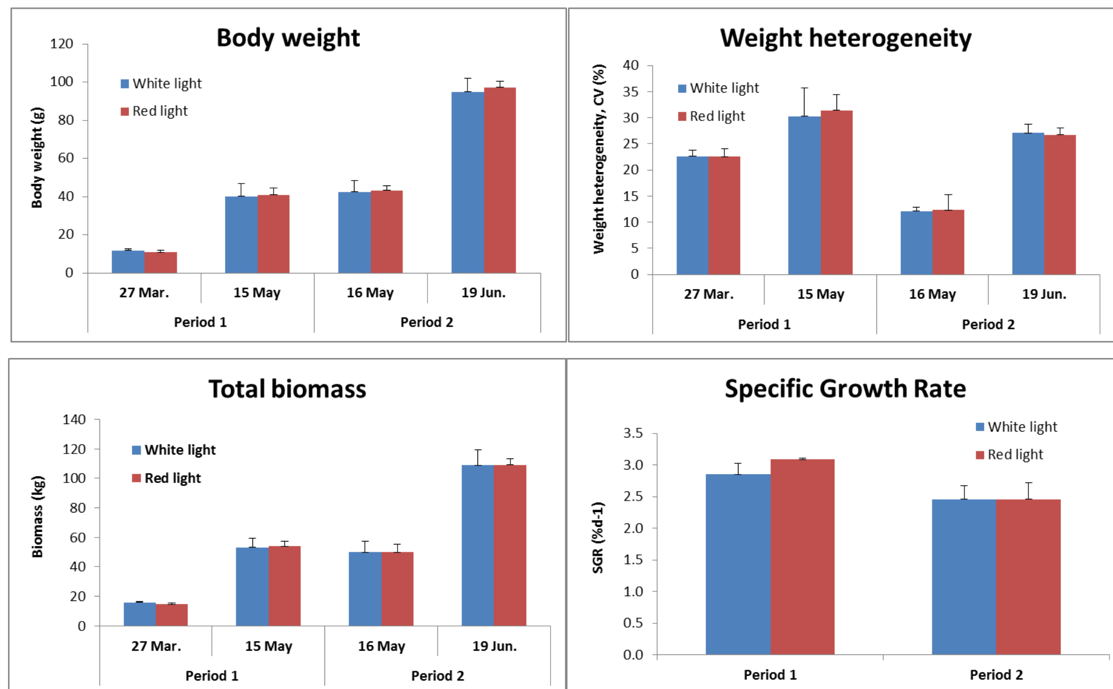


Figure. 3.1. Growth performances (mean ± s.d. of final body weight, weight heterogeneity, biomass and specific growth rate) of pikeperch reared under a white (W) or a red (R) spectrum for periods 1 (27 Mar. to 15 May) and 2 (16 May to 19 Jun.), n = 3.

Physiological and immune stress response

Fish submitted to grading manipulations showed a significant increase ($p < 0.001$) in cortisol and plasma glucose levels on both days 49 and 83 (**Figure 3.2**) whatever the light spectrum and body weight. These levels were also influenced by the day of sampling since higher values ($p <0.05$) were observed at day 83.

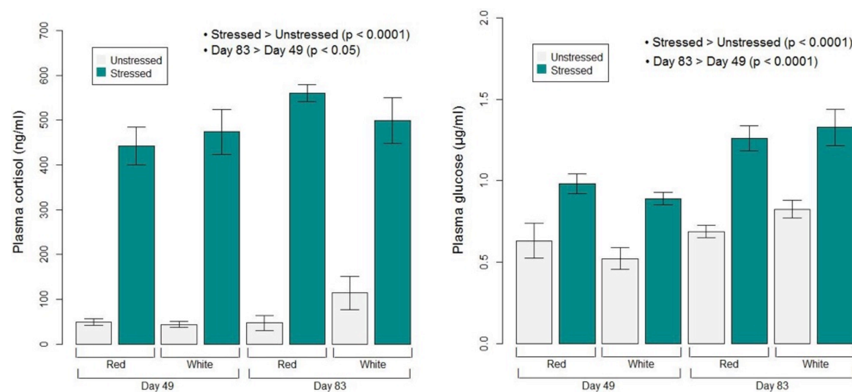


Figure. 3.2. Mean (± SEM) of plasma cortisol (left) and glucose (right) levels of pikeperch juveniles submitted or not to grading process (unstressed vs stressed fish), and reared under a red or a white light spectrum, on days 49 and 83 (n = 24). All significant results are indicated on the graphs.

While the use of a red spectrum led to increased lysozyme activity, higher values for the peroxidase activity were observed in fish reared under a white light (**Figure 3.3**). Moreover, the grading manipulations affected

the lysozyme activity. From day 49 to day 83, the lysozyme activity is decreased while the opposite tendency is observed for the peroxidase activity (**Figure 3.3**). No effect of the fish size was detected.

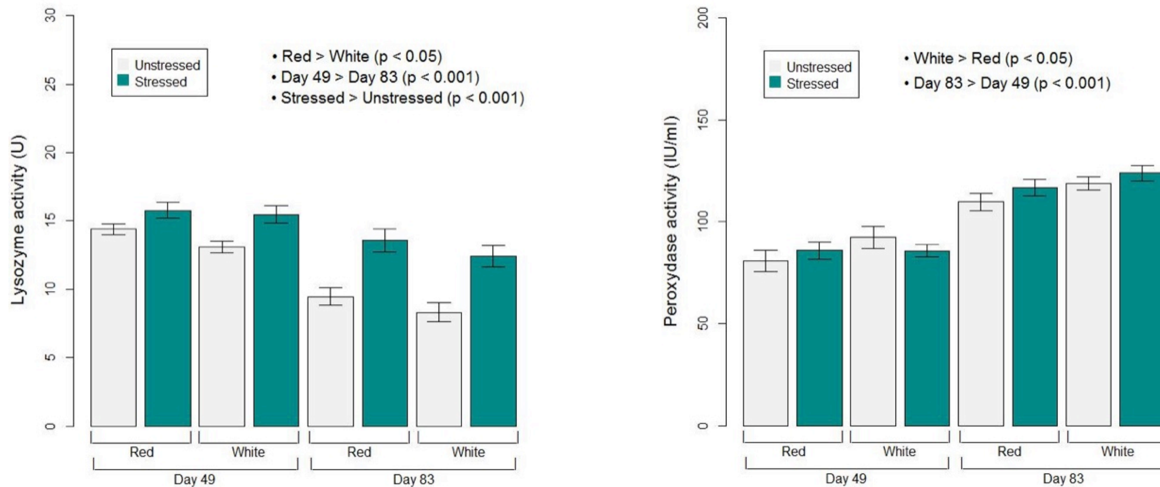


Figure 3.3. Mean (± SEM) lysozyme (left) and peroxidase (right) activity in plasma of pikeperch juveniles submitted or not to grading process (unstressed vs stressed fish), and reared under a red or a white light spectrum, on days 49 and 83, n = 21. All significant results are indicated on the graphs.

C. Discussion

The effects of light environment on growth and physiological-immune status of pikeperch or other fish species are poorly documented. The few previous studies have already pointed out that light spectrum is a determining factor affecting the physiological response of pikeperch, and, subsequently, the yield of pikeperch production. The results from our multifactorial experiment confirmed that rearing modalities including low light intensity were associated to high husbandry performances whatever the light spectrum; moreover, no clear relationship was established between stress level and husbandry performance (see Deliverables 22.1, and Baekelandt et al., 2018). Thus, in the present study, we intended to validate in commercial farm production conditions the effect of low light intensity of red and white spectrum on growth and physio-immunological status of pikeperch at different developmental stages.

During the first on growing phase (until day 83), good SGR results were observed for both experimental conditions, and values (around 3.0 and 2.5% day^{-1} for periods 1 and 2, respectively) were higher than those previously reported in lab conditions for pikeperch juveniles at comparable developmental stages (Wang et al., 2009, Baekelandt et al., 2018). Unfortunately, poor SGR values were recorded afterwards, perhaps due to the transfer of fish to different tanks, even if the same rearing conditions were maintained. Considering these poor results, the validation comparison of white and red light conditions was based on the results from the first ongrowing phase. During this phase of juvenile development, all husbandry parameters were statistically comparable between the experimental conditions confirming that light characteristics act mainly through light intensity. It has been reported that environmental colors may 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; Karakatsouli et al, 2007; Luchiari and Pirhonen, 2008). For instance, rearing of rainbow trout (*Oncorhynchus mykiss*) under blue light and of gilthead seabream (*Sparus aurata*) under red light has negative effects on growth and physiological status, including an increased stress status

(Karakatsouli et al, 2007). In pikeperch, it was shown that the use of red light improves specific growth rate and feed efficiency without any clear influence on stress status (Luchiari et al, 2009). In the present experiment, light spectrum (red or white) did not significantly affect the growth parameters or stress level measured through plasma cortisol and glucose levels in plasma. However, red light improved lysozyme activity while the industrial white light increased peroxidase activity, suggesting that the two tested rearing conditions act differently to immune functions, but it is not clear if they could induce a different level of immunocompetence. The only study found on immune regulation by the light spectrum in teleosteans focuses in goldfish (*Carassius auratus*) (Eslamloo et al, 2013). This study revealed that a red or a blue environmental light are chronically stressful and immunosuppressive with increase in plasma cortisol and decrease in lysozyme and plasma antiprotease. The fish immune responses might be indirectly altered by different background colors through effects on stress-induced hormones (Eslamloo et al, 2013).

In the present experiment, we also test the stress sensitivity of fish under red or white light conditions to grading manipulations that are unavoidable practices in pikeperch rearing. Increase in plasma cortisol and glucose was clearly observed 30 min post-stress with no statistical differences in red and white rearing conditions. These results show, more clearly than those of the multifactorial study in Task 22.1, that pikeperch juveniles are highly sensitive to emersion stress. 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). 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; Douxfils et al., 2014). Therefore, the stress responsiveness to frequent manipulations in pikeperch juveniles reared in farm intensive conditions may be the major factor affecting their immunocompetence, since a relationship between stress response and immune status has been established in other percid fish (Milla et al., 2010; Mathieu et al., 2013).

Since light appears to be an important factor to consider for pikeperch rearing, it was decided to lead another experiment to better define the link between the light environment (color and intensity) and the immune system (see *Experiment 2*).

4. **Supplementary-lab confirmation experiment:**

The objective was to deepen the understanding of the mechanisms by which light intensity affects the stress and immune response in pikeperch juveniles, and to determine the nictemeral variations of this response.

A. Materials and methods

Experimental design and rearing conditions

A stock of 960 pikeperch juveniles from Asialor farm (Dieuze, France) were transferred to URBE facilities at the University of Namur, Belgium. Fish were randomly distributed in 24 indoor 100 l-tanks. They were acclimated for 20 days under constant lightning conditions (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 20 ± 4 g body weight. They were fed twice daily with a commercial pellet diet (Skretting, France) at 2.0 % biomass during all the period. On day 1 at 8 am, tanks were exposed to new light conditions, including two light intensities (10 or 120 lux) and two light spectra (industrial white or red at 610 nm). Specimens were reared under these conditions with a 12 L:12 D daily cycle for a month.

Samplings and output variables

Samplings occurred during photophase (4 pm) and scotophase (4 am), on days 1 and 30. To avoid the stress artefact of nocturnal fishing on diurnal samplings, the number of tanks was doubled. Thus, each treatment group had 3 replicates. Fish were starved one day before sampling. 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 5 min and centrifuged at 3,000 g for 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.

Stress indicators

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).

Brain neurotransmitters

The contents of serotonin (5-HT), 5-hydroxylindol-3-acetic acid (5-HIAA), dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) in whole brain were assessed by High Performance Liquid Chromatography with electrochemical detection (HPLC-EC) as previously described in Gesto et al (2006) with some modifications. Tissues were weighed 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 0.250 ml of mobile phase used in the chromatography. They were then submitted to ultrasonic disruption. Homogenates were centrifuged (20,000 x g, 10 min, 4 °C) and supernatants were transferred in new tubes. Considering the tank as the experimental unit, supernatants from fish sampled in one tank were pooled together in respect to brain weight. Pools were filtered through 0.5 µm filters and an aliquot (35 µl) of the filtrate was injected in triplicate 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. Standard solutions were treated similarly to samples. Concentrations of the compounds were calculated by interpolation of their respective standard curves. Serotonergic and dopaminergic activities were respectively expressed as (5-HT) / (5-HIAA) and DA / DOPAC ratios.

Humoral immune variables

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 130 µl *Micrococcus lysodeikticus* (Sigma-Aldrich) solution (0.6 g l^{-1} in Na_2HPO_4 0.05 M pH 6.2). This assay was performed in triplicate. Absorbance was measured at 450 nm every 2 min for 16 min at room temperature. Lysozyme activity (units) is defined as the amount of enzyme decreasing the turbidity of 0.001 OD min^{-1}.

The total peroxidase activity in plasma was assessed following the method described in Quade and Roth (1997). Briefly, 10 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.

Gene expression analysis

Total RNA isolation was performed using Extract-all® reagent (Eurobio, Paris, France) following manufacturer's instructions. Considering the tank as the experimental unit, head kidney tissue from 5 fish was constituted. The 50 mg-pools 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 DEPC-treated water. After verification of RNA integrity and quantification of nucleic acid concentration with a Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, USA), each RNA sample was subjected to DNase treatment (DNase Ambion, Life Technologies) following manufacturer's recommendation to avoid gDNA contaminations. Then, 2 µg of total RNA were reverse-transcribed using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) using oligo(dT)18 and following the manufacturer's instructions. At the end of the reverse transcription reaction, cDNA was diluted 25 times in sterile water for RT-qPCR analysis and kept at -80 °C.

The relative expression of complement C3, C-type lysozyme (Lys), interleukine-1 (Il-1), Hepcidin c (Hepc), Tumor Necrosis Factor alpha (TNF-α) and the reference genes β-actin and elongation factor alpha were investigated by RT-qPCR using specific primers. Primer sequences were designed with primer3 software and efficiencies were validated when ranged between 90 and 105%. 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 sec^{-1}) and a final cooling step (4°C) using a StepOne plus real time PCR machine (Applied Biosystem). The relative mRNA levels of complement C3, C-type lysozyme, 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 (Larionov et al, 2005).

B. Results

Growth performances

Growth parameters were influenced by the light intensity but not by the light spectrum during this 1 month-experiment. The high light intensity affected both the final individual body weight and the SGR, whose values reached 1.6 ± 0.3 and 1.2 ± 0.4 % day^{-1} for the 10 and 100 lux light respectively (**Figure 4.1**).

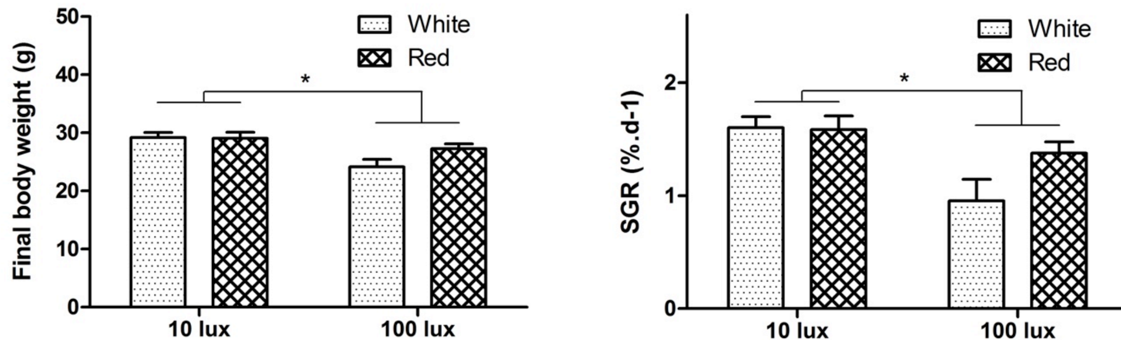


Figure 4.1. Mean (± SEM) final individual body weight (g) and Specific Growth Rate (SGR, % day^{-1}) of pikeperch reared under a low (10 lux) or a high (100 lux) light intensity of a red or white spectrum. (*) indicates a significant difference between treatments.

Physiological stress response

Plasma cortisol level (**Figure 4.2**) was highly influenced ($p < 0.001$) by the time of sampling with values reaching 50 to 100 ng ml^{-1} during the night (4 am) and 10 to 40 ng ml^{-1} during photophase (4 pm). No effect of the light intensity or spectrum was detected.

Concerning the brain serotonergic activity (5HIAA/5HT ratio) (**Figure 4.3**), change of light environment on day 1 stimulated this activity at 4 pm. On day 30, only the high light intensity led to increased serotonergic activity. No effect was observed on the dopaminergic activity.

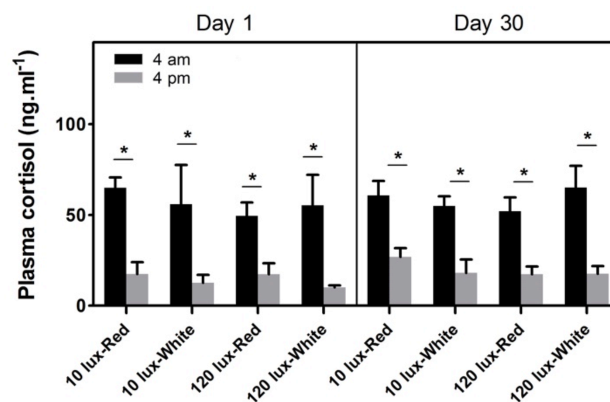


Figure. 4.2. Mean (± SEM) of plasma cortisol at 4 am (grey columns) and 4 pm (black columns) under 4 different light conditions, on day 1 and day 30. (*) indicates a significant difference ($p < 0.05$) between samplings at 4 am and 4 pm for 1 light condition and 1 day of sampling.

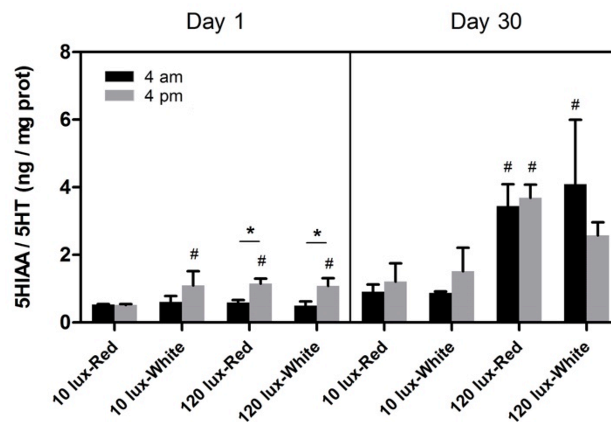


Figure. 4.3. Mean (± SEM) brain serotonergic activity (5HIAA/5HT ratio) at 4 am (grey columns) and 4 pm (black columns) under 4 different light conditions, on day 1 and day 30. (*) indicates a significant difference ($p < 0.05$) between samplings at 4 am and 4 pm for 1 light condition and 1 day of sampling. (#) indicates a significant difference ($p < 0.05$) between the indicated column and the control (10 lux, white light) at the same time and the same day of sampling.

Immune status

Plasma lysozyme activity was significantly decreased under a high light intensity after 30 days compared to the control (**Figure 4.4**). Values differed ($p < 0.05$) between scotophase and photophase on day 1 under a 100-lux light and the same trend was observed for the control. For plasma peroxidase activity, a day-night variation was observed with a peak of activity during scotophase (**Figure 4.5**).

TNF-α and Il-1 gene expressions also showed a cyclic activity with significant higher ($p < 0.05$) values obtained at 4 pm (**Figure 4.6**). Change of light intensity from a 10 to a 100-lux light on day 1 led to increased Hepc, Il-1 and TNF-α gene expressions. However, on day 30, the 100-lux light (white spectrum) led to significant increase ($p < 0.05$) in C3 and Lys expression (**Figure 4.6**).

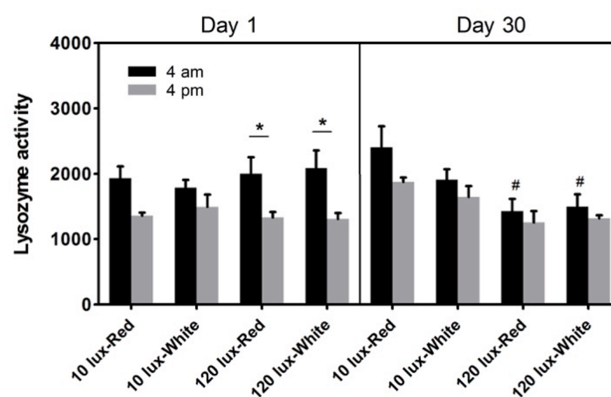


Figure. 4.4. Mean (± SEM) plasma lysozyme activity at 4 am (grey columns) and 4 pm (black columns) under 4 different light conditions, on day 1 and day 30. (*) indicates a significant difference ($p < 0.05$) between samplings at 4 am and 4 pm for 1 light condition and 1 day of sampling. (#) indicates a significant difference ($p < 0.05$) between the indicated column and the control (10 Lux, white light) at the same time and the same day of sampling.

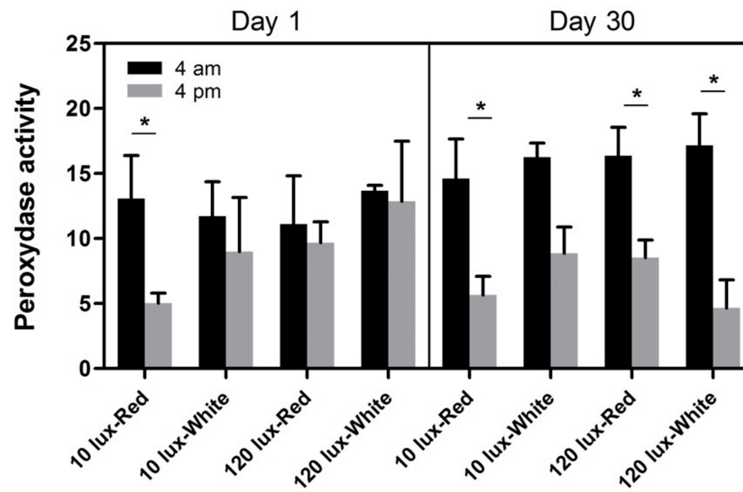
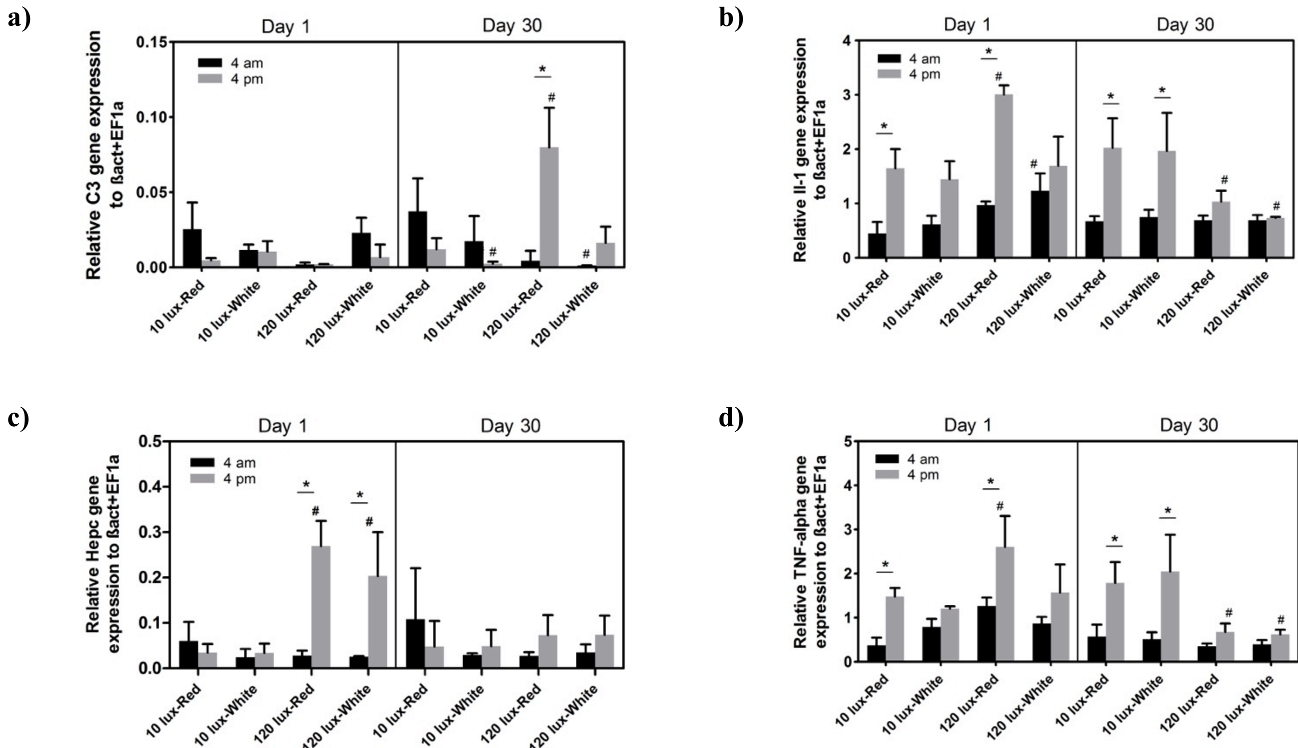


Figure. 4.5. Mean (± SEM) plasma peroxidase activity at 4 am (grey columns) and 4 pm (black columns) under 4 different light conditions, on day 1 and day 30. (*) indicates a significant difference ($p < 0.05$) between samplings at 4 am and 4 pm for 1 light condition and 1 day of sampling.



e)

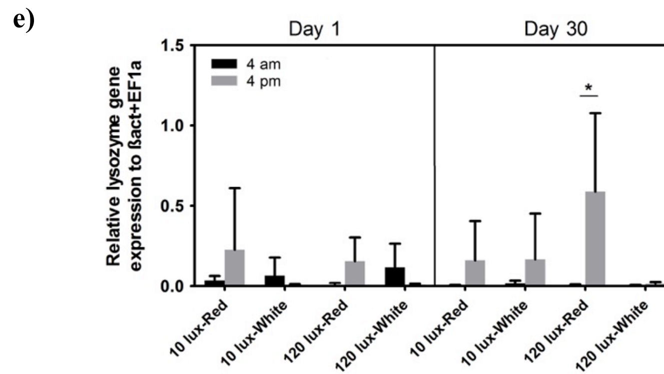


Figure 4.6. The relative expression (mean ± SEM) of *(a)* complement C3, *(b)* interleukine-1 (Il-1), *(c)* Hepcidin c (Hepc), *(d)* Tumor Necrosis Factor alpha (TNF-α) and *(e)* Lysozyme (Lys) in the head kidney tissue at 4 am (grey columns) and 4 pm (black columns) under 4 different light conditions, on day 1 and day 30 (n = 3). The relative mRNA levels were normalized with the geometric mean of ef1-α and β-actin. (*) indicates a significant difference ($p < 0.05$) between samplings at 4 am and 4 pm for 1 light condition and 1 day of sampling. (#) indicates a significant difference ($p < 0.05$) between the indicated column and the control (10 lux, white light) at the same time and the same day of sampling.

C. Discussion

In previous studies (Luchiari et al, 2006; 2009; Baekelandt et al, 2018) light characteristics were defined as determining factors affecting physiology status of pikeperch. However, the mechanism by which the light environment affects physiological and immune status of pikeperch, and more extensively of all fish species, is poorly documented. In this experiment, the effects of two light spectra (red and white) and 2 light intensities (10 and 120 lux at water surface) were tested on the physio-immunological status of pikeperch. As shown from the multifactorial study, this experiment showed that light intensity, and to a lesser extent light spectrum, are crucial factors regulating growth and endocrine and immune functions in pikeperch.

First of all, the results showed a cyclic day-night activity for cortisol and for various immune markers. Such circadian activities have already been described in several teleost species and are the results of the cyclic production of melatonin which is under the control of the day-night cycle. The melatonin hormone is produced and secreted by the pineal gland during the dark phase of the photoperiod (Esteban et al, 2006; Falcón et al, 2010). Through the secretion of this hormone, the pineal gland processes photoperiodic information and provides information such as time of the day and season for cells and organs (Kulczykowska et al, 2010; Brüning et al, 2015). In mammals, melatonin is largely described to act on important physiological functions, including thermoregulation, reproduction and immune functions (Carrillo-Vico et al, 2005; Cuesta et al, 2008). 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 (Boeuf and Le Bail, 1999; Downing and Litvak, 2002; Falcón et al, 2010; Mehner, 2012). Nevertheless, its potential role on immune functions in teleosteans is not well described. Evidence suggests that melatonin may act as an important immune regulator. It is known to possess an immunomodulatory activity, through a direct action via specific receptors on immune cells and tissues and/or an indirect action through several candidate hormones such as glucocorticoids, growth hormone, prolactin and somatolactin (Cuesta et al, 2006, Falcón et al, 2010).

In most fish species, plasma cortisol exhibits a circadian rhythm that is species specific (Brüning et al, 2015). Considering available results in various fish species, it seems that cortisol release is not correlated to the rhythmicity profile of melatonin in blood. For instance, a peak of plasma cortisol occurs during the night in salmonids while it happens during photophase at 15:00 in the Senegalese sole (*Solea senegalensis*) and at light onset in goldfish (Brüning et al, 2015; Oliveira et al, 2013; Noeske and Spieler, 1983; Laidley and Leatherland, 1988). Such differences between species in terms of cortisol peak can be explained, at least partially, by the meal time that could be perceived as a pacemaker of cortisol rhythm (Kulczykowska and Sanchez-Vazquez, 2010, Oliveira et al, 2013).

High cortisol production has been described as a potent immunosuppressive agent in vertebrates with complex actions on immune cells (Tort et al, 2011; Mathieu et al, 2013). Thus, it is important to consider this hormone in the potential link between the light characteristics and the immune system in pikeperch. In this experiment, the circadian activity of some immune markers may be driven by the cyclic cortisol release, itself under the control of pineal gland secretions. However, the light intensity influenced several immune markers without any impact on cortisol release but in association to a high neuro-serotonergic activity, suggesting that the relationship between stress response and immune system may be primarily activated by the central neuro-endocrine pathways. Serotonergic activity has been described several times as a good indicator of acute and chronic stress in various fish species including the Senegalese sole and several salmonids (Winberg and Nilsson, 1993; Gesto et al, 2013; 2016; Conde-Sieira et al, 2014). When considering serotonergic activity level in the present study, the change of light environment (spectrum and/or intensity) induced a higher stress status on day 1. On day 30, this activity was significantly higher under a high light intensity whichever the light spectrum, suggesting that high light intensity was perceived, for fish initially maintained under a 10-lux light, as a long-term stressor. This 120-lux light also impacted immune status with immune stimulation on day 1 as seen through TNF-α and Hepc gene expression and lysozyme activity and with an immune suppression on day 30 including decrease in Il-1 and TNF-α gene expressions. Since cortisol release was not impacted by the light environment, it would be suggested that neurotransmitter parameters may be better stress indicators to further understand the relationship between stress response and immune functions.

5. General conclusions

Collectively, the results from the farm-validation experiments and the supplementary-lab study confirmed the findings from the multifactorial experiment (Deliverable 22.1) that low light intensity conditions are recommended to optimize growth rate and to reduce stress sensitivity in pikeperch. The results also showed that pikeperch are somewhat sensitive to grading manipulations despite the optimization of rearing conditions, and such stress response may be one of the factors that weaken the immune status of pikeperch during the young developmental stages.

6. References

Baekelandt, S., Redivo, B., Mandiki, S. N. M., Bournonville, T., Houndji, A., Bernard, B., El Kertaoui, N., Schmitz, M., Fontaine, P., Gardeur, J.-N., Ledoré, Y., Kestemont, P. (2018). Multifactorial analyses revealed optimal aquaculture modalities improving husbandry fitness without clear effect on stress and immune status of pikeperch *Sander lucioperca*. *Gen. Comp. Endocrinol.*, *258*, 194–204.

Boeuf, G., & Bail, P. Le. (1999). Does light have an influence on fish growth? *Aquaculture*, *177*, 129–152.

Brüning, A., Hölker, F., Franke, S., Preuer, T., & Kloas, W. (2015). Spotlight on fish: Light pollution affects circadian rhythms of European perch but does not cause stress. *Science of the Total Environment*, *511*, 516–522.

Carrillo-vico, A., Guerrero, J. M., Lardone, P. J., & Reiter, R. J. (2005). A Review of the Multiple Actions of Melatonin on the Immune System. *Endocrine*, *27*(2), 189–200.

Conde-sieira, M., Muñoz, J. L. P., López-patiño, M. A., Gesto, M., Soengas, J. L., & Míguez, J. M. (2014). Oral administration of melatonin counteracts several of the effects of chronic stress in rainbow trout. *Domest. Anim. Endocrinol., 46*, 26–36.

Cuesta, A., Vargas-Chaco, L., Garcıa-Lopez, A., et al. (2007). Effect of sex-steroid hormones, testosterone and estradiol, on humoral immune parameters of gilthead seabream. *Fish Shellfish Immunol.* 23:693–700.

Cuesta, A. (2008). In vivo actions of melatonin on the innate immune parameters in the teleost fish gilthead seabream. *Journal of Pineal Research*, *45*, 70–78.

Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K., & Pedersen, P. B. (2013). Farming different species in RAS in Nordic countries : Current status and future perspectives. *Aquac. Eng*., *53*, 2–13.

Douxfils, J., Mandiki, S. N. M., Marotte, G., Wang, N., Silvestre, F., Milla, S., Henrotte, E., Vandecan, M., Rougeot, C., Mélard, C., Kestemont, P. (2011). Does domestication process affect stress response in juvenile Eurasian perch *Perca fluviatilis*? *Comp. Biochem. Physiol.*, *159*, 92–99.

Douxfils, J., Deprez, M., Mandiki, S. N. M., Milla, S., Henrotte, E., Mathieu, C., Silvestre, F., Vandecan, M., Rougeot, C., Mélard, C., Dieu, M., Raes, M., Kestemont, P. (2012). Physiological and proteomic responses to single and repeated hypoxia in juvenile Eurasian perch under domestication - Clues to physiological acclimation and humoral immune modulations. *Fish Shellfish Immunol.*, *33*, 1112–1122.

Downing, G. (2002). Impact of spectral composition on larval haddock, *Melanogrammus aeglefinus* L., growth and survival. *Aquac. Res.*, *33*, 251–259.

Downing, G., Litvak, M.K., 2002. Effects of light intensity, spectral composition and photoperiod on development and hatching of haddock (*Melanogrammus aeglefinus*) embryos. Aquaculture 213, 265–278. Esteban, M. A., Cuesta, A., Rodríguez, A., & Meseguer, J. (2006). Effect of photoperiod on the fish innate immune system: A link between fish pineal gland and the immune system. *Journal of Pineal Research*, *41*(3), 261–266.

Eslamloo, K., Akhavan, S. R., Eslamifar, A., & Henry, M. A. (2015). Effects of background colour on growth performance, skin pigmentation, physiological condition and innate immune responses of goldfish, *Carassius auratus*. *Aquaculture Research*, *46*(1), 202–215.

Falcon, J., Migaud, H., Munoz-Cueto, J. A., & Carrillo, M. (2010). Current knowledge on the melatonin system in teleost fish. *General and Comparative Endocrinology*, *165*(3), 469–482.

Fast, M. D., Hosoya, S., Johnson, S. C., & Afonso, L. O. B. (2008). Cortisol response and immune-related effects of Atlantic salmon (*Salmo salar* Linnaeus) subjected to short- and long-term stress. *Fish Shellfish Immunol.*, *24*, 194–204.

Fevolden, S., Røed, K. H., & Fjalestad, K. T. (2002). Selection response of cortisol and lysozyme in rainbow trout and correlation to growth. *Aquaculture*, *205*, 61–75.

Gesto, M., Tintos, A., Soengas, J. L., and Míguez, J. M. (2006). Effects of acute and prolonged naphthalene exposure on brain monoaminergic neurotransmitters in rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. *Physiol. C Toxicol. Pharmacol*. 144, 173–183.

Gesto, M., López-patiño, M. A., Hernández, J., Soengas, J. L., & Míguez, J. M. (2013). The response of brain serotonergic and dopaminergic systems to an acute stressor in rainbow trout: a time course study. *J. Exp. Biol.*, *216*, 4435–4442.

Gesto, M., Álvarez-otero, R., Conde-sieira, M., Otero-rodiño, C., Usandizaga, S., Soengas, J. L., Míguez, J. M., & López-patiño, M. A. (2016). A simple melatonin treatment protocol attenuates the response to acute stress in the sole *Solea senegalensis*. *Aquaculture*, *452*, 272–282.

Jentoft, S., Aastveit, A. H., Torjesen, P. A., & Andersen, Ø. (2005). Effects of stress on growth, cortisol and glucose levels in non-domesticated Eurasian perch (*Perca fluviatilis*) and domesticated rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol.*, *141*, 353–358.

Karakatsouli, N., Papoutsoglou, S. E., Pizzonia, G., Tsatsos, G., Tsopelakos, A., Chadio, S., Kalogiannis, D., Dalla, C., Polissidis, A., Papadopoulou-Daifoti, Z. (2007). Effects of light spectrum on growth and physiological status of gilthead seabream *Sparus aurata* and rainbow trout *Oncorhynchus mykiss* reared under recirculating system conditions. *Aquacultural Engineering*, *36*(3), 302–309.

Kulczykowska E, Gozdowska M, Martos-Sitcha JA, Kalamarz H, Nietrzeba M, Martínez-Rodríguez G. (2010). Melatonin, vasotocin and isotocin as biomarkers of the condition of fish. Comp. Biochem. Physiol. A. 157:S18.

Laidley CW, Leatherland JF (1988) Cohort sampling, anaesthesia and stocking-density effects on plasma cortisol, thyroid hormone, metabolite and ion levels in rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Biol.* 33:73–88

Larionov, A., Krause, A., & Miller, W. (2005). A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics*, *6*, 62.

Luchiari, A. C., Freire, F., Koskela, J., & Pirhonen, J. (2006). Light intensity preference of juvenile pikeperch *Sander lucioperca* (L.). *Aquac. Res.*, *37*, 1572–1577.

Luchiari, A. C., & Pirhonen, J. (2008). Effects of ambient colour on colour preference and growth of juvenile rainbow trout *Oncorhynchus mykiss* (Walbaum). *J. Fish. Biol.*, *72*, 1504–1514.

Luchiari, A. C., Freire, F. A. M., Pirhonen, J., & Koskela, J. (2009). Longer wavelengths of light improve the growth, intake and feed efficiency of individually reared juvenile pikeperch *Sander lucioperca* (L .). *Aquac. Res.*, *40*, 880–886.

Mehner, T. (2012). Diel vertical migration of freshwater fishes - proximate triggers, ultimate causes and research perspectives. *Freshwater Biology*, *57*(7), 1342–1359.

Noeske, T.A., Spieler, R.E., (1983). Photoperiod and diel variations of serum cortisol, thyroxine and protein in goldfish, *Carassius auratus* L. J. *Fish Biol*. 23, 705–710.

Politis, S. N., Butts, I. A. E., & Tomkiewicz, J. (2014). Light impacts embryonic and early larval development of the European eel, *Anguilla anguilla*. *J. Exp. Mar. Biol. Ecol.*, *461*, 407–415.

Pottinger, T. G., & Carrick, T. R. (1999). A comparison of plasma glucose and plasma cortisol as selection markers for high and low stress-responsiveness in female rainbow trout. *Aquaculture*, *175*, 351–363.

Siwicki A, Studnicka M. (1987). The phagocytic ability of neutrophils and serum lysozyme activity in experimentally infected carp, *Cyprinus carpio* L. The Journal of Fish Biology, 31, 57-60.

Tort, L. (2011). Stress and immune modulation in fish. *Dev. Comp. Immunol.*, *35*, 1366–1375.

Trinder, P. (1969). Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Ann. Clin. Biochem.*, *6*, 24–27.

Volckaert, F. M., Hellemans, B., Batargias, C., Louro, B., Massault, C., Van Houdt, J.K.J., Haley, C., de Koning, D.-J., and Canario, A.V.M. (2012). Heritability of cortisol response to confinement stress in European sea bass *Dicentrarchus labrax*. Genet. Sel. Evol. *44*, 15.

Winberg, S., & Nilsson, G. E. (1993). Roles of brain monoamine neurotransmitters in agonistic behaviour and stress reactions, with particular reference to fish. *Comp. Biochem. Physiol.*, *106*, 597–614.

Zingel, P., & Paaver, T. (2010). Effects of turbidity on feeding of the young-of-the-year pikeperch (*Sander lucioperca*) in fishponds. *Aquac. Res.*, *41*, 189–197.

Co-funded by the Seventh Framework Programme of the European Union